The Neural Mechanisms Mediating the Augmentation of Heroin Seeking Induced by Chronic Food Restriction

Tracey D'Cunha

A Thesis
In the Department
of
Psychology

Presented in Partial Fulfillment of the Requirement
For the Degree of
Doctor of Philosophy (Psychology) at
Concordia University
Montreal, Quebec, Canada

July 2018

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis prepared

By: Tracey D'Cunha

Entitled: The Neural Mechanisms Mediating the Augmentation of Heroin Seeking Induced by Chronic Food Restriction.

and submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Psychology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair
Dr. Ian Irwin

_____ External Examiner
Dr. Paul Clarke

_____ External to Program
Dr. Andreas Bergdahl

_____ Examiner
Dr. Wayne Brake

_____ Examiner
Dr. Nadia Chaudhri

_____ Supervisor
Dr. Uri Shalev

Approved by _____
Dr. Karen Li, Graduate Program Director

August 10, 2018 _____
Dr. André Roy, Dean, Faculty of Arts and Science

ABSTRACT

The Neural Mechanisms Mediating the Augmentation of Heroin Seeking Induced by Chronic Food Restriction

Tracey D'Cunha, Ph.D.

Concordia University, 2018

Addiction can be defined as a chronic relapsing disorder that is characterized by a loss of control over drug consumption. One of the major obstacles in the treatment of drug addiction is relapse, with the majority of individuals relapsing within the first year of drug abstinence. In humans, restricted food intake can modulate the main triggers of relapse thereby increasing drug craving and relapse. In animal models of relapse, caloric restriction will also increase drug seeking. The experiments presented in this thesis investigated the neuronal mechanisms that mediate the augmentation of heroin seeking induced by chronic food restriction in the rat. The mesolimbic dopamine pathway is heavily implicated in reward and motivation. Therefore, the experiments presented in Chapter 3 explored the role of dopamine in the mesolimbic pathway, specifically the nucleus accumbens, in the augmentation of heroin seeking induced by chronic food restriction. Extracellular dopamine in the nucleus accumbens core and shell subregions was differentially altered during the heroin-seeking test in chronically food restricted rats. Blockade of dopamine D1-like receptors in the nucleus accumbens shell decreased heroin seeking, whereas blockade of D1-like receptors in the nucleus accumbens core selectively reduced heroin seeking in the food restricted rats.

Hormones involved in energy balance and food intake, such as leptin and ghrelin are implicated in drug-related behaviors. Thus, the experiments in Chapter 4 investigated the role of leptin and ghrelin in heroin seeking induced by chronic food restriction. As expected, chronic food restriction decreased plasma levels of leptin and increased plasma levels of ghrelin. Furthermore, administration of leptin or a ghrelin receptor antagonist into the ventral tegmental area exclusively decreased heroin seeking in the food restricted rats. These results suggest that leptin and ghrelin may modulate drug seeking by acting upstream from the mesolimbic dopamine pathway.

Finally, in addition to the dense innervations from dopamine projections, the nucleus accumbens also receives a multitude of glutamatergic innervations from a variety of brain regions.

Hence, in Chapter 5 we investigated the role of glutamate in the nucleus accumbens on the augmentation of heroin seeking induced by chronic food restriction. Contrary to our predictions, there were no changes in extracellular glutamate in the nucleus accumbens during ongoing heroin seeking. Moreover, administration of a glutamate receptor antagonist had no effect on heroin seeking induced by chronic food restriction. Taken together, these findings demonstrate that the augmentation of heroin seeking induced by chronic food restriction is mediated by dopamine transmission in the nucleus accumbens and can be modulated by hormones involved in metabolic processes, such as leptin and ghrelin.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Uri Shalev, PhD, for your guidance, encouragement, and patience throughout this process. You have taught me how to critically assess science, and motivated me to always be curious and never stop asking questions. I am very privileged that you trained me with great scientific rigor throughout my Masters and PhD. To all the members of the Shalev Lab, studying behavioral neuroscience is not a feat that can be accomplished alone. A special thank you to Firas Sedki, who was my partner in crime in the lab. It was always reassuring to know that when we made mistakes in the lab, we could go to Uri's office together to break the bad news. Hopefully, by the end of our degrees he was excited to see us, as we were no longer bringing him bad news but new data! I would also like to extend my gratitude to all of the undergraduates who helped me run the numerous experiments through the years, including Melissa Russo, Damaris Rizzo, Emilie Daoud, Soraya le Noble, Alexandrea Usypchuk, Audrey Bishop, Gabrielle Mourra, Laurie Hamel, Suzanne Trieu Chao, and Jessica Ianuzzi. I thank you for being my many hands and agreeing to highly complicated experiments with long hours spent in the lab. I would also like to extend a special thank you to Katuschia Germé from the Pfaus lab, who willingly came into the HPLC room and worked tirelessly to help me set-up the assay for amino acids.

I would also like to express my utmost appreciation to all the students and staff at the Centre for Studies in Behavioral Neurobiology (CSBN). I cannot express how grateful I am, that I was able to drag (kicking and screaming) Heshmat Rajabi back to the HPLC room to help me set up the assay for monoamines. Your willingness to drop everything and always help troubleshoot was greatly appreciated. I am also very appreciative of the amazing staff at Concordia's animal care facility, Aileen Murray, Jean-Francois Trudel, and Jason Price. Thank you for taking care of my animals, and putting up with my numerous special requests to accommodate my experimental protocols. A special thank you to Dave Munro for helping with numerous engineering solutions to run especially the microdialysis experiments more efficiently, and also Steve Cabilio for help with optimizing the programming for behavioral testing. I would also like to thank Isabelle Bouvier for all her help throughout my time at the CSBN, helping me navigate through all the administrative processes, especially applying for grants and awards. To my fellow graduate students, there is light at the end of the tunnel! The CSBN's grad lounge provided a collaborative space that has helped my foster not just professional collaborations with my colleagues but also lifelong friendships. To my fellow cohort, especially Lindsay Sparks and Waqqas Shams, thank you for all of your support both

in and out of school. Finally, I would like to thank my PhD committee members, Wayne Brake, PhD, Nadia Chaudhri, PhD, Andreas Bergdahl, PhD, and Paul Clarke, PhD, for taking time out of their busy schedules to be on my committee and provide feedback on my thesis. I would also like to thank my colleagues at EMD Serono for being very supportive as I transitioned from being a student to entering the workforce, and for encouraging me as I completed the last stage of my PhD.

Finally, last but not least, I would like to thank my family. My parents, Peter and Sandra D'Cunha, who always held out hope that I would not be a forever student and would someday eventually get a job. Thank you for always supporting me to stay in school and hanging up all of my graduation portraits! I would also like to thank my brother Ronald, who supported me throughout the years, even if you don't understand why anyone would stay in school for this long. To conclude, I save the best for last, my soon-to-be-husband (and husband by the time I defend this thesis!) Sean Reed. You have been there right from the beginning of this roller coaster of a ride called graduate school. As a fellow PhD, you inspire and motivate me to be a better scientist and provide valuable insights on all my research. As a partner, you are the key to my success. Your support, love, and encouragement are what have propelled me to excel, and to you I dedicate this thesis.

CONTRIBUTION OF AUTHORS

1. **Augmentation of heroin-seeking following chronic food restriction in the rat: Differential role for dopamine transmission in the nucleus accumbens shell and core**

   Tracey D'Cunha. Conceived and designed the experiments. Performed surgeries, made microdialysis probes, completed drug preparation and administration, ran samples through HPLC, and behavioral data collection. Conducted data analysis, interpreted data, wrote the manuscript.

   Emilie Daoud. Helped with surgeries, microdialysis probe construction, histology, and data collection in Experiments 1 and 2.

   Damaris Rizzo. Helped with surgeries, histology, and data collection in Experiments 3 and 4.

   Audrey Bishop. Helped with surgeries, microdialysis probe construction, histology, and data collection in Experiment 2.

   Melissa Russo. Helped with surgeries, histology, and data collection in Experiments 3 and 4.

   Gabrielle Mourra. Helped with surgeries, histology, and data collection in Experiment 3.

   Laurie Hamel. Helped with surgeries and data collection in Experiment 1.

   Firas Sedki. Helped with surgeries, and behavioral data collection in all experiments. Contributed to the interpretation of the results and revisions of the manuscript.

   Uri Shalev. Conceived and designed the experiments. Contributed to data analysis, interpretation of the results, and writing of the manuscript.

2. **A role for leptin and ghrelin in the augmentation of heroin seeking induced by chronic food restriction**

   Tracey D'Cunha. Conceived and designed the experiments. Performed surgeries, collected blood samples, completed drug preparation and administration, ran ELISAs, and behavioral data collection. Conducted data analysis, interpreted data, wrote the manuscript.

   Melissa Russo. Helped with surgeries, blood sample collection, and data collection.

   Soraya le Noble. Helped with surgeries, blood sample collection, and data collection.

   Damaris Rizzo. Helped with surgeries and data collection.

   Emilie Daoud. Helped with surgeries and data collection.

Firas Sedki. Helped with surgeries, blood sample collection, ELISAs, and behavioral data collection.

Uri Shalev. Conceived and designed the experiments. Contributed to data analysis, interpretation of the results, and writing of the manuscript.

3. **The role of glutamate in the augmentation of heroin seeking induced by chronic food restriction**

Tracey D'Cunha. Conceived and designed the experiments. Performed surgeries, made *in vivo* microdialysis probes, completed drug preparation and administration, ran samples through HPLC, and behavioral data collection. Conducted data analysis, interpreted data, wrote the manuscript.

Melissa Russo. Helped with surgeries, microdialysis probe construction, histology, and data collection in all experiments.

Soraya le Noble. Helped with surgeries, microdialysis probe construction, histology, and data collection in all experiments.

Alexandra Usypchuk. Helped with surgeries, microdialysis probe construction, and data collection in Experiments 1 and 2.

Katuschia Germé. Helped with set-up of HPLC for amino acid detection, protocol for running amino acids in HPLC, preparation of solutions for HPLC, and creation of software analysis methods for quantification of samples in EZ Chrom Chromatography Data System.

Jessica Iannuzzi. Helped with surgeries and data collection in Experiments 1 and 2.

Damaris Rizzo. Helped with surgeries and data collection in all experiments.

Suzanne Trieu Chao. Helped with surgeries and data collection in Experiments 1 and 2.

Firas Sedki. Helped with surgeries, and behavioral data collection in all experiments.

Uri Shalev. Conceived and designed the experiments. Contributed to data analysis, interpretation of the results, and writing of the manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1. General Introduction

Chapter 3. Augmentation of heroin-seeking following chronic food restriction in the rat: differential role for dopamine transmission in the nucleus accumbens shell and core

Chapter 4. A role for leptin and ghrelin in the augmentation of heroin seeking induced by chronic food restriction

Chapter 5. The role of glutamate in the augmentation of heroin seeking induced by chronic food restriction

Appendix: The role of dopamine in the basolateral amygdala and dorsolateral striatum in the augmentation of heroin seeking induced by chronic food restriction

LIST OF TABLES

Chapter 3. Augmentation of heroin-seeking following chronic food restriction in the rat: differential role for dopamine transmission in the nucleus accumbens shell and core

Chapter 4. A role for leptin and ghrelin in the augmentation of heroin seeking induced by chronic food restriction

Chapter 5. The role of glutamate in the augmentation of heroin seeking induced by chronic food restriction

ABBREVIATIONS

ACF	animal care facility
aCSF	artificial cerebrospinal fluid
AgRP	agouti-related protein
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor
ANOVA	analysis of variance
BLA	basolateral amygdala
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPP	conditioned place preference
CRF	corticotrophin releasing factor
DA	dopamine
DAT	dopamine transporter
DLS	dorsolateral striatum
DMH	dorsomedial nucleus of the hypothalamus
ED	electrochemical detection
ELISA	enzyme-linked immunosorbent assay
FDR	food restricted
FI	fixed interval
GABA	gamma-aminobutyric acid
GHS-R1a	growth hormone secretagogue receptor
GLT-1	glutamate transporter
GOAT	ghrelin-O-acyltransferase
HPLC	high performance liquid chromatography
i.c.v.	intracerebroventricular
iGluR	ionotropic glutamate receptor
ILC	infralimbic cortex
LepR	leptin receptor
LH	lateral hypothalamus
LTD	long-term depression
LTP	long-term potentiation

mGluR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex
MSNs	medium spiny neurons
mThal	medial thalamic nuclei
NAc	nucleus accumbens
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NPY	neuropeptide Y
NR2B	N-methyl-D-aspartate receptor 2B subunit
PFC	prefrontal cortex
PLC	prelimbic cortex
PMSF	phenylmethylsulfonyl fluoride
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus of the hypothalamus
PVT	paraventricular nucleus of the thalamus
SMLA	superactive mouse leptin antagonist
STAT3	signal-transducer-and-activator-of-transcription-3
vHipp	ventral hippocampus
VMH	ventromedial nucleus of the hypothalamus
VP	ventral pallidum
vSub	ventral subiculum
VTA	ventral tegmental area

CHAPTER 1: GENERAL INTRODUCTION

In the last 20 years, North America has seen a steady increase in the use of prescription opioids for pain management. Globally, Canada has the 2nd highest use of prescription opioids, behind only the United States of America (Murphy, Goldner, & Fischer, 2015). Between 1998 and 2007, the spending per Canadian on opioid drugs more than doubled (Rehm, Fischer, & Gittins, 2009). Since 2010, overall levels of prescription opiate use have increased by nearly 70%, specifically in non-medical use (Fischer, Keates, Buhringer, Reimer, & Rehm, 2014). Numerous patients are being routinely prescribed relatively strong opioids for post-operative pain relief (Helmerhorst, Teunis, Janssen, & Ring, 2017). While most patients have the full intention of taking these medications for pain relief as directed by their physician, the possibility remains that they may transition to subsequent abuse and dependence on these substances (Helmerhorst et al., 2017).

In fact, recent reports indicate that just under 1% of the general population of adults in Canada has abused prescription opiates. More alarmingly, is that approximately 3% of students in grades 7 to 12 have reported abusing prescription pain relief drugs in the previous year as recreational drug use (Murphy et al., 2015). With this rise of prescription opiate abuse, especially among young individuals, there is an increased risk for individuals to transition to heroin use. Recently it has been revealed that the trajectory to heroin use among younger and older individuals follows very divergent paths. Young heroin users (ages 20-29) report transitioning from opioid pills to snorting and then injecting heroin, whereas older heroin users (age 30 and up) report transitioning to heroin from non-opioid drugs such as cannabis, methamphetamine, and cocaine (Mars, Bourgois, Karandinos, Montero, & Ciccarone, 2014).

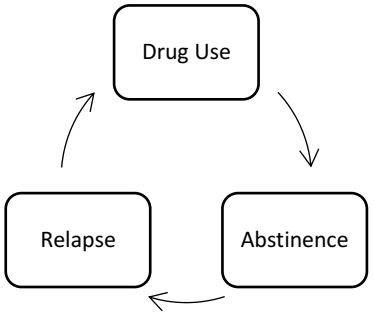

Figure 1.1 The cycle of addiction is characterized by drug use followed by periods of abstinence, and then relapse to resume drug use.

This increase in heroin use is a major concern for society. It is estimated that the economic burden placed on the Canadian population for substance abuse (excluding tobacco) is approximately $22.8 billion annually (Etches, 2013). In addition to this economic burden, there are marked health and social impacts, especially premature deaths. The extent of prescription opiate-related harms has also increased in Canada, as approximately 5000 – 10,000 Canadians have died prematurely as a result of prescription opiate-related overdose (Fischer et al., 2014). In 2016, there were almost 3000 apparent opioid-related deaths in Canada, and the latest data for the first half of 2017 has almost 1500 deaths with the number expected to increase (*Special Advisory Committee on the Epidemic of Opioid Overdoses. National report: Apparent opioid-related deaths in Canada*, 2017). Although the first use of a drug may be voluntary, continued use may lead to urges that become compulsive. Even when an individual can abstain from drug use, 40-60% will relapse within the first year (McLellan, Lewis, O'Brien, & Kleber, 2000). Therefore, addiction is considered a chronic relapsing disorder that is characterized by compulsive drug seeking and a loss of control over drug consumption (O'Brien & McLellan, 1996). The cycle of addiction (Figure 1.1) is a life-long battle with the vicious cycle of drug use, abstinence, and relapse. Relapse remains one of the major obstacles in the treatment of drug addiction (O'Brien, 1997).

In particular, heroin users exhibit a life characterized by repeated cycles of drug abuse and abstinence. At the end of a study that followed heroin users for over 3 decades, 40% of the subjects reported heroin use in the previous year (Hser, Hoffman, Grella, & Anglin, 2001). Moreover, 20% of the subjects were still regularly using heroin. Detoxification programs and prolonged periods of abstinence do not guarantee a safeguard against relapse. Even 15 years into drug abstinence, 25% of subjects in the study had relapsed (Hser et al., 2001). Relapse occurring after such extended periods of abstinence cannot be explained by physical withdrawal symptoms, since these are acute and dissipate 24-72 hours following drug cessation (Alper, Lotsof, Frenken, Luciano, & Bastiaans, 1999). Furthermore, death among individuals in the study was estimated to be 50-100 times higher than the general population of the same age (Hser et al., 2001). The causes of relapse and the barriers faced for an individual to remain abstinent from drug use is key knowledge that is necessary to help develop drug treatment programs.

Triggers to Relapse

There are three main factors that may trigger relapse to drug use, even when individuals have abstained for decades. First, re-exposure to the previously abused drug, also known as drug priming, will increase the subjective craving and desire for the drug, leading to relapse (de Wit, 1996). Second, a variety of stressors can increase drug craving and subsequent relapse (Sinha, 2001). For example, clinical populations of drug users report that stressful life events and situations increased drug craving (Hyman, Fox, Hong, Doebrick, & Sinha, 2007; Sinha, 2001). Guided imagery of stressful life events, tailored to an individual's personal experience, also increased subjective self-reports of drug craving (Sinha, 2009). Stressors can include both acute and chronic forms of stress (Brown, Vik, Patterson, Grant, & Schuckit, 1995; Preston & Epstein, 2011). Lastly, cues that have previously been paired with drug availability and drug consumption also increase drug craving and relapse (Childress et al., 1993). It is thought that these drug-associated cues trigger craving through Classical or Pavlovian conditioning (Stewart, 1983), and drug craving is the conditioned response following exposure to the cues. Both proximal cues, including drug paraphernalia, and distal cues, such as the environment related to drug taking, elicit arousal and craving (Childress et al., 1993).

Contrary to prior beliefs, the strength of these triggers leading to drug relapse does not fade with time. Instead evidence shows that drug craving actually increases over the abstinence period, a phenomenon termed the incubation of drug craving (Grimm, Hope, Wise, & Shaham, 2001). This incubation of drug craving in humans has been demonstrated for a variety of substances including cigarettes, alcohol, and methamphetamine (Li, Caprioli, & Marchant, 2015). Therefore, exposure to one of the three triggers to relapse at any point in the abstinence period is a risk that may trigger drug craving and subsequent relapse.

Dietary Manipulations and Drug Use in Humans

A period of abstinence from drug use is a vulnerable time where the risk for relapse increases, or incubates, and the individual may be more susceptible to the factors that trigger relapse. Dietary manipulations, such as restricted food intake, during the abstinence period can modulate the three main triggers to relapse. Furthermore, restricted food intake can affect both drug seeking and drug taking. For example, restricted food intake during times of war led to increased intake of coffee and tobacco products (Franklin, Shiele, Brozek, & Keys, 1948).

Malnourished Peruvian Indians also increase coca leaf chewing during times of food shortage (Hanna & Hornick, 1977). Furthermore, the severity of dieting in young women is positively associated with the prevalence of alcohol, cigarette, and marijuana use (Krahn, Kurth, Demitrack, & Drewnowski, 1992). Therefore, caloric restriction and diet should be an area of consideration in the development of treatment programs for drug addiction, since there is a high comorbidity rate between eating disorders and substance user disorders (Harrop & Marlatt, 2010; Holderness, Brooks-Gunn, & Warren, 1994).

In fact, empirical evidence indicates that food restriction increases the risk for relapse in abstinent smokers. Specifically, individuals that were assigned to a treatment group that had a combined abstinence program along with a nutrition plan to avoid weight gain following smoking cessation, reported higher levels of craving than the abstinence only treatment group (Hall, Tunstall, Vila, & Duffy, 1992). Furthermore, when individuals that were calorically restricted during abstinence did relapse, they smoked more cigarettes than the group on the abstinence only program did. These results were unanticipated as the authors thought that the additional intervention for potential weight gain would help rather than hinder the individual's progress for smoking cessation.

Unbalanced diet and insufficient nutrition is a widespread problem in many individuals abusing drugs. Active heroin users report eating infrequently and display a loss of interest in food (Neale, Nettleton, Pickering, & Fischer, 2012). However, it is reassuring that following treatment programs and with cessation of drug use, the individuals experience a return of their appetite. This increase in appetite usually results in weight gain, which in most individuals can trigger an increase in anxiety. Increased anxiety levels surrounding the issue of becoming overweight can lead to maladaptive behaviors to control appetite (Neale et al., 2012). The major concern here is that controlling appetite during abstinence via caloric restriction may have the unintended negative consequences of increasing drug craving and subsequent relapse. Collectively, these results bring to light that food restriction during the abstinence period may inadvertently hinder treatment by increasing drug craving and relapse, even though the intention is to maintain a healthy body weight.

Animal Models of Relapse

Accordingly, treatment programs for drug addiction need to take into consideration the nutrition of individuals in the program, as dieting and food restriction during drug abstinence

may increase drug craving and subsequent relapse. Studies are needed to investigate the mechanisms that mediate how dieting and food restriction affect drug craving. The problem is that clinical studies in humans are largely based on correlative data and self-reports on what triggers drug craving, making it difficult to infer causal relationships. Furthermore, although more controlled laboratory studies can uncover the causal links to what causes drug craving, craving on its own does not always lead to drug relapse. Ethical considerations preclude researchers from trying to trigger relapse in an abstinent user in order to study the mechanisms that triggered the relapse. Overall, ethical constraints limit the type of research that can be conducted on human subjects. Thus, animal models provide a useful approach to studying the underlying neurobiology of what triggers relapse to drug use. Animal models also allow for greater control of experimental parameters and approaches that are deemed unethical in human subjects (Shaham, Shalev, Lu, de Wit, & Stewart, 2003).

Reinstatement Procedure

One of the most commonly used animal models of relapse is the reinstatement procedure, as it has both face and predictive validity (Epstein, Preston, Stewart, & Shaham, 2006; Shalev, Grimm, & Shaham, 2002). Reinstatement is defined as the resumption of behavior that has been previously extinguished (Bouton & Swartzentruber, 1991). In the reinstatement of conditioned place preference (CPP) paradigm, animals are exposed to two different environments. One environment is repeatedly paired with drug injections, while another contextually distinct environment is not paired with the drug. Preference for the drug-paired environment develops through Pavlovian conditioning due to the drug's reinforcing effects. This preference is then extinguished with repeated pairings in the absence of the drug. Finally, a trigger to relapse such as drug re-exposure with a priming injection, reinstates the preference for the previously drug-paired environment (Mueller & Stewart, 2000). One drawback to this procedure is that animals receive passive exposure to the drug as it is administered by the experimenter. Evidence indicates that there are differences in behavior and tolerance to the drug when the drug is self-administered by the animal, versus when it is administered by the experimenter (Weise-Kelly & Siegel, 2001).

In contrast, the reinstatement procedure where the animal can self-administer the drug allows for greater ecological validity. In this procedure animals are first trained to self-administer a drug of abuse by performing an operant task, such as lever-pressing or nose-poking. Once the

animal establishes stable self-administration of the drug, the drug is removed, and the behavior is extinguished. Extinction is reached when the animal demonstrates a low level of responding and reaches a predetermined criterion. Next, a trigger is used to elicit renewed drug seeking under extinction conditions. The drug-seeking test is done under extinction conditions in order to avoid the psychomotor effects of the drug on the behavior. As in humans, the same three factors that trigger relapse – drug re-exposure, drug-associated cues, and stress – also reinstate drug seeking in animals (de Wit & Stewart, 1981; Meil & See, 1996; Shaham & Stewart, 1995).

Renewal Procedure

Although the reinstatement procedure is a well validated animal model of relapse, it is unable to dissociate the respective contribution of the discrete (proximal) cues from the contextual (distal) cues towards drug seeking. The renewal procedure builds on the reinstatement procedure but allows for the study of the mechanisms underlying relapse to drug seeking elicited by drug-associated contextual cues (Crombag & Shaham, 2002). The first phase of the renewal procedure, like in reinstatement procedure, is self-administration training for a substance, which is then followed by an extinction phase. However, in the renewal procedure animals are trained to self-administer a drug in a context with distinct contextual cues, referred to as context A. Extinction occurs under the same conditions as self-administration but with the removal of the drug, and this occurs in context B. Contexts A and B differ in their tactile, visual, and olfactory properties (Crombag & Shaham, 2002). On test day, the experimental group is brought back to context A for a drug-seeking test under extinction conditions, where they demonstrate a renewal of drug seeking. Animals in the control group are tested in the same context as extinction training, context B, and do not show an increase in drug seeking. This renewal of drug seeking is not due to the change in a context, as a novel context does not increase drug seeking (Crombag & Shaham, 2002). As a model for the human condition, this procedure is useful as the drug-taking environment differs from the context of detoxification and treatment programs. This procedure provides a useful way of investigating the role of contextual cues in drug seeking, and can potentially help in the development of treatment programs since returning to the original drug-taking context following a rehabilitation program may increase the risk for relapse in a recovering addict.

Punishment-induced Abstinence

One of the weaknesses of the reinstatement and renewal procedures is that they do not capture the negative consequences of drug use. The punishment-based model does directly assess the motivation to seek drugs when there are negative consequences to those actions (Marchant, Li, & Shaham, 2013). As in the other procedures, the first phase of this procedure is self-administration training for a drug of abuse. Next, the drug remains available to the animal with the addition of a negative consequence when drug is consumed, such as a mild electric foot-shock (Marchant et al., 2014). These negative consequences typically result in a rapid suppression of drug taking. Once animals have suppressed drug taking, the resumption of drug seeking is induced by a trigger, such as re-exposure to the drug-associated context. Once again, this procedure has the advantage of mimicking the human condition by incorporating the negative consequences of drug taking into the model (Marchant et al., 2013). Some of the issues with this paradigm are that there is a great deal of variability between animals on their foot-shock sensitivity. Foot-shock punishment can also not completely capture the psychological and social negative consequences of drug addiction in humans. Furthermore, the drug is still available, and we can therefore not eliminate the psychomotor effects of having the drug in the body (Marchant et al., 2013).

Forced-withdrawal and Voluntary Abstinence-based Procedures

Another criticism of the reinstatement procedure is that extinction training does not mimic the human condition of abstinence. Abstinence-based models were published in the late 1990s and try to address this weakness of the reinstatement model (Neisewander, O'Dell, Tran-Nguyen, Castañeda, & Fuchs, 1996; Tran-Nguyen et al., 1998). There are three main phases of abstinence-based procedures: self-administration training, abstinence, and testing. In the first phase animals are trained to self-administer a drug of abuse by lever-pressing or nose-poking for the delivery of a drug infusion that is paired with a discrete cue. Next, animals are housed in the animal facility for varying periods of time during the abstinence phase. Last, animals are brought back to the drug self-administration context for a drug-seeking test. Testing takes place under extinction conditions where responses on the previously drug-associated lever (or nose poke) result in a contingent presentation of the drug-associated discrete cue but no delivery of the drug. This increase in non-reinforced responding on the lever (or nose poke) is the operational measure

for the relapse to drug seeking. Although in the literature it is often referred to as the abstinence phase, or sometimes as "forced" abstinence, withdrawal is a more accurate term, as the animals do not voluntarily abstain from drug use, but rather have it imposed by the experimenter (Venniro, Caprioli, & Shaham, 2016).

An extension to this procedure is the incubation of drug craving (Grimm et al., 2001; Shalev, Morales, Hope, Yap, & Shaham, 2001). Here, animals are tested during the early days of withdrawal and following varying time periods (either in a repeated measures design or a between groups design where one group is tested early in withdrawal and a different group is tested later in the withdrawal period). As previously mentioned, incubation of drug craving is also demonstrated in human subjects with drug dependence problems (Bedi et al., 2011; Li et al., 2014; Wang, Shi, et al., 2013). One advantage of all procedures that incorporate a withdrawal period is that they have higher ecological validity and are closer to the human condition as compared to extinction training in the reinstatement procedure.

However, one criticism of withdrawal-based procedures is that although they are a better model for translation to the human condition, drug abstinence in humans is usually a voluntary behavior, and in these procedures, withdrawal is imposed on the animals by the experimenter. To address the issue of experimenter-imposed withdrawal, Caprioli and colleagues (2015) modified the procedure to try and make the withdrawal phase voluntary for the animals. This newly developed procedure is referred to as the voluntary abstinence incubation of craving (Caprioli et al., 2015). This procedure has 4 phases: self-administration training for a palatable food reward, followed by self-administration training for a drug, then voluntary abstinence, and finally a drug-seeking test. First, animals are trained to self-administer a palatable food pellet that is paired with a distinct discrete cue. Next, animals are trained to self-administer a drug of abuse that is paired with a discrete cue that is different from the palatable food-associated cue. Once stable self-administration is established, the voluntary abstinence phase begins where animals are given mutually exclusive choice sessions between the palatable food and the drug. Finally, animals are tested for cue-induced drug seeking in extinction tests during early and late abstinence, with the palatable food unavailable. Interestingly, animals almost exclusively choose the palatable food reward during choice sessions during voluntary abstinence. Nevertheless, animals show robust levels of drug seeking during the cue-induced test. The authors state that this is a strength of the procedure as it mimics the human condition, whereby when alternative non-drug rewards are

taken away it can increase the incidence of relapse in abstaining drug users (Caprioli et al., 2015). Based on all the aforementioned animal models of drug addiction and relapse, the incorporation of a withdrawal phase offers the advantage of ecological validity as it closely mimics the human condition.

Dietary Manipulations in Animal Models of Reward and Addiction

Dietary manipulations, specifically caloric or food restriction, can affect drug reward, drug intake, and drug seeking in humans as well as in animal models of addiction. Both acute food deprivation, defined as a brief period with absolutely no access to food, and chronic food restriction, defined as an extended period of time with limited access to food, increase drug self-administration to a variety of drugs (Carroll, France, & Meisch, 1979; Carroll & Meisch, 1981; Carroll & Meisch, 1984). In addition to effecting drug consumption, food restriction can also increase drug reward and drug seeking. Early studies on reward have demonstrated that animals will reliably self-stimulate for electrical brain stimulation in certain brain regions (Olds & Milner, 1954). One way to evaluate rewarding stimulation is through the determination of intracranial self-stimulation response rates against the resulting stimulation frequency (rate-frequency curve). An increase in the rewarding efficacy of the brain stimulation is reflected by leftward shifts on the rate-frequency curve. The threshold frequency that is necessary to maintain intracranial self-stimulation in the lateral hypothalamus is decreased by both drugs of abuse and chronic food restriction. However, the effects of food restriction on the threshold to maintain brain stimulation reward is complex, as it is dependent on the placement of the stimulating electrode within the lateral hypothalamus (Fulton, Woodside, & Shizgal, 2000). There is also an additive effect of chronic food restriction and drugs of abuse on brain stimulation reward. Chronic food restriction results in further reductions of the threshold frequency on top of the leftward shift induced by the administration of the drug alone (Abrahamsen, Bermanm, & Carr, 1995; Abrahamsen & Carr, 1996; Cabeza de Vaca & Carr, 1998; Carr, 2007; Fulton, Woodside, & Shizgal, 2000).

In more direct assessments of drug seeking, food deprivation and food restriction will increase drug reward and drug seeking. Using the CPP paradigm, both food deprivation and food restriction increased the conditioned reinforcing properties of both morphine and amphetamine (Gaiardi, Bartoletti, Bacchi, Gubellini, & Babbini, 1987; Jung et al., 2016; Stuber, Evans, Higgins, Pu, & Figlewicz, 2002). We have also demonstrated that acute food deprivation (24 –

48 h) reinstates extinguished drug-seeking (Shalev, Highfield, Yap, & Shaham, 2000; Tobin, Newman, Quinn, & Shalev, 2009).

Unlike animal models, acute food deprivation and chronic food restriction differentially affect drug seeking in humans. Although acute food deprivation increases both drug taking and drug seeking in animals (Carroll, France, & Meisch, 1979; Carroll & Meisch, 1981; Carroll & Meisch, 1984; Shalev, Highfield, Yap, & Shaham, 2000; Tobin, Newman, Quinn, & Shalev, 2009), prolonged food restriction, and not acute deprivation, is related to increased drug taking and seeking in humans (Cheskin, Hess, Henningfield, & Gorelick, 2005; Zacny & de Wit, 1992). Therefore, we focused on the effects of chronic food restriction on drug seeking in a withdrawal-based animal model of relapse. Although the voluntary abstinence-based procedure parallels the human condition the most, to study the effects of chronic food restriction on heroin seeking we used an extended forced-withdrawal period, as the voluntary abstinence-based procedure could introduce confounds. Since the alternative non-drug reward in the voluntary abstinence procedure is a palatable food reward pellet, it may differentially motivate the food restricted animals versus the free-fed animals. Using the forced withdrawal-based procedure we found that 14 days of chronic food restriction during the withdrawal phase significantly augments heroin seeking in rats with a history of heroin self-administration (D'Cunha, Sedki, Macri, Casola, & Shalev, 2013). However, it remains unclear what neural mechanisms underlie this chronic food restriction-induced augmentation of heroin seeking. There are a variety of factors that may be involved in this behavior, which will be outlined in the subsequent sections of this introduction.

The Role of Dopamine in Drug Seeking

One of the most studied and heavily implicated neurotransmitter systems in the neurobiology of reward and addiction is the mesocorticolimbic dopamine (DA) pathway (Di Chiara, Acquas, Tanda, & Cadoni, 1993; Kalivas & Duffy, 1995; Kelley & Berridge, 2002; Wise, 2009). The cell bodies in this pathway originate in the ventral tegmental area (VTA) and send projections to various areas in the brain including the prefrontal cortex (PFC), nucleus accumbens (NAc), and basolateral amygdala (BLA; Ungerstedt, 1971). Of particular interest is the NAc, as both natural rewards as well as drugs of abuse stimulate DA transmission in this region (Di Chiara et al., 1993). The NAc is subdivided into 2 subregions based on their innervations, projections, and function: the core, which surrounds the anterior commissure, and the shell, which encases the medial portion of the core (Ikemoto, 2007). While both the shell and

core receive projections from the VTA, the medial portion of the NAc shell receives the majority of its projections from the posteromedial VTA, whereas the lateral portion of the shell and the core receive the majority of their projections from the anteromedial VTA (Ikemoto, 2007). In turn, both the shell and the core send their projections to the ventral pallidum (VP), which is part of the basal ganglia and is important for motor control (Alexander & Crutcher, 1990). However, like its afferents, the subregions of the NAc also project to different regions of the VP. The shell projects primarily to the ventromedial part of the VP, whereas the core projects primarily to the dorsolateral part of the VP (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). Furthermore, the NAc shell but not the core, projects to the extended amygdala, and is thought to be a transition area between the striatal complex and the extended amygdala (Heimer et al., 1991). In addition, the NAc MSNs also send projections to non-dopaminergic neurons in the VTA, but it is unknown if the targets are GABAergic or glutamatergic neurons (Xia et al., 2011). Therefore, as the NAc receives information from the VTA and other inputs, it is positioned in an anatomical region where it may mediate and integrate sensory and limbic inputs about reward and thus affect behavioral output (Mogenson, Jones, & Yim, 1980).

Drugs of abuse all result in increased levels of DA in the NAc (Bozarth, 1986). However, due to pharmacological differences among drug classes, they increase DA by different mechanisms. Opiates, such as morphine and heroin, indirectly increase extracellular DA in the NAc by disinhibition of DA neurons in the VTA through the attenuation of GABAergic synapses on these cells (Johnson & North, 1992). On the other hand, psychostimulants, like cocaine, inhibit the actions of the DA transporter (DAT) allowing DA to remain in the synapse longer (Koob, 1992). There is also a differential response in DA transmission between the shell and the core. Specifically, extracellular DA is elevated more in the shell than in the core in response to intravenous administration of a variety of drugs, such as amphetamine, cocaine, or morphine (Pontieri, Tanda, & Di Chiara, 1995).

Once released, DA acts on two different classes of G-protein coupled receptors. The DA D1-like class of receptors include the D_1 and D_5 receptors which are coupled to the G_s alpha subunit and activate adenylyl cyclase, thereby mediating excitatory neurotransmission. The DA D2-like class of receptors include D_2, D_3, and D_4 receptors which are coupled to the G_i/G_o subunits and inhibit the actions of adenylyl cyclase, thereby mediating inhibitory neurotransmission (Sokoloff & Schwartz, 1995). Neurotransmission through DA receptors in the

NAc plays a pivotal role in the consumption of natural rewards and drugs of abuse with differential effects in the shell versus the core. For instance, both D1 receptor antagonist SCH23390 and D2 receptor antagonist eticlopride decrease food and cocaine self-administration when administered into the NA core. However, SCH23390 and eticlopride administered into the NAc shell selectively decreased cocaine self-administration and had no effect on food self-administration (Bari & Pierce, 2005).

DA transmission in the NAc is not only affected by the consumption of rewards but is also implicated in the motivation to seek reward, both for natural rewards and drugs of abuse. Cues that have been previously paired with the availability of a reward stimulate DA release (Bassareo, De Luca, & Di Chiara, 2007; Bassareo, Musio, & Di Chiara, 2011; Schultz, Dayan, & Montague, 1997). Specifically, cues previously associated with drugs of abuse elevate DA in the NAc shell but not the core. Whereas, cues previously associated with food availability elevate DA in the NAc core but not the shell (Bassareo et al., 2011). Furthermore, blocking DA receptors in the NAc will reduce seeking for natural rewards and drugs of abuse. For example, administration of the D1 receptor antagonist SCH23390 or the D2 antagonist raclopride into the NAc blocked cue-evoked reinstatement of food seeking (Guy, Choi, & Pratt, 2011). Similarly, administration of SCH23390 into the shell or core reduced cue-induced sucrose seeking 30 days into the withdrawal phase (Grimm et al., 2011). In the same manner, administration of SCH23390 into the NAc blocked the reinstatement of CPP for morphine induced by acute food deprivation (Sadeghzadeh, Babapour, & Haghparast, 2015). Additionally, SCH23390 administered into the core or shell also decreased context-induced reinstatement or renewal of alcohol seeking, and renewal of punished alcohol seeking (Chaudhri, Sahuque, & Janak, 2009; Marchant & Kaganovsky, 2015). Administration of the D2 receptor antagonist sulpride into the shell and not the core dose-dependently decreased cocaine-primed reinstatement (Anderson, Schmidt, & Pierce, 2006).

Evidence suggests that there is a specific role for D1-like and not D2-like receptors in opioid seeking induced by drug-associated cues and stress. Systemic administration of the D1 receptor antagonist SCH23390, and not the D2 receptor antagonist raclopride, attenuated acute food deprivation-induced reinstatement of heroin seeking (Tobin et al., 2009). Specifically, these results are mediated by D1 transmission in the NAc shell and not the core, as SCH23390 administration into the NAc shell, but not core, attenuated acute food deprivation-induced

reinstatement of heroin seeking (Tobin, Sedki, Abbas, & Shalev, 2013). There is also a critical role for D1 transmission in the NAc shell in morphine seeking following prolonged withdrawal, as morphine seeking is attenuated by administration of SCH23390 in the NAc shell but not the D2 receptor antagonist eticlopride (Gao, Li, Zhu, Brimijoin, & Sui, 2013). Furthermore, blocking D1 receptors in the shell (but not the core) attenuated context-induced reinstatement of heroin seeking, whereas blocking D1 receptors in the core (but not the shell) attenuated discrete cue-induced reinstatement of heroin seeking (Bossert, Poles, Wihbey, Koya, & Shaham, 2007). Taken together, this line of evidence suggests that DA transmission through the NAc shell and/or core, specifically through D1 receptors, may be involved in the augmentation of heroin seeking induced by chronic food restriction. Therefore, in Chapter 3 we examined extracellular concentrations of dopamine in the NAc to assess changes in DA during heroin seeking. Next, we assessed the effect of blocking D1 receptors in the NAc shell and core on the augmentation of heroin seeking induced by chronic food restriction during a prolonged period of withdrawal.

The Role of the Endocrine System in Reward-related Behavior

Leptin is a hormone that signals satiety and is produced by the *ob* gene and released by peripheral adipocytes into the blood (Ahima, Saper, Flier, & Elmquist, 2000; Friedman & Halaas, 1998). In times of starvation and limited food availability, adipose tissue is decreased thereby placing the animal in a state of negative energy balance and leading to lower levels of circulating leptin. This reduction in circulating leptin is detected by various nuclei in the hypothalamus, including the arcuate nucleus, ventromedial nucleus of the hypothalamus (VMH), lateral hypothalamus (LH), dorsomedial nucleus of the hypothalamus (DMH), and paraventricular nucleus of the hypothalamus (PVN). When these hypothalamic nuclei detect a reduction in leptin it results in a concerted hormonal, behavioral, and metabolic response to increase food intake and body weight. Conversely, when endogenous levels of leptin are increased in times of food availability, the outcome is a decrease in food intake, thereby reducing adipose tissue and subsequently body weight (Friedman & Halaas, 1998). It should be noted that chronically elevated levels of endogenous leptin (often seen in obese animals and humans) can result in leptin resistance, which blocks the outcome of reduced food intake and subsequent body weight loss. Similarly, exogenously administered leptin (intracerebroventricularly, i.c.v.) reduces food intake and subsequent body weight. Once the exogenous source of leptin is removed, food intake is increased to re-establish body weight (Halaas et al., 1997).

From an evolutionary perspective, it is therefore not surprising that leptin is directly involved in the rewarding aspects of food intake and energy balance. In fact, in addition to its hypothalamic targets, leptin receptor mRNA has also been found in the DA neurons of the mesolimbic pathway that originate in the VTA (Hommel et al., 2006). Functionally, leptin administration into the VTA decreased the firing rate of DA neurons and also decreased food intake (Hommel et al., 2006). Leptin also has direct effects on the motivation to work for a reward, which can be assessed using a progressive ratio schedule of reinforcement. In a progressive ratio schedule of reinforcement, the response requirement to receive a reward increases following each delivered reward. Using this procedure, acute i.c.v. administration of leptin decreased sucrose self-administration in rats that were maintained on an *ad libitum* diet (Figlewicz, Bennett, Naleid, Davis, & Grimm, 2006). Furthermore, it has been demonstrated that chronic replacement of leptin in food restricted rats can block the development of a sucrose conditioned place preference (Figlewicz, Higgins, Ng-Evans, & Havel, 2001). Collectively, these results suggest that leptin can modulate the neural circuitry underlying food reward and directly affect behavior.

Leptin plays a pivotal role in the rewarding aspects of food intake and energy balance, thus it can also affect the reinforcing properties of non-food rewards, such as electrical brain stimulation and drugs of abuse. As previously mentioned, both drugs of abuse and chronic food restriction lower the threshold frequency for electrical brain stimulation reward, which is interpreted as an increase in the rewarding properties of the stimulation. Intracerebroventricular infusions of leptin reversed the chronic food restriction-induced sensitization of brain stimulation reward, reflected by rightward shifts on the rate-frequency curve (Fulton et al., 2000). Furthermore, leptin can affect drug seeking as i.c.v. leptin attenuated heroin reinstatement induced by acute food deprivation (Shalev, Yap, & Shaham, 2001). Additionally, intra-VTA leptin blocked the ability of cocaine to establish a conditioned place preference and blocked cue-induced cocaine seeking in a self-administration paradigm (You et al., 2016). Thus, leptin has a direct effect on drug-seeking behaviors and may therefore play a role in the augmentation of heroin seeking induced by chronic food restriction.

Ghrelin, a peptide released from the gut, binds to the growth hormone secretagogue receptor (GHS-R1a) and has the opposite actions of leptin by acting as a hunger signal (Kojima et al., 1999). Plasma levels of ghrelin spike prior to a meal and then quickly fall following food

consumption (Toshinai et al., 2001). Ghrelin and its receptors can also affect the reinforcing properties of food. Administration of the GHS-R1a antagonist, JMV 2959, decreased body weight gain and consumption of a palatable chocolate drink, but surprisingly had no effect on regular chow intake (Egecioglu et al., 2010). Furthermore, systemic administration of JMV 2959 also decreased the breakpoint for the self-administration of sucrose pellets under a progressive ratio schedule of reinforcement (Skibicka, Hansson, Egecioglu, & Dickson, 2012).

Like leptin, ghrelin's actions also extend beyond the reinforcing and rewarding properties of food. Rats that lack the GHS receptors required four times the current intensity to acquire electrical intracranial self-stimulation in the LH as compared to their wild type counterparts (Wellman et al., 2012). Ghrelin itself induces a conditioned place preference and increased DA in the NAc (Jerlhag, 2008), which is likely because ghrelin receptors are located in the VTA (Guan et al., 1997). Administration of ghrelin directly into the VTA increased the activity of mesolimbic VTA DA neurons and also increased food intake in rats (Abizaid et al., 2006; Skibicka, Hansson, Alvarez-Crespo, Friberg, & Dickson, 2011). Moreover, intra-VTA administration of ghrelin increased the breakpoint for sucrose self-administration under a progressive ratio schedule of reinforcement, whereas, intra-VTA administration of JMV 2959 decreased the breakpoint for sucrose self-administration (Skibicka et al., 2011).

As expected, ghrelin and its receptors can also modulate the reinforcing properties of drug reward. Ghrelin receptor antagonism attenuated drug-induced locomotion, accumbal DA release, and conditioned place preference for a variety of drugs including alcohol, cocaine, amphetamine, and nicotine (Jerlhag, Egecioglu, Dickson, & Engel, 2010; Jerlhag et al., 2009; Jerlhag & Engel, 2011). Central administration of ghrelin also increased the breakpoint of heroin reinforcement under a progressive ratio schedule (Maric, Sedki, Ronfard, Chafetz, & Shalev, 2011). Furthermore, systemic administration of the ghrelin receptor antagonist JMV 2959 dose-dependently reduced morphine-induced DA release in the NAc (Sustkova-Fiserova, Jerabek, Havlickova, Kacer, & Krsiak, 2014), and inhibited the ability of morphine to induce a condition place preference (Jerabek et al., 2017).

Taken together, these findings demonstrate that the hormones leptin and ghrelin are involved in the reinforcing and rewarding properties of drugs of abuse. Therefore, they may modulate the augmentation of heroin seeking induced by chronic food restriction. Thus, in Chapter 4 we measured how plasma levels of leptin and ghrelin are altered following chronic

food restriction in heroin experienced rats. Next, we examined the role of central and intra-VTA administration of leptin in heroin seeking following food restriction. Finally, we examined the effect of intra-VTA administration of the ghrelin receptor antagonist JMV 2959 on heroin seeking following food restriction.

The Role of Glutamate in Drug Seeking

As previously mentioned, the mesolimbic DA pathway, especially the NAc, is heavily implicated in the neurobiology of reward-seeking and drug addiction (Di Chiara et al., 1993; Kalivas & Duffy, 1995; Kelley & Berridge, 2002; Wise, 2009). The NAc is referred to as the limbic-motor interface as it integrates internal and external sensory information coming from various inputs to regulate goal-directed behavior (Mogenson et al., 1980). The NAc is primarily composed of GABAergic medium spiny neurons (MSNs) which compose 90-95% of the total neuronal population, with the remaining 5-10% being GABAergic and cholinergic interneurons (Kawaguchi, Wilson, Augood, & Emson, 1995; Wilson & Groves, 1980). The NAc receive dense dopaminergic innervation from the VTA, as well as a multitude of glutamatergic afferents from the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), medial thalamic nuclei (mThal), and the ventral hippocampus (vHipp; Brog, Apongse, Deutch, & Zahm, 1993). It is hypothesized that these glutamatergic afferents to the NAc individually encode discrete elements of behavior that help shape reward-seeking behavior. In addition, pathway-specific disruptions are linked to various aspects of drug-seeking behavior.

The PFC is involved in executive function and plays a critical role in encoding extinction learning (Peters, Kalivas, & Quirk, 2009). The dorsal mPFC, known as the prelimbic cortex (PLC) sends dense glutamatergic projections to the NAc core, and has been demonstrated to play a role in cocaine-primed reinstatement (McFarland, Lapish, & Kalivas, 2003), heroin-primed and cue-induced heroin reinstatement (LaLumiere & Kalivas, 2008). Inactivation of the PLC-NAc core pathway via GABA agonists reduced both drug-primed and cue-induced reinstatement of heroin seeking, indicating that this projection is critical for initiating drug seeking (LaLumiere & Kalivas, 2008). Furthermore, blocking DA signaling in the PLC and glutamate signaling in the contralateral NAc core attenuated cue-induced cocaine seeking (McGlinchey, James, Mahler, Pantazis, & Aston-Jones, 2016). Thus, it has been suggested that this projection from the PLC to the NAc core is the final common pathway in the relapse circuit for both cocaine and heroin seeking (Scofield et al., 2016). Conversely, the ventral mPFC, or infralimbic cortex (ILC) to the

NAc shell is thought to inhibit drug seeking. Inactivation of this projection via GABA agonists reinstated extinguished cocaine seeking (Peters, LaLumiere, & Kalivas, 2008). In contrast, administration of GABA agonists (muscimol and baclofen) into the ILC and a DA D1 receptor antagonist (SCH 23390) into either the contralateral or ipsilateral NAc shell decreased context-induced heroin reinstatement (Bossert et al., 2012).

The BLA is suggested to encode the valence of reward-associated stimuli and convey this information to the NAc (Ambroggi, Ishikawa, Fields, & Nicola, 2008; Everitt, Cardinal, Parkinson, & Robbins, 2003). Both BLA and NAc neurons respond to reward-associated cues, however, the excitations in the BLA occur prior to the NAc (Ambroggi et al., 2008). Specifically, it is thought that the integration of glutamatergic signals from the BLA in conjunction with DA signaling from the VTA in the NAc promotes motivated behavioral responding (Ambroggi et al., 2008; Stuber et al., 2011). This projection is also implicated in addiction and drug seeking, as optogenetic inhibition of this pathway inhibited cue-induced reinstatement of cocaine seeking (Stefanik & Kalivas, 2013).

It is well established that the hippocampus is involved in memory (Squire, 1992) and the encoding of spatial information (O'Keefe & Dostrovsky, 1971). Unlike glutamatergic afferents from other regions, the vHipp – specifically the ventral subiculum (vSub) – uniquely projects to the medial NAc shell (Brog et al., 1993). This projection from the vHipp to the NAc shell is selectively potentiated 2 weeks after repeated non-contingent administration of cocaine, as measured by increased AMPA/NMDA ratios (Britt et al., 2012). Moreover, recent evidence has demonstrated that the pathway from the vSub to the NAc shell is associated with drug seeking in the renewal procedure. For example, inactivation of the vSub via administration of GABA agonists (muscimol and baclofen) decreased context-induced renewal of heroin seeking (Bossert & Stern, 2014). Additionally, inactivation of the vSub with GABA agonists and administration of a D1R antagonist in the contralateral or ipsilateral NAc shell also decreased context-induced renewal of heroin seeking (Bossert & Stern, 2014).

Lastly, the final major glutamatergic projection to the NAc is from the mThal, specifically the paraventricular nucleus of the thalamus (PVT; Brog et al., 1993). Less is known about the role of this pathway, but these projections do converge on the same targets as dopaminergic projections from the VTA, suggesting that DA may modulate glutamate transmission in this region (Pinto, Jankowski, & Sesack, 2003). Recent evidence suggests that

this pathway also plays a pivotal role in drug seeking. Morphine withdrawal induced neuronal activation in PVT neurons projecting to the NAc, indicating this projection may be implicated in opiate dependence (Zhu, Wienecke, Nachtrab, & Chen, 2016). Reducing the activity of this pathway via chemogenetic inhibition blocked cocaine-primed reinstatement but enhanced cue-induced cocaine reinstatement (Wunsch et al., 2017). More evidence is needed to clarify the precise role this projection plays in mediating drug seeking, but since the dopaminergic projections coincide with the PVT's glutamatergic afferents to the NAc it is highly likely that this pathway plays a role in addiction and drug seeking.

Glutamate plays a pivotal role in mediating synaptic plasticity, which is required for animals to learn and adapt to a constantly changing environment. Drug addiction can be perceived as a maladaptive learning process, and drugs of abuse can have a lasting impact on synaptic plasticity (Kalivas, 2009). Specifically, drugs of abuse modulate glutamate transmission, which can have a direct impact on synaptic plasticity and result in reinforcing drug-seeking behaviors that are maladaptive and to the detriment of the animal (Kalivas & O'Brien, 2008). Kalivas (2009) has proposed a glutamate homeostasis hypothesis of addiction based on the effects of cocaine on striatal synaptic physiology. For example, cocaine administration increased extracellular glutamate levels in the NAc following cocaine behavioral sensitization (Pierce, Bell, Duffy, & Kalivas, 1996). As previously mentioned, there can be differences in the neuroadaptations that occur following passive drug administration versus drug self-administration (Weise-Kelly & Siegel, 2001). Interestingly, short durations of cocaine self-administration had no effect on extracellular glutamate in the NAc core, but basal glutamate levels were reduced following chronic cocaine self-administration. Once chronic cocaine self-administration has been established, extracellular glutamate levels within the NAc core rose to over 200% of basal levels during ongoing cocaine self-administration (Miguens et al., 2008). Similar findings are present in both the NAc core and shell, however, rats that were trained to self-administer saline and then given yoked-cocaine administration did not show these changes (Suto, Ecke, You, & Wise, 2010).

Glutamate neurotransmission in the NAc is not only affected during ongoing drug self-administration but also during drug seeking. Extracellular glutamate levels in the NAc are elevated during both cocaine-primed reinstatement (McFarland et al., 2003), and cue-induced heroin reinstatement (LaLumiere & Kalivas, 2008). Moreover, an odor previously paired with

cocaine availability also increased extracellular glutamate levels in both the NAc core and shell (Suto, Elmer, Wang, You, & Wise, 2013). These changes in extracellular glutamate levels may be due to higher levels of presynaptic release or changes in glutamate uptake by glial glutamate transporters in the synaptic cleft. Indeed, chronic exposure to various drugs of abuse such as cocaine (Reissner et al., 2015) and heroin (Shen, Scofield, Boger, Hensley, & Kalivas, 2014a) reduced the expression of the glutamate transporter (GLT-1). As chronic drug exposure downregulates GLT-1, there is a disruption in the equilibrium between extrasynaptic and synaptic glutamate levels which promotes spillover of synaptic glutamate out of the synaptic cleft and into the extracellular space in the NAc. This spillover of glutamate can result in glutamate activating postsynaptic glutamate receptors resulting in synaptic potentiation, which is associated with drug seeking and relapse (Kalivas, 2009; Scofield et al., 2016).

Once in the synapse, glutamate can act on 2 different classes of receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic GluRs are synaptic ligand-gated ion channels that mediate fast excitatory neurotransmission and include 3 receptor subtypes: α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA), N-methyl-D-aspartate (NMDA), and Kainate receptors. On the other hand, mGluRs are G-protein coupled receptors that mediate slower synaptic processes. They are composed of 8 receptor subtypes categorized into 3 groups: Group I consist of mGluR1 and mGluR5, which are primarily postsynaptic; Group II includes mGluR2 and mGluR3; and Group III includes mGluR4 and mGluR6 – mGluR8. Group II and III mGluRs are all largely presynaptic inhibitory autoreceptors on glutamatergic terminals, whose activation reduce vesicular release (Conn & Pin, 1997; Gass & Olive, 2008; Guo, Wang, Xiang, & Zhao, 2009; Niswender & Conn, 2010).

Drugs of abuse have a long-lasting impact on glutamate receptors. Numerous studies have focused on the role of iGluRs and particularly AMPA receptors (AMPAR) in drug reward and addiction. During basal conditions, MSNs are extremely hyperpolarized and have low spontaneous activity. Thus, the activity of these MSNs relies heavily on excitatory glutamatergic inputs from cortical and limbic regions (O'Donnell & Grace, 1993), which is mediated primarily by AMPARs (Wolf, 2010; Wolf & Ferrario, 2010).

AMPARs are assembled as tetramers, from the subunits GluA1, GluA2, GluA3, and GluA4. When AMPARs are composed of subunits with a short C-terminus – GluA2 or GluA3 – they are constitutively cycled into and out of synapses. Alternatively, AMPARs composed of

subunits with a long C-terminus – GluA1 – are inserted into synapses in an activity-dependent manner, and are regulated by a series of homeostatic mechanisms. There are many homeostatic mechanisms that regulate and maintain AMPARs in the synapse. Synaptic scaling is a form of homeostatic plasticity in which prolonged blockade of activity leads to increased excitatory synaptic transmission, whereas prolonged increases in activity produces the opposite effect (Turrigiano, 2008; Turrigiano & Nelson, 2004). The major postsynaptic mechanism of synaptic scaling involves alterations in AMPAR levels, such that synaptic AMPAR levels are increased in response to activity blockade, and are decreased after prolonged increases in activity (Sun & Wolf, 2009). NAc MSNs demonstrate bidirectional synaptic scaling as both long-term potentiation (LTP; an increase in synaptic strength) and long-term depression (LTD; a reduction in synaptic strength; Wolf, 2010).

As previously mentioned, both non-contingent (Pierce et al., 1996) and contingent cocaine self-administration (Miguens et al., 2008; Suto et al., 2010) increased extracellular glutamate levels in the NAc. This phenomenon is only seen during ongoing cocaine exposure. In contrast, basal levels of extracellular glutamate are decreased after both non-contingent and contingent cocaine, due to the reduced activity of the cysteine-glutamate antiporter, which is the main regulator of extracellular glutamatergic tone (Kalivas, 2009). This reduction in basal glutamate levels in the NAc may result in increased expression of synaptic AMPAR through the mechanism of synaptic scaling. Accordingly, both non-contingent administration of cocaine in behavioral sensitization models (Ferrario et al., 2010), and contingent administration of cocaine in the incubation of cocaine craving, resulted in increased AMPAR in the NAc (Conrad et al., 2008). This increase in AMPAR in the NAc does not occur during cocaine exposure but rather requires a prolonged withdrawal period to develop (Wolf, 2010). Thus, this increase in surface AMPAR is not found in cocaine-experienced rats when measured on the 1st day of withdrawal, but only develops and is evident in the NAc during a prolonged withdrawal period. This pattern of changes in AMPAR parallels behavior (i.e. drug seeking), as drug seeking incubates over the withdrawal period and higher levels are observed following weeks of withdrawal, not withdrawal day 1 (Conrad et al., 2008).

Specifically, the increase in surface AMPAR in the NAc is increased in levels of GluA2-lacking AMPARs associated with the incubation of cue-induced cocaine seeking (Conrad et al., 2008). AMPARs lacking the GluA2 subunit have a higher permeability to Ca^{2+}, which results in

higher conductance. As a result, a small fluctuation in the number of these receptors along a synapse can have robust changes in synaptic strength and overall firing activity (Wolf & Ferrario, 2010). In line with these findings, blocking AMPARs in the NAc will consistently block drug seeking (Cornish & Kalivas, 2000; Di Ciano & Everitt, 2001; LaLumiere & Kalivas, 2008; Suto et al., 2004). For instance, administration of the AMPAR antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) in the NAc attenuated cocaine-primed reinstatement of drug seeking (Cornish & Kalivas, 2000). Similarly, CNQX administered into the NAc core attenuated both heroin-primed and cue-induced reinstatement of heroin seeking (LaLumiere & Kalivas, 2008).

One of the major problems with extrapolating these findings is that the glutamate homeostasis hypothesis of drug addiction has been formulated from experiments and conclusions drawn using psychostimulants, particularly cocaine. There are likely limitations to generalizing these findings to opiate addiction as there are both behavioral and neurobiological distinctions between psychostimulant and opiate addiction (Badiani, Belin, Epstein, Calu, & Shaham, 2011). Therefore, more research is required to establish the role of glutamate transmission in the NAc on opioid addiction. Furthermore, recent evidence indicates that chronic food restriction can also alter AMPARs in the NAc. Specifically, chronic food restriction increased levels of the GluA1 subunit of AMPARs in the NAc (Peng, Cabeza de Vaca, Ziff, & Carr, 2014; Peng, Ziff, & Carr, 2011). Therefore, glutamate transmission and AMPAR in the NAc may be involved in the augmentation of heroin seeking following chronic food restriction.

In order to explore the role of glutamate in heroin seeking, we first measured changes in extracellular glutamate in the NAc shell and core during ongoing heroin seeking. Next, since both chronic food restriction and prolonged drug withdrawal can modulate AMPARs in the NAc, we examined how blocking AMPARs in the NAc core and shell would affect the augmentation of heroin seeking induced by chronic food restriction.

Rationale for Current Studies

The underlying neural mechanisms mediating the augmentation of heroin seeking induced by chronic food restriction remain undetermined. Therefore, this thesis explored three potential systems that may be involved in this behavior. The goal of the first set of experiments in Chapter 3 was to study the role of DA and DA D1-like receptors in the NAc in the augmentation of heroin seeking following chronic food restriction. Our hypothesis was that

chronic food restriction would increase extracellular levels of DA in the NAc during heroin seeking. Furthermore, blocking DA D1-like receptors in the NAc would attenuate heroin seeking and block the augmentation of heroin seeking induced by chronic food restriction. To test our hypotheses, we first assessed extracellular DA concentrations in the NAc shell and core during baseline conditions and then during the heroin-seeking test in freely behaving rats. In a separate set of experiments, we administered the D1-like receptor antagonist SCH 39166 into either the NAc shell or core immediately prior to the heroin-seeking test.

In Chapter 4, the next set of experiments explored the role of the endocrine system in the augmentation of heroin seeking induced by chronic food restriction. Particularly, we were interested in the roles of two key hormones involved in food intake and energy balance: leptin, a key satiety signal, and ghrelin, a key hunger signal. Our hypotheses were that chronic food restriction would alter levels of leptin and ghrelin which would then affect heroin seeking. Specifically, if we stimulate the leptin system or block the ghrelin system we will block the augmentation of heroin seeking induced by chronic food restriction. To test this hypothesis, we first measured circulating levels of plasma leptin and ghrelin in heroin-experienced, sated and food restricted rats. Next, in separate experiments we administered either leptin or the ghrelin receptor antagonist JMV 2959, to evaluate their effects on the augmentation of heroin seeking induced by chronic food restriction.

Finally, in the last set of experiments in Chapter 5 we investigated the role of glutamate and the glutamate AMPA receptors in the augmentation of heroin seeking induced by chronic food restriction. Our hypothesis was that chronic food restriction would: (1) increase extracellular levels of glutamate in the NAc during heroin seeking; and (2) alter glutamate transmission in the NAc by modulating AMPA receptors. To test these hypotheses, we first measured extracellular concentrations of glutamate in the NAc shell and core during baseline conditions and then during the heroin-seeking test. In a separate set of experiments, we administered the AMPA receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) into either the NAc shell or core immediately prior to the heroin-seeking test. Together, these studies will provide insight on the neural mechanisms underlying the augmentation of heroin seeking induced by chronic food restriction.

CHAPTER 2: GENERAL METHODOLOGY

Subjects

A total of 562 (Chapter 3: n = 196, Chapter 4: n = 206, Chapter 5: n = 160) male Long Evans rats (Charles River, St. Constant, Quebec, Canada, or Raleigh, North Carolina, USA) were used in the various experiments. Rats were pair-housed in clear shoebox cages and allowed 1 week to acclimate to the animal colony prior to undergoing any surgical procedures. Rats were kept on a reverse 12 h light-dark cycle (lights OFF 9:30am) at 21˚C. Following recovery from surgery (described below) rats were individually housed in operant conditioning chambers with *ad libitum* access to food and water during heroin self-administration training.

Following heroin self-administration training, rats were returned to the animal colony for the withdrawal and food restriction phase (described below). Rats in the *in vivo* microdialysis experiments were housed in clear Plexiglass chambers with a grid floor for baseline sampling prior to the heroin-seeking test (see Chapters 3 & 5 for more details). Following baseline microdialysis collection, rats were transferred back to the operant conditioning chambers for the heroin-seeking test. Rats in the intracranial injection experiments were brought back to the operant conditioning chambers on the last day of the withdrawal phase for the heroin-seeking test. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and approval for all procedures was granted by the Concordia University Animal Research Ethics Committee.

Surgical Procedures

Rats in all experiments underwent intravenous catheterization to allow for heroin self-administration. Catheters were constructed out of silastic tubing (Inner Diameter: 0.51 mm, Outer Diameter: 0.94 mm; Dow Corning, Midland, MI, USA) that were cut to 12 cm. Intravenous catheterization was completed under ketamine and xylazine (90 and 13 mg/kg, ip). Once rats were fully anesthetized, 2 incisions were made: one on the skull and one on the neck. The jugular vein was then isolated, and a small incision was made. Approximately 3 cm of one end of the catheter was then inserted into this incision on the jugular vein and secured in place with 3 silk sutures. This was then followed by threading the other end of the catheter subcutaneously to the skull where it was attached to a modified 22-gauge cannula (Plastics One, Roanoke, VA, USA).

During the intravenous catheterization, rats also received intracranial cannulae to allow for microinjections. Rats in the *in vivo* microdialysis experiments received a unilateral cannula (counterbalanced between the right and left hemispheres) aimed at either the NAc shell or core to allow for *in vivo* microdialysis (see Chapters 3 & 5 for specific details). Rats in the intracranial injection studies received either a unilateral guide cannula aimed at the lateral ventricles or bilateral guide cannulae aimed at the NAc shell, NAc core, or VTA (for specific details refer to Chapters 3 – 5). Guide cannulae were mounted adjacent to the modified catheter cannula on the skull using jeweller's screws and dental cement. Following surgery rats were given penicillin (450,000 IU/rat, sc) and the analgesic ketoprofen (5 mg/kg, sc, CDMV, Quebec, Canada). Throughout self-administration, rats were flushed daily with heparin and gentamicin in sterile saline (7.5IU + 12.0 μg per day per rat) to prevent catheter blockage.

Apparatus

Operant conditioning chambers (Med Associates Inc., St. Albans, VT, USA, 32.0 cm × 24.0 cm × 25.0 cm; or Coulbourn Instruments, Holliston, MA, 29.0 cm × 29.0 cm × 25.5 cm) were used for heroin self-administration and the heroin-seeking tests. Operant conditioning chambers were located within individual sound attenuating boxes, and each chamber contained two levers located 5.0 cm (Med Associates) or 11.0 cm (Coulbourn Instruments) above the grid floor. One lever was designated as the "active" drug-paired lever and the other as the "inactive" non-drug paired lever (counterbalanced left and right designations between rats). The chamber also contained a cue-light and an audio indicator (Sonalert, 2.9 KHz, 10-20 dB above background level) located above the active lever, and a red house-light positioned on the top of the wall opposite the levers. During the heroin self-administration rats were attached to a liquid swivel (Instech Laboratories, Plymouth Meeting, PA, USA or Lomir Biomedical Inc., Quebec, Canada) which was connected to an infusion pump (Med Associates Inc.) via Tygon tubing (Inner Diameter: 0.50 mm, Outer Diameter: 1.52 mm; Saint-Gobain Performance Plastics, Granville, NY, USA) which was shielded by a metal spring. In the *in vivo* microdialysis experiments (Chapter 3 & 5) baseline microdialysis sampling took place in a clear Plexiglass chamber (30.0 cm × 28.0 cm × 25.0 cm) with a metal grid floor located in the animal care facility.

Drugs

For drug self-administration heroin (diacetylmorphine HCl; National Institute on Drug Abuse, Baltimore, Maryland, USA) was dissolved in sterile saline and delivered in a concentration of 0.10 mg/kg/infusion. All other compounds used for intracranial injections are detailed in their respective chapter methodology.

General Procedure

The experiments presented in the following chapters all followed the same general procedure (specific details are described in each chapter). There were three main phases in all experiments: heroin self-administration training, a withdrawal phase, and a heroin-seeking test phase.

Self-Administration Training

Following recovery from the surgical procedures, rats were habituated to the operant conditioning chambers for 24 h prior to 10 days of heroin self-administration training. During self-administration, rats underwent daily three-3 h sessions separated by a 3 h period under a fixed interval (FI) 20 s schedule of reinforcement. The first self-administration session of the day began with the onset of the dark phase and was marked by the extension of the levers, the illumination of a red house-light and the activation of a white-light/tone complex cue above the active lever. The white-light/tone complex remained on for 30 s or until the rat pressed the active lever. Responses on the inactive, non-drug paired, lever had no programmed consequences. Responses on the active, drug-paired lever, resulted in the activation of the infusion pump (5 s for the Coulbourn Chambers or 12 s for the Med Associates Chambers; 0.13 ml/infusion) and the initiation of a 20-s timeout period during which responses on the active lever were recorded but not reinforced. At the end of the 3 h self-administration session the active lever retracted, and the house-light turned off. To aid in lever discrimination, the inactive lever remained extended in the operant chamber.

Withdrawal Phase

At the end of the heroin self-administration training, rats were transferred back to the animal care facility and housed individually in clear shoebox cages for a 24 h drug washout period. Rats were matched on the number of infusions, active lever responses, and body weight

during the last 5 days of training and divided into a food restricted (FDR) group or left free fed in the sated group. Rats were weighed daily. The FDR rats received approximately 15 g of standard rat chow daily at 1:30 pm, and the food ration was adjusted daily in order to bring their body weight down to approximately 90% of their original body weight at the start of the withdrawal phase.

Heroin-Seeking Test

In the *in vivo* microdialysis experiments in Chapters 3 & 5, rats had a microdialysis probe lowered into the targeted brain region the day preceding the heroin-seeking test. Baseline dialysate samples were collected in the animal care facility for approximately one hour before rats were moved to the operant conditioning chambers for the heroin-seeking test. In the other experiments from Chapters 3 – 5 rats received intracranial injections in the animal care facility approximately 10 – 30 minutes (dependent on the compound that was injected, see specific experiment for further details) prior to being brought back to the operant conditioning chambers for the heroin-seeking test.

For the heroin-seeking test, rats were brought back to the operant chambers on the 14th day of food restriction. All FDR rats had empty food hoppers in their operant chambers. The heroin-seeking test took place under extinction conditions over a 3 h session. Responses on the active lever resulted in the same consequences as self-administration training (presentation of the white-light/tone complex for 20 s, during which the house-light was turned off) with the exception that no heroin infusions were administered. As during self-administration training, responses on the inactive lever had no programmed consequences.

Histology

At the end of the experiment rats were euthanized with carbon dioxide gas and decapitated. Brains were fixed with 4% paraformaldehyde solution for one week before being sliced on a cryostat in 40 μm coronal sections. Sections were then mounted onto slides and stained with cresyl violet. Cannula and microdialysis probe locations were determined under a light microscope with reference to a brain atlas (Paxinos & Watson, 2005).

CHAPTER 3: AUGMENTATION OF HEROIN-SEEKING FOLLOWING CHRONIC FOOD RESTRICTION IN THE RAT: DIFFERENTIAL ROLE FOR DOPAMINE TRANSMISSION IN THE NUCLEUS ACCUMBENS SHELL AND CORE

ABSTRACT

Caloric restriction during drug abstinence increases the risk for relapse in addicts. In rats, chronic food restriction during a period of withdrawal following heroin self-administration augments heroin seeking. The mechanisms underlying this effect are largely unknown. Here, we investigated the role of nucleus accumbens (NAc) shell and core dopamine (DA) in food restriction-induced augmentation of heroin seeking. Rats were trained to self-administer heroin (0.1 mg/kg/infusion) for 10 days. Next, rats were moved to the animal colony for a withdrawal period, during which rats were food restricted to 90% of their original body weight (FDR group) or given unrestricted access to food (sated group). On day 14 of food restriction, rats were returned to the operant conditioning chambers for a heroin-seeking test under extinction conditions. Extracellular DA levels were assessed using in vivo microdialysis. In separate experiments, the DA D1-like receptor antagonist SCH39166 (12.5, 25.0, or 50.0 ng/side) was administered into the NAc before the heroin-seeking test. In the NAc shell, pre-test exposure to the heroin-associated context increased DA only in FDR rats; but in the NAc core, DA increased regardless of feeding condition. Food restriction significantly augmented heroin seeking and increased DA in the NAc shell and core during the test. Intra-NAc shell administration of SCH39166 decreased heroin seeking in all rats. In contrast, in the NAc core, SCH39166 selectively decreased the augmentation of heroin-seeking induced by chronic food restriction. Taken together, these results suggest that activation of the DA D1-like receptor in the NAc core is important for food restriction-induced augmentation of heroin seeking.

INTRODUCTION

In humans, a reciprocal association exists between the abuse of drugs like tobacco, cocaine and heroin, and food intake. Drug use decreases food intake, and caloric restriction can increase drug consumption, craving and relapse. For example, the level of dietary restriction has been positively correlated with the use of alcohol, cigarettes, and marijuana in young women (Krahn et al., 1992). Furthermore, the risk for relapse in abstinent smokers is higher in subjects that are concurrently calorie restricted (Hall et al., 1992). Prolonged food restriction seems to be a critical factor, since shorter food restriction period (24-72 h) did not change cigarette smoking (Cheskin et al., 2005) or responses to intravenous fentanyl administration (Zacny & de Wit, 1992). In animal models of addiction and relapse, caloric restriction reliably augments drug taking and seeking. Both acute food deprivation (complete removal) and chronic food restriction (prolonged, restricted availability) increase self-administration of a variety of substances, including opiate and psychostimulant drugs (Carroll & Meisch, 1981; Carroll, Stotz, Kliner, & Meisch, 1984), and the conditioned rewarding properties of morphine in the conditioned place preference paradigm (CPP; Gaiardi et al., 1987; Jung et al., 2016). Recently, we found that chronic (14 days) food restriction augments heroin seeking in rats under prolonged withdrawal (D'Cunha et al., 2013).

The neural mechanisms underlying the augmentation of heroin seeking induced by chronic food restriction remain unknown. However, drug- and food-associated cues have been found to elicit significant elevations in extracellular dopamine (DA) levels in the nucleus accumbens (NAc) shell and core (Bassareo et al., 2011). In addition, chronic food restriction increases DA receptor signalling in the NAc (Carr, Tsimberg, Berman, & Yamamoto, 2003).

Here, we investigated the role of DA transmission in the NAc in food restriction-induced augmentation of heroin seeking. We used *in vivo* microdialysis to determine changes in extracellular DA in the NAc shell and core. Previous studies suggest that DA D1-like receptors are important for context- and discriminative cue-induced reinstatement of heroin, cocaine, and natural rewards seeking (Marchant & Kaganovsky, 2015). Moreover, we have recently reported that blockade of DA D1-, but not D2-like receptors, attenuated acute food deprivation-induced reinstatement of heroin seeking (Tobin et al., 2013). In addition, chronic food restriction increases synaptic plasticity in NAc cells expressing DA D1-like, but not D2-like, receptors

(Carr et al., 2010; Carr et al., 2003). Consequently, here we administered a DA D1-like receptor antagonist into the NAc shell and core to determine if DA transmission via the D1-like receptors is causally related to the augmentation of heroin seeking induced by chronic food restriction.

MATERIALS AND METHODS

Subjects

Male Long Evans rats (Charles River, St. Constant, Quebec, Canada; n = 196) were used in six different experiments. Rats were pair-housed until surgery, and then individually housed after surgery (see Supplementary Online Material for further details on housing).

All rats were treated in accordance with the guidelines of the Canadian Council on Animal Care and approval for all procedures was granted by the Concordia University Animal Research Ethics Committee.

Surgical procedures

Intravenous catheterization was completed under ketamine and xylazine (90.0 and 13.0 mg/kg, ip) as previously described (Sedki, D'Cunha, & Shalev, 2013). Following surgery rats were given penicillin (450,000 IU/rat, sc) and the analgesic ketoprofen (5 mg/kg, sc, CDMV, Quebec, Canada). For rats in the microdialysis experiments, unilateral guide cannulae, counterbalanced between the right and left hemispheres, were implanted targeting the NAc shell (Experiment 1) or NAc core (Experiment 2) during the intravenous surgical procedure (Ito, Dalley, Howes, Robbins, & Everitt, 2000). For DA receptor antagonist administration, bilateral guide cannulae targeting the NAc shell (Experiment 3) or NAc core (Experiment 4) were implanted during the intravenous surgical procedure (Tobin et al., 2013). See also Supplementary Online Material.

Apparatus

Operant conditioning chambers (Med Associates Inc., St. Albans, VT; or Coulbourn Instruments, Holliston, MA) equipped with two levers were used. See also Supplementary Online Material.

Drugs

Heroin (diacetylmorphine HCl; National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in physiological saline and delivered at a dose of 0.1 mg/kg/infusion. The DA D1-like receptor antagonist SCH39166 (Tocris Bioscience, Minneapolis, MN, USA) was diluted in sterile saline to produce the following doses: 0.0, 12.5, 25.0, 50.0 ng/side. These doses were previously shown to impair the acquisition of morphine conditioned place preference with minimal motor side-effects (Fenu, Spina, Rivas, Longoni, & Di Chiara, 2006).

Procedure

Different rats were used for each of the experiments, which followed a similar procedure. There were three phases: heroin self-administration training in the operant conditioning chambers, a withdrawal phase in the animal care facility (ACF), and a testing phase in the operant conditioning chambers, or in clear Plexiglas chambers (context control experiments).

Training

Following two post-surgery recovery days, rats underwent daily three 3-h sessions separated by a 3 h period, under a fixed-interval-20 (FI-20) schedule of heroin reinforcement (0.1 mg/kg/infusion), as previously described (D'Cunha et al., 2013), over 10 days. Active lever responses resulted in a heroin infusion and initiation of a 20-s timeout period during which a tone-light compound cue was activated. Active lever responses made during the timeout were recorded but not reinforced. Inactive lever responses were recorded but had no programmed consequences. See also Supplementary Online Material.

Withdrawal and Food Restriction

Following the heroin self-administration phase, rats were transferred back to the ACF and housed in individual cages. After a 24 h drug washout period, rats were matched for number of infusions, active lever responses, and body weight during the last 5 days of training and assigned to a food restricted (FDR) or sated group. FDR rats were fed daily at 1:30 pm and the food ration was adjusted daily to bring the body weight of the FDR group to approximately 90% of their first withdrawal day body weight.

In vivo microdialysis and heroin-seeking tests

Approximately 14 h prior to the heroin-seeking test, microdialysis probes (Bioanalytical Systems Inc., West Lafayette, IN, USA, or made in the laboratory; Sorge, Rajabi, & Stewart, 2005) were lowered into the targeted brain region, under light isoflurane anaesthesia. Probes targeted at the NAc shell (Experiment 1) had a 2.0 mm semipermeable active membrane (280 μm OD), whereas probes targeted at the NAc core (Experiment 2) had a 1.5 mm active membrane. Baseline collection started in the ACF, and dialysate samples were collected every 10 min over 1 h at a flow rate of 1.0 μl/min. Next, rats were transported from the animal facility to the drug self-administration training room (Experiments 1A, 2A) or to an unfamiliar room for the context-change control experiments (1B, 2B). Microdialysis pumps were plugged into a battery pack so that the flow rate and sampling continued during transfer. Rats were then transferred to the operant conditioning chambers for the heroin-seeking test (Experiments 1A, 2A) or to a Plexiglas chamber (Experiments 1B, 2B). After the move to the testing chamber, but prior to the initiation of the test, one dialysate sample was collected (corresponding with the context change). Testing took place under extinction conditions over a 3-h session, and dialysate samples were collected every 10 min. Active lever responses resulted in the same consequences as in training except that no heroin infusions occurred. No levers or cues were presented in the Plexiglas chambers. See also Supplementary Online Material.

Intracranial injections and heroin-seeking tests

Rats were administered the D1-like receptor antagonist, SCH39166, approximately 10 min prior to the heroin-seeking test (Experiments 3, 4). Testing took place under extinction conditions over one 3-h session. See also Supplementary Online Material.

Analytical Chemistry

Extracellular DA was isolated in the dialysate samples using high performance liquid chromatography (HPLC) and quantified using electrochemical detection as previously described (Sorge et al., 2005). See also Supplementary Online Material.

Statistical Analysis

Statistical analyses were conducted using SPSS (IBM, SPSS Statistics, version 20). Data were analyzed using ANOVA followed by post-hoc tests with a Bonferroni correction for multiple comparisons where appropriate. See also Supplementary Online Material.

RESULTS

For experiments 1 and 2, 60 rats were trained, but 10 rats were excluded due to technical problems or incorrect probe placement (Figure 3.1A, B). For experiments 3 and 4, 136 rats were trained, but 24 rats were removed due to incorrect cannulae placements (Figure 3.1C, D). All rats acquired reliable heroin self-administration behaviour (Table 3.1). In all experiments, at test day the food-restricted rats were at approximately 90% of their body weight at the start of the withdrawal phase (Table 3.1).

Experiment 1A: Changes in extracellular DA in the NAc shell

Behaviour: Exposure to 14 days of food restriction resulted in a robust overall augmentation of active lever responses made by the FDR (n = 10) compared to the sated group (n = 8; Figure 3.2A inset). Active lever responses recorded in 10 min bins (Figure 3.2A) were higher in the FDR rats (*feeding condition*: $F_{(1,16)} = 7.769$, $p = 0.013$, $\eta^2 = 0.327$), with a statistically significant decrease over time ($F_{(17,272)} = 9.131$, $p < 0.001$, $\eta^2 = 0.344$) but no interaction of *feeding condition* x *time* ($F_{(17,272)} = 1.388$, $p = 0.142$, $\eta^2 = 0.052$). There were no statistically significant differences between groups in the number of inactive lever responses.

Microdialysis: There were no statistically significant differences in absolute baseline dialysate concentrations of DA (Table 3.2). Chronic food restriction resulted in increased extracellular DA throughout the sampling period (Figure 3.2B; *feeding condition*: $F_{(1,16)} = 4.699$, $p = 0.046$, $\eta^2 = 0.227$), and there were no statistically significant effects for *time* ($F_{(18,288)} = 1.202$, $p = 0.258$, $\eta^2 = 0.065$) or *feeding condition* x *time* ($F_{(18,288)} = 1.158$, $p = 0.296$, $\eta^2 = 0.063$). Planned comparisons revealed a statistically significant increase in extracellular DA in FDR rats compared to baseline following exposure to the heroin self-administration context ($t(9) = -2.316$, $p = 0.046$, $d = 1.544$; Figure 3.2B). Extracellular DA at the initiation of the heroin-seeking test was elevated in the FDR rats compared to baseline levels ($t(9) = -3.951$, $p = 0.003$, $d = 2.634$) and to sated rats ($t(16) = 3.080$, $p = 0.007$, $d = 1.54$; Figure 3.2B).

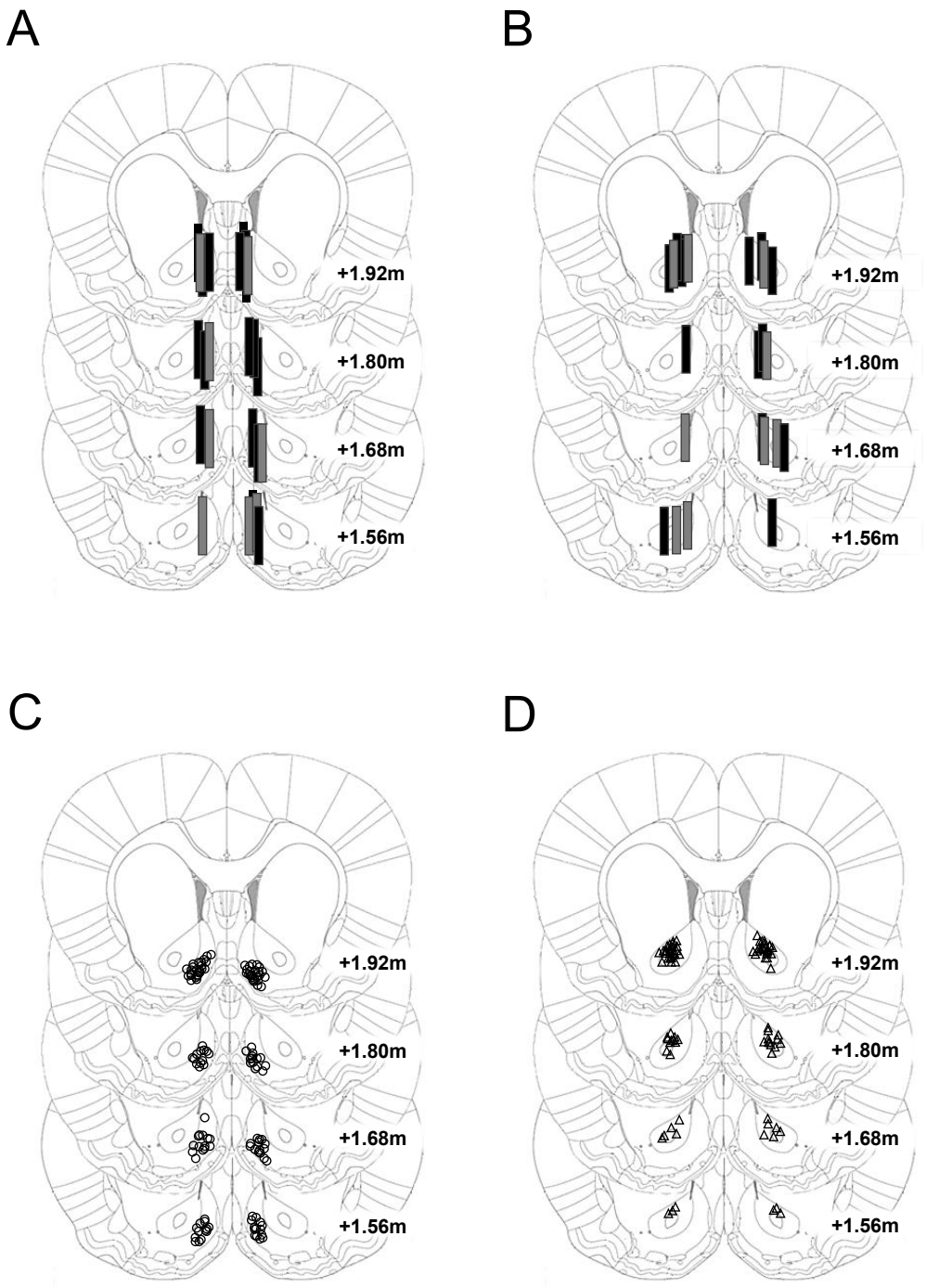

Figure 3.1. Cannula placements for all experiments. Approximate locations of active region of microdialysis probe targeting the NAc shell (A) for Experiment 1A (n = 18; black line) and Experiment 1B (n = 9; gray line), or the NAc core (B) for Experiment 2A (n = 14; black line) and Experiment 2B (n = 9; gray line). Approximate anatomical position for microinjector tips targeting the NAc shell (C) for Experiment 3 (n = 66; open circles) or NAc core (D) for Experiment 4 (n = 46; open triangles). Images modified from the brain atlas of Paxinos & Watson (2005) of Figures 17 – 20 (+1.56 - +1.92 mm anterior to Bregma).

Table 3.1. Mean ± SEM of the number of infusions taken, and the number of active and inactive lever responses made on the last training day (9 h) in each experiment, as well as body weight for the FDR and sated rats on the 14th day of food restriction (the drug seeking test).

	Mean ± SEM				
	Self-administration training day 10			Food-restriction day 14	
Experiment	Infusions	Active lever responses	Inactive lever responses	Body weight (g) (FDR)	Body weight (g) (Sated)
1A	40.18 ± 5.37	120.76 ± 30.13	4.76 ± 1.85	315.60 ± 5.09	422.25 ± 8.82
1B	40.78 ± 8.13	108.22 ± 34.92	4.56 ± 0.91	295.00 ± 6.81	401.75 ± 24.95
2A	34.50 ± 4.02	79.79 ± 13.38	12.57 ± 6.25	309.78 ± 6.19	431.400 ± 9.21
2B	54.44 ± 14.09	230.00 ± 96.91	4.00 ± 1.00	305.25 ± 10.91	421.60 ± 13.32
3	46.38 ± 3.05	186.06 ± 53.48	9.87 ± 1.09	316.36 ± 2.46	428.41 ± 4.52
4	39.63 ± 2.78	134.70 ± 20.14	16.04 ± 4.73	315.25 ± 3.08	442.77 ± 4.65

Experiment 1B: Specificity of changes in extracellular DA in the NAc shell to the drug context

In a separate group of rats, following heroin self-administration training and 14 days of food restriction as in Experiment 1, rats were transferred to an unfamiliar room and into a Plexiglas chamber (novel context). Absolute baseline dialysate concentrations of DA were not statistically significantly different between FDR (n = 5) and sated rats (n = 4; Table 3.2). There were no changes in extracellular DA in either the FDR or sated groups as compared to baseline (Figure 3.2C; *feeding condition*: $F_{(1,7)} = 0.339$, $p = 0.579$, $\eta^2 = 0.046$; *time*: $F_{(19,133)} = 1.399$, $p = 0.138$, $\eta^2 = 0.144$; *feeding condition* x *time*: $F_{(19,133)} = 1.325$, $p = 0.178$, $\eta^2 = 0.136$).

Experiment 2A: Changes in extracellular DA in the NAc core

Behaviour: FDR rats (n = 9) displayed a considerable increase in the total number of responses on the active lever compared to sated rats (n = 5; Figure 3.3A inset). Active lever responses recorded in 10 min bins in FDR rats were substantially higher than in sated rats over the first 20 min of the test session (Figure 3.3B; *feeding condition*: $F_{(1,12)} = 5.218$, $p = 0.041$, $\eta^2 = 0.303$; *time*: $F_{(17,204)} = 6.281$, $p < 0.001$, $\eta^2 = 0.297$; *feeding condition* x *time*: $F_{(17,204)} = 2.858$, $p < 0.001$, $\eta^2 = 0.135$). There were no statistically significant differences in the number of inactive lever responses between groups.

Microdialysis: There were no statistically significant differences in absolute baseline dialysate concentrations of DA (Table 3.2). Extracellular DA in the NAc core increased following the move to the operant conditioning chamber but decreased back to baseline during the test session (Figure 3.3B; *time*: $F_{(19,228)} = 7.630$, $p = 0.007$, $\eta^2 = 0.075$). There were no statistically significant effects for *feeding condition* ($F_{(1,12)} = 0.979$, $p = 0.342$, $\eta^2 = 0.367$) or *feeding condition* x *time* ($F_{(19,228)} = 1.174$, $p = 0.282$, $\eta^2 = 0.056$). Planned comparisons found that both the FDR rats ($t(8) = -3.166$, $p = 0.01$, $d = 2.239$), and the sated rats ($t(4) = -3.959$, $p = 0.017$, $d = 3.959$) demonstrated a statistically significant increase in extracellular DA following exposure to the operant chamber compared to baseline levels. However, extracellular DA remained elevated compared to baseline following the initiation of the heroin-seeking test in only the FDR rats ($t(8) = -3.226$, $p = 0.012$, $d = 2.281$) before returning to basal levels.

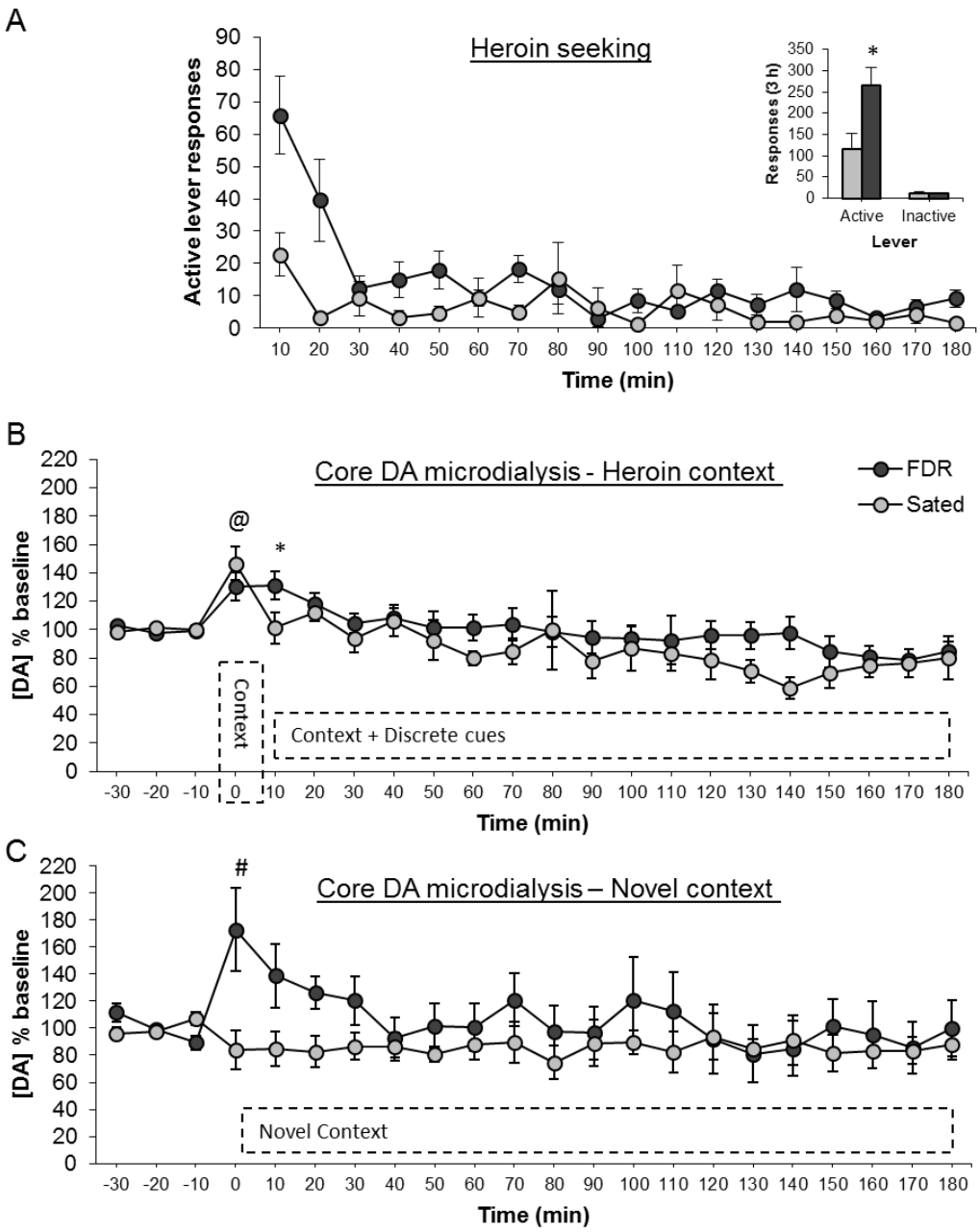

Figure 3.2. Chronic food restriction-induced augmentation of heroin seeking and extracellular dopamine in the NAc shell. (A) Total number of active and inactive lever responses for FDR (n = 10) and sated (n = 8) rats (inset), and active lever responses in 10-min time intervals during the 3 h heroin-seeking test in Experiment 1, * $p = 0.013$ compared to sated rats. (B) Extracellular dopamine following re-exposure to the drug environment and during the heroin seeking test in the FDR and sated rats, * $p < 0.05$ compared to baseline levels, # $p = 0.007$ compared to sated rats. (C) Extracellular dopamine following exposure to a novel context in the FDR (n = 5) or sated (n = 4) rats.

Table 3.2. Mean ± SEM of the absolute concentrations of baseline dopamine levels of the FDR and sated rats.

	Mean ± SEM absolute [DA] in pg/µl		Statistics
Experiment	FDR	Sated	*t-test* & Cohen's *d* effect size
1A	0.125 ± 0.022	0.147 ± 0.025	t (14) = -0.646, p = 0.529, d = -0.345
1B	0.107 ± 0.016	0.089 ± 0.005	t (7) = 0.949, p = 0.374, d = 0.717
2A	0.092 ± 0.015	0.109 ± 0.026	t (12) = -0.626, p = 0.543, d = -0.361
2B	0.093 ± 0.013	0.090 ± 0.018	t (7) = 0.143, p = 0.890, d = 0.108

Experiment 2B: Specificity of changes in extracellular DA in the NAc core to the drug context

Absolute baseline dialysate concentrations of DA in the NAc core did not differ between FDR (n = 4) and sated (n = 5) groups. FDR rats displayed a short-term increase in NAc core extracellular DA after the move to the novel chamber (Figure 3.3C; *feeding condition*: $F_{(1,7)}$ = 1.177, $p = 0.314$, $\eta^2 = 0.144$; *time*: $F_{(19,133)} = 2.484$, $p = 0.001$, $\eta^2 = 0.202$; *feeding condition* x *time*: $F_{(19,133)} = 2.786$, $p < 0.001$, $\eta^2 = 0.227$). Planned comparisons revealed that the increase from baseline in extracellular DA in the FDR rats following exposure to the novel context did not reach statistical significance ($t(3) = -2.376$, $p = 0.098$, $d = 2.744$), but was statistically significantly higher than the in sated rats ($t(7) = 2.823$, $p = 0.026$, $d = 2.134$).

Experiment 3: Administration of the DA D1-like receptor antagonist, SCH39166, into the NAc Shell

The final analysis included the following 8 groups: FDR-0.0 ng (n = 8), FDR-12.5 ng (n = 8), FDR-25.0 ng (n = 8), FDR-50.0 ng (n = 8), sated-0.0 ng (n = 9), sated-12.5 ng (n = 9), sated-25.0 ng (n = 8), sated-50.0 ng (n = 8). Overall, the FDR groups responded more on the active lever during the 3-h heroin-seeking test compared to the sated groups (Figure 3.4A; *feeding condition*: $F_{(1,58)} = 20.35$, $p < 0.001$, $\eta^2 = 0.234$). Administration of SCH39166 into the NAc shell statistically significantly decreased active lever responding regardless of food restriction condition (*SCH39166 dose*: $F_{(3,58)} = 2.765$, $p = 0.05$, $\eta^2 = 0.095$). No statistically significant effect for *feeding condition* x *SCH39166 dose* interaction was observed ($F_{(3,58)}$ = 0.100, $p = 0.959$, $\eta^2 = 0.003$). Finally, no statistically significant effects were observed for inactive lever responding during the test.

Experiment 4: Administration of the DA D1-like receptor antagonist, SCH39166, into the NAc Core

The final analysis included the following 6 groups: FDR-0.0 ng (n = 8), FDR-12.5 ng (n = 9), FDR-25.0 ng (n = 7), sated-0.0 ng (n = 7), sated-12.5 ng (n = 7), sated-25.0 ng (n = 8). Overall, the FDR groups responded more on the active lever during the 3-h heroin-seeking test compared to the sated groups (Figure 3.4B; *feeding condition*: $F_{(1,40)} = 22.703$, $p < 0.001$, η^2 = 0.314), but there was no main effect for *SCH39166 dose*: $F_{(2,40)} = 0.891$, $p = 0.418$, $\eta^2 = 0.025$).

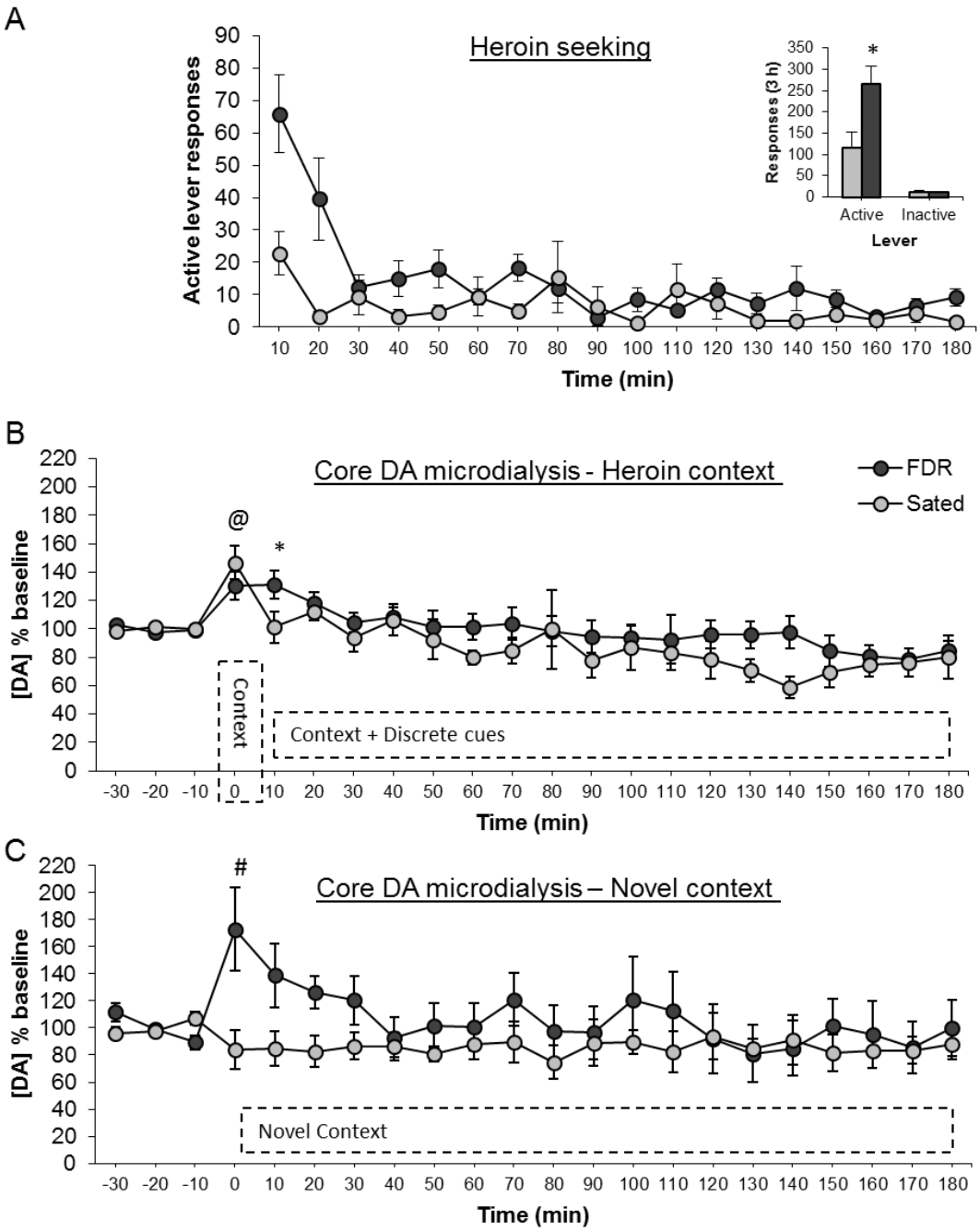

Figure 3.3. Chronic food restriction-induced augmentation of heroin seeking and extracellular dopamine in the NAc core. (A) Total number of active and inactive lever responses for FDR (n = 9) and sated (n = 5) rats (inset), and active lever responses in 10-min time intervals during the 3-h heroin-seeking test in Experiment 2. * $p = 0.041$ compared to sated rats. (B) Extracellular dopamine following re-exposure to the drug environment and during the heroin seeking test in the FDR and sated rats, @ $p < 0.05$ FDR and sated groups compared to baseline, * $p = 0.012$ FDR group compared to baseline. (C) Extracellular dopamine following exposure to a novel context in FDR (n = 4) and sated (n = 5) rats, # $p = 0.026$ compared to sated rats.

Importantly, there was a statistically significant interaction between *feeding condition* x *SCH39166 dose* ($F_{(2,40)} = 3.86$, $p = 0.029$, $\eta^2 = 0.107$). Following Bonferroni corrections, post-hoc analyses revealed a statistically significant higher number of responses in the FDR-0.0 ng group compared to the sated-0.0 ng group ($t(40) = 4.967$, $p < 0.0001$, $d = 1.990$), but no differences between the FDR-12.5 ng and sated-12.5 ng groups, or between the FDR-25.0 ng and sated-25.0 ng groups. In addition, the number of active lever responses made by the FDR-0.0 ng group was statistically significantly higher than the FDR-12.5 ng group ($t(40) = 2.847$, $p = 0.02$, $d = 0.978$), but not the FDR-25.0 ng ($t(40) = 2.283$, $p = 0.08$, $d = 1.690$). Finally, no statistically significant effects were observed for inactive lever responding during the test.

DISCUSSION

Chronic food restriction augmented heroin seeking following two weeks of withdrawal, as we have previously demonstrated (D'Cunha et al., 2013). Re-exposure to the self-administration context increased extracellular DA levels in the NAc shell and core. However, the increase in extracellular DA levels in the NAc core occurred in both the sated and FDR rats, while in the NAc shell, exposure to the drug context selectively increased extracellular DA levels in FDR rats. Exposure to a novel context increased extracellular DA levels in the NAc core, but not shell, only in the FDR rats. Initiation of the heroin-seeking test maintained the increase in extracellular DA levels in the NAc shell and core of FDR rats. DA levels then quickly returned to baseline in the NAc core, while extracellular DA levels in the NAc shell remained elevated. Finally, administration of the DA D1-like receptor antagonist SCH39166 into the NAc shell decreased heroin seeking in both feeding conditions. In contrast, intra-NAc core SCH39166 decreased heroin seeking selectively in the FDR group.

Effects of exposure to heroin-associated context on extracellular DA in NAc shell and core in FDR and sated rats

To the best of our knowledge, ours is the first report on changes in extracellular DA levels following exposure to a heroin-associated context, independent of the discrete drug-associated cues or instrumental contingency, in rats with a history of heroin self-administration.

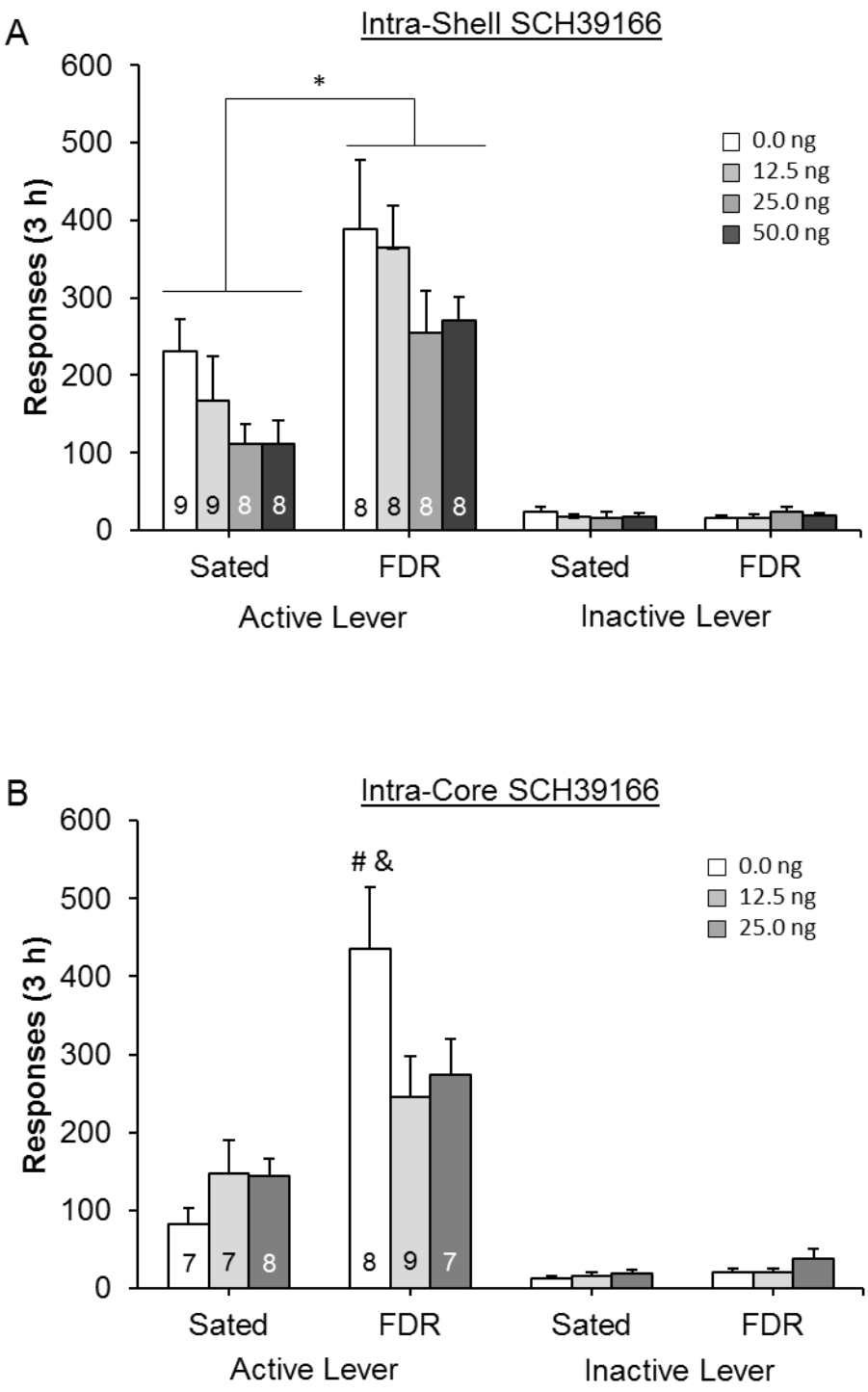

Figure 3.4. The effect of injections of the dopamine D1-like receptor antagonist SCH 39166 into the NAc shell (A) or NAc core (B) on the augmentation of heroin seeking induced by chronic food restriction. * $p = 0.05$ compared to the sated groups. # $p < 0.0001$ FDR-0.0 ng group compared to sated-0.0 ng group. & $p = 0.02$ FDR-0.0 ng group compared to FDR-12.5 ng group.

Previous reports on conditioned changes in DA levels in the mesolimbic pathway terminals are inconsistent. Increases in extracellular DA in the NAc following exposure to an amphetamine- or cocaine-associated context have been reported (Duvauchelle, Ikegami, Asami, et al., 2000; Duvauchelle, Ikegami, & Castaneda, 2000; Stuber et al., 2002). In contrast, other investigations did not find changes in extracellular or tissue DA levels following exposure to cocaine- or morphine-conditioned contextual stimuli (Brown & Fibiger, 1992; Walter & Kuschinsky, 1989). Unfortunately, in most of the studies cited above, no distinctions were made between the NAc shell and core. Importantly, even when changes in NAc extracellular DA levels were found following exposure to the drug context (Duvauchelle, Ikegami, Asami, et al., 2000; Stuber et al., 2002), those changes were not temporally or quantitatively associated with the conditioned behavioural response.

Stuber et al (2002) reported a conditioned increase in extracellular DA levels in the NAc core following exposure to an amphetamine-paired environment in sated rats, but not in food restricted rats (to 90% of their baseline bodyweight). This finding contrasts with the statistically significant increase in extracellular DA levels in the NAc core that we observed in both the sated and FDR rats following exposure to the heroin self-administration environment. However, in FDR rats, an increase in NAc core DA also occurred in a novel context, suggesting that the transfer between environments induced a non-specific DA response. This response might be more related to arousal than to conditioned drug effects (Brown & Fibiger, 1992). Discrepancies compared to previous reports on NAc DA response to drug-associated context could also result from the use of passive, Pavlovian conditioning, while instrumental conditioning was used here.

Mesolimbic DA is thought to be critically involved in the generation of incentive salience to drug-associated stimuli, leading to approach and engagement with the drug-related stimuli (Berridge, 2007). Since the generation of incentive salience is strongly modulated by physiological states (Berridge, 2007), this could explain the heroin-context specific increase in NAc shell DA in FDR rats. In the current study, rats' behaviour in the operant conditioning chambers was not monitored during the 10-min period leading to the initiation of the heroin-seeking test. We therefore have no direct assessment of the association between the change in NAc DA levels and non-instrumental drug context-conditioned behaviours.

Effects of exposure to heroin-associated discrete cues on extracellular DA in NAc shell and core in FDR and sated rats

The increase in extracellular DA levels in the NAc shell and core that was selective to the FDR groups following the initiation of the heroin-seeking test, coincided with the intense active lever pressing over the first 10-min time bin. This suggests that food restriction-induced augmentation of heroin seeking is associated with increased NAc extracellular DA levels. As mentioned above, food restriction can sensitize neural systems that are involved in incentive motivational processes, including DA systems (Berridge & Robinson, 1998), resulting in cue-induced elevation of NAc core and shell DA in FDR rats.

Bassareo et al. (2011, 2015) found elevated DA levels in the NAc following exposure to sucrose- and morphine-conditioned stimuli, albeit only in the shell. The differences between our results and those reported by Bassareo et al. (2011) may be due to the use of Pavlovian/contextual conditioned cues by Bassareo et al. (2011), versus the instrumental/discrete cues in the present report. However, an explanation based on the difference in the conditioning procedures and the nature of the cues seems unlikely, since a selective increase in DA in the NAc shell, but not core, was reported by Bassareo et al. (2015) using an instrumental conditioning procedure with sucrose-associated discrete cues. Importantly, comparisons with Bassareo et al.'s (2015) study should be made with caution, considering the non-drug reward used in their procedure and the known differential effects of non-drug and drug conditioned stimuli on DA transmission in the NAc (Bassareo et al., 2011). Thus, the reasons for the less selective increase in NAc DA in FDR rats versus the NAc shell selective effect reported by Bassareo et al. (2011, 2015) are not clear.

Sated rats in the current study showed no statistically significant changes in DA levels in NAc shell or core during the heroin-seeking test. This finding is in line with the unaltered DA levels in the NAc following response-contingent exposure to cocaine-associated cues reported by Ito et al. (2000), and Neisewander et al. (1996). Interestingly, rats in Ito et al.'s (2000) study were mildly food restricted throughout the experiment. This apparent lack of food-restriction effect on drug-cue-induced NAc DA could be explained by their experimental procedure that involved cocaine-seeking tests under a second-order schedule of reinforcement. In contrast, rats in the current study experienced a prolonged period of withdrawal before the heroin-seeking test. It has been established that behavioural and neurochemical adaptations occur over drug

withdrawal periods, resulting in an enhanced response over time (incubation of drug craving; (Grimm et al., 2001; Pickens et al., 2011).

Finally, extracellular DA in the NAc shell remained higher in the FDR group compared to the sated one throughout most of the heroin-seeking test, while lever pressing rapidly extinguished in both groups. Moreover, the initiation of the test session was characterized by robust heroin seeking, in both feeding condition groups, that is typically observed following a withdrawal period (Fuchs, Lasseter, Ramirez, & Xie, 2008; Grimm et al., 2001; Neisewander et al., 1996; Shalev, Morales, et al., 2001). This dissociation between levels of DA in the NAc and the magnitude of drug seeking has been noted in previous reports with cocaine- and heroin-trained rats (Ito et al., 2000; Neisewander et al., 1996; Wise et al., 1995).

Taken together, our data indicate that there is a weak, or no relation between changes in extracellular DA levels in the NAc shell and heroin seeking following prolonged withdrawal, in both FDR and sated rats. In contrast, changes in DA levels in the NAc core seem to parallel the changes in lever pressing throughout the heroin-seeking test, but only in the FDR rats, suggesting a differential role for DA in NAc core in sated and FDR rats.

Effects of intra-NAc shell and core injections of SCH39166 on heroin seeking in FDR and sated rats

Notably, active lever responses performed by sated rats with intra-NAc core vehicle injections (0.0 ng SCH39166) were considerably lower compared to rats with intra-NAc shell vehicle injections. This could prevent an effect of SCH39166 from being demonstrated (floor effect) in the sated intra-NAc core injected rats. Nevertheless, the sated vehicle-injected group performed a substantial number of lever responses (~85 lever presses), and under identical conditions, sated rats from the same cohorts did not show any attenuation of lever responding following treatment with SCH39166.

DA D1-like receptors in the NAc have been strongly implicated in context and discrete cue-induced drug seeking for alcohol (Chaudhri, Sahuque, Schairer, & Janak, 2010; Marchant & Kaganovsky, 2015), morphine (Gao et al., 2013), and heroin (Bossert et al., 2007). However, the involvement of D1-like receptors in the NAc shell vs. core seems to be dependent on the self-administered substance, as well as on the drug-seeking procedure. Renewal of extinguished or punished alcohol seeking was attenuated by antagonism of D1-like receptors in either NAc shell or core (Chaudhri et al., 2010; Marchant & Kaganovsky, 2015). In contrast, blockade of D1-like

receptors in the NAc shell, but not core, decreased context-induced reinstatement of heroin seeking, while intra-core injections decreased discrete cue-induced reinstatement of heroin seeking (Bossert et al., 2007). Gao et al (2013) reported that D1-like receptor antagonism in the shell attenuated morphine seeking using a withdrawal procedure (similar to the one used here); however, the role of D1-like receptors in the core was not assessed in that study. The implied critical role for D1-like receptors in the NAc shell in context- and cue-induced drug seeking corresponds with the attenuated heroin seeking, regardless of feeding condition, which we observed after intra-NAc shell injections of SCH39166.

Finally, considering the findings of Bossert et al. (2007), the selective attenuation of heroin seeking in FDR rats that were injected with SCH39166 into the NAc core suggests that food restriction-induced augmentation of heroin seeking is mediated by an enhancement of the incentive motivational properties (Robinson & Berridge, 2003) of the discrete, rather than contextual, heroin-associated cues.

There are several methodological issues that should be considered when interpreting the present findings. First, although the DA microdialysis sampling intervals were comparatively short (10 min), the temporal resolution of this technique makes it impossible to acquire very fast, phasic, changes in the DA signals that can be picked up using voltammetry (~100 ms). However, the microdialysis approach, as utilized here, successfully identified changes in extracellular DA levels over behaviourally relevant epochs, such as the switch from context exposure to the beginning of the heroin-seeking session (Figure 3.3B). Second, it is important to note that the current experimental design does not allow a clear dissociation between the contribution of contextual and discrete cues to heroin seeking once the test session commenced, a caveat that should be addressed in future studies (see Shalev et al., 2002 for a discussion of the relevant challenges in dissociating the effects of discrete and contextual cue on drug seeking). Third**,** the observed effects of the intracranial SCH39166 injections might be due to diffusion of the antagonist into surrounding tissue. However, this is most unlikely, as indicated by the differential effect of injections into the adjacent core and shell compartments of the NAc. Finally, we did not assess the involvement of NAc DA D2-like receptors in the food restriction effect, as explained in the introduction. Future investigations should support this rational by targeting these receptors.

Conclusion

Context-induced changes in DA levels in the NAc core of FDR rats following a period of withdrawal suggest a non-specific arousal effect. In contrast, the heroin-context selective increase in NAc shell DA levels in FDR rats, when first exposed to the test chambers, might reflect an enhancement of the incentive properties of the contextual cues. Future studies will address this hypothesis. In the NAc shell, food restriction-induced changes in DA presynaptic mechanisms, reflected by changes in extracellular DA levels during the heroin-seeking test, or postsynaptic adaptations in D1-like receptor function were not closely associated with heroin seeking driven by response-contingent discrete cues. In contrast, in the NAc core, changes in extracellular DA levels paralleled heroin seeking in FDR rats, with increased DA levels at the beginning of the test session, when lever pressing was most vigorous, that subsequently decreased as heroin seeking declined. In addition, intra-NAc core injections of SCH39166 selectively decreased heroin seeking in FDR rats. We therefore conclude that changes in DA presynaptic mechanisms and postsynaptic adaptations in NAc core cells that express DA D1-like receptors mediate food restriction-induced augmentation of heroin seeking. The results we report for the sated rats support current neurobiological addiction theories that suggest a minor role for DA transmission in the NAc core in drug seeking (Kalivas & Volkow, 2005). However, in food-restricted rats, DA in the NAc core plays a critical role in the augmentation of heroin seeking. An interesting future direction would be to investigate the generalization of this role to other chronic stressors.

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Subjects

A total of 196 rats (Charles River, St. Constant, Quebec, Canada) were used in this series of experiments. Upon arrival, rats were pair-housed in clear shoebox cages, and allowed 1 week to acclimate to the animal colony prior to surgical procedures. The rats were kept under a reverse 12 h light-dark cycle (lights OFF 9:30 am) at 21°C. Following recovery from surgery (described below) rats were individually housed in operant conditioning chambers with *ad libitum* food and water during heroin self-administration.

At the completion of heroin self-administration training, rats were returned to the animal colony for the withdrawal and food restriction phase (described below). Prior to in vivo microdialysis sampling, rats were housed in clear Plexiglas chambers with a grid floor for baseline sampling prior to the heroin seeking test (described below). Following baseline microdialysis collection rats were transferred back to the operant conditioning chambers for the heroin-seeking test. Rats in the experiments with the DA D1-like receptor antagonist, were brought to the operant conditioning chambers for the heroin seeking test.

Surgical procedures

Intravenous surgery

Intravenous catheterization was completed under ketamine and xylazine (90 and 13 mg/kg, ip) as previously described (Sedki, D'Cunha, et al., 2013). Silastic catheters (Dow Corning, Midland, MI, USA) were inserted into the right jugular vein and secured with silk sutures. The tip of the catheter was attached to a modified 22-gauge cannula (Plastics One, Roanoke, VA) that was mounted on the rat's skull with jeweler's screws and dental cement. Following surgery rats were given penicillin (450,000 IU/rat, sc) and the analgesic ketoprofen (5 mg/kg, sc, CDMV, Quebec, Canada). Throughout self-administration rats were flushed daily with heparin and gentamicin in sterile saline (7.5 IU + 12.0 µg per day per rat) to prevent catheter blockage.

Intracranial surgery

For rats in the microdialysis experiments, unilateral guide cannulae were implanted

targeting one of the following regions (coordinates in mm relative to Bregma): nucleus accumbens (NAc) shell AP +1.6, ML ±1.0, DV -5 (Experiment 1A and 1B); NAc core AP +1.6, ML ±1.5, DV -4.5 (Experiment 2A and 2B; (Paxinos & Watson, 2005b), during the intravenous surgical procedure. Cannulae were mounted adjacent to the modified catheter cannula on the skull using jewelers' screws and dental cement. Cannulae placements were counterbalanced between the right and left hemispheres. Rats in the DA D1-like receptor antagonist experiments were instead implanted with bilateral guide cannulae targeting one of the following regions (coordinates in mm relative to Bregma): nucleus accumbens (NAc) shell AP +1.7, ML ±3.7, DV -4.8 with a 20° angle (Experiment 3); or, NAc core AP +1.8, ML ±2.5, DV -4.5 with a 6° angle (Experiment 4).

Apparatus

Operant conditioning chambers (Med Associates Inc., St. Albans, VT; or Coulbourn Instruments, Holliston, MA) were used for heroin self-administration and the heroin-seeking tests. Operant chambers were located within individual sound attenuating boxes, and each chamber contained two levers located 5 cm above the grid floor. Responses on the "active" retractable lever activated the infusion pump (Med Associates). Conversely, responses on the "inactive" lever were recorded but had no programmed consequences. Prior to the in vivo microdialysis baseline sampling (or testing for context control experiments described subsequently in Experiments 1B & 2B), rats were habituated to a clear Plexiglas chamber (30 cm × 28 cm × 25 cm) with a metal grid floor in the animal facility.

Procedure

Training

Rats were habituated to the operant conditioning chambers for 24 h prior to 10 days of heroin self-administration training. Rats underwent daily three-3 h sessions separated by a 3 h period, under a fixed-interval 20 s (FI-20) timeout schedule of reinforcement. The initial training session began shortly after the onset of the dark phase with the insertion of the active lever, illumination of the houselight, and activation of a compound tone/light cue (2.9 kHz, 10 dB above background/white light above the active lever), which remained on for 30 s or until the active lever was pressed. Responses on the active lever resulted in the delivery of 0.1 mg/kg of heroin (diacetylmorphine HCl; provided by the National Institute for Drug Abuse, Research Triangle

Park, NC, USA) in 0.13 ml over 12 s, and of the tone-light cue for 20 s during which the houselight was turned off. Responses on the active lever made during the timeout were recorded but not reinforced. At the end of each 3 h session the active lever retracted and the houselight was turned off.

In vivo microdialysis and heroin-seeking tests

Two days prior to the heroin seeking test, on the 12th day of food restriction, rats were habituated to the neutral clear Plexiglas chamber. Approximately 14 h prior to the heroin seeking test, on the 13th day of food restriction, probes were lowered into the targeted brain region, under light isoflurane anaesthesia. Probes were either purchased (Bioanalytical Systems Inc., West Lafayette, IN, USA) or made in the laboratory. Probes targeted at the NAc shell (Experiment 1) had a 2.0 mm semipermeable active membrane, whereas probes targeted at the NAc core (Experiment 2) had a 1.5 mm semipermeable active membrane. The active semipermeable membrane of the probe had a pore size of 13kD with an OD of 280 µm and ID of 200 µm. To prevent occlusion, probes were perfused with artificial cerebrospinal fluid (aCSF; 145mM Na^+, 2.7mM K^+, 1.22mM Ca^{2+}, 1.0mM Mg^{2+}, 150mM Cl^-, 2mM Na_2PO_4, pH 7.4 ± 0.1) at 1.0 µl/min for approximately 1 h. The flow rate was then lowered to 0.2 µl/min overnight. On the 14th day of food restriction, the flow rate was returned to 1.0 µl/min for approximately 1 h prior to baseline sampling. Baseline samples were then collected in 10 min time bins prior to the rat being moved to the operant conditioning chamber for the heroin-seeking test, where samples were further collected in 10 min time bins for the 3 h test.

Intracranial injections and heroin-seeking tests

Intracranial injections were made using a syringe pump (Harvard Apparatus, Holliston, MA) connected to a 10 µl Hamilton syringe. This syringe was attached via polyethelene-20 tubing to a 28-gage injector (Plastics One) that extended 2.0 mm below the guide cannula. For all intracranial injections, drug was delivered in a volume of 0.5 µl over 1 min with injectors remaining in place for 1 min following the injection. Two days prior to the heroin seeking test, on the 12th and 13th days of food restriction rats received mock injections to habituate them to the injection procedure. For mock injections the syringe pump was run for 1 min, and short injectors not extending beyond the guide cannulae, were inserted into the guide cannulae. No solution was

administered during the mock injections to minimize tissue damage in the target areas. Rats were then administered SCH 39166 approximately 10 min prior to the heroin-seeking test.

Analytical Chemistry

Extracellular DA and its metabolites were isolated in the dialysate samples using high performance liquid chromatography (HPLC) and quantified using electrochemical detection (ED) as previously described (Hernandez, Rajabi, Stewart, Arvanitogiannis, & Shizgal, 2008). Dialysate samples were loaded through manual injection ports (Rheodyne 7125; Rheodyne LLC, Rhonert Park, CA; 20 µl loop) into a reverse-phase column (15 cm × 0.46 cm, Spherisorb-ODS, 5µm; Higgins Analytical, Mountain View, CA). Following separation in the column the sample passed through dual-channel ESA (Chelmsford, MA) coulometric detectors (Coulochem 5100, with a model 5011 analytical cell), which were connected to a computer. Standard samples of solution containing DA was used to calibrate the equipment. Waters 515 HPLC pumps (Lachine, Quebec, Canada) were used to circulate the mobile phase (19% acetonitrile, 40mg 0.076M SDS, 0.1M EDTA, 0.058M $NaPO_4$, 0.03M citric acid, pH 3.35) at a flow rate of 1.2mL/min. EZChrom Chromatography Data System (Scientific Software Inc., San Ramon, CA) was used to analyze and integrate the data obtained for DA, DOPAC, and HVA.

Histology

After completion of the experiment rats were euthanized with carbon dioxide gas and decapitated. Brains were fixed with 4% paraformaldehyde solution for a week before being sliced in 40 µm coronal sections. Sections were then stained with cresyl violet and cannula and probe locations were determined under a microscope with reference to a brain atlas (Paxinos & Watson, 2005).

Statistical Analysis

All statistical analyses were conducted using SPSS Statistics software package. For the microdialysis experiments, total active and total inactive lever responses during the test session were analyzed separately using independent samples t-test (2-tailed) to compare FDR and sated groups. For the microdialysis experiments, active and inactive lever responses during the test session were analyzed separately using a mixed factorial analysis of variance (ANOVA) with a between subject factor of *feeding condition* (FDR, sated) and a within subject factor of *time* (18 x

10 min bins). To assess the effects of food restriction on NAc extracellular DA levels, baseline levels of DA were determined by averaging the 3 samples collected prior to the move to the operant or neutral chamber for each rat, and then converting the values of all test session samples to a percentage of baseline. Changes from baseline were analyzed separately for each experiment using a mixed factorial ANOVA with the between subjects factor of *feeding condition* and within subjects factor of *time* (baseline average, context change, and test samples 1-18). Statistically significant interactions were followed by post hoc tests with Bonferroni corrections.

To assess the effects of SCH 39166 on the augmentation of heroin seeking induced by chronic food restriction, active and inactive lever responses were analyzed separately using a two-way ANOVA with the between subjects factors of *feeding condition* (FDR, sated) and *SCH 39166 dose* (0.0,12.5, 25.0, 50.0 ng/side). Statistically significant main effects and interactions were followed by post hoc tests with Bonferroni corrections. Statistically significant results are reported for $p \leq 0.05$.

CHAPTER 4: A ROLE FOR LEPTIN AND GHRELIN IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

ABSTRACT

Caloric restriction increases the risk of relapse in abstinent drug users. Similarly, in an animal model of relapse we have found that chronic food restriction augments heroin seeking during withdrawal, but the neural mechanisms are not fully elucidated. Hormones involved in the regulation of energy balance and food intake, such as leptin and ghrelin, are implicated in drug-related behaviors. Thus, we investigated the role of leptin and ghrelin in the augmentation of heroin seeking induced by chronic food restriction. Rats self-administered heroin for 10 days followed by 14 days of drug withdrawal. During this time some of the rats were food restricted to 90% of their original body weight while others were given free access to food. In Experiment 1 we measured the plasma concentrations of leptin and ghrelin following heroin self-administration and withdrawal. As expected, chronic food restriction significantly decreased plasma levels of leptin and elevated plasma levels of ghrelin. Next, in Experiment 2, leptin was administered centrally (0.0, 2.0, or 4.0 μg; i.c.v.) prior to a heroin-seeking test under extinction conditions. Surprisingly, central administration of leptin had no statistically significant effect on heroin seeking. Dopamine neurons in the ventral tegmental area (VTA) express a high density of both leptin and ghrelin receptors, suggesting a direct effect on reward and motivation. Hence, we administered leptin (Experiment 3; 0.000, 0.125, or 0.250 μg/side) or the ghrelin receptor antagonist JMV 2959 (Experiment 4; 0.0, 2.0, 10.0 μg/side) directly into the VTA prior to the heroin-seeking test. Intra-VTA administration of leptin (Experiment 3) and JMV 2959 (Experiment 4) both dose-dependently decreased heroin seeking specifically in the food-restricted rats. We conclude that leptin and ghrelin transmission in the VTA modulates the augmentation of heroin seeking induced by chronic food restriction. Subsequent studies will investigate if this is a result of direct interactions with the mesolimbic dopamine pathway in the VTA.

Keywords: heroin, self-administration, food restriction, leptin, ghrelin, ventral tegmental area

INTRODUCTION

Dependent drug users are characterized by compulsive drug seeking, and cycling through stages of drug use, abstinence, and relapse (O'Brien, 1997; O'Brien & McLellan, 1996). The biggest obstacle in breaking this cycle of drug addiction is relapse, especially in heroin users. In dependent heroin users followed for three decades, 25% had relapsed even after 15 years of abstinence (Hser et al., 2001). Relapse to drug use is triggered by three factors: 1) exposure to the self-administered drug (de Wit, 1996), 2) cues associated with drug availability and drug effects (Carter & Tiffany, 1999; Childress et al., 1993), and 3) stress (Sinha, 2001).

These triggers to relapse are modulated by restricted food intake, or caloric restriction. For example, the risk for relapse in abstinent smokers was remarkably higher when subjects underwent concurrent caloric restriction (Hall et al., 1992). The effects of caloric restriction on increased drug seeking is also evident in animal models of relapse. We found that chronic food restriction during two weeks of drug withdrawal will augment heroin seeking in rats that previously self-administered heroin (D'Cunha et al., 2013).

The neural mechanisms underlying the augmentation of heroin seeking induced by chronic food restriction are not fully elucidated. Recently, we have demonstrated that dopamine (DA) in the mesolimbic pathway plays a key role in the augmentation of heroin seeking induced by chronic food restriction. Specifically, chronic food restriction increased levels of extracellular DA in the nucleus accumbens and blocking DA receptors in the nucleus accumbens reduced the augmentation of heroin seeking induced by chronic food restriction.

Dopamine in the mesolimbic pathway is modulated by the hormones leptin and ghrelin, which regulate food intake and energy homeostasis (Abizaid et al., 2006; Hommel et al., 2006). Leptin is released by peripheral adipocytes (Friedman & Halaas, 1998), and central administration of leptin decreases food intake and increases energy expenditure (Ahima et al., 2000). Additionally, central administration of leptin attenuates the reinstatement of heroin seeking induced by acute food deprivation (Shalev, Yap, et al., 2001). Moreover, leptin receptors can be found in the mesolimbic dopamine pathway, particularly in the ventral tegmental area (VTA; Fulton et al., 2006). Intra-VTA leptin reduces cocaine-induced dopamine (DA) release in the mesolimbic pathway terminals, specifically in the nucleus accumbens (NAc), and decreases the expression of cocaine conditioned place preference, suggesting an attenuation in cocaine

reward (You et al., 2016). Taken together, the findings described above suggest that leptin is a likely modulator of the augmentation of heroin seeking induced by chronic food restriction.

Ghrelin is released by the gut and binds to the growth hormone secretagogue receptor (GHS-R1a; Kojima et al., 1999). Plasma ghrelin is elevated during food restriction and rapidly decreases following meal intake (Toshinai et al., 2001). As with leptin, the effects of ghrelin extend beyond energy balance and food intake and can affect drug use and drug seeking. Central administration of ghrelin increases alcohol intake (Jerlhag et al., 2009), and increases the breakpoint for heroin under a progressive ratio schedule of reinforcement (Maric, Sedki, Ronfard, Chafetz, & Shalev, 2012). Like leptin, ghrelin receptors are abundant in the VTA of the mesolimbic pathway, and the VTA is a target for ghrelin as shown in binding studies (Abizaid et al., 2006). Ghrelin in the VTA increases DA neuronal activity, and intra-VTA infusions of ghrelin increase food intake in rats (Abizaid et al., 2006). Therefore, ghrelin may mediate heroin seeking induced by chronic food restriction.

The current study was designed to elucidate the role of leptin and ghrelin in chronic food restriction-induced augmentation of heroin seeking. In experiment 1 we characterized basal plasma levels of circulating leptin and ghrelin in sated and food restricted rats, as well as changes in their levels following re-feeding. Moreover, since it is demonstrated that ghrelin levels fluctuate throughout the day, and peak prior to meals (Drazen, Vahl, D'Alessio, Seeley, & Woods, 2006), we wanted to assess if the increase in plasma ghrelin, that usually accompanies the onset of the dark phase, would be shifted in the chronically food restricted rats to precede their daily meal that was delivered in the middle of the dark phase. Next, in experiment 2 we tested the hypothesis that centrally administered leptin will attenuate food restriction-induced augmentation of heroin seeking. As a follow-up to experiment 2, in experiment 3, we selectively targeted leptin receptors in the VTA to elucidate their involvement on heroin seeking. Finally, experiment 4 examined whether ghrelin receptors (GHS-R1a) in the VTA are involved in the augmentation of heroin seeking induced by chronic food restriction.

Materials and Methods

Subjects

A total of 206 rats (325-350 g on arrival; Charles River, St. Constant, Quebec, Canada or Raleigh, North Carolina, USA) were used in the 4 experiments. Rats were pair-housed in clear

shoebox cages and allowed 1 week to acclimate to the animal colony prior to undergoing any surgical procedures. Rats were kept on a reverse 12 h light-dark cycle (lights OFF 9:30 am) at 21°C. Following recovery from surgery (described below) rats were individually housed in operant conditioning chambers with free access to food and water during heroin self-administration. At the end of heroin self-administration phase rats were returned to the animal colony for the withdrawal and food restriction phase (described below). On the last day of the withdrawal phase rats were brought back to the operant conditioning chambers for the heroin-seeking test. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and the approval for all procedures was granted by the Concordia University Animal Research Ethics Committee.

Surgical procedures

Intravenous catheterization was completed under ketamine and xylazine (90.0 and 13.0 mg/kg, ip) anaesthesia as previously described (D'Cunha et al., 2013). Briefly, silastic catheters (Dow Corning, Midland, MI, USA) were inserted into the right jugular vein and secured with silk sutures. During the intravenous catheter surgery rats were also implanted with either a unilateral guide cannula (23 gauge; Plastics One, Roanoke, VA) aimed 2 mm above one of the lateral ventricles: AP = -0.5 mm, ML = ± 1.4 mm, DV = -3.0 mm relative to Bregma (Experiments 1A and 1B), or bilateral guide cannulae (23 gauge) targeting the VTA (coordinates relative to Bregma): AP = -5.8 mm, ML = ±2.2 mm, DV = -7.5 mm with a 12° angle. Following surgery rats were given penicillin (450,000 IU/rat, sc) and analgesic ketoprofen (5.0 mg/kg, sc; CDMV, Quebec, Canada). Throughout self-administration rats were flushed daily with heparin and gentamicin in sterile saline (7.5 IU + 12.0 µg per day per rat) to prevent catheter blockage. Following recovery from surgery in experiment 2, intracerebroventricular (i.c.v.) guide cannula placement was verified by demonstrating a short latency (< 60 s) vigorous drinking response to angiotensin II (100.0 nmol, i.c.v.).

Apparatus

Experiments were conducted in operant conditioning chambers (Coulbourn Instruments, Whitehall, PA, USA; 29.0 cm × 29.0 cm × 25.5 cm), placed in individual sound-attenuating cubicles. Each chamber contained two retractable levers located 9.0 cm above the grid floor.

Responses on the ‘active’ lever activated the infusion pump (Coulbourn Instruments),

whereas responses on the 'inactive' lever were recorded but had no programmed consequences. Rats were attached to the infusion pump via a liquid swivel (Lomir Biomedical, QC, Canada) and Tygon tubing shielded with a metal spring.

Drugs

Heroin (diacetylmorphine HCl; National Institute on Drug Abuse, Baltimore, MD, USA) was dissolved in physiological saline and delivered at a concentration of 0.1 mg/kg per infusion. Recombinant murine leptin (Peprotech; Roanoke, VA) was dissolved in sterile water to concentrations of 0.25, 0.50, 1.0, or 2.0 µg/µl. Doses of hormone administered were based on previous reports (Fulton et al., 2000; Shalev, Yap, et al., 2001; You et al., 2016). GHS-R1a antagonist JMV 2959 (EMD Millipore, Billerica, Massachusetts, USA) was dissolved in 0.9% sterile saline to a concentration of 4.0 or 20.0 µg/µl. Administered doses were based on previous work (Skibicka et al., 2011).

Procedure

Experiments 2-4 all consisted of 3 phases: heroin self-administration, withdrawal and food restriction, and finally the heroin-seeking test.

Training

Following recovery from surgery rats were housed in the operant conditioning chambers for a 24-h habituation period and 10 days of heroin self-administration training. Rats were given three 3-h training sessions per day separated by a 3 h period under a fixed interval (FI) 20 s schedule of reinforcement. The first self-administration session started at the onset of the dark phase and was marked by the insertion of both levers, the illumination of a red houselight and the activation of a white-light/tone complex cue (2.9 kHz; 10 dB above background level) above the active lever. The white-light/tone complex remained on for 30 s or until the active lever was pressed. A response on the active lever resulted in the activation of the infusion pump (5 s; 0.13 ml/infusion) and the white-light/tone complex, and the houselight was turned off. Subsequent responses on the active lever for the following 20 s were recorded but not reinforced. Responses on the inactive lever had no programmed consequences. At the end of each 3 h session, the active lever was retracted and the houselight was turned off. To help with lever discrimination, the inactive lever remained extended until 1 h before the 1st session on the following day.

Withdrawal and Food Restriction

After the heroin self-administration training, rats were transferred back to the animal colony and individually housed throughout the withdrawal period. Following a 24 h drug washout period, rats were matched for average number of infusions, active lever responses, and body weight during the last 5 days of training and assigned to either the food restricted (FDR) or sated group. FDR rats were given approximately 15 g of standard rat chow daily at 1:30 pm. The food ration was titrated daily to bring the FDR rats to 90% of their original body weight at the beginning of the withdrawal period.

Intracranial Injections

Intracranial injections were made using a syringe pump (Harvard Apparatus, Holliston, MA, USA) connected to a 10 µl Hamilton syringe. The syringe was attached via polyethylene-20 tubing to a 28-gauge injector (Plastics One) that extended either 2 mm beyond the guide cannula in experiment 2, or 1 mm beyond the guide cannula in experiments 3 and 4. In all experiments rats received mock injections on the 2 days preceding the heroin-seeking test to habituate them to the procedure. For mock injections the syringe pump was run for 1 minute, and short injectors, not extending beyond the guide cannulae, were inserted into the guide cannula. No solution was administered during the mock injections to minimize tissue damage in the target region.

Experiment 1: Characterization of plasma levels of leptin and acylated ghrelin following chronic food restriction and re-feeding

Blood was collected from a separate group of rats that underwent a similar procedure of heroin self-administration for 10 days, 14 days of withdrawal and food restriction, and a heroin-seeking test. A small nip was made at the tip of the tail, and blood was collected in Eppendorf tubes which contained 20 µl of heparin per 1 ml of blood collected. Plasma concentration levels of leptin and ghrelin were repeatedly quantified over time in food restricted and sated rats. At the onset of the dark phase (9:30 am), a baseline measure of blood was collected in chronically food restricted and sated rats. Next, rats that were food restricted were allowed 2 h of free access to rat chow in their home cage, followed by another tail blood collection from both 2 h re-fed and sated rats. Finally, 24 h following *ad libitum* access to food, a third and final tail blood collection was taken from the 24 h re-fed and sated rats. Since it was previously demonstrated that acylated ghrelin levels change throughout the day and peak prior to meal time (Drazen et al., 2006), in

another group of rats we also assessed plasma fluctuations of acylated ghrelin. First sample was collected at the onset of the dark phase (9:30 am), then again prior to the chronically food restricted rats' meal time (1:30 pm), and lastly at the onset of the light phase (9:30 pm) in both chronically food restricted and sated rats.

Plasma was separated by centrifugation at 10,000 rpm for 10 minutes. Following centrifugation, blood plasma was aliquoted and stored at -80˚C until processed. To protect the acylated ghrelin molecule, 1.0 µl of 1.0 N HCl and 1.0 µl of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) was added per 100 µl of blood plasma. Plasma leptin and ghrelin were determined in separate enzyme-linked immunosorbent assay (ELISA) kits (Millipore, MA, USA). The reported detection sensitivity for the ELISA kits was 0.04 ng/ml for leptin and 7.9 pg/ml for acylated ghrelin.

Experiment 2A: The effect of a single central administration of leptin on the augmentation of heroin seeking induced by chronic food restriction

On the 14th day of food restriction rats were injected with leptin (0.0 or 2.0 µg/rat, i.c.v.) approximately 30 min before the 3 h heroin-seeking test. Leptin was administered over 2 minutes at a rate of 1.0 µl/min and the injector was left in place for an additional 1 min. Sterile water was used as the vehicle.

Experiment 2B: The effect of repeated central administration of leptin on the augmentation of heroin seeking induced by chronic food restriction

We have previously reported that 24 h of re-feeding the FDR rats eliminates the augmentation of heroin seeking induced by chronic food restriction (D'Cunha et al., 2013). Interestingly, in Experiment 1 in the current study we found a statistically significant increase in plasma leptin 24 h following re-feeding. Consequently, we wanted to investigate if we could mimic the re-feeding effects with leptin administration approximately 24 h and 30 min prior to the heroin-seeking test. Moreover, we have previously demonstrated that a similar repeated treatment with leptin was highly effective in blocking acute food deprivation-induced reinstatement of heroin seeking (Shalev et al. 2001). Rats received either 0.0, 2.0, or 4.0 µg of leptin into the ventricles on the 13th day of food restriction and the same dose on the 14th day of food restriction, approximately 22 h and 30 min prior to the 3 h heroin-seeking test, respectively.

Administration of leptin was identical to experiment 2A and sterile water was used as the vehicle.

Experiment 3: The effects of intra-VTA leptin on the augmentation of heroin seeking induced by chronic food restriction

As previously mentioned there are leptin receptors located on neurons in the VTA of the mesolimbic dopamine pathway that modulate DA activity (Hommel et al., 2006). We suspected that i.c.v. administrated leptin affected mostly the hypothalamic nuclei around the 3rd ventricle, which are critically involved in the maintenance of energy balance, but may not be as important for the augmentation of heroin seeking induced by chronic food restriction. Therefore, the purpose of experiment 3 was to investigate the role of leptin administration directly into the VTA. Rats received a bilateral infusion of leptin (0.000, 0.125, 0.250 μg/side) into the VTA approximately 30 min prior to the heroin-seeking test on the 14th day of food restriction. Leptin was administered over 1 min at a flow rate of 0.5 μl/min. The injector was left in place for 1 min following each microinjection and sterile water was administered as the vehicle.

Experiment 4: The effects of intra-VTA GHS-R1a antagonist, JMV 2959, administration on the augmentation of heroin seeking induced by chronic food restriction

On the 14th day of food restriction rats were administered the GHS-R1a receptor antagonist JMV 2959 (0.0, 2.0, or 10.0 μg/side) bilaterally into the VTA approximately 10 min prior to the 3 h heroin-seeking test. JMV 2959 was administered at a flow rate of 0.5 μl/min over 1 min. Injector was left in place for an additional minute and 0.9% sterile saline was used as a vehicle.

Histology

At the end of experiments 3 and 4 rats were euthanized with carbon dioxide and decapitated. Brains were fixed with 4% paraformaldehyde (Paxinos & Watson, 2005) solution for a week before being sliced in 40 μm coronal sections. Sections were then stained with cresyl violet and cannula placements were determined under a light microscope with reference to a brain atlas (Paxinos & Watson, 2005).

Statistical Analysis

To characterize the changes of plasma levels of leptin and acylated ghrelin, separate mixed factorial analyses of variance (ANOVA) were used, with the between subjects factor of *feeding condition* (FDR, sated) and the within subjects factor of *time* (baseline, 2 h re-fed, 24 h re-fed, which correspond to the following sample times: 9:30 am, 1:30 pm, 9:30 pm). To assess the effects of leptin or JMV 2959 on the augmentation of heroin seeking induced by chronic food restriction, active and inactive lever responses made during the 3 h heroin-seeking test were analyzed separately using a two-way ANOVA with a between subjects factor of *feeding condition* (FDR, sated) and the second between subjects factor was either: experiment 2A *leptin dose* (0.0 or 2.0 µg/rat); experiment 2B *leptin dose* (0.0, 2.0, or 4.0 µg/rat); experiment 3 *leptin dose* (0.000, 0.125, 0.250 µg/side); experiment 4 *JMV 2959 dose* (0.0, 2.0, or 10.0 µg/side). Repeated measures ANOVAs were preceded by Mauchly's sphericity test, and a Greenhouse-Geisser correction was used when statistical significance was found. Statistically significant main effects and interactions are reported for $p \leq 0.05$, and were followed up with post-hoc analyses with a Bonferroni correction.

RESULTS

All rats acquired reliable heroin self-administration behavior. Mean ± SEM number of infusions and number of active and inactive lever responses made on the last day of heroin self-administration training for each experiment are shown in Table 4.1. For experiments 3 and 4, 102 out of 120 rats had correct histological placements and were included in the analysis (Figure 4.4). In all experiments, on test day the FDR rats were approximately 90% of their original body weight at the start of the withdrawal phase, or approximately 75% of the sated rats' body weight (Table 4.1).

Experiment 1: Characterization of plasma levels of leptin and acylated ghrelin following chronic food restriction and re-feeding

To assess the effects of chronic food restriction and re-feeding on plasma hormone levels, rats from a previous experiment that had an experience with heroin self-administration and food restriction were used. Following the heroin-seeking test, rats were maintained as either food restricted (FDR; n = 6) or sated (n = 6) and assessed for plasma hormone concentration levels at 3 time-points.

As seen in Figure 4.1A, plasma concentrations of leptin were significantly reduced in chronically food restricted rats (*feeding condition*, $F_{(1,10)} = 13.484$, $p = 0.004$, $\eta^2 = 0.574$). Plasma levels in FDR rats increased gradually following re-feeding (*time*, $F_{(2,20)} = 7.303$, $p = 0.004$, $\eta^2 = 0.337$; *feeding condition* × *time*, $F_{(2,20)} = 4.397$, $p = 0.026$, $\eta^2 = 0.203$). Pairwise comparisons with a Bonferroni correction revealed that within the FDR rats, there was a trend difference for plasma leptin levels from baseline compared to 2 h following re-feeding ($p = 0.064$), and a statistically significant increase from baseline to 24 h following re-feeding ($p = 0.003$). There were no statistically significant changes in plasma leptin concentrations in the sated rats over time.

For the analysis of plasma acylated ghrelin levels following re-feeding, Mauchly's sphericity test was significant ($p < 0.05$), therefore data were analyzed using the Greenhouse-Geisser correction. Plasma Ghrelin levels in FDR rats were higher than in sated rats but decreased rapidly following re-feeding (Figure 4.1B). The main effect of *feeding condition* was not statistically significant, although there was a large effect size, $F_{(1,10)} = 3.246$, $p = 0.102$, $\eta^2 = 0.245$. The effect of feeding status on plasma ghrelin levels was reflected in the statistically significant main effect of *time* ($F_{(2,20)} = 19.859$, $p = 0.001$, $\eta^2 = 0.490$) and *feeding condition* × *time* interaction ($F_{(2,20)} = 10.688$, $p = 0.007$, $\eta^2 = 0.264$). Pairwise comparisons with a Bonferroni correction revealed that within the FDR rats, there was a statistically significant decrease in plasma acylated ghrelin levels from baseline compared to 2 h following re-feeding ($p = 0.001$) and to 24 h following re-feeding ($p = 0.001$). There were no statistically significant changes in plasma acylated ghrelin levels in the sated rats over time.

For the analysis of changes in plasma acylated ghrelin levels at different time-points throughout the day, Mauchly's Test of Sphericity was significant ($p < 0.05$), therefore data were analyzed using the Greenhouse-Geisser correction. As seen in Figure 4.1C plasma ghrelin levels were higher in FDR rats compared to the sated group (*food restriction*, $F_{(1,10)} = 37.822$, $p < 0.001$, $\eta^2 = 0.791$). Plasma ghrelin levels in FDR rats fluctuated dramatically over time (*time*, $F_{(2,20)} = 25.263$, $p < 0.001$, $\eta^2 = 0.366$; *food restriction* × *time*, $F_{(2,20)} = 33.673$, $p < 0.001$, $\eta^2 = 0.488$). Pairwise comparisons with a Bonferroni correction revealed that within the FDR rats, there was a statistically significant increase in ghrelin from the onset of the dark phase at 9:30 am to prior to meal time at 1:30 pm ($p < 0.001$). There was also a significant decrease in plasma

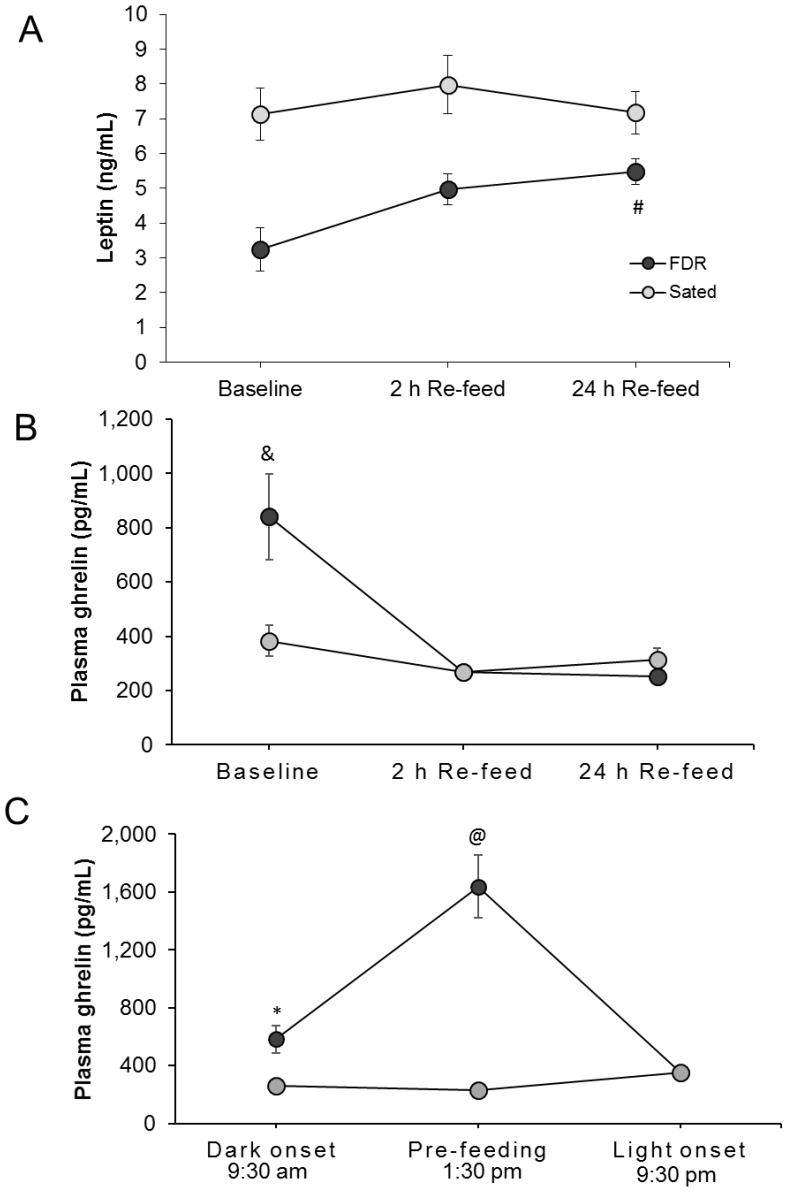

Figure 4.1. Plasma concentrations of leptin and acylated ghrelin in chronically food restricted and sated rats. (A) Concentration of plasma leptin in food restricted (FDR; n = 6) and sated (n = 6) rats under baseline conditions and following 2 h and 24 h of re-feeding the previously food restricted rats. # $p = 0.003$, 24 h re-feeding significantly higher than baseline within the FDR group. (B) Plasma concentration of acylated ghrelin in FDR (n = 6) and sated (n = 6) rats under baseline conditions and following 2 h and 24 h of re-feeding in the previously food restricted rats. & $p = 0.001$, FDR baseline significantly different from 2 h and 24 h re-feeding. (C) Plasma concentration of acylated ghrelin in FDR (n = 6) and sated (n = 6) rats at the onset of the dark phase (9:30 am), prior to meal time in the FDR group (1:30 pm) and at the onset of the light phase (9:30 pm). * $p < 0.02$, compared to 9:30 pm within the FDR group. @ $p < 0.001$, compared to 1:30 pm within the FDR group.

Table 4.1. Mean ± SEM of the number of infusions taken, and the number of active and inactive lever responses made on the last training day (9 h) in each experiment, as well as body weight for the FDR and sated rats on the 14th day of food restriction (the drug seeking test).

	Mean ± SEM				
	Self-administration training day 10			Food-restriction day 14	
Experiment	Infusions	Active lever responses	Inactive lever responses	Body weight (g) (FDR)	Body weight (g) (Sated)
2A	36.10 ± 4.42	123.15 ± 31.36	9.15 ± 3.10	326.60 ± 4.93	447.00 ± 13.46
2B	43.18 ± 6.44	139.13 ± 15.68	14.87 ± 4.02	322.61 ± 3.55	420.86 ± 6.21
3	35.31 ± 2.59	110.71 ± 17.82	13.44 ± 2.60	323.96 ± 3.43	431.23 ± 6.74
4	34.16 ± 3.36	103.64 ± 19.21	8.36 ± 1.37	327.77 ± 5.09	427.71 ± 11.25

ghrelin from 1:30 pm to the onset of the light phase at 9:30 pm ($p < 0.001$). Plasma ghrelin levels at the onset of the dark phase at 9:30 am were also significantly higher than at the onset of the light phase at 9:30 pm, ($p = 0.020$). There were no statistically significant changes in plasma ghrelin levels in the sated rats.

Experiment 2A: The effect of a single central administration of leptin on the augmentation of heroin seeking induced by chronic food restriction

The final analysis included 20 rats divided into 4 groups: FDR – 0.0 µg (n = 4), FDR – 2.0 µg (n = 6), Sated – 0.0 µg (n = 4), Sated – 2.0 µg (n = 6). As seen in Figure 4.2A, overall the FDR groups pressed more on the active lever during the 3-hour heroin-seeking test compared to the sated groups (*feeding condition,* $F_{(1,16)} = 7.083$, $p = 0.017$, $\eta^2 = 0.304$). A single pre-test i.c.v. administration of leptin had no effect on active lever responses (*leptin dose,* $F_{(1,16)} = 0.244$, $p = 0.628$, $\eta^2 = 0.010$), and there was no statistically significant interaction for *feeding condition* × *leptin dose* ($F_{(1,16)} = 0.010$, $p = 0.922$, $\eta^2 = 0.000421$).

Leptin injections resulted in a statistically significant increase in inactive lever responses (*leptin dose,* $F_{(1,16)} = 4.955$, $p = 0.041$, $\eta^2 = 0.184$). It should be noted that inactive lever responses were still much lower than active lever responses. There were no other significant effects observed for inactive lever responses during the heroin-seeking test (*feeding condition,* $F_{(1,16)} = 4.165$, $p = 0.058$, $\eta^2 = 0.155$; *feeding condition* × *leptin dose* ($F_{(1,16)} = 1.773$, $p = 0.202$, $\eta^2 = 0.066$).

Experiment 2B: The effect of repeated central administration of leptin on the augmentation of heroin seeking induced by chronic food restriction

The final analysis included 60 rats divided into 6 groups: FDR – 0.0 µg (n = 15), FDR – 2.0 µg (n = 8), FDR – 4.0 µg (n = 8), Sated – 0.0 µg (n = 13), Sated – 2.0 µg (n = 7), Sated – 4.0 µg (n = 9). As previously demonstrated, the FDR groups pressed significantly more on the active lever than the sated groups, (*feeding condition,* $F_{(1,54)} = 11.329$, $p = 0.001$, $\eta^2 = 0.165$; Figure 4.2B). Although both feeding-condition groups showed a decrease in active lever responses following leptin administration, there was no statistically significant main effect of *leptin dose* ($F_{(2,54)} = 1.421$, $p = 0.250$, $\eta^2 = 0.042$) or an interaction of *feeding condition* × *leptin dose* ($F_{(2,54)} = 0.153$, $p = 0.858$, $\eta^2 = 0.0044$). Inactive lever responses were not statistically significantly affected by *feeding condition,* ($F_{(1,54)} = 1.484$, $p = 0.228$, $\eta^2 = 0.024$), treatment with *leptin dose*

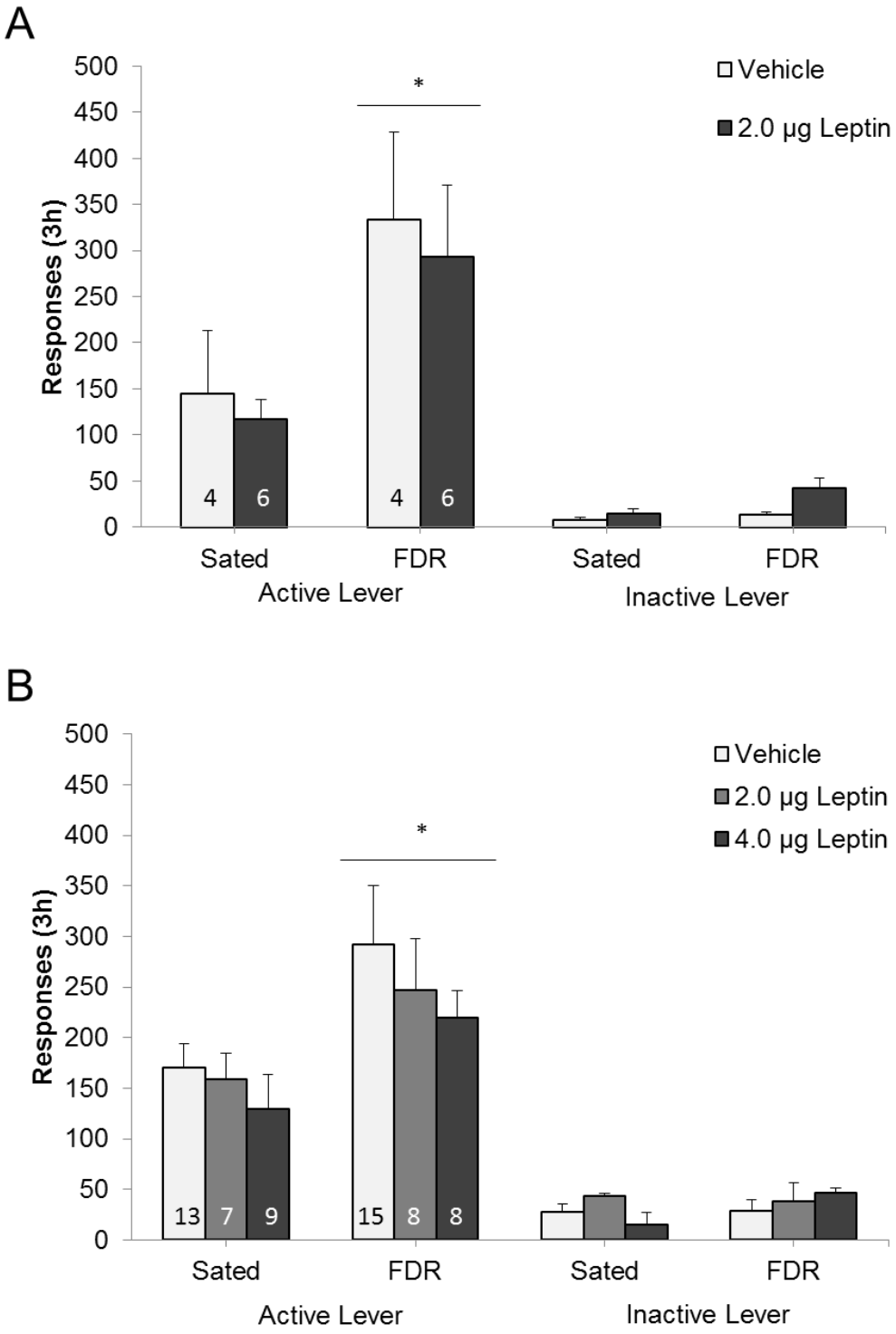

Figure 4.2. The effect of i.c.v. leptin injections on heroin seeking in food restricted (FDR) and sated rats. (A) Following a single leptin (2.0 µg) or vehicle administration, 30 min prior to the heroin-seeking test. (B) Leptin (2.0 or 4.0 µg) or vehicle injections were given twice, 24 h and 30 min prior to the heroin-seeking test. * $p < 0.05$, compared to the sated groups.

($F_{(2,54)} = 0.931$, $p = 0.401$, $\eta^2 = 0.030$) or interaction of *feeding condition* × *leptin dose* ($F_{(2,54)} = 2.020$, $p = 0.142$, $\eta^2 = 0.066$).

Although repeated administration of leptin into the ventricles had no statistically significant effect on heroin seeking, it was not due to a lack of efficacy of leptin. As seen in Figure 4.3, both 2.0 µg and 4.0 µg of leptin administered on FDR 13 and FDR 14 significantly reduced 24 h food intake in the sated rats as compared to vehicle. A one-way ANOVA performed on FDR 14 food consumption data, revealed a main effect of *leptin dose* ($F_{(2,26)} = 5.006$, $p = 0.014$, $\eta^2 = 0.278$). Post-hoc analyses with Bonferroni corrections revealed that both the Sated – 2.0 µg and the Sated – 4.0 µg groups consumed less food than the Sated – 0.0 µg group ($p = 0.034$, $p = 0.055$, respectively). Similarly, a one-way ANOVA performed on FDR 15 food consumption data (~24 h after the last leptin injection) revealed a statistically significant main effect of *leptin dose* ($F_{(2,26)} = 5.665$, $p = 0.009$, $\eta^2 = 0.304$). Post-hoc analyses with Bonferroni corrections revealed that both the Sated – 2.0 µg and the Sated – 4.0 µg groups consumed less food than the Sated – 0.0 µg group ($p = 0.031$, $p = 0.027$, respectively).

Experiment 3: The effect of intra-VTA leptin administration on the augmentation of heroin seeking induced by chronic food restriction

Only rats with correct cannula placements located in the VTA were included in the statistical analysis (Figure 4.4A). The final analysis included the following groups of rats: FDR – 0.000 µg (n = 9), FDR – 0.125 µg (n = 8), FDR – 0.250 µg (n = 9), Sated – 0.000 µg (n = 9), Sated – 0.125 µg (n = 9), and Sated – 0.250 µg (n = 8).

Food restriction augmented heroin seeking on test day, as indicted by the higher number of active lever responses compared to the sated rats (*feeding condition,* $F_{(1,46)} = 21.181$, $p < 0.001$, $\eta^2 = 0.261$). Intra-VTA leptin injections reversed the food restriction effect, without changing drug seeking in the sated groups (*leptin dose* $F_{(2,46)} = 1.688$, $p = 0.196$, $\eta^2 = 0.042$; *food restriction* × *leptin*, $F_{(2,46)} = 5.328$, $p = 0.008$, $\eta^2 = 0.131$; Figure 4.5). Post-hoc analyses with Bonferroni corrections revealed that the FDR – 0.000 µg had statistically significantly more active lever presses than all of the sated groups during the heroin-seeking test ($p < 0.002$). Furthermore, the FDR – 0.000 µg group had significantly more active lever presses than the FDR – 0.250 µg group ($p = 0.012$) but not the FDR – 0.125 µg group ($p = 0.507$). There were no

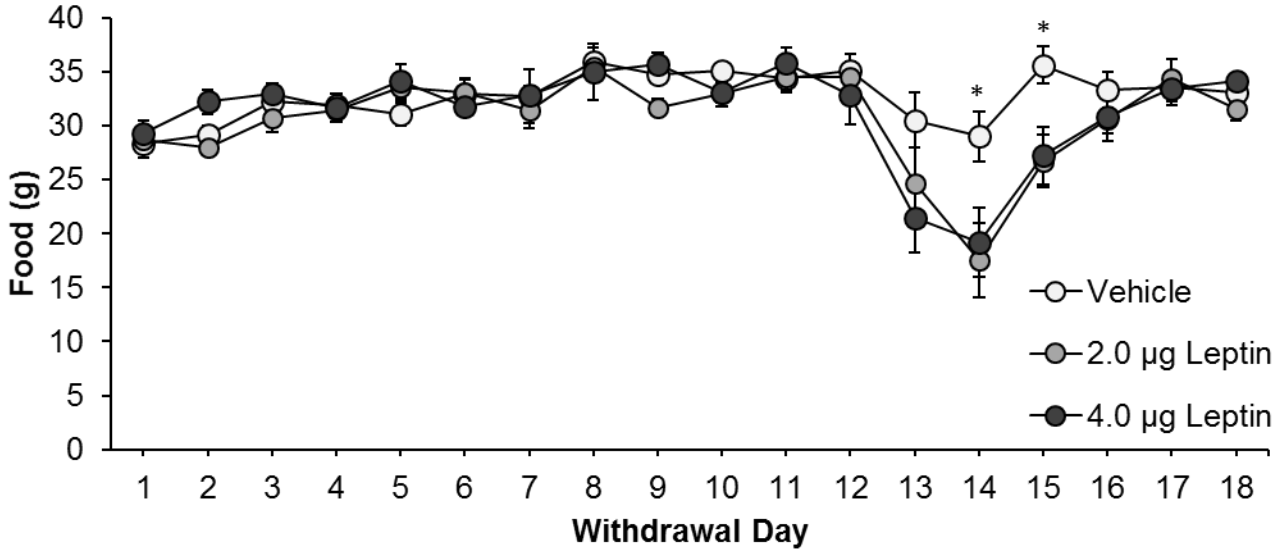

Figure 4.3. The effects of i.c.v. administration of leptin on food intake in sated rats. Leptin (2.0 or 4.0 µg) or vehicle was injected twice, once on day 13, and again on day 14 of withdrawal approximately 30 min before the heroin-seeking test. * $p < 0.05$ compared to either the 2.0 or 4.0 µg treated groups.

statistically significant effects on inactive lever responses (*feeding condition,* $F_{(1,46)} = 1.158$, $p = 0.288$, $\eta^2 = 0.021$; *leptin dose* $F_{(2,46)} = 1.802$, $p = 0.176$, $\eta^2 = 0.066$; *food restriction × leptin*, $F_{(2,46)} = 1.783$, $p = 0.180$, $\eta^2 = 0.066$).

Experiment 4: The effects of intra-VTA ghrelin receptor antagonist (JMV 2959) administration on the augmentation of heroin seeking induced by chronic food restriction

Only rats with correct cannula placements located in the VTA as assessed by histological verification were included in the final statistical analyses (Figure 4.4B). The final analyses included the following groups of rats: FDR – 0.0 µg (n = 9), FDR – 2.0 µg (n = 8), FDR – 10.0 µg (n = 9), Sated – 0.0 µg (n = 8), Sated – 2.0 µg (n = 8), and Sated – 10.0 µg (n = 8). Food restriction significantly augmented heroin seeking, as the FDR groups pressed more on the active lever than the sated groups (*feeding condition,* $F_{(1,44)} = 8.834$, $p = 0.005$, $\eta^2 = 0.140$; Figure 4.6). Intra-VTA injections of JMV 2959 reversed the food restriction effect, without affecting drug seeking in the sated groups (*JMV 2959 dose,* $F_{(2,44)} = 1.116$, $p = 0.337$, $\eta^2 = 0.048$*; feeding condition × JMV 2959 dose*, $F_{(2,44)} = 4.095$, $p = 0.023$, $\eta^2 = 0.129$). Post-hoc analyses with Bonferroni corrections revealed that the FDR – 0.0 µg group had significantly more active lever presses than the Sated – 0.0 µg ($p = 0.009$), the Sated – 2.0 µg ($p = 0.015$) and the FDR – 10.0 µg ($p = 0.046$) groups. Analysis of the inactive lever responses found only a significant effect of *JMV 2959 dose* ($F_{(2,44)} = 3.45$, $p = 0.041$, $\eta^2 = 0.136$), driven by an overall increase in inactive lever response in rats treated with the 10.0 µg dose. There were no other significant effects observed for inactive lever responses during the heroin-seeking test (*feeding condition,* $F_{(1,44)} = 0.003$, $p = 0.953$, $\eta^2 = 0.0000638$; *feeding condition × JMV 2959 dose*, $F_{(2,44)} = 1.936$, $p = 0.156$, $\eta^2 = 0.071$).

DISCUSSION

The neural mechanisms involved in the augmentation of heroin seeking following chronic food restriction have yet to be elucidated. In the current study, as we have previously demonstrated (D'Cunha et al., 2013), chronic food restriction over a period of drug withdrawal significantly augmented heroin seeking in rats with a history of heroin self-administration. Our data demonstrate that i.c.v. administration of leptin, either as single or repeated infusions had no selective effect on food restriction-induced augmentation of heroin seeking. Notably, although the treatment effect was not statistically significant, repeated i.c.v. administration of the high

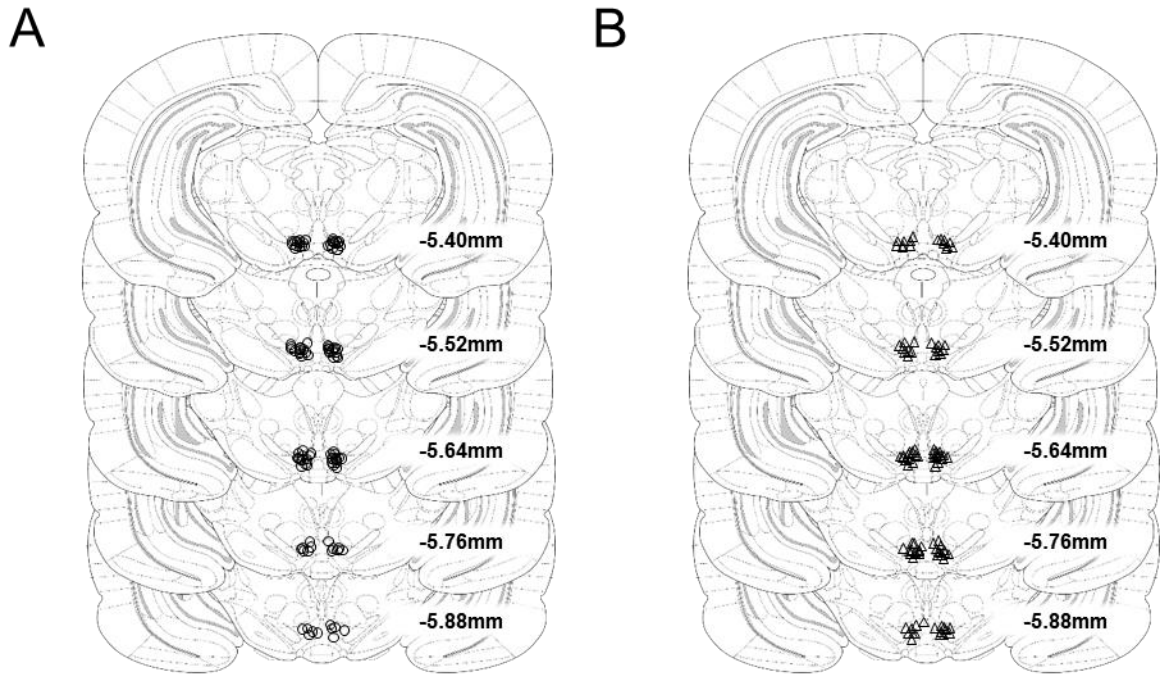

Figure 4.4. Cannula placements for Experiments 3 & 4. Approximate anatomical position for microinjector tips of guide cannula targeting the VTA for (A) Experiment 3 (n = 52; open circles) or (B) Experiment 4 (n = 50; open triangles). Images modified from the brain atlas of Paxinos & Watson (2005), Figures 78 – 82 (-5.40 to -5.88 mm posterior to Bregma).

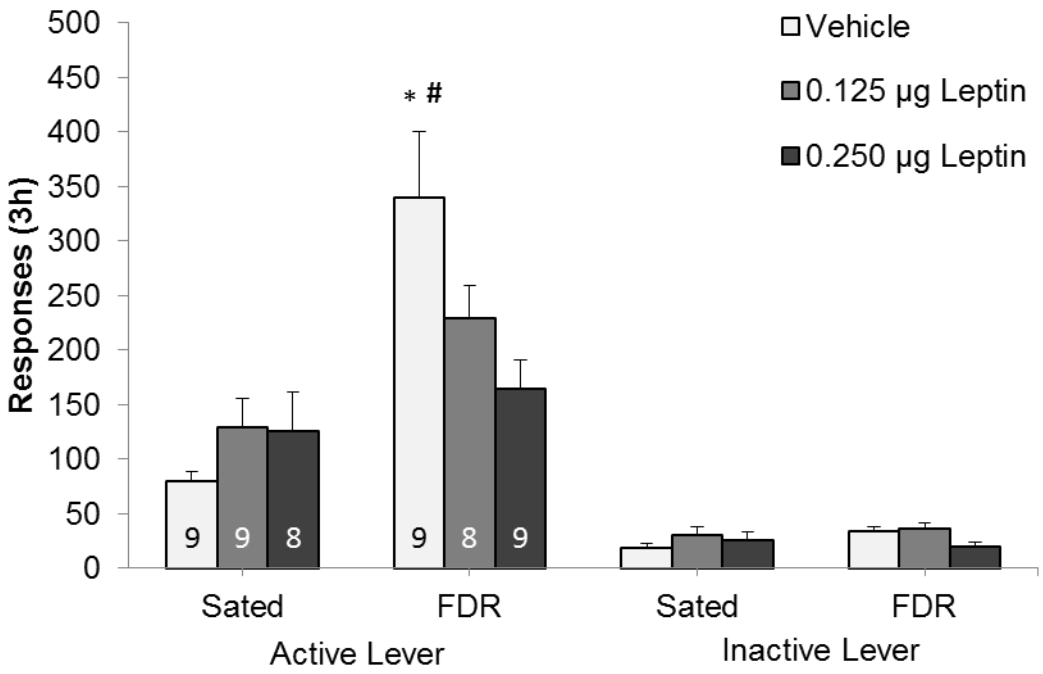

Figure 4.5. The effect of intra-VTA leptin injections on heroin seeking in food restricted (FDR) and sated rats. Leptin (0.125 or 0.250 µg/side) or vehicle was injected 30 min before the heroin-seeking test. * $p < 0.05$, compared to sated - Vehicle rats. # $p < 0.05$, FDR – vehicle vs. FDR – 0.250 µg Leptin.

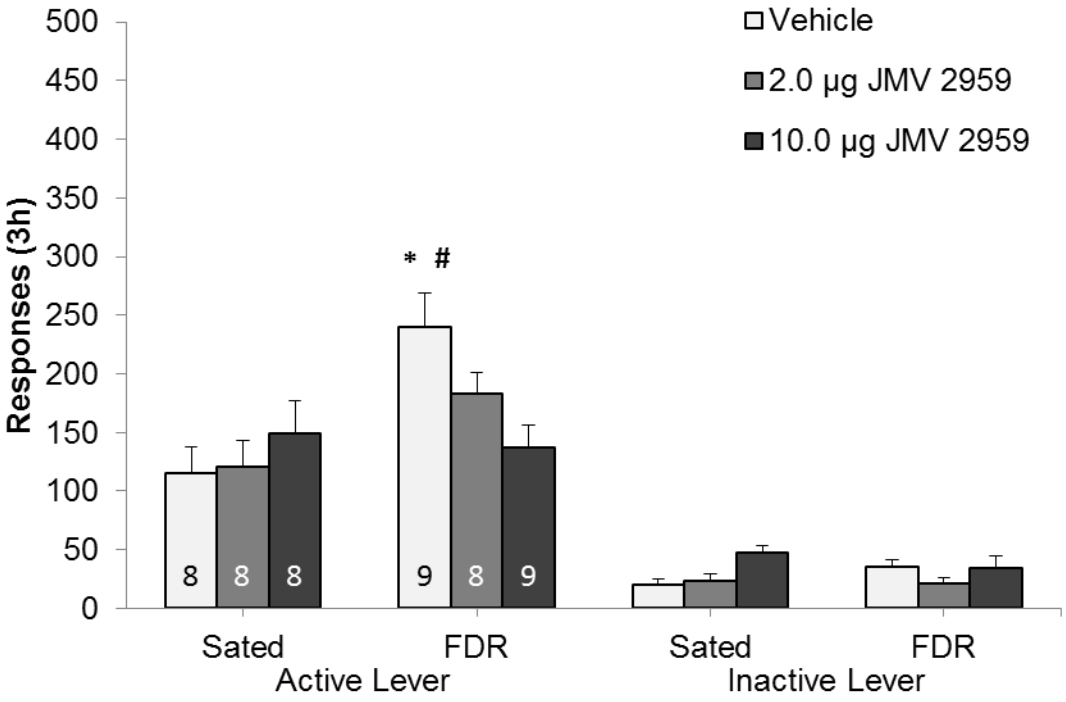

Figure 4.6. The effect of intra-VTA injections of the ghrelin receptor antagonist, JMV 2959, on heroin seeking in food restricted (FDR) and sated rats. JMV 2959 (2.0 or 10.0 µg/side) or vehicle was injected 30 min before the heroin-seeking test. * $p < 0.05$, FDR – Vehicle vs. Sated – Vehicle, and Sated – 2.0 µg JMV 2959. # $p < 0.05$, FDR – vehicle vs. FDR – 10.0 µg JMV 2959.

dose of leptin (4.0 μg) attenuated overall heroin seeking with a considerable effect size (Vehicle vs. 4.0 μg leptin-treated rats: Cohen's $d = 0.61$; 95%CI [-30.19, 31.4]). Most importantly, administration of leptin directly into the VTA decreased heroin seeking selectively in the FDR rats. Lastly, administration of the ghrelin receptor antagonist JMV 2959 directly into the VTA also selectively decreased heroin seeking in the FDR rats.

We also found that, as expected, leptin and ghrelin levels in rats with a history of heroin self-administration are modulated by chronic food restriction. Taken together our results suggest that the changes in hormones levels may act directly in the VTA to alter heroin seeking induced by chronic food restriction.

Changes in plasma concentrations of leptin and acylated ghrelin in the FDR and sated rats

We found that in agreement with previous reports (Johansson et al., 2008; Kinzig, Hargrave, & Tao, 2009), 14 days of chronic food restriction following 10 days of heroin self-administration significantly decreased plasma leptin levels. Absolute basal levels of plasma leptin in the current study were higher than previously reported. Possible reasons for these discrepancies include acute food deprivation of sated rats prior to blood collection, differences in time of blood collection (light vs. dark phase in the current study), and lower body weight of the rats at baseline in the previous studies. Nevertheless, the pattern of change in plasma leptin levels that we recorded is consistent with the effects of chronic food restriction that were observed in earlier studies. In addition, plasma leptin levels significantly increased following 2 or 24 h of free access to chow. This was an unexpected finding as leptin is thought of as a long-term energy balance signal that is strongly correlated with the amount of adipose tissue (Friedman & Halaas 1998).

Chronically food restricted rats also had significantly higher plasma acylated ghrelin levels compared to the sated rats, a finding that is consistent with previous reports (Abou Heif, Deif, & Abdel Aziz, 2009; Kinzig et al., 2009). In contrast, (Johansson et al., 2008) report no changes in plasma ghrelin following 12 days of food restriction. Here again, discrepancies may be accounted for by differences in methodology, with samples collected during the light phase, and comparisons to acutely food deprived (12 h) control rats in the Johansson et al. (2008) study. We did not see a peak in plasma ghrelin, that was reported by Drazen et al. (2006), in the sated rats at the onset of the dark phase. This intriguing discrepancy could be the result of the plasma ghrelin analysis method that targeted acylated ghrelin in the current study, while no information

is available for the method used by Drazen et al. (2006). We did however observe that plasma ghrelin levels were the highest prior to the delivery of the daily meal in the FDR rats, similar to the findings reported by Drazen et al., (2006). Finally, re-feeding the previously food restricted rats for 2 h or 24 h statistically significantly decreased plasma ghrelin levels. We therefore have demonstrated a considerable modulation of circulating levels of leptin and ghrelin in our experimental subjects, that coincided with the augmentation of heroin seeking in FDR rats.

Effects of central administration of leptin on heroin seeking in the FDR and sated rats

Contrary to our hypothesis, i.c.v. administration of leptin did not statistically significantly decrease heroin seeking in the FDR rats. These results contrast with our previous report that the same doses of leptin attenuated acute food deprivation-induced reinstatement of heroin seeking (Shalev, Yap, et al., 2001). There are a few possible reasons for these discrepancies. Acute food deprivation is defined as having no food available for a short duration of time (24 – 48 h), whereas chronic food restriction occurs over an extended period of time with limited food availability. Not only are there distinctions in the metabolic consequences between acute food deprivation and chronic food restriction (Bi, Robinson, & Moran, 2003), but the neural mechanisms impacted by these manipulations are distinct. For example, administration of a corticotrophin releasing factor (CRF) antagonist attenuated acute food deprivation-induced reinstatement of heroin seeking (Shalev, Finnie, Quinn, Tobin, & Wahi, 2006) but had no effect on food restriction-induced augmentation of heroin seeking (Sedki, Abbas, et al., 2013). Another explanation for the differences may be that the current study investigated heroin seeking following a withdrawal period, whereas Shalev and colleagues (2001) investigated reinstatement of heroin seeking following extinction training. The neural mechanisms mediating these procedures are, at least partially, distinct (Fuchs, Branham, & See, 2006).

Our findings also disagree with the demonstration that leptin can reverse the sensitization of brain stimulation reward in the lateral hypothalamus that is induced by chronic food restriction (Fulton et al., 2000). In addition, acute central leptin decreases the breakpoint for sucrose self-administration under a progressive ratio schedule of reinforcement (Figlewicz et al., 2006). However, both these reports found that leptin decreased ongoing rewarded behavior, either by brain stimulation or sucrose, while our procedure is assessing drug seeking under extinction conditions. Interestingly, intravenous leptin attenuated cocaine seeking under “surprising” extinction conditions (You et al., 2016). The discrepancy between our results and You and

colleagues (2016) could be explained by the different procedures used (prolonged withdrawal versus a "surprise extinction" during the self-administration phase, respectively), but this explanation seems unlikely given that intra-VTA leptin injections effectively attenuated drug seeking in both studies (see below). Alternative explanations could be the different classes of self-administered drugs (opiate versus psychostimulant drugs, respectively), or the somewhat different route of leptin administration (intravenous vs. i.c.v.).

Finally, the small effect for repeated leptin treatment on heroin seeking could be explained by an insufficient dose or reduced sensitivity of the rats in our study. However, Experiment 2B argues against this possibility. It is well established that leptin reduces food intake in a variety of species (reviewed in Bruijnzeel, Corrie, Rogers, & Yamada, 2011; Halaas et al., 1997; Hommel et al., 2006; Morton, Cummings, Baskin, Barsh, & Schwartz, 2006). Accordingly, we have found that centrally administered leptin significantly reduced food intake in the sated rats; therefore, the minor effects on heroin seeking are not due to the efficacy of the leptin treatment.

Effects of intra-VTA leptin administration on heroin seeking in FDR and sated rats

We demonstrated that intra-VTA leptin significantly decreased the augmentation of heroin seeking induced by chronic food restriction. Although leptin's primary role is in the hypothalamus to regulate food intake and energy metabolism (Ahima et al., 2000), it also acts on extrahypothalamic targets, specifically the VTA to affect food intake and reward function (Bruijnzeel et al., 2011). Within the VTA there is a high level of leptin receptor expression located on dopaminergic neurons, with lower levels of expression on gamma-aminobutyric acid-ergic (GABAergic) neurons (Figlewicz, 2003; Fulton et al., 2006; Hommel et al., 2006). Leptin administration to the midbrain *in vitro* and systemic administration in anesthetized rats *in vivo* both significantly decrease the firing rate of DA neurons in the VTA (Hommel et al., 2006). These inhibitory actions of leptin on VTA DA neurons are thought to be mediated through a change in the intrinsic properties of these neurons (Hommel et al., 2006), and by presynaptic inhibition of glutamatergic release onto DA neurons (Thompson & Borgland, 2013). More recently, Shen et al. (2016) reported that selective knockdown of leptin receptors in the VTA resulted in increased extracellular DA levels in the NAc. In addition, intra-VTA administration of leptin inhibited the increase in extracellular DA in the NAc induced by cocaine in rats (You et al., 2016), while i.c.v. injections of a leptin antagonist, superactive mouse leptin antagonist

(SMLA), resulted in upregulation of DA and its metabolites in the NAc in mice (Shen, Jiang, Liu, Wang, & Ma, 2016). Importantly, the leptin-signal driven changes in DA transmission described above were associated with altered reward-related behaviours. Thus, You and colleagues (2016) found that intra-VTA leptin decreased conditioned place preference for cocaine, and i.c.v. administration of a leptin receptor antagonist increased cocaine conditioned place preference (Shen, et al. 2016). We have recently identified a selective increase in extracellular DA levels in the NAc of food-restricted rats during heroin seeking tests (D'Cunha et al., 2017). Moreover, we have found that blocking DA D1-like receptors in the NAc decreased the augmentation of heroin seeking induced by chronic food restriction (D'Cunha et al., 2017).

Taken together with the previous findings described above, our current findings suggest that chronic food restriction-induced reduction in plasma leptin levels results in a decrease in the inhibitory signal of leptin on VTA DA neurons' activity and disinhibition of these neurons. This, in turn, would make the FDR rats more responsive to drug-associated cues, leading to increased dopaminergic transmission in the terminals of the mesolimbic pathway, such as the NAc (D'Cunha et al., 2017), and to augmented heroin seeking. Accordingly, when leptin is administered directly into the VTA of FDR rats it reduces DA neurons excitability, effectively attenuating the impact of re-exposure to the heroin-taking context and cues. It has been previously suggested that under food restriction the DA system becomes highly responsive to leptin and its effects on appetitive motivation (Fernandes et al., 2015). Such an adaptation would help explain why we only see decreased heroin seeking in the FDR, but not sated, rats following intra-VTA leptin administration.

The same mechanism could explain our previous findings that acute re-feeding (2 or 24 h before testing) reversed the augmentation of heroin seeking in food-restricted rats (D'Cunha et al., 2017). Thus, as demonstrated here, re-feeding increases plasma leptin in FDR rats, which would result in reduced/normalized excitability of VTA DA neurons and a reduction in DA levels in the NAc, eventually leading to decreased heroin seeking. Interestingly, with both the re-feeding and intra-VTA leptin injections approaches, heroin seeking was reduced to the level demonstrated by the sated rats, but not completely eliminated. This suggests a "ceiling effect" or a limit to the inhibitory signal of leptin in the VTA, maybe due to receptor saturation.

It is unclear why central administration and direct infusion of leptin into the VTA resulted in different behavioral responses. One possibility is that leptin administered into the ventricles is

unable to reach the local levels for sufficient receptor activation in the VTA to trigger significant behavioral effects. We tested heroin seeking 30 minutes following i.c.v. leptin infusion, and although this is reported to be enough time for leptin to diffuse the distance from the ventricles to the VTA (Maness, Kastin, Farrell, & Banks, 1998), it is likely in very low concentrations. These low concentrations may be due to significant degradation of leptin by endogenous enzymes, or leptin may be removed with the turnover of cerebrospinal fluid. It is recommended that nuclei be in close proximity to the ventricles or relatively large amounts of leptin should be delivered to ensure that sufficient concentrations reach the target (Maness et al., 1998). Therefore, the majority of the leptin infused in the ventricles may be absorbed in nuclei closer to the ventricles such as the hypothalamus. If indeed most i.c.v. leptin targets the hypothalamic nuclei, we would predict that these nuclei play a minimal role in drug seeking as their primary function is to regulate feeding and energy homeostasis (Ahima et al., 2000). Although speculative, we would predict that intra-hypothalamic leptin administration would have little or no effect on heroin seeking in the FDR rats.

Effects of blocking ghrelin receptors in the VTA on heroin seeking in FDR and sated rats

Administration of the ghrelin receptor antagonist, JMV 2959, into the VTA selectively decreased heroin seeking in chronically food-restricted rats, suggesting an important role for ghrelin receptors activation in the augmentation of heroin seeking induced by chronic food restriction. This contrasts with previous findings in our laboratory that central administration of a ghrelin receptor antagonist had no effect on heroin self-administration, or on the reinstatement of heroin seeking induced by acute food deprivation (Maric et al., 2012). This may be explained by, first, the use of different ghrelin receptor antagonists: Maric et al. (2012) used the GHS-R1a antagonist D-Lys3-GHRP-6, whereas here we used JMV 2959. Although both these antagonists block GHS-R1a, D-Lys3-GHRP-6 is a peptide-based antagonist (Traebert, Riediger, Whitebread, Scharrer, & Schmid, 2002), whereas JMV 2959 is a small molecule-type antagonist (Moulin et al., 2007). Direct comparison of these two compounds has demonstrated that D-Lys3-GHRP-6 has higher selectivity in attenuating alcohol, compared to water and food intake, but tolerance rapidly developed to its effects (Gomez & Ryabinin, 2014). Second, Maric et al. (2012) administered the antagonist centrally, whereas in the current study we directly targeted the VTA. Finally, the neural adaptations associated with acute food deprivation, used in the previous study,

versus chronic food restriction, used in this study, are distinct (Bi et al., 2003); as are the differences between the neural mechanisms mediating the reinstatement of extinguished drug seeking versus drug seeking following drug withdrawal and abstinence (Fuchs et al., 2006).

We propose that intra-VTA administration of JMV 2959 directly altered dopaminergic transmission in the downstream terminals of the mesolimbic circuit, consequentially affecting heroin seeking. It is well documented that ghrelin transmission in the VTA modulates DA activity in the mesolimbic pathway (Abizaid et al., 2006). Approximately 35-60% of neurons in the VTA that express GHS-R1a are dopaminergic, with the remainder being GABAergic (Abizaid et al., 2006; van Zessen, van der Plasse, & Adan, 2012). However, it seems that ghrelin also increases the activation of VTA DA neurons by increasing excitatory presynaptic glutamatergic input and decreasing inhibitory GABAergic input through rearrangement of the synaptic input (Abizaid et al., 2006). Since chronic food restriction increases plasma ghrelin levels, it is possible that this lowering of the activation threshold in VTA neurons could increase DA levels in the terminal regions of the mesolimbic pathway, such as the NAc, upon exposure to drug cues. Indeed, we have recently reported that FDR rats are more responsive to drug-associated cues, leading to increased DA transmission in the NAc and augmented heroin seeking (D'Cunha et al., 2017). Therefore, we suggest that administration of JMV 2959 blocked this ghrelin-mediated increase in VTA DA firing rates, subsequently reducing DA levels in the NAc and heroin seeking in the FDR rats. This process would also explain why JMV 2959 had no effect on heroin seeking in the sated rats, which demonstrated lower levels of circulating ghrelin.

It is tempting to speculate that chronic food restriction-induced changes in both leptin and ghrelin levels in the VTA, would lead to altered DA transmission in the VTA and downstream mesolimbic targets, following exposure to heroin-associated cues. These changes in leptin and ghrelin in the VTA may be directly linked to the dopaminergic changes that we have observed in the NAc of food restricted rats during the heroin-seeking test (D'Cunha et al., 2017). Future studies should investigate this putative link between leptin and ghrelin in the VTA and downstream regulation of DA in the NAc in chronically food restricted rats.

An important consideration when interpreting the current results is that the ghrelin receptor, GHS-R1a, has constitutive activity (Holst, Cygankiewicz, Jensen, Ankersen, &

Schwartz, 2003; Holst & Schwartz, 2004). The physiological role of this activity remains unclear, although it is hypothesized to be related to ligand-independent release of growth hormone (Holst & Schwartz, 2004). One option to ensure that our results are mediated by interfering with the action of ghrelin on its receptor and not by blocking the constitutive activity of GHS-R1a would be to block ghrelin from activating the receptor by preventing the conversion to its active form. This approach has been successfully implemented, using a ghrelin-O-acyltransferase (GOAT) inhibitor, which prevented the addition of the acyl functional group (Abizaid & Horvath, 2012). However, ghrelin acylation occurs in the gut and this technique would not be able to dissociate the central role of ghrelin from its peripheral effects. Importantly, recent evidence suggests that the GHS-R1a receptor antagonist that we used, JMV 2959, does not affect growth hormone secretion, yet effectively reduces ghrelin-induced food intake (M'Kadmi et al., 2015). These findings suggest dissociated pathways that are involved in ghrelin actions on its receptor and the constitutive activity of the GHS-R1a (M'Kadmi et al., 2015). Lastly, we did not observe an effect of intra-VTA JMV 2959 administration on heroin seeking in sated rats, suggesting that the constitutive activity of GHS-R1a in the VTA has no role in heroin seeking.

Conclusion

Surprisingly, central administration of leptin did not significantly attenuate heroin seeking induced by chronic food restriction. However, intra-VTA leptin selectively reduced heroin seeking in chronically food restricted rats. Similarly, intra-VTA administration of the ghrelin receptor antagonist JMV 2959, also reduced heroin seeking in only the FDR rats. Taken together, these results indicate that chronic food restriction alters leptin and ghrelin transmission in the VTA, which then modify downstream activity in the mesolimbic dopamine pathway. Future studies will directly address if changes in leptin and ghrelin transmission modulate DA transmission in the NAc, resulting in food restriction-induced augmentation of heroin seeking.

CHAPTER 5: THE ROLE OF GLUTAMATE IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

ABSTRACT

Caloric restriction and limited food intake increases the risk of relapse during drug abstinence. Our laboratory has demonstrated a similar finding in an animal model of relapse, where chronic food restriction augments heroin seeking following prolonged withdrawal in rats that previously self-administered heroin. Moreover, food-restricted (FDR) rats have elevated extracellular dopamine (DA) levels in the nucleus accumbens (NAc), and administration of a DA D1 receptor antagonist into the NAc decreased heroin seeking in the FDR rats. The NAc receives a multitude of glutamatergic innervations from a variety of brain regions, and dopamine can modulate glutamatergic activity in the NAc. Therefore, in this study we investigated extracellular levels of glutamate in the NAc subregions of the shell and core during ongoing heroin seeking. Next, we investigated the role of AMPA receptors in the augmentation of heroin seeking induced by chronic food restriction. First, rats were trained to self-administer heroin for 10 days. Rats were then moved to the animal colony for 14 days of drug withdrawal. During this time some rats were given unrestricted access to food and others were subjected to a mild chronic food restriction until they reached approximately 90% of their original body weight. On the 14th day of food restriction rats were returned to the operant conditioning chambers for a heroin-seeking test under extinction conditions. Extracellular glutamate in the NAc was assessed using *in vivo* microdialysis during baseline conditions and during the 3h heroin-seeking test. Food restriction significantly augmented heroin seeking compared to the sated rats. There were no significant changes in extracellular glutamate levels between the FDR and sated rats. Bilateral administration of the AMPA receptor antagonist NBQX (0.0, 0.3, 1.0 μg/side) into either the NAc shell or core had no statistically significant effect on heroin seeking, however it may have reduced heroin seeking in the sated rats, particularly following intra-NAc core injections. Taken together, these results suggest that glutamate transmission in the NAc through AMPA receptors is not involved in the augmentation of heroin seeking induced by chronic food restriction. Future studies will investigate if NMDA or metabotropic glutamate receptors play a role in food-restriction induced augmentation of heroin seeking.

Keywords: heroin, self-administration, food restriction, glutamate, AMPA receptor, nucleus accumbens

INTRODUCTION

Even after decades of drug abstinence, over a quarter of heroin addicts relapsed back to drug use (Hser et al., 2001). Dependent drug users cycle through phases of drug use, abstinence, and relapse (O'Brien, 1997; O'Brien & McLellan, 1996), with relapse being the biggest obstacle to overcome. Exposure to the self-administered drug (de Wit, 1996), cues associated with drug availability (Carter & Tiffany, 1999; Childress et al., 1993), and stress (Sinha, 2001) are the major factors that trigger drug craving and subsequent relapse. These three triggers are also modulated by dietary manipulations such as caloric restriction.

Caloric restriction, or food restriction, increases both drug craving and drug seeking, leading to eventual relapse to drug use. For example, abstinent smokers undergoing a concurrent dietary program under caloric restriction reported higher rates of drug craving and relapse compared to non-restricted subjects (Hall et al., 1992). Similar findings on the effects of caloric restriction in animal models of relapse have also been validated. We have demonstrated that chronic food restriction during drug withdrawal will augment heroin seeking in rats with a history of heroin self-administration (D'Cunha et al., 2013).

Recently, we have established that dopamine (DA) in the nucleus accumbens (NAc), a terminal target region of the mesolimbic pathway, is critically involved in the augmentation of heroin seeking induced by chronic food restriction. Extracellular DA levels in both the NAc subregions, the shell and core, were significantly elevated during heroin seeking in the food restricted (FDR) rats (D'Cunha et al., 2017). Furthermore, administration of the DA D1 receptor antagonist SCH 39166 into the NAc shell generally decreased heroin seeking, whereas administration into the NAc core selectively decreased heroin seeking in the FDR rats. These results suggest that activation of the DA D1-like receptors in the NAc core is important for food restriction-induced augmentation of heroin seeking.

In addition to the DAergic afferents into the NAc, the NAc receives a multitude of glutamatergic afferents from a variety of brain regions (Brog et al., 1993). Previous work has demonstrated that these dopaminergic inputs can interact with glutamatergic activity in the NAc (Yagishita et al., 2014). Therefore, it is possible that glutamatergic and dopaminergic inputs into the NAc may interact to mediate heroin seeking.

There is also ample evidence that glutamatergic transmission in the mesolimbic circuit, specifically the NAc, is critically involved in drug seeking. For example, presentation of an odor

previously paired with cocaine availability increased extracellular glutamate levels in both the NAc core and shell (Suto et al., 2013), and extracellular glutamate concentrations were also elevated in the NAc core during cue-induced reinstatement of heroin seeking (LaLumiere & Kalivas, 2008). Moreover, reducing evoked glutamate release in both the NAc core and shell significantly attenuated context-induced reinstatement of heroin seeking (Bossert, Gray, Lu, & Shaham, 2006). Taken together, these findings suggest that glutamate transmission in the NAc may be involved in the augmentation of heroin seeking induced by chronic food restriction.

To test this hypothesis, we first measured fluctuations in extracellular levels of glutamate in the NAc core and shell during ongoing heroin seeking in both FDR and sated rats. Next, to assess the role of glutamate receptors in the NAc core and shell, we administered the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist NBQX prior to the test for heroin seeking.

MATERIALS AND METHODS

Subjects

Male Long Evans rats (n = 160; Charles River, Raleigh, North Carolina, USA) weighing between 325 – 350 g upon arrival were used in four different experiments. Rats were pair-housed in clear Plexiglass cages and allowed one week to habituate to the animal colony. Rats were kept under reverse 12 h light-dark cycle (lights OFF 9:30am) at 21°C. Following recovery from surgery (described below) rats were individually housed in operant conditioning chambers with *ab libitum* access to food and water during heroin self-administration.

At the completion of heroin self-administration training, rats were returned to the animal colony for the withdrawal and food restriction phase (described below). Prior to *in vivo* microdialysis sampling, rats were housed in clear Plexiglass chambers with a grid floor for baseline sampling prior to the heroin-seeking test (described below). Following baseline microdialysis collection rats were transferred back to the operant conditioning chambers for the heroin-seeking test. Rats in the experiments with the AMPA receptor antagonist were brought to the operant conditioning chamber for the heroin-seeking test.

Surgical procedures

Intravenous surgery

Rats were implanted with an intravenous catheter under ketamine and xylazine (90.0 and 13.0 mg/kg, ip) anesthesia, as previously described (D'Cunha et al., 2013). Silastic catheters (Dow Corning, Midland, MI, USA) were inserted into the right jugular vein and secured in place with silk sutures. The other end of the catheter was subcutaneously guided to the rat's head where it was attached to a modified 22-gauge cannula (Plastics One, Roanoke, VA) which was mounted to the rat's skull using jeweller's screws and dental cement. Following surgery rats were given penicillin (450 000 IU/rat, sc) and the analgesic ketoprofen (5.0 mg/kg, sc; CDMV, Quebec, Canada). Throughout heroin self-administration training rats were flushed daily with heparin and gentamicin in sterile saline (7.5 IU + 12.0 µg per day per rat) to prevent catheter blockage.

Intracranial surgery

For the microdialysis experiments, rats were also implanted with a unilateral guide cannula targeting one of the following regions (coordinates relative to Bregma): nucleus accumbens (NAc) shell AP: +1.6 mm, ML: ±1.5 mm, DV: -5.0 mm (Experiment 1); NAc core AP: +1.6 mm, ML: ±1.0 mm, DV: -4.5 mm (Experiment 2). Cannulae were mounted adjacent to the modified catheter cannula on the skull using jeweller's screws and dental cement. Cannula placements were counterbalanced between right and left hemispheres.

For the AMPA receptor antagonist experiments, in addition to the intravenous catheter, rats were implanted with bilateral guide cannula targeting one of the following regions (coordinates relative to Bregma): NAc shell AP: +1.7 mm, ML: ±3.7 mm, DV: -4.8 mm, 20° angle (Experiment 3); or NAc core AP: +1.8 mm, ML: ±2.5 mm, DV: -4.5 mm, 6° angle.

Apparatus

Operant conditioning chambers (Med Associates Inc., St. Albans, VT or Coulbourn Instruments, Holliston, MA) were used for heroin self-administration training and the heroin-seeking tests. Operant conditioning chambers were located within a sound-attenuating cubicle, and each chamber contained two levers located 5.0 cm above the grid floor. Responses on the 'active' lever activated the infusion pump (Med Associates). Conversely, responses on the

'inactive' lever were recorded but had no programmed consequences. Prior to *in vivo* microdialysis baseline sampling rats were habituated to a neutral clear Plexiglass chamber (30 × 28 × 25 cm) with a grid floor in the animal facility.

Drugs

Heroin (diacetylmorphine HCl; National Institute on Drug Abuse, Baltimore, MD, USA) was dissolved in physiological saline and delivered at a concentration of 0.1 mg/kg per infusion. The AMPA receptor antagonist NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide) disodium salt (Abcam, Cambridge, MA, USA) was diluted in sterile saline to produce the following doses: 0.0, 0.3, 1.0 µg/side. These doses were previously shown to reduce cue-induced alcohol seeking (Sciascia, Reese, Janak, & Chaudhri, 2015).

Procedure

Experiments consisted of 3 main phases: heroin self-administration training in the operant conditioning chambers, withdrawal and food restriction in shoebox cages in the animal facility, and a heroin-seeking test in the operant conditioning chambers.

Training

Rats were habituated to the operant conditioning chambers for 24 h prior to the 10 days of heroin self-administration training. Rats underwent 3-3 h sessions of self-administration separated by a 3 h period under a fixed interval (FI) 20 s schedule of reinforcement. The initiation of the self-administration session began with the onset of the dark phase and the insertion of the active lever, illumination of the houselight, and activation of a compound tone (2.9 kHz, 10 dB above background noise) and light cue above the active lever, that remained on for 30 s or until the active lever was pressed. Responses on the active lever resulted in the delivery of 0.1 mg/kg infusion of heroin in a volume of 0.13 ml over 12 s. Once the active lever was pressed it resulted in the initiation of the tone-light cue for 20 s and the houselight was turned off. Any additional responses on the active lever during that 20-s interval were recorded but not reinforced. At the end of each 3 h session, the active lever was retracted and the houselight was turned off.

Withdrawal and Food Restriction

Following heroin self-administration training, rats were transferred back to the animal facility for 24 h of drug wash-out. Rats were matched for body weight, infusions, and active lever presses over the last 5 days of training and assigned to either the food restricted (FDR) or sated group. FDR rats were given approximately 15.0 g of rat chow daily at 1:30 pm and the food ration was adjusted daily to bring the FDR group to approximately 90% of their body weight at the start of the withdrawal period.

In vivo Microdialysis and Heroin-Seeking Test (Experiments 1 & 2)

Two days prior to the heroin-seeking test, on the 12th day of food restriction, rats were habituated to a neutral clear Plexiglass chamber. Approximately 14 h prior to testing, on the 13th day of food restriction, microdialysis probes were lowered into the targeted brain region under light isoflurane anesthesia. Probes were made in the laboratory and had either a 2.0 mm (Experiment 1) or 1.5 mm (Experiment 2) semipermeable active membrane. To prevent occlusion, probes were continually perfused with artificial cerebrospinal fluid (aCSF; 145 mM NA^{+}, 2.7 mM K^{+}, 1.22 mM Ca^{2+}, 1.0 mM Mg^{2+}, 150 mM Cl^{-}, 2 mM Na_2PO_4, pH 7.4 ± 0.1) at 1.0 µl/min for 1 h. The flow rate was then lowered to 0.2 µl/min overnight and returned to 1.0 µl/min on the 14th day of food restriction approximately 1 h prior to baseline sample collection.

At 8:10 am baseline dialysate sample collection began, and samples were collected every 10 min thereafter. At 9:30 am rats were transported in the clear Plexiglass chambers from the animal facility to the heroin self-administration room. Microdialysis pumps were plugged into a battery pack on a cart to ensure flow rate and sampling was uninterrupted. Rats were transferred to the operant conditioning chambers for the heroin-seeking test. After the move from the neutral chamber to the operant box, one dialysate sample was collected (corresponding to the context change) prior to the initiation of the testing session. Testing took place under extinction conditions over the 3 h session, and dialysate samples were collected in 10 min intervals. Active lever responses resulted in the same consequences as training with the exception that no heroin infusions occurred.

Intracranial Injections and Heroin-Seeking Test (Experiments 3 & 4)

Intracranial injections were made using a syringe pump (Harvard Apparatus, Holliston, MA, USA) connected to a 10 µl Hamilton syringe. The syringe was attached via polyethylene-20

tubing to a 28-gauge injector (Plastics One) that extended either 2.0 mm beyond the guide cannula. For all intracranial injections, NBQX was delivered in a volume of 0.5 µl over 1 min and injectors remained in place for 1 min following the injection. Two days prior to the heroin-seeking test, rats received mock injections to habituate them to the injection procedure. For mock injections, the syringe pump was run for 1 min and short injectors that did not extend beyond the tip of the guide cannulae were inserted. No solution was administered during mock injections to minimize tissue damage in the target regions. Rats were bilaterally administered the AMPA receptor antagonist, NBQX (0.0, 0.3, 1.0 µg/side), approximately 10 min prior to being placed in the operant conditioning chambers for the heroin-seeking test. Testing took place under extinction conditions over a 3-h session, as in experiments 1 & 2.

Analytical Chemistry

Glutamate was isolated and quantified in the dialysate samples using high performance liquid chromatography (HPLC) and fluorescence detection as in Placenza, Rajabi, & Stewart, (2008). Briefly, dialysate samples were derivatized with o-phthaladehyde and loaded through a manual injection port (Rheodyne 7125; Rheodyne LLC, Rhonert Park, CA; 20 µl loop). A C-18 reverse phase column (5.0 µm, 15.0 cm, 4.0 mm ID, Higgins Analytical) was used to separate components in the sample. Glutamate was detected using Ultrafluor (Lab Alliance, Fisher Scientific, Montreal, Quebec, Canada) with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Water 515 HPLC pumps were used to circulate the phosphate buffer mobile phase at a rate of 1.1 ml/min through the HPLC system. After each sample the column was flushed with a 40% acetonitrile wash buffer for 2 min. Peaks were integrated and quantified by the EZ Chrom Chromatography Data System (Scientific Software Inc., San Ramon, CA, USA).

Histology

At the end of the experiments rats were euthanized with carbon dioxide gas and decapitated. Brains were collected and fixed in 4% paraformaldehyde solution for one week before being sliced in 40.0 µm coronal sections. Sections were mounted and stained with cresyl violet. Cannula and probe locations were determined under a microscope with reference to a brain atlas (Paxinos & Watson, 2005).

Statistical Analysis

For the microdialysis experiments 1 & 2, active and inactive lever responses were analyzed separately using a mixed factorial analysis of variance (ANOVA) with the between subjects factor of *food restriction* (FDR, Sated) and the within subjects factor of *time* (10 min bins of responses). To assess the effects of food restriction on glutamate transmission, baseline levels of glutamate were determined by averaging 3 samples collected prior to the move to the operant chamber for each rat, and then converting the values of all samples to a percentage of baseline. Changes from baseline were analyzed separately for each experiment using a mixed factorial ANOVA with the between subjects factor of *food restriction* (FDR, Sated) and the within subjects factor of *time* (baseline average, context change, and test samples 1 – 18). Statistically significant main effects and interactions were followed by post-hoc analyses with Bonferroni corrections. Significant results are reported for $p \leq 0.05$.

To access the effects of NBQX on the augmentation of heroin seeking induced by chronic food restriction, active and inactive lever responses were analyzed separately using a univariate ANOVA with the between subjects factors of *food restriction* (FDR, Sated) and *NBQX dose* (0.0, 0.3, 1.0 μg/side). Statistically significant main effects and interactions were followed by post-hoc analyses with Bonferroni corrections. Statistically significant results are reported for $p \leq 0.05$.

RESULTS

For experiments 1 & 2, 40 rats were trained, but rats were excluded if they pulled out probes during the test session, or if there were technical problems with the analysis of the dialysate samples in the HPLC. In addition, only rats with correct histological placements were included in the final analysis (Figure 5.1), which left a total of 32 rats in experiments 1 & 2. For experiments 3 & 4, 120 rats were trained but only 102 had correct histological placements and were included in the final analysis (Figure 5.1). All rats acquired reliable heroin self-administration (Table 5.1). In all experiments the FDR rats were approximately 90% of their body weight at the start of the withdrawal place or approximately 75% of the sated rats' body weight (Table 5.1).

Experiment 1: Changes in extracellular glutamate in the NAc shell

Fourteen days of chronic food restriction resulted in the augmentation of heroin seeking. FDR rats (n = 8) made significantly more responses on the active lever compared to the sated

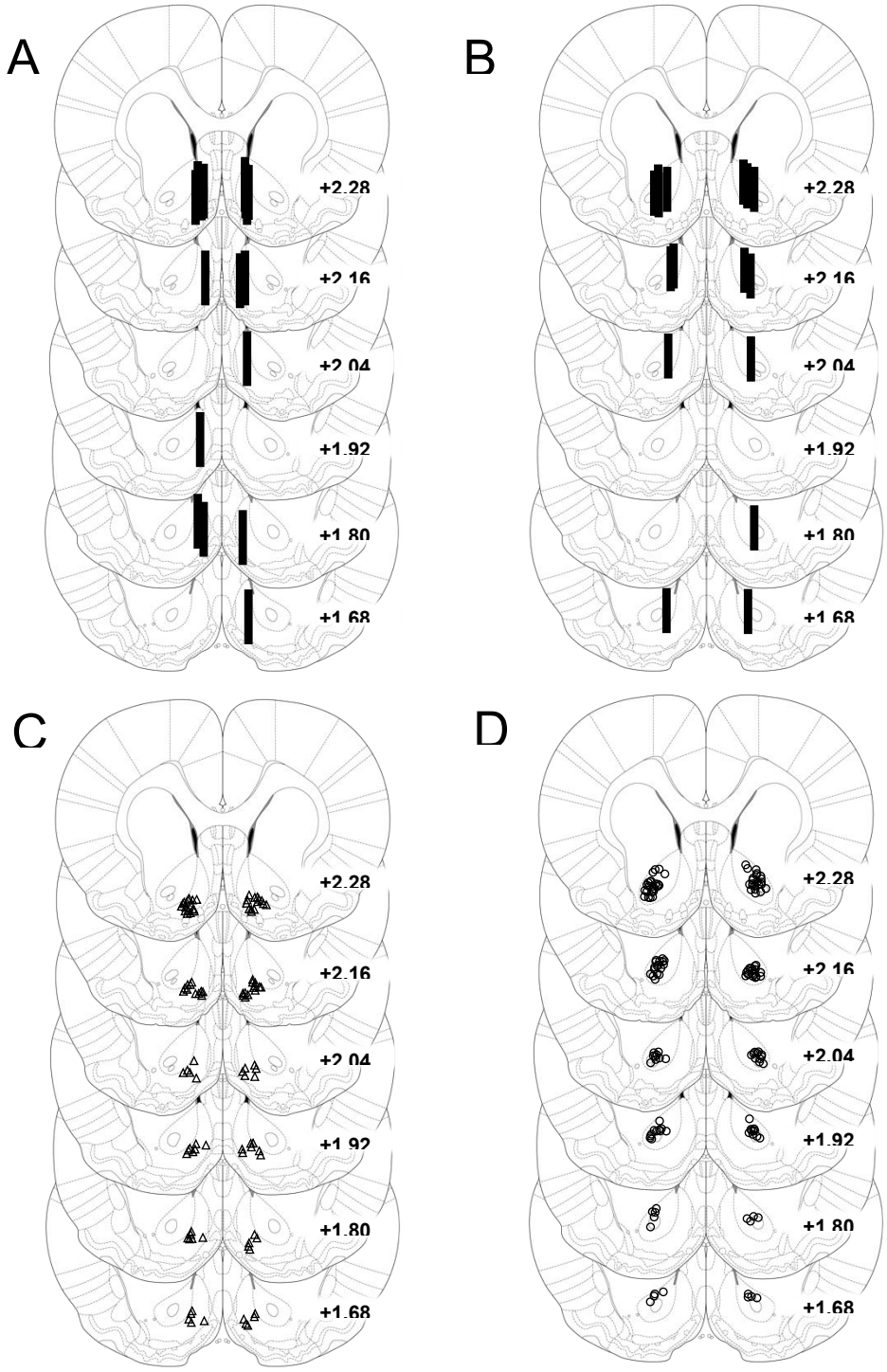

Figure 5.1. Cannula placements for all experiments. Approximate locations of active region of microdialysis probe targeting the NAc shell (A) for Experiment 1 (n = 17; black rectangles), or the NAc core (B) for Experiment 2 (n = 16; black rectangles). Approximate anatomical position for microinjector tips targeting the NAc shell (C) for Experiment 3 (n = 50; open triangles) or NAc core (D) for Experiment 4 (n = 52; open circles). Images modified from the brain atlas of Paxinos and Watson (2005) of Figures 14 – 19 (+1.68 to +2.28 mm anterior to Bregma).

Table 5.1. Mean ± SEM of the number of infusions taken, and the number of active and inactive lever responses made on the last training day (9 h) in each experiment, as well as body weight for the FDR and sated rats on the 14th day of food restriction (the drug seeking test).

	Mean ± SEM				
	Self-administration training day 10			Food-restriction day 14	
Experiment	Infusions	Active lever responses	Inactive lever responses	Body weight (g) (FDR)	Body weight (g) (Sated)
1	34.77 ± 2.18	86.12 ± 13.34	9.59 ± 3.90	310.88 ± 6.87	402.67 ± 4.54
2	55.06 ± 12.90	224.06 ± 96.84	15.19 ± 7.60	301.00 ± 4.86	456.29 ± 15.22
3	48.48 ± 3.90	175.76 ± 30.34	19.52 ± 5.43	325.96 ± 3.08	421.39 ± 8.79
4	43.39 ± 3.37	110.50 ± 14.62	10.73 ± 1.45	331.54 ± 2.77	456.23 ± 5.44

rats (n = 9; Figure 5.2A inset). Active lever responses recorded in 10 min time bins were higher in the FDR rats (*feeding condition*: $F_{(1,15)} = 6.104$, $p = 0.026$, $\eta^2 = 0.289$; Figure 5.2A). There was also a statistically significant effect of *time* ($F_{(17,255)} = 11.240$, $p < 0.001$, $\eta^2 = 0.373$) and a statistically significant interaction of *feeding condition* × *time* ($F_{(17,255)} = 3.886$, $p = 0.003$, $\eta^2 = 0.129$). There were no statistically significant differences between groups on responses made on the inactive lever (Figure 5.2A inset).

There were no statistically significant differences in absolute baseline dialysate concentrations of glutamate (Table 5.2). Chronic food restriction had no effect on extracellular glutamate during the heroin-seeking test (*feeding condition*: $F_{(1,15)} = 0.226$, $p = 0.614$, $\eta^2 = 0.015$; Figure 5.2B). Furthermore, there were no statistically significant effects for *time* ($F_{(19,285)} = 1.211$, $p = 0.267$, $\eta^2 = 0.071$) or *feeding condition* × *time* ($F_{(19,285)} = 0.732$, $p = 0.746$, $\eta^2 = 0.043$).

Experiment 2: Changes in extracellular glutamate in the NAc core

Like experiment 1, FDR rats (n = 9) displayed higher levels of heroin seeking, assessed by active lever responses, compared to the sated rats (n = 7; Figure 5.3A inset). Active lever responses recorded in 10 min bins were higher in the FDR rats compared to the sated rats (*feeding condition*: $F_{(1,14)} = 20.024$, $p = 0.001$, $\eta^2 = 0.589$; Figure 5.3A). There was also a statistically significant main effect of *time* ($F_{(17,238)} = 14.182$, $p < 0.001$, $\eta^2 = 0.434$) and a statistically significant interaction of *feeding condition* × *time* ($F_{(17, 238)} = 4.469$, $p = 0.005$, $\eta^2 = 0.137$). There were no statistically significant differences between groups on responses made on the inactive lever (Figure 5.3A inset).

Absolute baseline dialysate levels of glutamate were not statistically significantly different between the FDR and sated rats (Table 5.2). There was no significant effect of food restriction on extracellular glutamate levels during the heroin-seeking test (*feeding condition*: $F_{(1,13)} = 0.305$, $p = 0.590$, $\eta^2 = 0.023$; Figure 5.3B). Moreover, there were no statistically significant effects of *time* ($F_{(19,247)} = 0.257$, $p = 0.999$, $\eta^2 = 0.0194$) or interaction of *feeding condition* × *time* ($F_{(19, 247)} = 0.360$, $p = 0.994$, $\eta^2 = 0.026$).

Experiment 3: Administration of AMPA receptor antagonist, NBQX, into the NAc shell

The final analysis included the following 6 groups: FDR – 0.0 µg (n = 10), FDR – 0.3 µg (n = 9), FDR – 1.0 µg (n = 8), Sated – 0.0 µg (n = 8), Sated – 0.3 µg (n = 8), Sated – 1.0 µg (n =

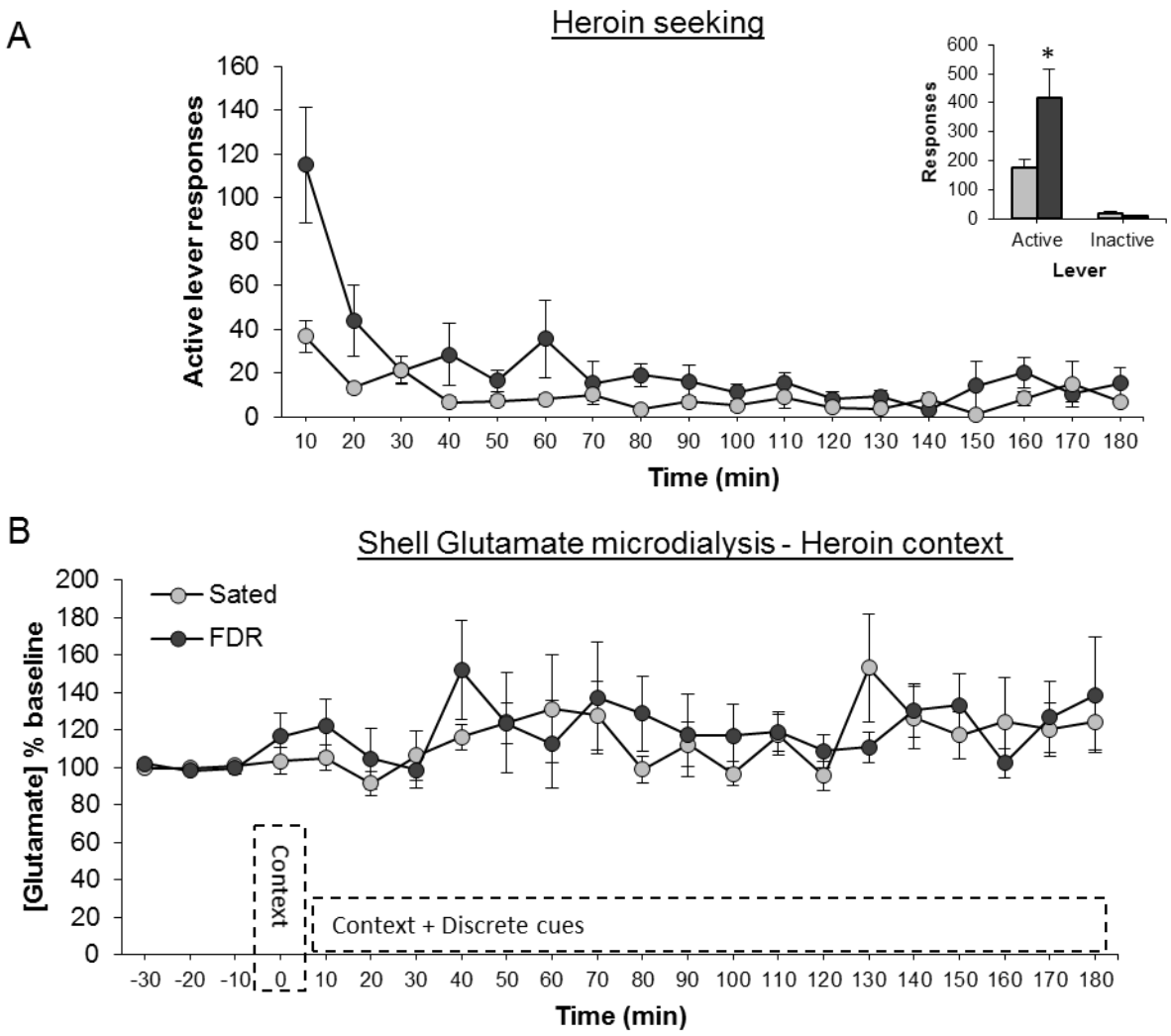

Figure 5.2. Chronic food restriction-induced augmentation of heroin seeking and extracellular glutamate in the NAc shell. (A) Total number of active and inactive lever responses for FDR (n = 8) and sated (n = 9) rats (inset), and active lever responses in 10-min time intervals during the 3 h heroin-seeking test in Experiment 1, *p = 0.026 compared with sated rats. (B) Extracellular glutamate following re-exposure to the drug environment and during the heroin-seeking test in the FDR and sated rats.

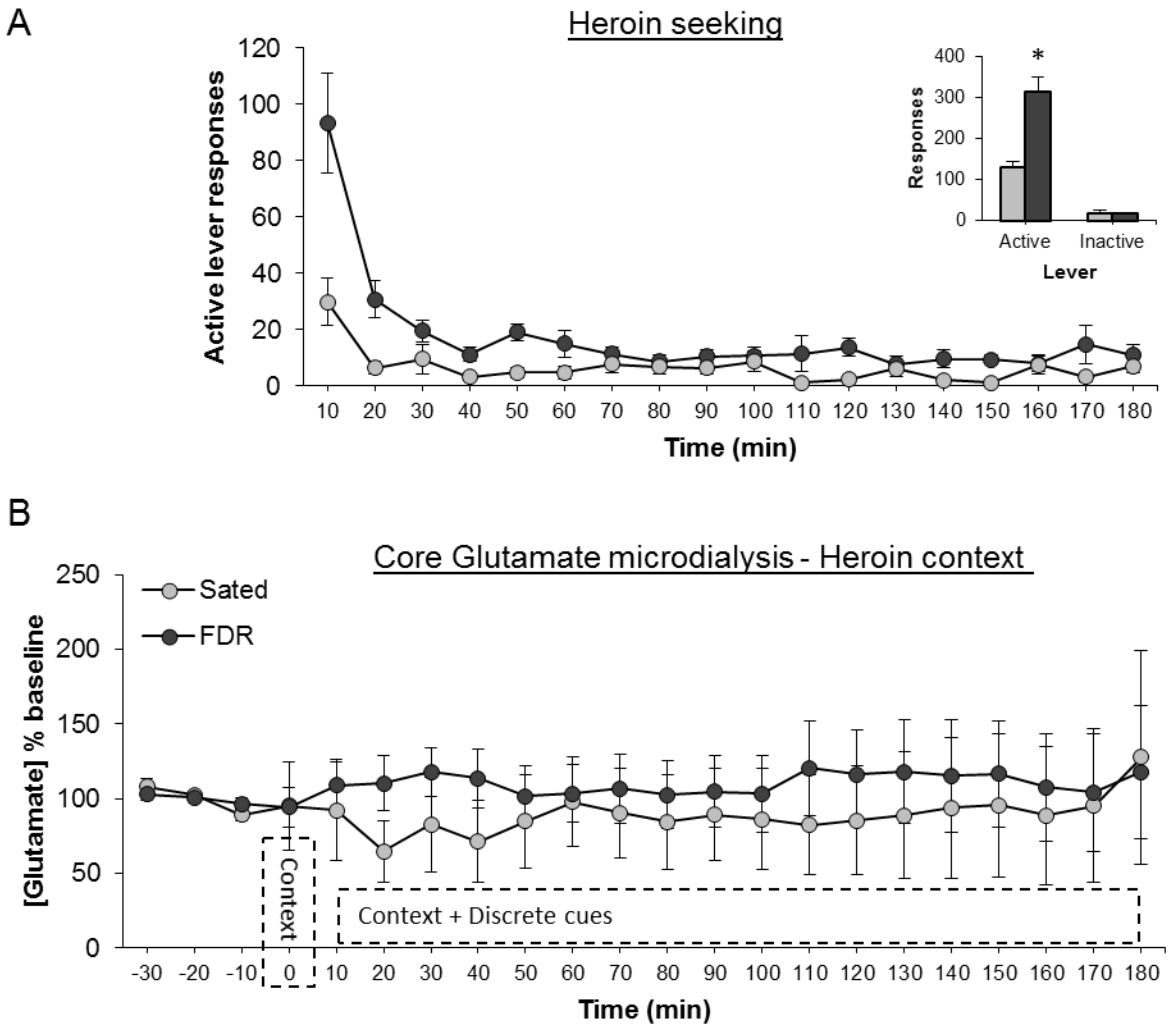

Figure 5.3. Chronic food restriction-induced augmentation of heroin seeking and extracellular glutamate in the NAc core. (A) Total number of active and inactive lever responses for FDR (n = 9) and sated (n = 7) rats (inset), and active lever responses in 10-min time intervals during the 3 h heroin-seeking test in Experiment 2, **p* = 0.001 compared with sated rats. (B) Extracellular glutamate levels following re-exposure to the drug environment and during the heroin-seeking test in the FDR and sated rats.

Table 5.2. Mean ± SEM of the absolute concentrations of baseline glutamate levels of the FDR and sated rats.

	Mean ± SEM absolute [Glutamate] in pg/µl		Statistics
Experiment	FDR	Sated	*t-test* & Cohen's *d* effect size
1	31.21 ± 2.69	42.42 ± 9.14	t (15) = -1.115, p = 0.282, d = -0.58
2	101.74 ± 76.46	185.98 ± 191.71	t (14) = -1.210, p = 0.246, d = -4.88

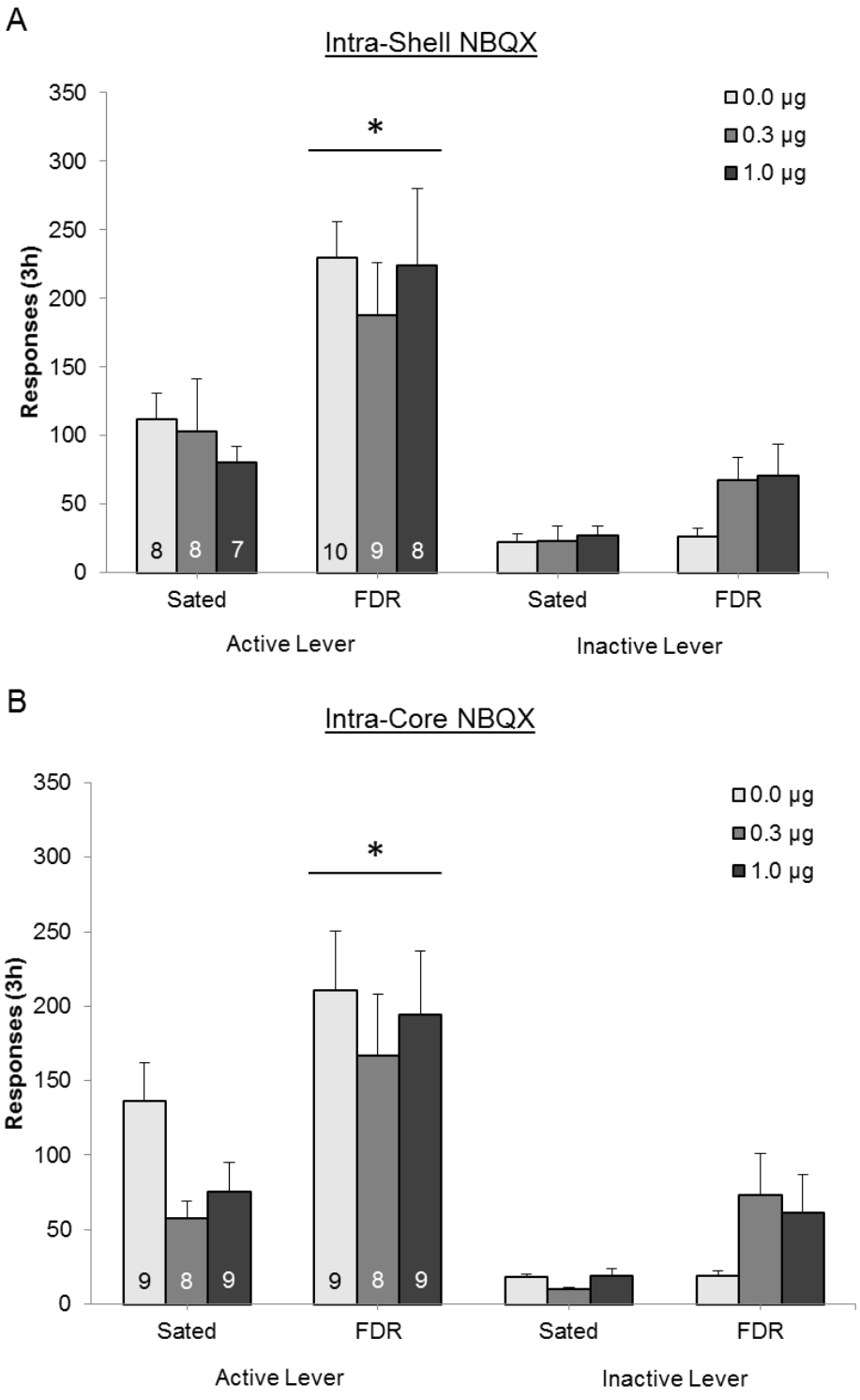

Figure 5.4. The effect of injections of the glutamate AMPA receptor antagonist NBQX into the NAc shell (A) or NAc core (B) on the augmentation of heroin-seeking induced by chronic food restriction, $*p < 0.001$ compared to the sated groups.

7). Overall, the FDR groups responded more on the active lever during the 3-h heroin-seeking test compared with the sated groups (*feeding condition*: $F_{(1,44)} = 15.819$, $p < 0.001$, $\eta^2 = 0.259$; Figure 5.4A). Administration of AMPA receptor antagonist, NBQX, into the NAc shell had no effect on heroin seeking (*NBQX dose*: $F_{(2,44)} = 0.292$, $p = 0.748$, $\eta^2 = 0.010$). Moreover, there was no statistically significant interaction of *feeding condition* × *NBQX dose* ($F_{(2,44)} = 0.342$, $p = 0.712$, $\eta^2 = 0.011$). Even though the interaction of *feeding condition* × *NBQX dose* was not statistically significant, there appears to be a reduction in heroin seeking in the Sated – 1.0 μg compared to Sated – 0.0 μg rats based on a relatively large effect size, Cohen's $d = 0.725$. Surprisingly, there was a main effect of *feeding condition* on inactive lever responses during the heroin-seeking test ($F_{(1,44)} = 8.272$, $p = 0.006$, $\eta^2 = 0.139$). Overall, the FDR groups had higher inactive lever responses ($M = 54.91 \pm 7.319$) compared to the sated groups ($M = 23.91 \pm 7.912$). There were no other statistically significant effects on inactive lever responses. Although there was no significant interaction, visual examination of Figure 5.4A shows that the FDR groups that received NBQX had higher inactive lever responses compared to the FDR-vehicle group.

Experiment 4: Administration of AMPA receptor antagonist, NBQX, into the NAc core

The final analysis included the following 6 groups: FDR – 0.0 μg (n = 9), FDR – 0.3 μg (n = 8), FDR – 1.0 μg (n = 9), Sated – 0.0 μg (n = 9), Sated – 0.3 μg (n = 8), Sated – 1.0 μg (n = 9). As previously reported, the FDR groups had significantly more active lever responses during the heroin-seeking test compared with the sated groups (*feeding condition*: $F_{(1,46)} = 14.391$, $p < 0.001$, $\eta^2 = 0.223$; Figure 5.4B). Administration of NBQX into the NAc core had no statistically significant effect on heroin seeking (*NBQX dose*: $F_{(2,46)} = 1.787$, $p = 0.179$, $\eta^2 = 0.055$), and there was no significant interaction of *feeding condition* × *NBQX dose* ($F_{(2,46)} = 0.265$, $p = 0.768$, $\eta^2 = 0.008$). However, although the interaction of *feeding condition* × *NBQX dose* was not statistically significant, administration of NBQX in the NAc core of the sated rats reduced heroin seeking as evidenced by large effect sizes: Sated – 0.0 μg compared to Sated – 0.3 μg (Cohen's $d = 1.290$) and Sated – 0.0 μg compared to Sated – 1.0 μg (Cohen's $d = 0.883$). Interestingly, similar to experiment 3, there was a statistically significant main effect of *feeding condition* on inactive lever responses during the heroin-seeking test, $F_{(1,46)} = 8.199$, $p = 0.006$, $\eta^2 = 0.133$. Overall, the FDR groups had higher inactive lever responses ($M = 51.35 \pm 8.763$) compared to the sated groups ($M = 15.87 \pm 8.763$). Similar to experiment 3, although there was no significant interaction of *feeding condition* × *NBQX dose*, visual examination of Figure 5.4B shows that the

FDR groups that received the NBQX had higher inactive lever responses compared to the FDR-vehicle group. There were no other statistically significant effects on the inactive lever responses.

DISCUSSION

As previously demonstrated (D'Cunha et al., 2013), 2 weeks of chronic food restriction during drug withdrawal significantly augmented heroin seeking. However, there were no significant fluctuations in extracellular levels of glutamate in either the NAc shell or core during heroin seeking in the FDR and sated rats. Finally, administration of the AMPA receptor antagonist NBQX into the NAc shell or core had no statistically significant impact on heroin seeking in the FDR rats, but may have reduced heroin seeking in the sated rats.

Extracellular glutamate in the NAc shell and core of FDR and Sated rats

Contrary to our expectations, chronic food restriction had no effect on extracellular glutamate levels in either the NAc shell or core subregions. Furthermore, there were no changes in extracellular glutamate levels in the sated rats. This is in direct contrast to numerous other reports that demonstrated increased extracellular glutamate during drug seeking and following exposure to drug-associated cues (Hotsenpiller, Giorgetti, & Wolf, 2001; LaLumiere & Kalivas, 2008; McFarland et al., 2003; Suto et al., 2013). For example, extracellular glutamate levels are significantly elevated in the NAc core during cue-induced reinstatement of heroin seeking (LaLumiere & Kalivas, 2008). Moreover, glutamate levels in both the NAc shell and core are significantly elevated in the presence of both discrete and environmental drug-associated cues (Hotsenpiller et al., 2001; Suto et al., 2013).

The NAc receives numerous glutamatergic innervations from a variety of brain regions including the prefrontal cortex (PFC), ventral hippocampus, and medial thalamic nuclei (Brog et al., 1993). Extracellular glutamate levels increased during cocaine-primed reinstatement and inhibiting the activation of the glutamate projection from the PFC blocks this rise in glutamate (McFarland et al., 2003). A leading hypothesis is that there is impaired communication from these glutamatergic inputs from the PFC (a region critical to executive function) to the NAc, which is why drug addicted individuals are unable to effectively regulate drug seeking (Chen et al., 2013; Kalivas, 2009).

Kalivas (2009) proposes that glutamate homeostasis in the NAc is critical to maintaining the correct levels of glutamate in the synaptic cleft, and prolonged drug use leads to alterations in the homeostatic mechanisms maintaining adequate glutamate levels. Heroin self-administration followed by extinction training does in fact result in a reduced ability for the NAc to efficiently eliminate glutamate from the synaptic cleft via the glutamate transporter, GLT-1 (Shen, Scofield, Boger, Hensley, & Kalivas, 2014b). This excess of glutamate in the synaptic cleft can result in increased stimulation of post-synaptic receptors, such as AMPA, which results in synaptic potentiation, eventually leading to drug craving and relapse (Shen et al., 2014b; Wolf, 2010).

The major difference between these studies and ours that may account for the discrepancy in findings is the animal model of relapse that was used. We investigated heroin seeking following a period of withdrawal whereas most of the aforementioned studies investigated drug seeking following extinction training. Previous work indicates that the neural mechanisms mediating drug seeking following a withdrawal period versus extinction training are distinct (Fuchs et al., 2006). Therefore, the changes in the ability of GLT-1 to remove glutamate from the synaptic cleft of the NAc may only be altered following extinction training. Although our heroin-seeking test is also an extinction session, these changes to glutamate homeostasis in the NAc may be a neuroadaptation that develops over time with repeated extinction sessions. Thus, a probable explanation for the lack of observed changes in extracellular glutamate during ongoing heroin seeking is that our procedure does not lead to the same neuroadaptations seen with extinction training. As a result, we suggest that changes in tonic glutamate levels in the NAc, do not mediate the augmentation of heroin seeking induced by chronic food restriction.

Effects of Intra-NAc Shell and Core Injections of NBQX on Heroin Seeking in FDR and Sated Rats

Administration of the AMPA receptor (AMPAR) antagonist NBQX into the NAc shell and core had no statistically significant effect on the augmentation of heroin seeking induced by chronic food restriction. However, relatively large effect sizes suggest that NBQX administration into the NAc core and possibly the shell reduced heroin seeking in the sated rats. These results complement numerous other reports that blockade of AMPAR in the NAc decreased drug seeking (Cornish & Kalivas, 2000; Di Ciano & Everitt, 2001; LaLumiere & Kalivas, 2008). The reduction in heroin seeking seen in the sated rats directly parallels evidence that administration of the AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; which is similar to

NBQX) into the NAc core reduced cue-induced reinstatement of heroin seeking (LaLumiere & Kalivas, 2008). This evidence corresponds with the finding that drug self-administration and withdrawal results in the upregulation of AMPAR on medium spiny neurons (MSNs) in the NAc (Wolf, 2010). In the early phase of drug withdrawal following cocaine self-administration, AMPAR are initially downregulated. However, over the period of prolonged drug withdrawal levels of synaptic AMPARs in the NAc shell and core are significantly increased (Conrad et al., 2008). In particular, there is an upregulation of GluA2-lacking AMPARs, which are calcium permeable, in the NAc following long access to cocaine self-administration and withdrawal (Loweth, Tseng, & Wolf, 2014). Incorporation of these receptors into the post-synaptic membrane can strengthen synapses and alter post-synaptic signalling by increasing the responsiveness of MSNs to incoming glutamatergic inputs (Loweth et al., 2014).

The increase in AMPAR in the NAc following prolonged drug withdrawal is also demonstrated following chronic food restriction. Like prolonged withdrawal from cocaine, chronic food restriction increased GluA2-lacking calcium-permeable AMPARs in the NAc (Ouyang et al., 2017). Specifically, food restriction increased the surface expression of GluA1 subunits and increased phosphorylation at the Ser845 site on the GluA1 subunit, which is thought to facilitate the trafficking of GluA1 to the post-synaptic density of the MSNs in the NAc. In addition, administration of Naspm, a specific antagonist for calcium-permeable AMPARs decreased the amplitude of excitatory post-synaptic potentials in the NAc shell. Naspm also suppressed the enhanced locomotion induced by administration of the DA D1R agonist SKF-82958 in food restricted, but not sated, rats. Consequently, synaptic incorporation of these calcium-permeable AMPARs in the D1-type MSNs of the NAc may increase the responsiveness of food restricted rats to both natural rewards and drugs of abuse (Carr, 2016; Jung et al., 2016; Ouyang et al., 2017; Zheng, Cabeza de Vaca, Jurkowski, & Carr, 2015).

There are several possible explanations for the finding that blockade of AMPARs in the NAc had no effect on the augmentation of heroin seeking induced by chronic food restriction. First, since both drug withdrawal (Conrad et al., 2008; Loweth et al., 2014; Wolf, 2010) and chronic food restriction (Carr, 2016; Ouyang et al., 2017) can increase calcium-permeable AMPARs in the NAc, the combination of heroin withdrawal and food restriction in our procedure may have resulted in a synergistic elevation of AMPARs in the NAc. As a result, perhaps the doses of NBQX used were not sufficient to saturate of the synaptic AMPARs in the

NAc and cause a reduction in heroin seeking. Our doses of NBQX were based in a previous report that demonstrated a reduction in cue-induced alcohol seeking, but the animals in that experiment were not food restricted (Sciascia et al., 2015). Future studies should perhaps try a higher dose of NBQX in the NAc to assess its effects on heroin seeking induced by chronic food restriction.

Second, as we have previously mentioned, the discrepancy between our results and the other reports may also be accounted for by the distinct procedures used to study drug seeking. Previous reports that found that blockade of AMPAR in the NAc reduced drug seeking used the reinstatement model with extinction training, which has been demonstrated to have distinct neural mechanisms that mediate it compared to a period of withdrawal (Fuchs et al., 2006). Furthermore, the majority of evidence reported above used cocaine as the training drug. Psychostimulants, like cocaine, lead to divergent neuroadaptations and drug-associated behaviors that are not always paralleled in other drug classes, such as opiates (Badiani et al., 2011). Therefore, glutamate transmission in the NAc may not mediate the behavioral outcome in our procedure because the rats in our experiments self-administered heroin and went through a withdrawal period.

In addition to AMPAR, glutamate binds to a variety of other receptor subtypes. One possibility is a role for NMDA ionotropic glutamate receptors in the augmentation of heroin seeking induced by chronic food restriction. Previous research does suggest that NMDARs are involved in opiate seeking (Ma, Chu, Guo, Han, & Cui, 2007; Shen, Moussawi, Zhou, Toda, & Kalivas, 2011; Shen et al., 2014a). For example, morphine-primed reinstatement of CPP is associated with increased levels of the NMDAR 2B (NR2B) subunit in the NAc, and administration of a selective NR2B antagonist attenuated the reinstatement of CPP induced by a priming injection of morphine (Ma et al., 2007). Similarly, heroin self-administration results in an upregulation of NR2B-containing NMDARs in the NAc core, and blocking these receptors prevented cue-induced heroin seeking (Shen et al., 2011). However, these findings are reported in procedures that have been shown to have distinct neuroadaptations compared to the one that was used in our experiments (Fuchs et al., 2006; Weise-Kelly & Siegel, 2001).

Finally, metabotropic glutamate receptors may also play a role in the augmentation of heroin seeking induced by chronic food restriction. Blockade of the group I metabotropic glutamate receptor, $mGluR_5$ in the NAc has been shown to attenuate both cue-induced cocaine

and heroin seeking (Lou, Chen, Liu, Ruan, & Zhou, 2014; Wang, Moussawi, Knackstedt, Shen, & Kalivas, 2013). In a procedure similar to ours, administration of an mGluR5 antagonist into the NAc shell dose-dependently reduced cue-induced heroin seeking following 2 weeks of withdrawal after heroin self-administration (Lou et al., 2014). Furthermore, administration of the group II metabotropic glutamate receptors ($mGluR_{2/3}$) agonist LY379268 into the NAc shell and core decreased context-induced reinstatement of heroin seeking (Bossert et al., 2006). Activation of $mGluR_{2/3}$ in the NAc decreases DA release, and we have previously demonstrated that DA transmission in the NAc is involved in heroin seeking induced by chronic food restriction (D'Cunha et al., 2017). However, the activation of $mGluR_{2/3}$ decreased glutamate levels as assessed by microdialysis, and as mentioned before we did not find that extracellular glutamate levels were altered during heroin seeking in either the FDR or sated rats, suggesting that these receptors may not play a critical role in food restriction-induced augmentation of heroin seeking.

Conclusion

Contrary to many other reports, we found no changes in extracellular glutamate levels in the NAc shell and core in either the FDR or sated rats. Furthermore, there are no indications that chronic food restriction had an effect on the function of post-synaptic AMPA receptors in the NAc, as administration of the AMPA receptor antagonist NBQX had no effect on heroin seeking in the FDR rats. Although it was not statistically significant, relatively large effect sizes suggest that in agreement with other reports, NBQX administered in the NAc core and possibly the NAc shell reduced heroin seeking in the sated rats. In conclusion, our results suggest that glutamate neurotransmission through AMPA receptors in the NAc are not involved in the augmentation of heroin seeking induced by chronic food restriction. Future studies will investigate the role of NMDA receptors, as well as the metabotropic classes of glutamate receptors to assess their involvement in heroin seeking.

CHAPTER 6: GENERAL DISCUSSION

This thesis explored the neural mechanisms that mediate the augmentation of heroin seeking induced by chronic food restriction. In Chapter 3, we demonstrated that chronic food restriction resulted in differential changes in extracellular DA in the NAc shell and core when rats were re-exposed to the heroin-associated context. Administration of the DA D1 receptor antagonist SCH 39166 into the NAc shell reduced heroin seeking regardless of feeding condition. However, in the NAc core SCH 39166 selectively decreased heroin seeking in the FDR rats. Results from this chapter suggest that DA transmission through the D1 receptors in the NAc core is critical to the augmentation of heroin seeking induced by chronic food restriction. In the following sections it will be discussed how food restriction-induced alterations in DA transmission in the NAc can modify the motivational state of the animal, thereby directly affecting drug seeking.

In Chapter 4, we observed that leptin and ghrelin can modulate heroin seeking induced by chronic food restriction. Contrary to our predictions, central administration of leptin had little effect on heroin seeking in either the FDR or sated rats. However, intra-VTA leptin administration dose-dependently reduced the augmentation of heroin seeking induced by chronic food restriction. Similarly, the administration of the ghrelin receptor antagonist JMV 2959 into the VTA also selectively and dose-dependently reduced heroin seeking in the FDR rats. Taken together, these results suggest that leptin and ghrelin transmission in the VTA can modulate heroin seeking induced by chronic food restriction. It is plausible that the effects of leptin and ghrelin in the VTA are mediated through interactions with the mesolimbic DA pathway, which will be discussed in subsequent sections of this discussion.

Finally, in Chapter 5, contrary to our hypotheses we found that chronic food restriction did not alter extracellular glutamate levels in the NAc during heroin seeking. Unexpectedly, administration of the AMPA receptor antagonist NBQX into the NAc had no effect on heroin seeking in the FDR group, and resulted in a trend for attenuation in the sated rats. It remains a possibility that glutamate in regions other than the NAc may be involved in the augmentation of heroin seeking induced by chronic food restriction.

Collectively, these data promote a framework where heroin seeking induced by chronic food restriction is mediated through dopaminergic modulation within the NAc. Moreover, hunger and satiety signals coming into the VTA through leptin and ghrelin can also modulate

heroin seeking, likely by affecting downstream dopaminergic transmission in the mesolimbic pathway. Although glutamate transmission through AMPA receptors in the NAc does not seem to be involved in heroin seeking induced by chronic food restriction, other glutamate receptors in the NAc, such as NMDA receptors or mGluRs, should to be evaluated in the future to help elucidate glutamate's role in this behavior.

The Role of Dopamine in Heroin Seeking

There are multiple theories on the role of DA in learning and motivation. One hypothesis posits that DA transmission mediates the impact of the hedonic properties of rewards in behavior (Wise, 1982). This is theorized to operate by DA modulating the motivational properties of rewards that are primary unconditioned reinforcers, in addition to affecting the motivational properties of secondary conditioned reinforcers (Wise, 1982). However, this theory is opposed by evidence that DA cell body lesions and administration of DA antagonists do not completely abolish the rewarding properties of electric brain stimulation, suggesting that DA is not solely responsible for signaling reward in the brain (Wise & Rompre, 1989). An alternative approach, known as the incentive salience model, explains this discrepancy by arguing that DA is not responsible for signaling the hedonic properties of a stimulus (Berridge & Robinson, 1998; Robinson & Berridge, 1993). Instead, this theory proposes that there are 2 distinct aspects to reward-related stimuli: the "wanting" or incentive salience of the stimuli and the "liking" or hedonic response that is elicited by the stimuli. The "wanting" or incentive salience is mediated by the mesolimbic DA pathway, whereas the "liking" is mediated by opioid systems in the brain (Berridge, 2007; Berridge & Robinson, 1998; Robinson & Berridge, 1993). Furthermore, DA transmission in the NAc can mediate and magnify the "wanting" of a reward that is triggered by a reward-associated cue (Wyvell & Berridge, 2001). Thus, the elevated DA levels in the NAc of the FDR rats may drive an increased "wanting" of the drug following exposure to the drug-associated cues, leading to the augmentation of heroin seeking. Blocking D1 receptors in the NAc may then block the "wanting" of the drug which would decrease heroin seeking.

Another hypothesis, Wolfram Schultz's Reward Prediction Error Theory, proposes that DA encodes the reward prediction error, defined as the difference between predicted and received rewards (Schultz et al., 1997). When the received reward is better than predicted, there is a positive reward prediction error and DA neurons show phasic excitatory activity. However, if the received reward matches the prediction then the DA neurons remain at baseline firing rates.

When the received reward is less than predicted, or is completely omitted, there is a negative reward prediction error and DA neurons show a transient suppression in firing. Additionally, over time the reward-associated DA neural activity transfers from the reward itself to the preceding reward-predicting stimuli (Schultz, 1998). This type of information processing is efficient for learning about long chains of events. Positive reward prediction errors (when we receive a better than expected reward), drive us toward ever-increasing rewards, as once the prediction error is updated each subsequent reward needs to be larger to elicit a positive prediction error (Schultz, 2016). Hence, drugs of abuse and then drug-associated cues result in positive prediction errors and drive motivation to obtain a larger reward to continually activate the DA neurons. In our experiments, due to methodological limitations we are unable to quantify changes in phasic DA to specifically assess reward prediction error by means of *in vivo* microdialysis. It may be possible that *in vivo* microdialysis could detect some aspects of the phasic DA signal overlaid on the tonic DA signal. If this is the case, then the possibility exists that the increases in extracellular concentrations of DA in the NAc core of chronically food-restricted rats may display some aspects of a positive reward prediction error which would lead to the continued heroin-seeking demonstrated by this group. In addition, when DA D1 receptors are blocked in the NAc core of the FDR rats, it may suppress the positive reward prediction error signal thereby attenuating heroin seeking.

The NAc is only one of the major inputs of the mesolimbic DA pathway. In addition to the NAc, the DA neurons of the mesolimbic pathway in the VTA send projections to other regions including the basolateral amygdala (BLA). The BLA in turn has direct projections to the NAc (Zorrilla & Koob, 2013), which modulate DA released within the NAc core (Jones et al., 2010). As a result, the BLA is thought to play a critical role in drug seeking. For example, inactivation of the BLA attenuates the reinstatement of cue-induced heroin seeking (Fuchs & See, 2002). Moreover, extracellular DA in the BLA is elevated during cue-induced cocaine seeking following a period of withdrawal and in the reinstatement procedure (Tran-Nguyen et al., 1998; Weiss et al., 2000). Despite this evidence, preliminary findings we obtained (presented in the Appendix) found that there were no changes in extracellular DA levels in the BLA during the augmentation of heroin seeking induced by chronic food restriction. The discrepancy between our preliminary results and those previously mentioned is likely due to the difference in drugs of abuse, as the neural mechanisms mediating psychostimulant addiction and opiate

addiction are at least partially distinct (Badiani et al., 2011). Based on these findings, DA in the BLA may not be directly involved in heroin seeking induced by chronic food restriction.

In addition to the mesolimbic DA pathway, the nigrostriatal DA pathway also plays a critical role in reward and motivation (Wise, 2009). Dopaminergic cell bodies in this pathway originate in the substantia nigra and project predominantly to the dorsal striatum. One subdivision of the dorsal striatum, the dorsolateral striatum (DLS), has been implicated in drug seeking. Dopamine in the DLS, and not in the dorsomedial portion of the striatum, is elevated during cue-induced reinstatement of cocaine seeking, and in response to the presentation of cocaine-paired cues (Ito, Dalley, Robbins, & Everitt, 2002). Extracellular DA levels in the DLS are also elevated during cocaine seeking following 14 days of withdrawal (Gabriele, Pacchioni, & See, 2012), and administration of a DA D1-like receptor antagonist into the DLS attenuated context-induced reinstatement of heroin seeking (Bossert, Wihbey, Pickens, Nair, & Shaham, 2009).

Consequently, it remains plausible that DA in the nigrostriatal pathway, specifically the DLS may be involved in heroin seeking following chronic food restriction. However, preliminary results we obtained (presented in the Appendix) revealed that there are no statistically significant changes in extracellular DA in the DLS during the augmentation of heroin seeking induced by chronic food restriction. One reason for these inconsistencies may be that the dorsal striatum is thought to mediate stimulus-response or habit learning, while the ventral striatum mediates goal-directed behavior (Everitt & Robbins, 2013). Perhaps heroin seeking remains a goal-oriented behavior in FDR rats, which is why we see significant increases in DA in the ventral striatum, i.e. the NAc, and not the DLS.

Although our preliminary results indicate that there are no significant changes in extracellular DA in the BLA or DLS during heroin seeking, the role of DA D1-like receptors in these regions remains to be tested. Fluctuations in extracellular DA may implicate presynaptic adaptations that have emerged but would not reflect postsynaptic changes at the level of the DA receptors. Therefore, future studies should address the role of DA D1-like receptors in the BLA and DLS in the augmentation of heroin seeking induced by chronic food restriction.

In addition to other brain regions, DA also acts on multiple receptor subtypes. Thus far, our research has focused on the involvement of D1-like receptors in heroin seeking. Previous research indicates that administration of the D2 antagonist raclopride completely blocks cue-

induced reinstatement of cocaine seeking (Cervo, Carnovali, Stark, & Mennini, 2003). Moreover, administration of the D2 receptor antagonist sulpiride into the NAc shell, but not the core, dose-dependently attenuated the reinstatement of cocaine seeking induced by a priming injection (Anderson et al., 2006). Similarly, intra-shell administration of the D2 receptor antagonist eticlopride also attenuates cocaine-primed reinstatement (Bachtell, Whisler, Karanian, & Self, 2005). In contrast, previous work from our laboratory indicates that D2-like receptors may not be involved in heroin seeking. In particular, we have shown that systemic administration of the D1-like receptor antagonist SCH 23390 and not a D2 or D3 receptor antagonist, dose-dependently decreased food deprivation-induced reinstatement of heroin seeking (Tobin et al., 2009). As previously mentioned, these discrepancies may be because psychostimulant and opiate addiction are mediated by overlapping but distinct neural circuits, and lead to different neuroadaptations (Badiani et al., 2011). Furthermore, the neural mechanisms mediating drug seeking in a reinstatement model versus a withdrawal-based procedure are distinct (Fuchs et al., 2006). This is also supported by evidence that intra-shell administration of a D1 antagonist SCH 23390, but not the D2 antagonist eticlopride, blocked cue-induced morphine seeking in a withdrawal-based procedure similar to ours (Gao et al., 2013). We therefore conclude that although we did not directly assess other DA receptors, it is likely that D1-like and not D2-like receptors are involved in heroin seeking following chronic food restriction.

Interactions of Dopamine and the Endocrine System

The hypothalamus is pivotal in regulating feeding and energy balance and is the primary site of action for circulating hormones such as leptin and ghrelin (DiLeone, 2009). Leptin, which is released by peripheral adipocytes, binds to numerous hypothalamic nuclei (Ahima et al., 2000; Friedman & Halaas, 1998). Once bound to the leptin receptor (LepR), it leads to dimerization and subsequent activation of Janus kinase (Jak) and phosphorylation of signal-transducer-and-activator-of-transcription-3 (STAT3; Fulton et al., 2006). These signaling pathways result in the inhibition of the neuropeptide Y (NPY)/ agouti-related protein (AgRP) orexigenic neurons and excitation of the pro-opiomelanocortin (POMC) anorexigenic neurons within the arcuate nucleus of the hypothalamus, which then signals satiety leading to decreased food intake and subsequent body weight loss (DiLeone, 2009; van Zessen et al., 2012). In contrast, the hormone ghrelin is released from the gut and binds to its receptors, GHS-R1As, which are located on the NPY/AgRP neurons in the arcuate nucleus to stimulate feeding by increasing meal frequency

(Horvath, Diano, & Tschop, 2003; Tschop, Smiley, & Heiman, 2000). Moreover, ghrelin is also critically involved in food anticipatory activity (Verhagen et al., 2011), as ghrelin receptor knockout animals show a marked reduction in food anticipatory activity (Blum et al., 2009).

Feeding and food intake is an evolved behavior that engages and integrates many physiological systems and neural circuits (DiLeone, 2009). As it is a highly complex behavior that is key to an organism's survival, these systems and circuits work in a concerted effort to regulate the various aspects of feeding, such as the motivational drive, the anticipation of food, and assessing the current metabolic state (van Zessen et al., 2012). These homeostatic signaling circuits from the hypothalamus eventually converge with the circuits that mediate reward and motivation via the mesolimbic DA pathway (DiLeone, 2009). The hypothalamic nuclei that leptin and ghrelin bind to, especially the arcuate nucleus, the lateral hypothalamus, and the ventromedial hypothalamus, all send projections to the VTA, either onto the DA cell bodies or the GABAergic interneurons (van Zessen et al., 2012). When circulating signals such as leptin or ghrelin bind to these hypothalamic sites they may result in downstream modulation of the mesolimbic DA circuit.

However, it is documented that DA depletion in the mesolimbic circuit is not sufficient completely eliminate feeding behavior (Salamone, Correa, Mingote, & Weber, 2005; Salamone, Cousins, & J., 1997; Salamone, Wisniecki, Carlson, & Correa, 2001). This is likely because feeding behavior is critical to survival, and evolutionary pressures have resulted in many redundant circuits to ensure adequate food intake. Therefore, DA may not be essential in mediating food intake and consumption but rather acts to integrate and drive motivation towards food rewards (Salamone et al., 1997). It is suggested that the hypothalamic circuits mediate food seeking and food intake, and the mesolimbic DA system only has modulatory effects on these behaviors. Similarly, the mesolimbic DA system mediates drug reinforcement and reward, whereas hypothalamic systems modulate it (DiLeone, 2009). These theories are supported by our results from Chapter 4.

As previously mentioned, centrally administered leptin had no effect on heroin seeking induced by chronic food restriction. This is probably because most of the leptin when administered into the ventricles was taken up by hypothalamic sites, and perhaps these pathways do not modulate heroin seeking induced by chronic food restriction. Although we did not directly assess the effects of ghrelin in our procedure, we have previously demonstrated that central

administration of a ghrelin receptor antagonist had no effect on heroin seeking following acute food deprivation (Maric et al., 2011). Thus, it is likely that leptin and ghrelin's actions on hypothalamic circuits that mediate food intake do not mediate heroin seeking in chronically food restricted rats.

On the other hand, direct administration of leptin or a ghrelin receptor antagonist into the VTA dose-dependently reduced the augmentation of heroin seeking induced by chronic food restriction. Our results therefore suggest that the effects of leptin and ghrelin are mediated by their actions directly on the mesolimbic DA system. It is well established that both leptin and ghrelin receptors are expressed in the VTA (Abizaid et al., 2006; Fulton et al., 2006; Hommel et al., 2006). Briefly, LepR have been located on both DA cell bodies in the VTA and GABAergic interneurons (Fulton et al., 2006; Hommel et al., 2006). Evidence indicates that leptin administration directly into the VTA decreases the firing rate of DA neurons (Hommel et al., 2006) which then results in a reduction of DA levels in the terminal regions of the mesolimbic pathway, such as the NAc (You et al., 2016). We demonstrated in Chapter 3 that blockade of D1 receptors in the NAc would attenuate heroin seeking induced by chronic food restriction. Therefore, it is possible that leptin's effects are upstream of these changes in DA neurotransmission in the NAc.

Similar to leptin, ghrelin's receptors are also located in the VTA, and ghrelin acts directly on DA neurons by increasing their activity (Abizaid et al., 2006). Since administration of a ghrelin receptor antagonist in the VTA attenuated heroin seeking induced by chronic food restriction, ghrelin may also be acting upstream in the VTA and modulating DA neurotransmission in the NAc to drive heroin seeking. Future studies should address if and how leptin and ghrelin are modulating DA transmission in the mesolimbic pathway to alter heroin seeking induced by chronic food restriction. One potential method to investigate if leptin and ghrelin's actions are mediated directly upstream from DA in the NAc would be to administer leptin or a ghrelin receptor antagonist into one hemisphere of the VTA and a D1 receptor antagonist in the contralateral hemisphere NAc to assess if they are part of one circuit. Furthermore, since we also found changes in plasma levels of leptin and ghrelin following heroin self-administration and chronic food restriction, it would be interesting to investigate the corresponding molecular intracellular effects of leptin and ghrelin in the VTA.

Interactions of Dopamine and Glutamate Systems

The dense dopaminergic innervations that the NAc receives from the VTA as part of the mesolimbic DA system is well established in reward and addiction (Di Chiara et al., 1993; Ungerstedt, 1971). Therefore, it is not surprising that we found that DA transmission in the NAc is involved in the augmentation of heroin seeking induced by chronic food restriction. In addition to the DA afferents, the NAc also receives dense glutamatergic innervations from a variety of other limbic and cortical regions (Brog et al., 1993). It was unexpected that we found no changes in glutamate transmission in the NAc, which is in contrast to numerous other reports that find changes (Hotsenpiller et al., 2001; LaLumiere & Kalivas, 2008; McFarland et al., 2003; Suto et al., 2013). Moreover, blocking AMPA receptors in our procedure had no effect on drug seeking in the FDR rats, which is also in contrast to other reports that find that administration of an AMPAR antagonist decreased drug seeking (Cornish & Kalivas, 2000; Di Ciano & Everitt, 2001; LaLumiere & Kalivas, 2008; Suto et al., 2004). One possibility for these discrepancies may be accounted for by differences in methodology, such as testing drug seeking following extinction training as opposed to a withdrawal period, or differences in the type of substance studied, i.e., psychostimulants versus opiates.

Prior to exposure to any drugs of abuse, naïve animals have AMPARs that are primarily composed of GluA1 subunits associated with GluA2 subunits. This combination of subunit type (GluA1 + GluA2) results in AMPARs that are Ca^{2+} impermeable. This is also accompanied with decreased intrinsic excitability of MSNs in the NAc (Conrad et al., 2008; Wolf & Ferrario, 2010). In the NAc core of drug-naïve animals, these GluA2-containing Ca^{2+} impermeable AMPARs are responsible for 90-95% of the evoked excitatory post-synaptic current (EPSC) (Wolf & Ferrario, 2010). However, following cocaine self-administration and prolonged drug withdrawal beyond 3 weeks, there is a marked increase in the synaptic levels of Ca^{2+} permeable AMPARs in the NAc core (Conrad et al., 2008). These AMPARs lack the GluA2 subunit and are primarily homomers of GluA1 subunits, which makes them permeable to Ca^{2+} (Wolf, 2016). This elevation of GluA2-lacking AMPARs results in higher conductance and increased intrinsic excitability of NAc MSNs. Thus, even a minor change in the insertion or removal of these receptors from the synapse can lead to robust changes in the synaptic strength and properties of excitatory transmission in the NAc (Wolf & Ferrario, 2010), resulting in strengthened responsiveness of MSNs to incoming glutamatergic drive (Wolf, 2016).

This increased sensitivity to incoming glutamatergic drive accompanied by elevations of DA in the NAc following drug exposure may provide insights on how maladaptive behaviors like drug seeking are established. As previously mentioned, DA's role is hypothesized to facilitate learning of contingencies related to obtaining rewards. Dopaminergic terminals are anatomically well-positioned to modulate synaptic plasticity in the NAc MSNs as evidenced by the fact that both dopaminergic and glutamatergic innervations converge onto the same dendritic spines of MSNs (Sesack, Carr, Omelchenko, & Pinto, 2003). Specifically, stimulation of D1Rs may prime for the insertion of GluA2-lacking AMPARs by facilitating their externalization to allow for translocation to the synapse (Wolf, 2010). Therefore, when drugs of abuse artificially increase DA in the NAc, it may lead to inappropriate modulation of Ca^{2+} permeable AMPARs being trafficked to the synapse and the strengthening of excitatory synapses (Wolf, 2010). In combination with the increased intrinsic excitability of MSNs in the NAc core, these adaptations result in an augmented drug-seeking response when animals are re-exposed to drug-associated cues. Thus, incoming glutamatergic transmission into the NAc core plays a key role in translating motivational signals into the motor output of drug seeking (Wolf, 2016).

Drug-induced neural adaptations in plasticity, particularly in glutamate transmission in the NAc contribute to the inability to inhibit drug seeking (Kalivas, Lalumiere, Knackstedt, & Shen, 2009). Both psychostimulant and opiate addiction share the common adaptation of altered glutamatergic transmission in the NAc, which is a key neurobiological substrate of reward and motivation (Hearing, Graziane, Dong, & Thomas, 2018). However, caution must be taken when generalizing the glutamate homeostasis hypothesis of addiction (Kalivas, 2009) to opioids, as the majority of evidence used to formulate that hypothesis is based on studies conducted with psychostimulants (particularly cocaine), and there is abundant data that demonstrates that psychostimulant and opioid addiction are distinct (Badiani et al., 2011). Perhaps in our procedure we saw no changes in NAc glutamatergic transmission because the alterations in synaptic plasticity following heroin exposure do not parallel the adaptations found following cocaine exposure. In particular, there is insufficient data to conclude if there are increased AMPA/NMDA ratios (indicative of synapses being in a potentiated state) (Kalivas, 2009) or if there is increased expression of GluA2-lacking AMPARs in both the NAc and VTA following opiate exposure (Wolf, 2010; Wolf & Tseng, 2012). Even though psychostimulants and opioids result in differing neural adaptations in NAc MSNs, they may produce similar behavioral output

of drug seeking (Hearing et al., 2018). Consequently, more evidence is needed on the precise mechanisms of synaptic plasticity in opioid addiction.

Although there is insufficient data on the effects of heroin self-administration and withdrawal on neuroadaptations of AMPARs in the NAc, recent evidence indicates that chronic food restriction itself may alter AMPARs. Specifically, chronic food restriction significantly increased GluA1 but not GluA2 subunits in the postsynaptic density of the NAc. These results suggest that there is increased incorporation of GluA2-lacking calcium-permeable AMPARs in the NAc following chronic food restriction (Ouyang et al., 2017). This is supported by electrophysiological evidence that spontaneous excitatory postsynaptic currents in the MSNs of the NAc shell of FDR rats have a higher amplitude and frequency compared to the sated rats, which is indicative of synaptic insertion of calcium-permeable AMPARs (Carr, 2016; Ouyang et al., 2017). Moreover, administration of Naspm, an antagonist specific to calcium-permeable AMPARs, into the NAc shell decreased the amplitude of excitatory postsynaptic currents. Naspm also reduced the enhanced locomotor response to a D1R agonist but not a D2R agonist in only the FDR rats (Ouyang et al., 2017). As with psychostimulant self-administration and withdrawal, the increased synaptic incorporation of AMPAR in the NAc is proposed to enhance DA encoded stimulus reward magnitude. In conjunction with increased signaling downstream of D1Rs, intracellular calcium signaling may increase stimulus-induced synaptic plasticity involving the upregulation of calcium-permeable AMPARs in the postsynaptic density of the NAc (Carr, 2016). These neuroadaptations of AMPARs in the NAc may increase synaptic strengthening through LTP resulting in increased reward-directed behavior, specifically increasing the likelihood to approach food and drugs of abuse (Carr, 2016).

Future experiments need to address the changes in glutamate transmission in the NAc following exposure to opioids in combination with chronic food restriction. One possibility is to investigate the role of other iGluRs, such as NMDA, in heroin seeking induced by chronic food restriction. This could be assessed through administration of an NMDA receptor antagonist in the NAc prior to the heroin-seeking test. Another possibility is to investigate the role of mGluRs in heroin seeking following chronic food restriction. As previously mentioned, the presynaptic mGluR2/3 receptors act as autoreceptors to regulate glutamate in the synapse. In fact, administration of an mGluR2/3 agonist reduced cue-induced reinstatement of heroin seeking

(Bossert, Ghitza, Lu, Epstein, & Shaham, 2005). These possibilities should be further investigated to elucidate the mechanisms and synaptic changes within opioid addiction.

Neural Circuitry Underlying the Augmentation of Heroin Seeking induced by Chronic Food Restriction

As previously mentioned, both leptin and ghrelin can modulate the excitability of VTA DA neurons which would subsequently alter DA levels in the terminals regions of the mesolimbic pathway, such as the NAc (Abizaid et al., 2006; Hommel et al., 2006). The increased excitability of VTA DA neurons by decreased leptin levels and increased ghrelin levels may have a concerted effect on the FDR rats, making them more responsive to drug-associated cues. Indeed, in FDR rats the higher levels of heroin seeking paralleled the increased DA we measured in the NAc during the heroin-seeking test in Chapter 3. Based on these findings, we suggest that chronic food restriction may sensitize the neural systems that are involved in incentive motivational processes, i.e. the mesolimbic DA pathway (Berridge & Robinson, 1998), resulting in cue-induced elevation of NAc core and shell DA. This idea is further substantiated by the fact that the sated group of rats had no significant changes in NAc DA and displayed lower levels of heroin seeking compared to the FDR rats.

Learning the predictive relationships, or contingencies, between salient stimuli and responses that will lead to beneficial outcomes is a key aspect of motivation (Di Chiara, 2002). Motivation can be defined as the process by which organisms react to stimuli in relation to their predicted outcomes to promote the survival of the organism and ultimately the species (Di Chiara, 2002; Dickinson & Balleine, 1994; Toates, 1998). Adaptations in the brain in response to chronic food restriction likely evolved as an adaptive function during times of food scarcity. Therefore, it is likely that chronic food restriction increases the motivational state of the organism by enhancing the incentive motivational effects of food-related cues (Carr, 1996, 2011). Increasing the rewarding efficacy of food when the organism has an energy deficit is of adaptive value (Bindra, 1978). Thus, it is not surprising that the incentive-motivating effects of external stimuli are dependent on the internal state of the organism (Stewart, de Wit, & Eikelboom, 1984). This enhancement of food reward and salience of associated cues may generalize to drugs of abuse because of their shared neural substrate (Di Chiara et al., 1993; Kelley & Berridge, 2002). We would therefore expect that chronic food restriction alters the incentive salience of both food-related and drug-related stimuli, which is mediated by

adaptations in the mesolimbic DA pathway (Berridge, 2007; Berridge & Robinson, 1998; Robinson & Berridge, 1993).

In summary, chronic food restriction may alter the motivational state of the animal due to the negative energy balance state. This state of negative energy balance may subsequently sensitize the neural circuitry that mediates reward and result in the attribution of more incentive salience to reward-related stimuli. The increased attribution of incentive salience to reward-related stimuli may extend to cues previously paired with drug availability and drug effects, which are a main trigger to increase drug seeking. Therefore, chronic food restriction may ultimately increase drug seeking in animals with a history of drug self-administration. Translated to the human condition, food restriction or dieting will result in a negative state of energy balance which may increase the incentive salience or "wanting" of drug-associated cues thereby increasing drug craving and the vulnerability to relapse in an abstinent drug addict.

Conclusion

In summary, the experiments presented in this thesis provide insights into the neural mechanisms underlying the augmentation of heroin seeking induced by chronic food restriction. Briefly, DA transmission in the NAc through D1 receptors mediates augmented heroin seeking induced by chronic food restriction. Furthermore, hormones involved in metabolic processes may modulate this behavior by acting upstream on the cell bodies of the mesolimbic pathway in the VTA. Unexpectedly, there were no changes in extracellular glutamate levels in the NAc during heroin seeking and administration of an AMPAR antagonist had no effect on the augmentation of heroin seeking induced by chronic food restriction.

Taken together, these findings provide insights on the interactions between dietary manipulations and the neural circuitry mediating drug seeking. It is well established that chronic food restriction and limited food intake can increase drug seeking in animal models of relapse. In addition, this increase in drug seeking is accompanied by neural adaptations in the mesolimbic DA pathway and alterations in hormones that mediate metabolic processes, which may interact to modify reward-related processing. Therefore, the findings from these preclinical experiments should be extrapolated to inform and guide clinical research in humans. It is clear that chronic food restriction can have lasting impacts on the neural circuitry and directly influence behavior.

REFERENCES

Abizaid, A., & Horvath, T. L. (2012). Ghrelin and the central regulation of feeding and energy balance. *Indian J Endocrinol Metab, 16*(Suppl 3), S617-626. doi:10.4103/2230-8210.105580

Abizaid, A., Liu, Z. W., Andrews, Z. B., Shanabrough, M., Borok, E., Elsworth, J. D., . . . Horvath, T. L. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *The Journal of clinical investigation, 116*(12), 3229-3239. doi:10.1172/JCI29867

Abou Heif, H. M., Deif, M. M., & Abdel Aziz, H. K. (2009). Effect of food restriction on ghrelin in adult male rats and its relation to male reproductive hormones. *Andrologia, 42*(2), 97-105.

Abrahamsen, G. C., Bermanm, Y., & Carr, K. D. (1995). Curve-shift analysis of self-stimulation in food-restricted rats: relationship between daily meal, plasma corticosterone and reward sensitization. . *Brain Res, 695*, 186-194.

Abrahamsen, G. C., & Carr, K. D. (1996). Effects of corticosteriod synthesis inhibitors on the sensitization of reward by food restriction. . *Brain Research., 726*, 39-48.

Ahima, R. S., Saper, C. B., Flier, J. S., & Elmquist, J. K. (2000). Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol, 21*(3), 263-307. doi:10.1006/frne.2000.0197

Alexander, G. E., & Crutcher, M. D. (1990). Functional architecture of basal ganglia circuits: neural substrates of parallel processing. *Trends in neurosciences, 13*(7), 266-271.

Alper, K. R., Lotsof, H. S., Frenken, G. M. N., Luciano, D. J., & Bastiaans, J. (1999). Treatment of acute opioid withdrawal with ibogaine. *The American Journal on Addictions, 8*, 234-242.

Ambroggi, F., Ishikawa, A., Fields, H. L., & Nicola, S. M. (2008). Basolateral amygdala neurons facilitate reward-seeking behavior by exciting nucleus accumbens neurons. *Neuron, 59*(4), 648-661. doi:10.1016/j.neuron.2008.07.004

Anderson, S. M., Schmidt, H. D., & Pierce, R. C. (2006). Administration of the D2 dopamine receptor antagonist sulpiride into the shell, but not the core, of the nucleus accumbens attenuates cocaine priming-induced reinstatement of drug seeking. *Neuropsychopharmacology, 31*(7), 1452-1461. doi:10.1038/sj.npp.1300922

Bachtell, R. K., Whisler, K., Karanian, D., & Self, D. W. (2005). Effects of intra-nucleus accumbens shell administration of dopamine agonists and antagonists on cocaine-taking and cocaine-seeking behaviors in the rat. *Psychopharmacology (Berl), 183*(1), 41-53. doi:10.1007/s00213-005-0133-1

Badiani, A., Belin, D., Epstein, D., Calu, D., & Shaham, Y. (2011). Opiate versus psychostimulant addiction: the differences do matter. *Nature reviews. Neuroscience, 12*(11), 685-700. doi:10.1038/nrn3104

Bari, A. A., & Pierce, R. C. (2005). D1-like and D2 dopamine receptor antagonists administered into the shell subregion of the rat nucleus accumbens decrease cocaine, but not food, reinforcement. *Neuroscience, 135*(3), 959-968. doi:10.1016/j.neuroscience.2005.06.048

Bassareo, V., Cucca, F., Musio, P., Lecca, D., Frau, R., & Di Chiara, G. (2015). Nucleus accumbens shell and core dopamine responsiveness to sucrose in rats: role of response contingency and discriminative/conditioned cues. . *Eur J Neurosci, 41*, 802-809.

Bassareo, V., De Luca, M. A., & Di Chiara, G. (2007). Differential impact of pavlovian drug conditioned stimuli on in vivo dopamine transmission in the rat accumbens shell and core

and in the prefrontal cortex. *Psychopharmacology, 191*(3), 689-703. doi:10.1007/s00213-006-0560-7

Bassareo, V., Musio, P., & Di Chiara, G. (2011). Reciprocal responsiveness of nucleus accumbens shell and core dopamine to food- and drug-conditioned stimuli. *Psychopharmacology, 214*(3), 687-697. doi:10.1007/s00213-010-2072-8

Bedi, G., Preston, K. L., Epstein, D. H., Heishman, S. J., Marrone, G. F., Shaham, Y., & de Wit, H. (2011). Incubation of cue-induced cigarette craving during abstinence in human smokers. *Biological psychiatry, 69*(7), 708-711. doi:10.1016/j.biopsych.2010.07.014

Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology, 191*(3), 391-431. doi:10.1007/s00213-006-0578-x

Berridge, K. C., & Robinson, T. E. (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain research reviews, 28*, 309-369.

Bi, S., Robinson, B. M., & Moran, T. H. (2003). Acute food deprivation and chronic food restriction differentially affect hypothalamic NPY mRNA expression. *American journal of physiology. Regulatory, integrative and comparative physiology., 285*(5), R1030-1036.

Bindra, D. (1978). How adaptive behavior is produced: A perceptual motivational alternative to response reinforcement. *Behavioral and brain sciences, 1*, 41-91.

Blum, I. D., Patterson, Z., Khazall, R., Lamont, E. W., Sleeman, M. W., Horvath, T. L., & Abizaid, A. (2009). Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience, 164*(2), 351-359. doi:10.1016/j.neuroscience.2009.08.009

Bossert, J., Ghitza, U., Lu, L., Epstein, D., & Shaham, Y. (2005). Neurobiology of relapse to heroin and cocaine seeking: An update and clinical implications. *European journal of pharmacology, 526*(1-3), 36-50. doi:10.1016/j.ejphar.2005.09.030

Bossert, J. M., Gray, S. M., Lu, L., & Shaham, Y. (2006). Activation of group II metabotropic glutamate receptors in the nucleus accumbens shell attenuates context-induced relapse to heroin seeking. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 31*(10), 2197-2209. doi:10.1038/sj.npp.1300977

Bossert, J. M., Poles, G. C., Wihbey, K. A., Koya, E., & Shaham, Y. (2007). Differential effects of blockade of dopamine D1-family receptors in nucleus accumbens core or shell on reinstatement of heroin seeking induced by contextual and discrete cues. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 27*(46), 12655-12663. doi:10.1523/JNEUROSCI.3926-07.2007

Bossert, J. M., & Stern, A. L. (2014). Role of ventral subiculum in context-induced reinstatement of heroin seeking in rats. *Addict Biol, 19*(3), 338-342.

Bossert, J. M., Stern, A. L., Theberge, F. R., Marchant, N. J., Wang, H. L., Morales, M., & Shaham, Y. (2012). Role of projections from ventral medial prefrontal cortex to nucleus accumbens shell in context-induced reinstatement of heroin seeking. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 32*(14), 4982-4991. doi:10.1523/JNEUROSCI.0005-12.2012

Bossert, J. M., Wihbey, K. A., Pickens, C. L., Nair, S. G., & Shaham, Y. (2009). Role of dopamine D(1)-family receptors in dorsolateral striatum in context-induced reinstatement of heroin seeking in rats. *Psychopharmacology, 206*(1), 51-60. doi:10.1007/s00213-009-1580-x

Bouton, M. E., & Swartzentruber, D. (1991). Sources of relapse after extinction in Pavlovian and instrumental learning. *Clinical Psychology Review, 11*(2), 123-140.

Bozarth, M. A. (1986). Neural basis of psychomotor stimulant and opiate reward: Evidence suggesting the involvement of a common dopaminergic system. *Behavioural brain research, 22*, 107-116.

Britt, J. P., Benaliouad, F., McDevitt, R. A., Stuber, G. D., Wise, R. A., & Bonci, A. (2012). Synaptic and behavioral profile of multiple glutamatergic inputs to the nucleus accumbens. *Neuron, 76*(4), 790-803. doi:10.1016/j.neuron.2012.09.040

Brog, J. S., Apongse, A. S., Deutch, A. Y., & Zahm, D. S. (1993). The patterns of afferent innervation of the core and shell in the "accumbens" part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. *The Journal of Comparative Neurology, 338*, 255-278.

Brown, E. E., & Fibiger, H. C. (1992). Cocaine-induced conditioned locomotion: Absence of associated increases in dopamine release. *Neuroscience, 48*(3), 621-629.

Brown, S. A., Vik, P. W., Patterson, T. L., Grant, I., & Schuckit, M. A. (1995). Stress, vulnerability and adult alcohol relapse. *Journal of Studies on Alcohol, 56*(5), 538-545.

Bruijnzeel, A. W., Corrie, L. W., Rogers, J. A., & Yamada, H. (2011). Effects of insulin and leptin in the ventral tegmental area and arcuate hypothalamic nucleus on food intake and brain reward function in female rats. *Behav Brain Res, 219*(2), 254-264. doi:10.1016/j.bbr.2011.01.020

Cabeza de Vaca, S., & Carr, K. D. (1998). Food restriction enhances the central rewarding effect of abused drugs. *The Journal of Neuroscience, 18*(18), 7502-7510.

Caprioli, D., Venniro, M., Zeric, T., Li, X., Adhikary, S., Madangopal, R., . . . Shaham, Y. (2015). Effect of the Novel Positive Allosteric Modulator of Metabotropic Glutamate Receptor 2 AZD8529 on Incubation of Methamphetamine Craving After Prolonged Voluntary Abstinence in a Rat Model. *Biol Psychiatry, 78*(7), 463-473. doi:10.1016/j.biopsych.2015.02.018

Carr, K. D. (1996). Feeding, drug abuse, and the sensitization of reward by metabolic need. *Neurochemical research, 21*(11), 1455-1467.

Carr, K. D. (2007). Chronic food restriction: enhancing effects on drug reward and striatal cell signaling. *Physiology & behavior, 91*(5), 459-472. doi:10.1016/j.physbeh.2006.09.021

Carr, K. D. (2011). Food scarcity, neuroadaptations, and the pathogenic potential of dieting in an unnatural ecology: binge eating and drug abuse. *Physiology & behavior, 104*(1), 162-167. doi:10.1016/j.physbeh.2011.04.023

Carr, K. D. (2016). Nucleus Accumbens AMPA Receptor Trafficking Upregulated by Food Restriction: An Unintended Target for Drugs of Abuse and Forbidden Foods. *Curr Opin Behav Sci, 9*, 32-39. doi:10.1016/j.cobeha.2015.11.019

Carr, K. D., Chau, L. S., Cabeza de Vaca, S., Gustafson, K., Stouffer, M., Tukey, D. S., . . . Ziff, E. B. (2010). AMPA receptor subunit GluR1 downstream of D-1 dopamine receptor stimulation in nucleus accumbens shell mediates increased drug reward magnitude in food-restricted rats. *Neuroscience, 165*(4), 1074-1086. doi:10.1016/j.neuroscience.2009.11.015

Carr, K. D., Tsimberg, Y., Berman, Y., & Yamamoto, N. (2003). Evidence of increased dopamine receptor signaling in food-restricted rats. *Neuroscience, 119*(4), 1157-1167. doi:10.1016/s0306-4522(03)00227-6

Carroll, M. E., France, C. P., & Meisch, R. A. (1979). Food deprivation increases oral and intravenous drug intake in rats. *Science, 205*, 319-321.

Carroll, M. E., & Meisch, R. A. (1981). Determinants of increased drug self-administration due to food deprivation. *Psychopharmacology, 74*, 197-200.
Carroll, M. E., & Meisch, R. A. (1984). Increased drug-reinforced behavior due to food deprivation. In T. Thompson, P. B. Dews, & J. E. Barrett (Eds.), *Advances in behavioral pharmacology* (pp. 47-88). New York.
Carroll, M. E., Stotz, D. C., Kliner, D. J., & Meisch, R. A. (1984). Self-administration of orally-delivered methohexital in rhesus monkeys with phencyclidine or pentobarbital histories: effects of food deprivation and satiation. *Pharmacology, biochemistry, and behavior, 20*(1), 145-151.
Carter, B. L., & Tiffany, S. T. (1999). Meta-analysis of cue-reactivity in addiction research. *Addiction, 94*(3), 327-340.
Cervo, L., Carnovali, F., Stark, J. A., & Mennini, T. (2003). Cocaine-seeking behavior in response to drug-associated stimuli in rats: involvement of D3 and D2 dopamine receptors. *Neuropsychopharmacology, 28*(6), 1150-1159. doi:10.1038/sj.npp.1300169
Chaudhri, N., Sahuque, L. L., & Janak, P. H. (2009). Ethanol seeking triggered by environmental context is attenuated by blocking dopamine D1 receptors in the nucleus accumbens core and shell in rats. *Psychopharmacology (Berl), 207*(2), 303-314. doi:10.1007/s00213-009-1657-6
Chaudhri, N., Sahuque, L. L., Schairer, W. W., & Janak, P. H. (2010). Separable roles of the nucleus accumbens core and shell in context- and cue-induced alcohol-seeking. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 35*(3), 783-791. doi:10.1038/npp.2009.187
Chen, B. T., Yau, H. J., Hatch, C., Kusumoto-Yoshida, I., Cho, S. L., Hopf, F. W., & Bonci, A. (2013). Rescuing cocaine-induced prefrontal cortex hypoactivity prevents compulsive cocaine seeking. *Nature, 496*(7445), 359-362. doi:10.1038/nature12024
Cheskin, L. J., Hess, J. M., Henningfield, J., & Gorelick, D. A. (2005). Calorie restriction increases cigarette use in adult smokers. *Psychopharmacology, 179*(2), 430-436. doi:10.1007/s00213-004-2037-x
Childress, A. R., Hole, A. V., Ehrman, R. N., Robbins, S. J., McLellan, A. T., & O'Brien, C. P. (1993). Cue reactivity and cue reactivity interventions in drug dependence. *NIDA Research Monograph Series, 137*, 73-95.
Conn, P. J., & Pin, J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol, 37*, 205-237. doi:10.1146/annurev.pharmtox.37.1.205
Conrad, K. L., Tseng, K. Y., Uejima, J. L., Reimers, J. M., Heng, L. J., Shaham, Y., . . . Wolf, M. E. (2008). Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature, 454*(7200), 118-121. doi:10.1038/nature06995
Cornish, J. L., & Kalivas, P. W. (2000). Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. *The Journal of Neuroscience, 20*, RC89.
Crombag, H. S., & Shaham, Y. (2002). Renewal of drug seeking by contextual cues after prolonged extinction in rats. *Behavioral neuroscience, 116*(1), 169-173. doi:10.1037//0735-7044.116.1.169
D'Cunha, T. M., Daoud, E., Rizzo, D., Bishop, A. B., Russo, M., Mourra, G., . . . Shalev, U. (2017). Augmentation of Heroin Seeking Following Chronic Food Restriction in the Rat: Differential Role for Dopamine Transmission in the Nucleus Accumbens Shell and Core. *Neuropsychopharmacology, 42*(5), 1136-1145. doi:10.1038/npp.2016.250

D'Cunha, T. M., Sedki, F., Macri, J., Casola, C., & Shalev, U. (2013). The effects of chronic food restriction on cue-induced heroin seeking in abstinent male rats. *Psychopharmacology*. doi:10.1007/s00213-012-2810-1

de Wit, H. (1996). Priming effects with drugs and other reinforcers. *Experimental and Clinical Psychopharmacology, 4*, 5-10.

de Wit, H., & Stewart, J. (1981). Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology, 75*, 134-143.

Di Chiara, G. (2002). Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behavioural brain research, 137*, 75-114.

Di Chiara, G., Acquas, E., Tanda, G., & Cadoni, C. (1993). Drugs of abuse: biochemical surrogates of specific aspects of natural reward? *Biochemical Society symposium, 59*, 65-81.

Di Ciano, P., & Everitt, B. J. (2001). Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior. *Neuropsychopharmacology, 25*(3), 341-360. doi:10.1016/S0893-133X(01)00235-4

Dickinson, A., & Balleine, B. (1994). Motivational control of goal-directed action. *Animal Learning & Behavior, 22*, 1-18.

DiLeone, R. J. (2009). The influence of leptin on the dopamine system and implications for ingestive behavior. *International journal of obesity, 33 Suppl 2*, S25-29. doi:10.1038/ijo.2009.68

Drazen, D. L., Vahl, T. P., D'Alessio, D. A., Seeley, R. J., & Woods, S. C. (2006). Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology, 147*, 23-30.

Duvauchelle, C. L., Ikegami, A., Asami, S., Robens, J., Kressin, K., & Castaneda, E. (2000). Effects of cocaine context on NAcc dopamine and behavioral activity after repeated intravenous cocaine administration. *Brain Res, 862*, 49-58.

Duvauchelle, C. L., Ikegami, A., & Castaneda, E. (2000). Conditioned increases in behavioral activity and accumbens dopamine levels produced by intravenous cocaine. *Behav Neurosci, 114*, 1156-1166.

Egecioglu, E., Jerlhag, E., Salome, N., Skibicka, K. P., Haage, D., Bohlooly, Y. M., . . . Dickson, S. L. (2010). Ghrelin increases intake of rewarding food in rodents. *Addict Biol, 15*(3), 304-311. doi:10.1111/j.1369-1600.2010.00216.x

Epstein, D. H., Preston, K. L., Stewart, J., & Shaham, Y. (2006). Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. *Psychopharmacology, 189*(1), 1-16. doi:10.1007/s00213-006-0529-6

Etches, V. (2013). *Addressing Substance Misuse in Ottawa*. Ottawa: Ottawa Public Health.

Everitt, B. J., Cardinal, R. N., Parkinson, J. A., & Robbins, T. W. (2003). Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. *Ann N Y Acad Sci, 985*, 233-250.

Everitt, B. J., & Robbins, T. W. (2013). From the ventral to the dorsal striatum: devolving views of their roles in drug addiction. *Neurosci Biobehav Rev, 37*(9 Pt A), 1946-1954. doi:10.1016/j.neubiorev.2013.02.010

Fenu, S., Spina, L., Rivas, E., Longoni, R., & Di Chiara, G. (2006). Morphine-conditioned single-trial place preference: role of nucleus accumbens shell dopamine receptors in

acquisition, but not expression. *Psychopharmacology (Berl), 187*(2), 143-153. doi:10.1007/s00213-006-0415-2
Fernandes, M. F., Matthys, D., Hryhorczuk, C., Sharma, A., Mogra, S., Alquier, T., & Fulton, S. (2015). Leptin Suppresses the Rewarding Effects of Running via STAT3 Signaling in Dopamine Neurons. *Cell Metab, 22*(4), 741-749.
Ferrario, C., Li, X., Wang, X., Reimers, J. M., Uejima, J. L., & Wolf, M. E. (2010). The role of glutamate receptor redistribution in locomotor sensitization to cocaine. *Neuropsychopharmacology, 35*(3), 818-833.
Figlewicz, D. P. (2003). Adiposity signals and food reward: expanding the CNS roles of insulin and leptin. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology, 284*, R882-892.
Figlewicz, D. P., Bennett, J. L., Naleid, A. M., Davis, C., & Grimm, J. W. (2006). Intraventricular insulin and leptin decrease sucrose self-administration in rats. *Physiol Behav, 89*(4), 611-616. doi:10.1016/j.physbeh.2006.07.023
Figlewicz, D. P., Higgins, G. A., Ng-Evans, S. B., & Havel, P. J. (2001). Leptin reverses sucrose-conditioned place preference in food-restricted rats. *Physiology & behavior, 73*, 229-234.
Fischer, B., Keates, A., Buhringer, G., Reimer, J., & Rehm, J. (2014). Non-medical use of prescription opioids and prescription opioid-related harms: why so markedly higher in North America compared to the rest of the world? *Addiction, 109*(2), 177-181. doi:10.1111/add.12224
Franklin, J. C., Shiele, B. C., Brozek, J., & Keys, A. (1948). Observations on human behavior in experimental semistarvation and rehabilitation. *Journal of Clinical Psychology, 4*, 28-45.
Friedman, J. M., & Halaas, J. L. (1998). Leptin and the regulation of body weight in mammals. *Nature, 395*, 763-770.
Fuchs, R. A., Branham, R. K., & See, R. E. (2006). Different neural substrates mediate cocaine seeking after abstinence versus extinction training: a critical role for the dorsolateral caudate-putamen. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 26*(13), 3584-3588. doi:10.1523/JNEUROSCI.5146-05.2006
Fuchs, R. A., Lasseter, H. C., Ramirez, D. R., & Xie, X. (2008). Relapse to drug seeking following prolonged abstinence: the role of environmental stimuli. *Drug discovery today. Disease models, 5*(4), 251-258. doi:10.1016/j.ddmod.2009.03.001
Fuchs, R. A., & See, R. E. (2002). Basolateral amygdala inactivation abolishes conditioned stimulus- and heroin-induced reinstatement of extinguished heroin-seeking behavior in rats. *Psychopharmacology (Berl), 160*(4), 425-433.
Fulton, S., Pissios, P., Manchon, R. P., Stiles, L., Frank, L., Pothos, E. N., . . . Flier, J. S. (2006). Leptin regulation of the mesoaccumbens dopamine pathway. *Neuron, 51*(6), 811-822. doi:10.1016/j.neuron.2006.09.006
Fulton, S., Woodside, B., & Shizgal, P. (2000). Modulation of brain reward circuitry by leptin. *Science, 287*, 125-128.
Gabriele, A., Pacchioni, A. M., & See, R. E. (2012). Dopamine and glutamate release in the dorsolateral caudate putamen following withdrawal from cocaine self-administration in rats. *Pharmacol Biochem Behav, 103*(2), 373-379.
Gaiardi, M., Bartoletti, M., Bacchi, A., Gubellini, C., & Babbini, M. (1987). Increased sensitivity to the stimulus properties of morphine in food deprived rats. *Pharmacol Biochem Behav, 26*(4), 719-723.

Gao, J., Li, Y., Zhu, N., Brimijoin, S., & Sui, N. (2013). Roles of dopaminergic innervation of nucleus accumbens shell and dorsolateral caudate-putamen in cue-induced morphine seeking after prolonged abstinence and the underlying D1- and D2-like receptor mechanisms in rats. *J Psychopharmacol, 27*(2), 181-191. doi:10.1177/0269881112466181

Gass, J. T., & Olive, M. F. (2008). Glutamatergic substrates of drug addiction and alcoholism. *Biochem Pharmacol, 75*(1), 218-265. doi:10.1016/j.bcp.2007.06.039

Gomez, J. L., & Ryabinin, A. E. (2014). The effects of ghrelin antagonists [D-Lys(3)]-GHRP-6 or JMV2959 on ethanol, water, and food intake in C57BL/6J mice. *Alcohol Clin Exp Res, 38*(9), 2436-2444. doi:10.1111/acer.12499

Grimm, J. W., Harkness, J. H., Ratliff, C., Barnes, J., North, K., & Collins, S. (2011). Effects of systemic or nucleus accumbens-directed dopamine D1 receptor antagonism on sucrose seeking in rats. *Psychopharmacology*. doi:10.1007/s00213-011-2210-y

Grimm, J. W., Hope, B. T., Wise, R. A., & Shaham, Y. (2001). Incubation of cocaine craving after withdrawal. *Nature, 412*, 141-142.

Guan, X. M., Yu, H., Palyha, O. C., McKee, K. K., Feighner, S. D., Sririnathsinghji, D. J. S., . . . Howard, A. D. (1997). Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Molecular Brain Research, 48*, 23-29.

Guo, Y., Wang, H. L., Xiang, X. H., & Zhao, Y. (2009). The role of glutamate and its receptors in mesocorticolimbic dopaminergic regions in opioid addiction. *Neurosci Biobehav Rev, 33*(6), 864-873. doi:10.1016/j.neubiorev.2009.02.005

Guy, E. G., Choi, E., & Pratt, W. E. (2011). Nucleus accumbens dopamine and mu-opioid receptors modulate the reinstatement of food-seeking behavior by food-associated cues. *Behav Brain Res, 219*(2), 265-272. doi:10.1016/j.bbr.2011.01.024

Halaas, J. L., Boozer, C., Blair-West, J., Fidahusein, N., Denton, D. A., & Friedman, J. M. (1997). Physiological response to long-term peripheral and central leptininfusion in lean and obese mice. *Proc Natl Acad Sci U S A, 94*, 8878-8883.

Hall, S. M., Tunstall, C. D., Vila, K. L., & Duffy, J. (1992). Weight gain prevention and smoking cessation: cautionary findings. *American Journal of Public Health, 82*, 799-803.

Hanna, J. M., & Hornick, C. A. (1977). Use of coca leaf in southern Peru: adaptation or addiction. *Bulletin on narcotics, 29*(1), 63-74.

Harrop, E. N., & Marlatt, G. A. (2010). The comorbidity of substance use disorders and eating disorders in women: prevalence, etiology, and treatment. *Addictive behaviors, 35*(5), 392-398. doi:10.1016/j.addbeh.2009.12.016

Hearing, M., Graziane, N., Dong, Y., & Thomas, M. J. (2018). Opioid and Psychostimulant Plasticity: Targeting Overlap in Nucleus Accumbens Glutamate Signaling. *Trends Pharmacol Sci, 39*(3), 276-294. doi:10.1016/j.tips.2017.12.004

Heimer, L., Zahm, D. S., Churchill, L., Kalivas, P. W., & Wohltmann, C. (1991). Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience, 41*, 89-125.

Helmerhorst, G., Teunis, T., Janssen, S. J., & Ring, D. (2017). An epidemic of the use, misuse and overdose of opioids an deaths due to overdose, in the United States and Canada. *Bone and Joint Journal, 99-B*, 856-864. doi:10.1302/0301-620X.99B7

Hernandez, G., Rajabi, H., Stewart, J., Arvanitogiannis, A., & Shizgal, P. (2008). Dopamine tone increases similarly during predictable and unpredictable administration of rewarding

brain stimulation at short inter-train intervals. *Behavioural brain research, 188*(1), 227-232. doi:10.1016/j.bbr.2007.10.035
Holderness, C. C., Brooks-Gunn, J., & Warren, M. P. (1994). Co-morbidity of eating disorders and substance abuse review of the literature. *The International journal of eating disorders, 16*(1), 1-34.
Holst, B., Cygankiewicz, A., Jensen, T. H., Ankersen, M., & Schwartz, T. W. (2003). High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol, 17*(11), 2201-2210. doi:10.1210/me.2003-0069
Holst, B., & Schwartz, T. W. (2004). Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends Pharmacol Sci, 25*(3), 113-117. doi:10.1016/j.tips.2004.01.010
Hommel, J. D., Trinko, R., Sears, R. M., Georgescu, D., Liu, Z. W., Gao, X. B., . . . DiLeone, R. J. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron, 51*(6), 801-810. doi:10.1016/j.neuron.2006.08.023
Horvath, T. L., Diano, S., & Tschop, M. (2003). Ghrelin in hypothalamic regulation of energy balance. *Current Topics in Medicinal Chemistry, 3*(8), 921-927.
Hotsenpiller, G., Giorgetti, M., & Wolf, M. E. (2001). Alterations in behaviour and glutamate transmission following presentation of stimuli previously associated with cocaine exposurre. *European Journal of Neuroscience., 14*, 1843-1855.
Hser, Y. I., Hoffman, V., Grella, C. E., & Anglin, M. D. (2001). A 33-year follow-up of narcotics addicts. *Archives of general psychiatry, 58*(5), 503-508.
Hyman, S. M., Fox, H., Hong, K. I., Doebrick, C., & Sinha, R. (2007). Stress and drug-cue-induced craving in opioid-dependent individuals in naltrexone treatment. *Experimental and clinical psychopharmacology, 15*(2), 134-143. doi:10.1037/1064-1297.15.2.134
Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain research reviews, 56*(1), 27-78. doi:10.1016/j.brainresrev.2007.05.004
Ito, R., Dalley, J. W., Howes, S. R., Robbins, T. W., & Everitt, B. J. (2000). Dissociation of conditioned dopamine release in the nucleus accumbens core and shell in response to cocaine cues and during cocaine-seeking behavior in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 20*(19), 7489-7495.
Ito, R., Dalley, J. W., Robbins, T. W., & Everitt, B. J. (2002). Dopamine release in the dorsal striatum during cocaine-seeking behavior under the control of a drug-associated cue. *The Journal of Neuroscience, 22*(1), 6247-6253.
Jerabek, P., Havlickova, T., Puskina, N., Charalambous, C., Lapka, M., Kacer, P., & Sustkova-Fiserova, M. (2017). Ghrelin receptor antagonism of morphine-induced conditioned place preference and behavioral and accumbens dopaminergic sensitization in rats. *Neurochem Int, 110*, 101-113. doi:10.1016/j.neuint.2017.09.013
Jerlhag, E. (2008). Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. *Addict Biol, 13*(3-4), 358-363. doi:10.1111/j.1369-1600.2008.00125.x
Jerlhag, E., Egecioglu, E., Dickson, S. L., & Engel, J. A. (2010). Ghrelin receptor antagonism attenuates cocaine- and amphetamine-induced locomotor stimulation, accumbal dopamine release, and conditioned place preference. *psychopharmacology, 211*, 415-422.
Jerlhag, E., Egecioglu, E., Landgren, S., Salome, N., Heilig, M., Moechars, D., . . . Engel, J. A. (2009). Requirement of central ghrelin signaling for alcohol reward. *Proceedings of the*

National Academy of Sciences of the United States of America, 106(27), 11318-11323. doi:10.1073/pnas.0812809106

Jerlhag, E., & Engel, J. A. (2011). Ghrelin receptor antagonism attenuates nicotine-induced locomotor stimulation, accumbal dopamine release and conditioned place preference in mice. *Drug and Alcohol Dependence, 117*, 126-131.

Johansson, A., Fredriksson, R., Winnergren, S., Hulting, A. L., Schioth, H. B., & Lindblom, J. (2008). The relative impact of chronic food restriction and acute food deprivation on plasma hormone levels and hypothalamic neuropeptide expression. *Peptides, 29*(9), 1588-1595. doi:10.1016/j.peptides.2008.04.018

Johnson, S. W., & North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 12*(2), 483-488.

Jones, J. L., Day, J. J., Aragona, B. J., Wheeler, R. A., Wightman, R. M., & Carelli, R. M. (2010). Basolateral amygdala modulates terminal dopamine release in the nucleus accumbens and conditioned responding. *Biol Psychiatry, 67*(8), 737-744. doi:10.1016/j.biopsych.2009.11.006

Jung, C., Rabinowitsch, A., Lee, W. T., Zheng, D., de Vaca, S. C., & Carr, K. D. (2016). Effects of food restriction on expression of place conditioning and biochemical correlates in rat nucleus accumbens. *Psychopharmacology (Berl), 233*(17), 3161-3172. doi:10.1007/s00213-016-4360-4

Kalivas, P. W. (2009). The glutamate homeostasis hypothesis of addiction. *Nature reviews. Neuroscience, 10*(8), 561-572. doi:10.1038/nrn2515

Kalivas, P. W., & Duffy, P. (1995). Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. *Brain research, 675*, 325-328.

Kalivas, P. W., Lalumiere, R. T., Knackstedt, L., & Shen, H. (2009). Glutamate transmission in addiction. *Neuropharmacology, 56 Suppl 1*, 169-173. doi:10.1016/j.neuropharm.2008.07.011

Kalivas, P. W., & O'Brien, C. (2008). Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology, 33*(1), 166-180. doi:10.1038/sj.npp.1301564

Kalivas, P. W., & Volkow, N. D. (2005). The neural basis of addiction: a pathology of motivation and choice. *American Journal of Psychiatry, 162*, 1403-1413.

Kawaguchi, Y., Wilson, C. J., Augood, S. J., & Emson, P. C. (1995). Striatal interneurones: chemical, physiological and morphological characterization. *Trends in Neurosciences, 18*(12), 527-535.

Kelley, A. E., & Berridge, K. C. (2002). The neuroscience of natural rewards: relevance to addictive drugs. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 22*(9), 3306-3311. doi:20026361

Kinzig, K. P., Hargrave, S. L., & Tao, E. E. (2009). Central and peripheral effects of chronic food restriction and weight restoration in the rat. *Am J Physiol Endocrinol Metab, 296*(2), E282-290. doi:10.1152/ajpendo.90523.2008

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., & Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptidefrom stomach. *Nature, 402*, 656-660.

Koob, G. F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci, 13*(5), 177-184.

Krahn, D., Kurth, C., Demitrack, M., & Drewnowski, A. (1992). The relationship of dieting severity and bulimic behaviors to alcohol and other drug use in young women. *Journal of Substance Abuse, 4*, 341-353.

LaLumiere, R. T., & Kalivas, P. W. (2008). Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 28*(12), 3170-3177. doi:10.1523/JNEUROSCI.5129-07.2008

Li, P., Wu, P., Xin, X., Fan, Y. L., Wang, G. B., Wang, F., . . . Lu, L. (2014). Incubation of alcohol craving during abstinence in patients with alcohol dependence. *Addict Biol, 20*(3), 513-522. doi:10.1111/adb.12140

Li, X., Caprioli, D., & Marchant, N. J. (2015). Recent updates on incubation of drug craving: a mini-review. *Addict Biol, 20*(5), 872-876. doi:10.1111/adb.12205

Lou, Z. Z., Chen, L. H., Liu, H. F., Ruan, L. M., & Zhou, W. H. (2014). Blockade of mGluR5 in the nucleus accumbens shell but not core attenuates heroin seeking behavior in rats. *Acta Pharmacol Sin, 35*(12), 1485-1492. doi:10.1038/aps.2014.93

Loweth, J. A., Tseng, K. Y., & Wolf, M. E. (2014). Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. *Neuropharmacology, 76 Pt B*, 287-300. doi:10.1016/j.neuropharm.2013.04.061

M'Kadmi, C., Leyris, J. P., Onfroy, L., Gales, C., Sauliere, A., Gagne, D., . . . Marie, J. (2015). Agonism, Antagonism, and Inverse Agonism Bias at the Ghrelin Receptor Signaling. *J Biol Chem, 290*(45), 27021-27039. doi:10.1074/jbc.M115.659250

Ma, Y. Y., Chu, N. N., Guo, C. Y., Han, J. S., & Cui, C. L. (2007). NR2B-containing NMDA receptor is required for morphine-but not stress-induced reinstatement. *Exp Neurol, 203*(2), 309-319. doi:10.1016/j.expneurol.2006.08.014

Maness, L. M., Kastin, A. J., Farrell, C. L., & Banks, W. A. (1998). Fate of leptin after intracerebroventricular injection into the mouse brain. *Endocrinology, 139*(11), 4556-4562. doi:10.1210/endo.139.11.6319

Marchant, N. J., & Kaganovsky, K. (2015). A critical role of nucleus accumbens dopamine D1-family receptors in renewal of alcohol seeking after punishment-imposed abstinence. *Behav Neurosci, 129*(3), 281-291. doi:10.1037/bne0000050

Marchant, N. J., Li, X., & Shaham, Y. (2013). Recent developments in animal models of drug relapse. *Curr Opin Neurobiol, 23*(4), 675-683. doi:10.1016/j.conb.2013.01.003

Marchant, N. J., Rabei, R., Kaganovsky, K., Caprioli, D., Bossert, J. M., Bonci, A., & Shaham, Y. (2014). A critical role of lateral hypothalamus in context-induced relapse to alcohol seeking after punishment-imposed abstinence. *J Neurosci, 34*(22), 7447-7457. doi:10.1523/JNEUROSCI.0256-14.2014

Maric, T., Sedki, F., Ronfard, B., Chafetz, D., & Shalev, U. (2011). A limited role for ghrelin in heroin self-administration and food deprivation-induced reinstatement of heroin seeking in rats. *Addiction biology*. doi:10.1111/j.1369-1600.2011.00396.x

Maric, T., Sedki, F., Ronfard, B., Chafetz, D., & Shalev, U. (2012). A limited role for ghrelin in heroin self-administration and food deprivation-induced reinstatement of heroin seeking in rats. *Addict Biol, 17*(3), 613-622. doi:10.1111/j.1369-1600.2011.00396.x

Mars, S. G., Bourgois, P., Karandinos, G., Montero, F., & Ciccarone, D. (2014). "Every 'never' I ever said came true": transitions from opioid pills to heroin injecting. *Int J Drug Policy, 25*(2), 257-266. doi:10.1016/j.drugpo.2013.10.004

McFarland, K., Lapish, C. C., & Kalivas, P. W. (2003). Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking

behavior. . *The Journal of neuroscience : the official journal of the Society for Neuroscience, 23*(8), 3531-3537.
McGlinchey, E., James, M., Mahler, S., Pantazis, C., & Aston-Jones, G. (2016). Prelimbic to Accumbens Core Pathway is Recruited in a Dopamine-Dependent Manner to Drive Cued Reinstatement of Cocaine Seeking. *The Journal of Neuroscience, 36*(33), 8700-8711.
McLellan, A. T., Lewis, D. C., O'Brien, C. P., & Kleber, H. D. (2000). Drug Dependence, a Chronic Mental Illness: Implications for Treatment, Insurance, and Outcomes Evaluation. *JAMA, 284*(13), 1689-1695.
Meil, W. M., & See, R. E. (1996). Conditioned cued recovery of responding following prolonged withdrawal from self-administered cocaine in rats: an animal model of relapse. *Behavioural pharmacology, 7*(8), 754-763.
Miguens, M., Del Olmo, N., Higuera-Matas, A., Torres, I., Garcia-Lecumberri, C., & Ambrosio, E. (2008). Glutamate and aspartate levels in the nucleus accumbens during cocaine self-administration and extinction: a time course microdialysis study. *Psychopharmacology (Berl), 196*(2), 303-313. doi:10.1007/s00213-007-0958-x
Mogenson, G., Jones, D., & Yim, C. (1980). From motivation to action: functional interface between the limbic system and the motor system. *Progress in Neurobiology, 14*(2-3), 69-97.
Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S., & Schwartz, M. W. (2006). Central nervous system control of food intake and body weight. *Nature, 443*(7109), 289-295. doi:10.1038/nature05026
Moulin, A., Demange, L., Berge, G., Gagne, D., Ryan, J., Mousseaux, D., . . . Martinez, J. (2007). Toward potent ghrelin receptor ligands based on trisubstituted 1,2,4-triazole structure. 2. Synthesis and pharmacological in vitro and in vivo evaluations. *J Med Chem, 50*(23), 5790-5806. doi:10.1021/jm0704550
Mueller, D., & Stewart, J. (2000). Cocaine-induced conditioned place preference: reinstatement by priming injections of cocaine after extinction. *Behav Brain Res, 115*(1), 39-47.
Murphy, Y., Goldner, E. M., & Fischer, B. (2015). Prescription Opioid Use, Harms and Interventions in Canada: A Review Update of New Developments and Findings since 2010. *Pain Physician, 18*, E605-E614.
Neale, J., Nettleton, S., Pickering, L., & Fischer, J. (2012). Eating patterns among heroin users: a qualitative study with implications for nutritional interventions. *Addiction, 107*(3), 635-641. doi:10.1111/j.1360-0443.2011.03660.x
Neisewander, J. L., O'Dell, L. E., Tran-Nguyen, L. T. L., Castañeda, E., & Fuchs, R. A. (1996). Dopamine overflow in the nucleus accumbens during extinction and reinstatement of cocaine self-administration behavior. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 15*(5), 506-514.
Niswender, C., & Conn, P. J. (2010). Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annual Review of pharmacology and toxicology, 50*, 295-322.
O'Brien, C. P. (1997). A Range of Research-Based Pharmacotherapies for Addiction. *Science, 278*(5335), 66-70. doi:10.1126/science.278.5335.66
O'Brien, C. P., & McLellan, A. T. (1996). Myths about the treatment of addiction. *Lancet, 347*, 237-240.
O'Donnell, P., & Grace, A. A. (1993). Physiological and morphological properties of accumbens core and shell neurons recorded in vitro. *Synapse, 13*(2), 135-160.

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res, 34*(1), 171-175.
Olds, J., & Milner, P. (1954). Positive reinforcement produced by electrical stimulation of septal area and other regions of the brain. . *Journal of comparative and physiological psychology., 47*(6), 419-427.
Ouyang, J., Carcea, I., Schiavo, J. K., Jones, K. T., Rabinowitsch, A., Kolaric, R., . . . Carr, K. D. (2017). Food restriction induces synaptic incorporation of calcium-permeable AMPA receptors in nucleus accumbens. *Eur J Neurosci, 45*(6), 826-836. doi:10.1111/ejn.13528
Pavlov, I. (1927). *Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex*. London: Oxford University Press.
Paxinos, G., & Watson, C. (2005). *The Rat Brain in Stereotaxic Coordinates.* Burlington, MA: Elsevier Academic Press.
Peng, X. X., Cabeza de Vaca, S., Ziff, E. B., & Carr, K. D. (2014). Involvement of nucleus accumbens AMPA receptor trafficking in augmentation of D- amphetamine reward in food-restricted rats. *Psychopharmacology (Berl), 231*(15), 3055-3063. doi:10.1007/s00213-014-3476-7
Peng, X. X., Ziff, E. B., & Carr, K. D. (2011). Effects of food restriction and sucrose intake on synaptic delivery of AMPA receptors in nucleus accumbens. *Synapse, 65*(10), 1024-1031. doi:10.1002/syn.20931
Peters, J., Kalivas, P. W., & Quirk, G. J. (2009). Extinction circuits for fear and addiction overlap in prefrontal cortex. *Learning & memory, 16*(5), 279-288. doi:10.1101/lm.1041309
Peters, J., LaLumiere, R. T., & Kalivas, P. W. (2008). Infralimbic prefrontal cortex is responsible for inhibiting cocaine seeking in extinguished rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 28*(23), 6046-6053. doi:10.1523/JNEUROSCI.1045-08.2008
Pickens, C. L., Airavaara, M., Theberge, F., Fanous, S., Hope, B. T., & Shaham, Y. (2011). Neurobiology of the incubation of drug craving. *Trends in neurosciences, 34*(8), 411-420. doi:10.1016/j.tins.2011.06.001
Pierce, R. C., Bell, K., Duffy, P., & Kalivas, P. W. (1996). Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. *The Journal of Neuroscience, 16*(4), 1550-1560.
Pinto, A., Jankowski, M., & Sesack, S. (2003). Projections from the paraventricular nucleus of the thalamus to the rat prefrontal cortex and nucleus accumbens shell: ultrastructural characteristics and spatial relationships with dopamine afferents. *Journal of comparative neurology, 459*(2), 142-155.
Placenza, F. M., Rajabi, H., & Stewart, J. (2008). Effects of chronic buprenorphine treatment on levels of nucleus accumbens glutamate and on the expression of cocaine-induced behavioral sensitization in rats. *Psychopharmacology (Berl), 200*(3), 347-355. doi:10.1007/s00213-008-1210-z
Pontieri, F. E., 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. *Proceedings of the National Academy of Sciences of the United States of America, 92*(26), 12304-12308.

Preston, K. L., & Epstein, D. H. (2011). Stress in the daily lives of cocaine and heroin users: relationship to mood, craving, relapse triggers, and cocaine use. *Psychopharmacology, 218*(1), 29-37. doi:10.1007/s00213-011-2183-x

Rehm, J., Fischer, B., & Gittins, J. (2009). *An overview of non-medical use of prescription drugs and criminal justice issues in Canada*. Canada: Department of Justice.

Reissner, K., Gipson, C. D., Tran, P. K., Knackstedt, L., Scofield, M. D., & Kalivas, P. W. (2015). Glutamate transporter GLT-1 mediates N-acetylcysteine inhibition of cocaine reinstatement. *Addict Biol, 20*(2), 316-323.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain research reviews, 18*(3), 247-291.

Robinson, T. E., & Berridge, K. C. (2003). Addiction. *Annual Review of psychology, 54*, 25-53. doi:10.1146/annurev.psych.54.101601.145237

Sadeghzadeh, F., Babapour, V., & Haghparast, A. (2015). Role of dopamine D1-like receptor within the nucleus accumbens in acute food deprivation- and drug priming-induced reinstatement of morphine seeking in rats. *Behav Brain Res, 287*, 172-181. doi:10.1016/j.bbr.2015.03.055

Salamone, J. D., Correa, M., Mingote, S. M., & Weber, S. M. (2005). Beyond the reward hypothesis: alternative functions of nucleus accumbens dopamine. *Current opinion in pharmacology, 5*(1), 34-41. doi:10.1016/j.coph.2004.09.004

Salamone, J. D., Cousins, M. S., & J., S. B. (1997). Behavioral functions of nucleus accumbens dopamine: Empirical and conceptual problems with the anhedonia hypothesis. *Neuroscience & Biobehavioral Reviews, 21*, 341-359.

Salamone, J. D., Wisniecki, A., Carlson, B. B., & Correa, M. (2001). Nucleus accumbens dopamine depletions make animals highly sensitive to high fixed ratio requirements but do not impair primary food reinforcement. *Neuroscience, 105*(4), 863-870.

Schultz, W. (1998). Predictive reward signal of dopamine neurons. *Journal of neurophysiology, 80*(1), 1-27.

Schultz, W. (2016). Dopamine reward prediction-error signalling: a two-component response. *Nature Reviews. Neuroscience., 17*(3), 183-195.

Schultz, W., Dayan, P., & Montague, P. R. (1997). A neural substrate of prediction and reward. *Science, 275*, 1593-1599.

Sciascia, J. M., Reese, R. M., Janak, P. H., & Chaudhri, N. (2015). Alcohol-Seeking Triggered by Discrete Pavlovian Cues is Invigorated by Alcohol Contexts and Mediated by Glutamate Signaling in the Basolateral Amygdala. *Neuropsychopharmacology, 40*(12), 2801-2812. doi:10.1038/npp.2015.130

Scofield, M. D., Heinsbroek, J. A., Gipson, C. D., Kupchik, Y. M., Spencer, S., Smith, A. C., . . . Kalivas, P. W. (2016). The Nucleus Accumbens: Mechanisms of Addiction across Drug Classes Reflect the Importance of Glutamate Homeostasis. *Pharmacol Rev, 68*(3), 816-871. doi:10.1124/pr.116.012484

Sedki, F., Abbas, Z., Angelis, S., Martin, J., D'Cunha, T., & Shalev, U. (2013). Is it stress? The role of stress related systems in chronic food restriction-induced augmentation of heroin seeking in the rat. *Front Neurosci, 7*, 98. doi:10.3389/fnins.2013.00098

Sedki, F., D'Cunha, T., & Shalev, U. (2013). A procedure to study the effect of prolonged food restriction on heroin seeking in abstinent rats. *J Vis Exp*(81), e50751. doi:10.3791/50751

Sesack, S. R., Carr, D. B., Omelchenko, N., & Pinto, A. (2003). Anatomical substrates for glutamate–dopamine interactions: evidence for specificity of connections and extrasynaptic actions. . *Annals of the New York Academy of Sciences, 1003*, 36-52.

Shaham, Y., Shalev, U., Lu, L., de Wit, H., & Stewart, J. (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology, 168*(1-2), 3-20. doi:10.1007/s00213-002-1224-x

Shaham, Y., & Stewart, J. (1995). Stress reinstates heroin-seeking in drug-free animals: an effect mimicking heroin, not withdrawal. *Psychopharmacology, 119*, 334-341.

Shalev, U., Finnie, P. S., Quinn, T., Tobin, S., & Wahi, P. (2006). A role for corticotropin-releasing factor, but not corticosterone, in acute food-deprivation-induced reinstatement of heroin seeking in rats. *Psychopharmacology, 187*(3), 376-384. doi:10.1007/s00213-006-0427-y

Shalev, U., Grimm, J. W., & Shaham, Y. (2002). Neurobiology of relapse to heroin and cocaine seeking: a review. *Pharmacological Reviews, 54*(1), 1-42.

Shalev, U., Highfield, D., Yap, J., & Shaham, Y. (2000). Stress and relapse to drug seeking in rats: studies on the generality of the effect. *Psychopharmacology, 150*(3), 337-346. doi:10.1007/s002130000441

Shalev, U., Morales, M., Hope, B., Yap, J., & Shaham, Y. (2001). Time-dependent changes in extinction behavior and stress-induced reinstatement of drug seeking following withdrawal from heroin in rats. *Psychopharmacology, 156*(1), 98-107. doi:10.1007/s002130100748

Shalev, U., Yap, J., & Shaham, Y. (2001). Leptin attenuates acute food deprivation-induced relapse to heroin seeking. *Journal of Neuroscience, 21*, RC129.

Shen, H., Moussawi, K., Zhou, W., Toda, S., & Kalivas, P. W. (2011). Heroin relapse requires long-term potentiation-like plasticity mediated by NMDA2b-containing receptors. *Proc Natl Acad Sci U S A, 108*(48), 19407-19412. doi:10.1073/pnas.1112052108

Shen, H., Scofield, M. D., Boger, H., Hensley, M., & Kalivas, P. W. (2014a). Synaptic Glutamate Spillover Due to Impaired Glutamate Uptake Mediates Heroin Relapse. *The Journal of Neuroscience, 34*(16), 5649-5657.

Shen, H. W., Scofield, M. D., Boger, H., Hensley, M., & Kalivas, P. W. (2014b). Synaptic glutamate spillover due to impaired glutamate uptake mediates heroin relapse. *J Neurosci, 34*(16), 5649-5657. doi:10.1523/JNEUROSCI.4564-13.2014

Shen, M., Jiang, C., Liu, F., Wang, F., & Ma, L. (2016). Mesolimbic leptin signaling negatively regulates cocaineconditioned
reward. *Translational Psychiatry, 6*, e972.

Sinha, R. (2001). How does stress increase risk of drug abuse and relapse? *Psychopharmacology, 158*(4), 343-359. doi:10.1007/s002130100917

Sinha, R. (2009). Modeling stress and drug craving in the laboratory: implications for addiction treatment development. *Addiction biology, 14*(1), 84-98. doi:10.1111/j.1369-1600.2008.00134.x

Skibicka, K. P., Hansson, C., Alvarez-Crespo, M., Friberg, P. A., & Dickson, S. L. (2011). Ghrelin directly targets the ventral tegmental area to increase food motivation. *Neuroscience, 180*, 129-137. doi:10.1016/j.neuroscience.2011.02.016

Skibicka, K. P., Hansson, C., Egecioglu, E., & Dickson, S. L. (2012). Role of ghrelin in food reward: impact of ghrelin on sucrose self-administration and mesolimbic dopamine and

acetylcholine receptor gene expression. *Addict Biol, 17*(1), 95-107. doi:10.1111/j.1369-1600.2010.00294.x

Sokoloff, P., & Schwartz, J. C. (1995). Novel dopamine receptors half a decade later. *Trends Pharmacol Sci, 16*(8), 270-275.

Sorge, R. E., Rajabi, H., & Stewart, J. (2005). Rats are maintained chronically on buprenorphine show reduced heroin and cocaine seeking in tests of extinction and dug-induced reinstatement. *Neuropharmacology, 30*, 1681-1692.

Special Advisory Committee on the Epidemic of Opioid Overdoses. National report: Apparent opioid-related deaths in Canada. (2017). Ottawa: Govenment of Canada Retrieved from https://www.canada.ca/en/public-health/services/publications/healthy-living/apparent-opioid-related-deaths-report-2016-2017-december.html.

Squire, L. (1992). Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychological Review, 99*(2), 195-231.

Stefanik, M., & Kalivas, P. W. (2013). Optogenetic dissection of basolateral amygdala projections during cue-induced reinstatement of cocaine seeking. *Frontiers in behavioral neuroscience, 7*, 213.

Stewart, J. (1983). Conditioned and unconditioned drug effects in relapse to opiate and stimulant drug self-adminstration. *Progress in Neuropsychopharmacology & Biological Psychiatry, 7*(4-6), 591-597.

Stewart, J., de Wit, H., & Eikelboom, R. (1984). Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychological Review, 91*(2), 251-268.

Stuber, G., Sparta, D., Stamatakis, A., van Leeuwen, W., Hardjoprajitno, J., Cho, S., . . . Bonci, A. (2011). Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *Nature, 475*(7356), 377-380.

Stuber, G. D., Evans, S. B., Higgins, M. S., Pu, Y., & Figlewicz, D. P. (2002). Food restriction modulates amphetamine-conditioned place preference and nucleus accumbens dopamine release in the rat. *Synapse, 46*(2), 83-90. doi:10.1002/syn.10120

Sun, X., & Wolf, M. E. (2009). Nucleus accumbens neurons exhibit synaptic scaling that is occluded by repeated dopamine pre-exposure. *The European Journal of Neuroscience, 30*(4), 539-550.

Sustkova-Fiserova, M., Jerabek, P., Havlickova, T., Kacer, P., & Krsiak, M. (2014). Ghrelin receptor antagonism of morphine-induced accumbens dopamine release and behavioral stimulation in rats. *Psychopharmacology (Berl), 231*(14), 2899-2908. doi:10.1007/s00213-014-3466-9

Suto, N., Ecke, L. E., You, Z. B., & Wise, R. A. (2010). Extracellular fluctuations of dopamine and glutamate in the nucleus accumbens core and shell associated with lever-pressing during cocaine self-administration, extinction, and yoked cocaine administration. *Psychopharmacology, 211*(3), 267-275. doi:10.1007/s00213-010-1890-z

Suto, N., Elmer, G. I., Wang, B., You, Z. B., & Wise, R. A. (2013). Bidirectional modulation of cocaine expectancy by phasic glutamate fluctuations in the nucleus accumbens. *J Neurosci, 33*(21), 9050-9055. doi:10.1523/JNEUROSCI.0503-13.2013

Suto, N., Tanabe, L. M., Austin, J. D., Creekmore, E., Pham, C. T., & Vezina, P. (2004). Previous exposure to psychostimulants enhances the reinstatement of cocaine seeking by nucleus accumbens AMPA. *Neuropsychopharmacology, 29*(12), 2149-2159. doi:10.1038/sj.npp.1300533

Thompson, J. L., & Borgland, S. L. (2013). Presynaptic leptin action suppresses excitatory synaptic transmission onto ventral tegmental area dopamine neurons. *Biol Psychiatry, 73*(9), 860-868. doi:10.1016/j.biopsych.2012.10.026

Toates, F. (1998). The interaction of cognitive and stimulus-response processes in the control of behaviour. *Neuroscience & Biobehavioral Reviews, 22*, 59-83.

Tobin, S., Newman, A. H., Quinn, T., & Shalev, U. (2009). A role for dopamine D1-like receptors in acute food deprivation-induced reinstatement of heroin seeking in rats. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum, 12*(2), 217-226. doi:10.1017/S1461145708008778

Tobin, S., Sedki, F., Abbas, Z., & Shalev, U. (2013). Antagonism of the dopamine D1-like receptor in mesocorticolimbic nuclei attenuates acute food deprivation-induced reinstatement of heroin seeking in rats. *Eur J Neurosci, 37*(6), 972-981. doi:10.1111/ejn.12112

Toshinai, K., Mondal, M. S., Nakazato, M., Date, Y., Murakami, N., Kojima, M., . . . Matsukura, S. (2001). Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem Biophys Res Commun, 281*(5), 1220-1225. doi:10.1006/bbrc.2001.4518

Traebert, M., Riediger, T., Whitebread, S., Scharrer, E., & Schmid, H. A. (2002). Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. *J Neuroendocrinol, 14*(7), 580-586.

Tran-Nguyen, L. T. L., Fuchs, R. A., Coffey, G. P., Baker, D. A., O'Dell, L. E., & Neisewander, J. L. (1998). Time-dependent changes in cocaine-seeking behavior and extracellular dopamine levels in the amygdala during cocaine withdrawal. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 19*(1), 48-59.

Tschop, M., Smiley, D. L., & Heiman, M. L. (2000). Ghrelin induces adiposity in rodents. *Nature, 407*, 908-913.

Turrigiano, G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell, 135*(3), 422-435.

Turrigiano, G., & Nelson, S. (2004). Homeostatic plasticity in the developing nervous system. *Nature Reviews. Neuroscience., 5*(2), 97-107.

Ungerstedt, U. (1971). Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta physiologica Scandinavica. Supplementum, 367*, 1-48.

van Zessen, R., van der Plasse, G., & Adan, R. A. (2012). Contribution of the mesolimbic dopamine system in mediating the effects of leptin and ghrelin on feeding. *Proc Nutr Soc, 71*(4), 435-445. doi:10.1017/S0029665112000614

Venniro, M., Caprioli, D., & Shaham, Y. (2016). Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence. *Prog Brain Res, 224*, 25-52. doi:10.1016/bs.pbr.2015.08.004

Verhagen, L. A., Egecioglu, E., Luijendijk, M. C., Hillebrand, J. J., Adan, R. A., & Dickson, S. L. (2011). Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity. *Eur Neuropsychopharmacol, 21*(5), 384-392. doi:10.1016/j.euroneuro.2010.06.005

Walter, S., & Kuschinsky, K. (1989). Conditioning of morphine-induced locomotor activity and stereotyped behavior in rats. *Journal of neural transmission general section, 78*, 231-247.

Wang, G., Shi, J., Chen, N., Xu, L., Li, J., Li, P., . . . Lu, L. (2013). Effects of length of abstinence on decision-making and craving in methamphetamine abusers. *PLoS One, 8*(7), e68791. doi:10.1371/journal.pone.0068791

Wang, X., Moussawi, K., Knackstedt, L., Shen, H., & Kalivas, P. W. (2013). Role of mGluR5 neurotransmission in reinstated cocaine-seeking. *Addict Biol, 18*(1), 40-49. doi:10.1111/j.1369-1600.2011.00432.x

Weise-Kelly, L., & Siegel, S. (2001). Self-administration cues as signals: Drug self-administration and tolerance. *Journal of Experimental Psychology: Animal Behavior Processes, 27*(2), 125-136.

Weiss, F., Maldonado-Aviles, J., Parsons, L. H., Kerr, T. M., Smith, D. L., & Ben-Shahar, O. (2000). Control of cocaine-seeking behavior by drug-associated stimuli in rats: Effects on recovery of extinguished operant-responding and extracellular dopamine levels in amygdala and nucleus accumbens. *PNAS, 97*, 4321-4326.

Wellman, P. J., Clifford, P. S., Rodriguez, J. A., Hughes, S., Di Francesco, C., Melotto, S., . . . Gozzi, A. (2012). Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation. *Addict Biol, 17*(5), 908-919. doi:10.1111/j.1369-1600.2011.00392.x

Wilson, C. J., & Groves, P. M. (1980). Fine structure and synaptic connections of the common spiny neuron of the rat neostriatum: A study employing intracellular injection of horseradish peroxidase. *The Journal of Comparative Neurology, 194*(3), 599-615.

Wise, R. A. (1982). Neuroleptics and operant behavior: The anhedonia hypothesis. *Behavioral and brain sciences, 5*, 39-53.

Wise, R. A. (2009). Roles for nigrostriatal--not just mesocorticolimbic--dopamine in reward and addiction. *Trends in neurosciences, 32*(10), 517-524. doi:10.1016/j.tins.2009.06.004

Wise, R. A., Newton, P., Leeb, K., Burnette, B., Pocock, D., & Justice, J. B. (1995). Fluctuations in nucleus accumbens dopamine concentration during intravenous cocaine self-administration in rats. *Psychopharmacology, 120*, 10-20.

Wise, R. A., & Rompre, P. P. (1989). Brain dopamine and reward. *Annual Review of psychology, 40*, 191-225.

Wolf, M. E. (2010). Regulation of AMPA receptor trafficking in the nucleus accumbens by dopamine and cocaine. *Neurotox Res, 18*(3-4), 393-409. doi:10.1007/s12640-010-9176-0

Wolf, M. E. (2016). Synaptic mechanisms underlying persistent cocaine craving. *Nat Rev Neurosci, 17*(6), 351-365. doi:10.1038/nrn.2016.39

Wolf, M. E., & Ferrario, C. R. (2010). AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. *Neurosci Biobehav Rev, 35*(2), 185-211. doi:10.1016/j.neubiorev.2010.01.013

Wolf, M. E., & Tseng, K. Y. (2012). Calcium-permeable AMPA receptors in the VTA and nucleus accumbens after cocaine exposure: when, how, and why? *Front Mol Neurosci, 5*, 72. doi:10.3389/fnmol.2012.00072

Wunsch, A., Yager, L., Donckels, E., Le, C., Neumair, J., & Ferguson, S. (2017). Chemogenetic inhibition reveals midline thalamic nuclei and thalamo-accumbens projections mediate cocaine-seeking in rats. *The European Journal of Neuroscience, 46*(3), 1850-1862.

Wyvell, C. L., & Berridge, K. C. (2001). Incentive sensitization by previous amphetamine exposure: Increased cue-triggered "wanting" for sucrose reward. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 21*, 7831-7840.

Xia, Y., Driscoll, J. R., Wilbrecht, L., Margolis, E. B., Fields, H. L., & Hjelmstad, G. O. (2011). Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. *J Neurosci, 31*(21), 7811-7816. doi:10.1523/JNEUROSCI.1504-11.2011

Yagishita, S., Hayashi-Takagi, A., Ellis-Davies, G. C., Urakubo, H., Ishii, S., & Kasai, H. (2014). A critical time window for dopamine actions on the structural plasticity of dendritic spines. *Science, 345*(6204), 1616-1620. doi:10.1126/science.1255514

You, Z. B., Wang, B., Liu, Q. R., Wu, Y., Otvos, L., & Wise, R. A. (2016). Reciprocal Inhibitory Interactions Between the Reward-Related Effects of Leptin and Cocaine. *Neuropsychopharmacology, 41*(4), 1024-1033. doi:10.1038/npp.2015.230

Zacny, J. P., & de Wit, H. (1992). The effects of a restricted feeding regimen on cigarette smoking in humans. *Addictive Behaviors, 17*, 149-157.

Zheng, D., Cabeza de Vaca, S., Jurkowski, Z., & Carr, K. D. (2015). Nucleus accumbens AMPA receptor involvement in cocaine-conditioned place preference under different dietary conditions in rats. *Psychopharmacology (Berl), 232*(13), 2313-2322. doi:10.1007/s00213-015-3863-8

Zhu, Y., Wienecke, C. F., Nachtrab, G., & Chen, X. (2016). A thalamic input to the nucleus accumbens mediates opiate dependence. *Nature, 530*(7589), 219-222. doi:10.1038/nature16954

Zorrilla, E. P., & Koob, G. F. (2013). Amygdalostriatal projections in the neurocircuitry for motivation: a neuroanatomical thread through the career of Ann Kelley. *Neurosci Biobehav Rev, 37*(9 Pt A), 1932-1945. doi:10.1016/j.neubiorev.2012.11.019

APPENDIX: THE ROLE OF DOPAMINE IN THE BASOLATERAL AMYGDALA AND DORSOLATERAL STRIATUM IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

MATERIALS AND METHODS

Experiments follow the same methodology, procedures, and statistical analyses as Experiment 1A and 2A in Chapter 3.

Surgical Procedures

Intravenous catheterization surgery is outlined in Chapter 3 Materials and Methods. For rats in the *in vivo* microdialysis experiments, immediately following the intravenous catheterization, unilateral guide cannula were implanted targeting one of the following regions (coordinates in mm relative to Bregma): basolateral amygdala (BLA; Supplemental Experiment 1) AP -2.6, ML ±5.0, DV -5.6; or, dorsolateral striatum (DLS; Supplemental Experiment 2) AP +1.2, ML ±3.4, DV -2.6 (Experiment 4; Paxinos & Watson, 2005). Cannula were mounted adjacent to the modified catheter cannula on the skull using jeweller's screws and dental cement. Cannula placements were counterbalanced between the right and left hemispheres.

RESULTS

Rats were excluded if they pulled out probes during the test session, or if there were technical problems with the analysis of the dialysate samples in the HPLC. In addition, only rats with correct histological placements were included in the final analysis (Supplemental Figure S.1).

Supplemental Experiment 1: Changes in extracellular DA in the BLA

FDR rats (n = 13) demonstrated augmented heroin seeking as indicated by the significantly higher number of active lever responses made compared to the sated rats (n = 8) during the test (t (19) = 2.293, p = 0.033, d = 1.216; Supplemental Figure S.2 inset). Supplemental Figure S.2A shows active lever responding for the FDR and sated rats in 10-minute time intervals across the 3 h test session, with significant effects of *food restriction* ($F_{(1,19)}$ = 5.259, p = 0.033), *time* ($F_{(17,323)}$ = 9.481, p < 0.001), and *time* × *food restriction*

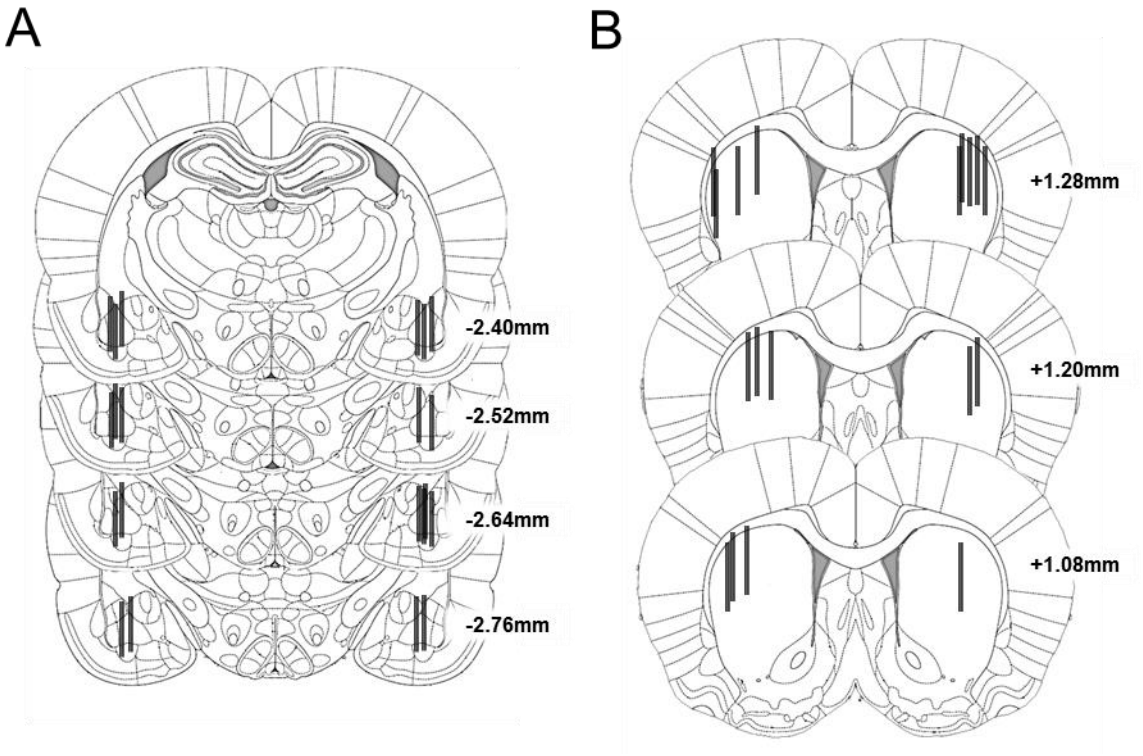

Figure S.1. Cannula placements for supplemental experiments. Approximate locations of active region of microdialysis probe targeting the BLA (A) for Supplemental Experiment 1 (n = 13; black rectangles), or the DLS (B) for Supplemental Experiment 2 (n = 18; black rectangles). Images modified from the brain atlas of Paxinos and Watson (2005) of Figures 53 – 56 (-2.40 to -2.76 mm anterior to Bregma) for the BLA and Figures 22 – 24 (+1.28 to +1.08 mm anterior to Bregma) for the DLS.

interaction ($F_{(17,323)} = 1.848$, $p = 0.022$). There were no statistically significant differences in the number of inactive lever responses.

Absolute baseline dialysate concentrations of DA in the FDR and sated groups were 0.0657 ± 0.00900 pg/µl and 0.0596 ± 0.00917 pg/µl, respectively (t (19) = 0.448, $p = 0.660$, $d = 0.229$). Overall, there were no changes in extracellular DA in the BLA compared to baseline in either group during the heroin seeking test (Supplemental Figure S.2B). There was a statistically significant main effect of *time* ($F_{(19,361)} = 1.977$, $p = 0.009$), which likely reflects slight variations in DA throughout the 3 h test session. However, there were no significant effects of *food restriction* ($F_{(1,19)} = 0.696$, $p = 0.414$), and no significant interaction of *time* × *food restriction* ($F_{(19,361)} = 1.524$, $p = 0.074$).

Supplemental Experiment 2: Changes in extracellular DA in the DLS

The FDR rats (n = 10) demonstrated significantly higher number of active lever responses compared to the sated rats (n = 8; t (16) = 2.742, $p = 0.014$, $d = 1.358$; Supplemental Figure S.3A inset) and there were no statistically significant differences in inactive lever responses. Supplemental Figure S.3A shows active lever responding for the FDR and sated rats in 10-minute time intervals across the 3 h test session, with significant effects of *food restriction* ($F_{(1,16)} = 7.517$, $p = 0.014$), *time* ($F_{(17,272)} = 6.995$, $p < 0.001$), and *time* × *food restriction* interaction ($F_{(17,272)} = 1.657$, $p = 0.051$). Absolute baseline dialysate concentrations of DA were 0.3466 ± 0.07689 pg/µl in the FDR rats, and 0.3880 ± 0.04242 pg/µl in the sated rats, t (16) = -0.438, $p = 0.667$, $d = 0.218$. Extracellular DA in the DLS appeared to increase slightly in both FDR and sated groups upon initiation of the heroin seeking test (Supplemental Figure S.3B), but the change was only significant in the sated group as compared to baseline (t (17) = -3.358, $p = 0.004$). In addition, extracellular DA levels remained slightly elevated in the sated group throughout the test session. Repeated measures ANOVA revealed a significant main effect of *time* ($F_{(19,304)} = 1.904$, $p = 0.014$) and a significant interaction of *time* × *food restriction* ($F_{(19,304)} = 2.215$, $p = 0.003$), but no effect of *food restriction* ($F_{(1,16)} = 1.693$, $p = 0.212$).

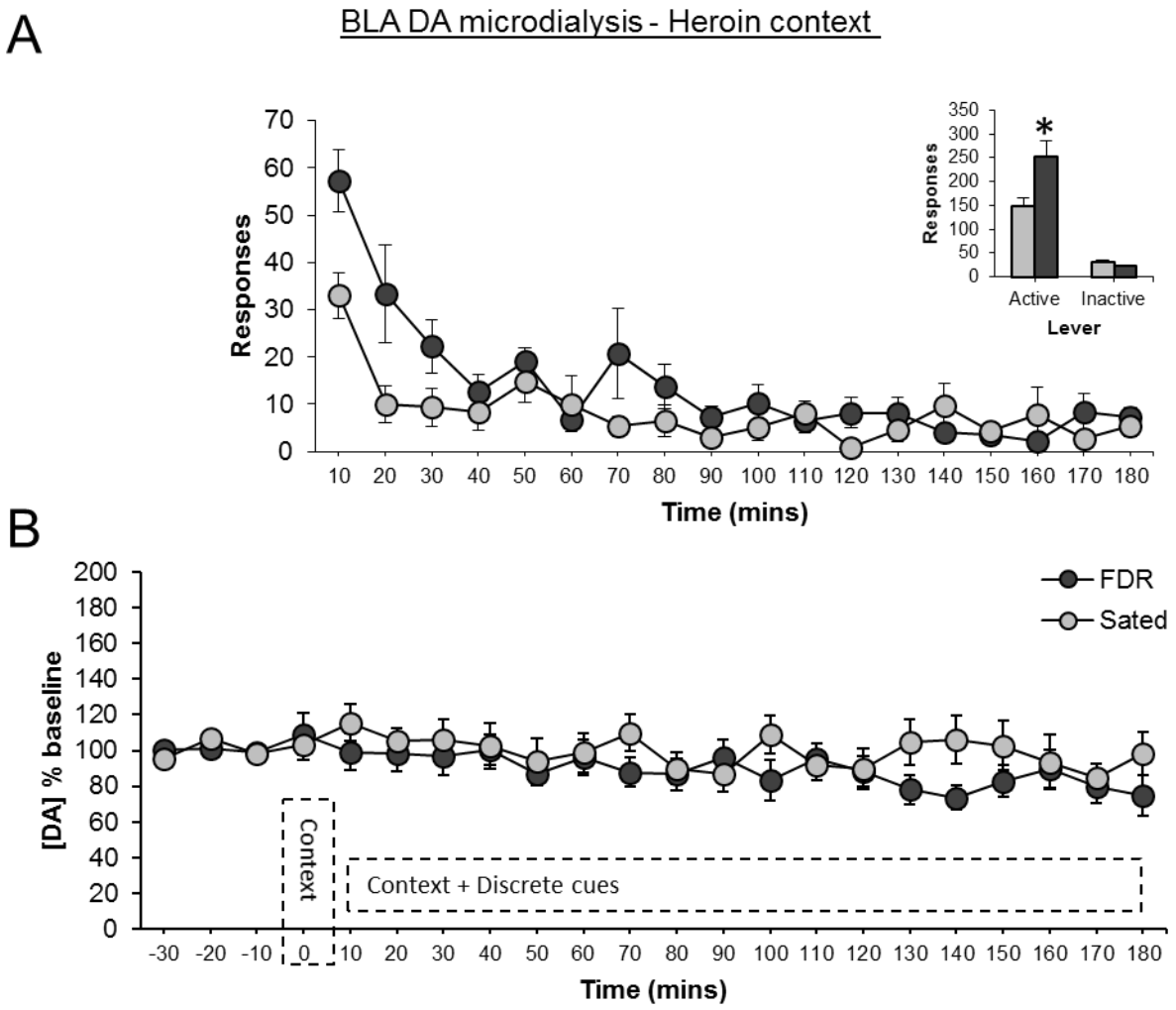

Figure S.2. Chronic food restriction-induced augmentation of heroin seeking and extracellular DA in the BLA (A) Total number of active and inactive lever responses for FDR (n = 13) and sated (n = 8) rats (inset), and active lever responses in 10-min time intervals during the 3 h heroin-seeking test in Supplemental Experiment 1, **p* = 0.033 compared with sated rats. (B) Extracellular DA following re-exposure to the drug environment and during the heroin-seeking test in the FDR and sated rats.

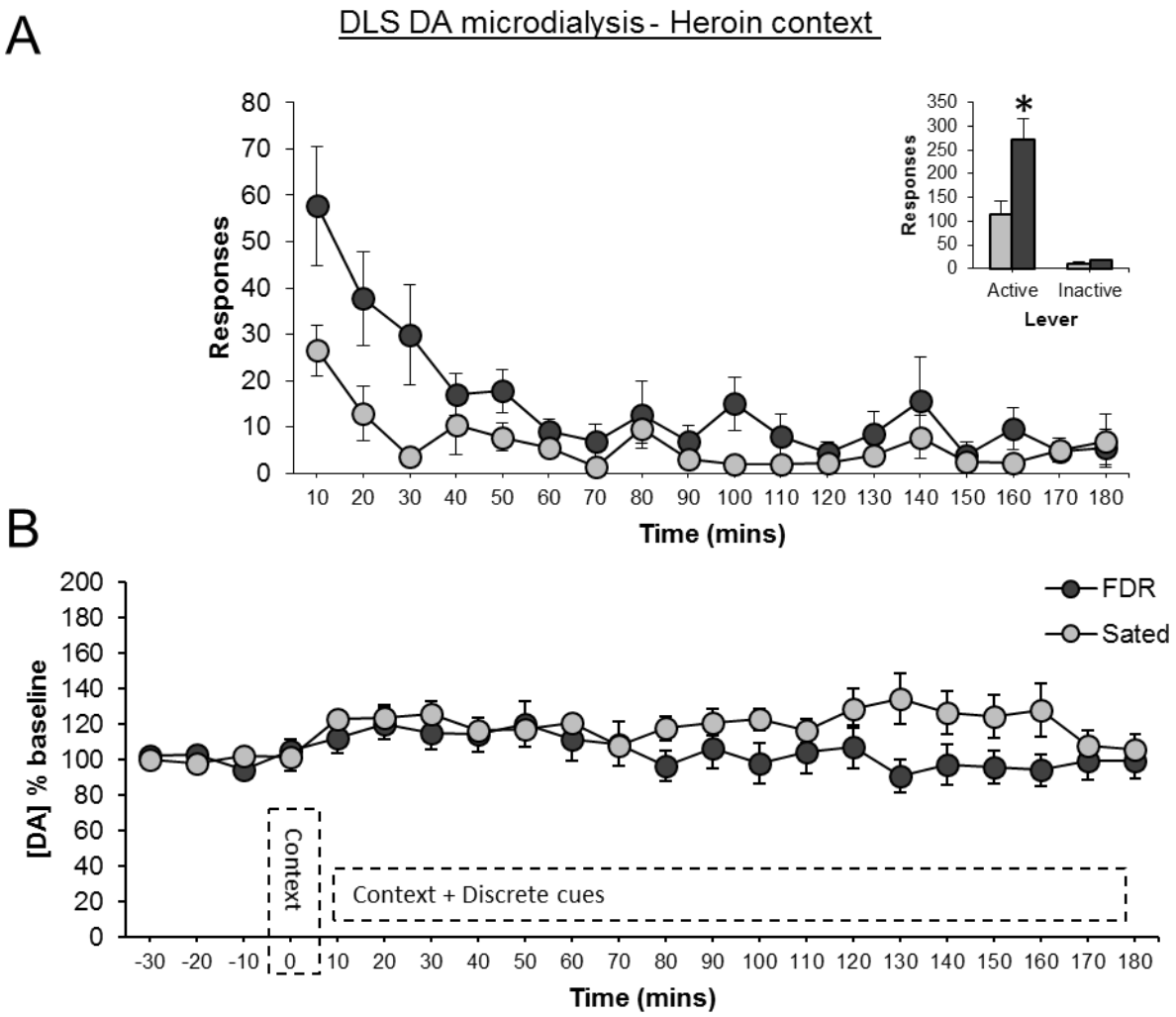

Figure S.3. Chronic food restriction-induced augmentation of heroin seeking and extracellular DA in the DLS (A) Total number of active and inactive lever responses for FDR (n = 10) and sated (n = 8) rats (inset), and active lever responses in 10-min time intervals during the 3 h heroin-seeking test in Supplemental Experiment 2, **p* = 0.014 compared with sated rats. (B) Extracellular DA following re-exposure to the drug environment and during the heroin-seeking test in the FDR and sated rats.