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Identification of Mechanisms Underlying the Reduction of Cocaine Seeking By Exercise

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Identification of Mechanisms Underlying the Reduction of Cocaine Seeking By Exercise

By

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B.S., Georgia State University, 2007

Advisor: David Weinshenker

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Neuroscience
2015

Abstract

Identification of Mechanisms Underlying the Reduction of Cocaine Seeking By Exercise
By Yvonne Ogbonmwan

Relapse represents one of the most significant problems in the long-term treatment of drug addiction. The current standard of care for substance use disorder combines behavioral and pharmacological treatments. Behavioral treatments provide some modest efficacy for addiction but more effective treatment is needed to significantly improve the outcomes of individuals diagnosed with substance use disorder. In recent human and animal studies, chronic exercise has shown to be a promising adjunct therapy for drug addiction. Additionally, while there are many pharmacological treatments for several classes of drugs of abuse, there are no FDA-approved pharmacotherapies to treat addiction to cocaine or other psychostimulants. The experiments described in this dissertation assess changes in drug-seeking behavior using an animal model of relapse and reinstatement, in response to chronic exercise or galanin receptor activation. We assessed the efficacy of chronic exercise as an interventional therapy in an animal model of cocaine addiction for both cocaine-primed and stress-induced reinstatement. We also investigated galanin receptor activation as a novel target for the treatment of cocaine addiction. Using rodent cocaine self-administration we show how galanin receptor activation alters responses to drugs of abuse. We also describe how galanin receptor activation alters extracellular levels of cocaine in the mesocorticolimbic brain circuit, which may underlie the behavioral effect of galanin receptor activation on responses to cocaine during reinstatement.

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Acknowledgements

I am deeply thankful of the relationships I have established with my mentors, colleagues and friends at Emory. Without their support, this dissertation would not have been possible. I am most grateful for the unwavering support, encouragement and patience from my mentor, Dr. David Weinshenker. David has thought me how to think critically about science and the world in general. His guidance has helped me develop and grow as a scientist.

I would also like to thank our co collaborator Dr. Philip Holmes for opening up his lab at the University of Georgia to me, sharing his expertise of behavioral paradigms and molecular assays. The assistance of Dr. Holmes and his former students, Natale Sciolino and Jessica Groves-Chapman, has been invaluable in designing and conducting the anatomical and neurochemical assays necessary for completing this dissertation.

Additionally, I would like to thank the other members of my thesis committee for their advice and insight: Dr. Shannon Gourley, Dr. John Hepler, and Dr. Gretchen Neigh. I feel very fortunate to have worked in the Neuroscience graduate program at Emory and the Department of Human Genetics and thank them for their support.

I am also greatly thankful of the past and current members of the Weinshenker lab without which my time at Emory would not have been as enjoyable. I am very fortunate to have forged my time as a researcher in a truly welcoming and intellectually stimulating environment that David has fostered in his lab. Past members include: Meriem Gaval-Cruz, Alisha Epps, Debra Cooper, Darlene Mitrano, Heather Mitchell. Current members include: Jason Schroeder, Canaan Jerome, Karl Schmidt, Dan Manvich, Natty Chalermpalanupap, Jackie Rorabaugh

Finally, I would like to acknowledge my funding sources, without which this research would not have been feasible. As student at Emory, I have been supported by a NIDA Ruth L. Kirschstein National Research Service Award Predoctoral Fellowship (1F31DA033091) and a NIDA T-32 Institutional Training Grant (DA015040).

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Abbreviations

5-HT	serotonin
α1R	alpha 1 adrenergic receptor
ANOVA	analysis of variance
AUC	area under the curve
BDNF	brain-derived neurotrophic factor
BNST	bed nucleus of the stria terminalis circuit
CBT	Conditioned Behavioral Therapy
CeA	central amygdala
CNS	central nervous system
CPP	conditioned place preference
CRF	corticotropin-releasing factor
DA	dopamine
dbcAMP	dibutyryl cAMP
DBH	dopamine beta (β)-hydroxylase
DAT	dopamine transporter
ET	exercise-training
FR1	fixed-ratio 1
GABA	γ-Aminobutyric acid
Gal	galanin
Gal -/-	galanin knockout mice
GalR1 -/-	galann receptor 1 knockout mice
GALR1	galanin receptor 1
GalR2 -/-	galanin receptor 2 knockout mice
GALR2	galanin receptor 2
HE	health education
i.c.v.	intracerebroventricular
i.p.	intraperitonal
LC	locus coeruleus
LgA	long access
NAc	nucleus accumbens
NE	norepinephrine
NET	norepinephrine transporter
NTS	nucleus of the solitarily tract
pERK	phosphorylated extracellular signal-related kinase
PFC	prefrontal cortex
PR	progressive ratio
SED	sedentary
SGZ	subgranular zone
ShA	short access
SNP	single nucleotide polymorphism
VTA	ventral tegmental area
WHEEL	continuous wheel access for 22 days
WHEEL-RM	wheel removed after 21 days

CHAPTER 1: BEHAVIORAL AND PHAMRACOLOGICAL TREATMENTS FOR COCAINE ADDICTION

1.1 Abstract

Psychostimulant addiction is a serious public health issue that affects millions of people in the United States. To combat this psychiatric illness, the current standard of care incorporates behavioral and pharmacological treatments. However, unlike other commonly abused drugs, there are no FDA-approved pharmacological therapies for the combating abuse of cocaine or other psychostimulants. In pursuit of the development of better treatments, there has been an immense amount of research conducted to better understand the pathology of addiction. Preclinical and clinical studies have demonstrated how cocaine-induced changes in neurotransmission hijack the reward system and drive the development of addiction. Various drugs that target dopamine, norepinephrine or the glutamate system have been investigated as potential treatments for psychostimulant abuse. However, none have emerged as effective therapies. More recent studies investigating pharmacological treatments have begun to explore galanin as a promising new target. Additionally, the effectiveness of behavioral treatments are discussed. Current behavioral therapies provide modest efficacy in preventing relapse, but is hindered by high dropout rates. New developments on designing better behavioral treatments are now focusing on exercise as an adjunct therapy. The benefits of exercise as an adjunct therapy are also discussed. Ultimately, addiction is a multifaceted disorder that will require both behavioral and pharmacological therapies to adequately treat it.

1.2 INTRODUCTION

Drug addiction is defined as intense craving for, and compulsive use of, a drug despite negative consequences. The focus of my thesis, cocaine addiction, poses a serious public health issue in the United States and globally. According to the 2012 National Survey on Drug Use and Health, 1.1 million individuals aged 12 and over reported past-year cocaine abuse or dependence (SAMHSA, 2013). Cocaine dependence is also associated with very serious medical and psychiatric needs. In 2011, cocaine misuse accounted for 40.3% of the 1.2 million emergency room visits that involved illicit drugs (DAWN, 2013). Moreover, data from the Treatment Episode Data Set suggest that stimulant abuse accounted for 14% of all admissions to substance abuse treatment services (TEDS SAMHSA, 2014). Thus, there is an urgent need for effective treatments for cocaine dependence. Preclinical and clinical studies investigating the neurobiology of drug addiction has given us a better understanding of the pathology of this disorder. Data collected from animal-based models of drug addiction and human studies point to some key areas on which to focus future neuroscience research dedicated to developing efficient treatments for substance use disorders.

1.3 ANIMAL MODELS OF DRUG ADDICTION

Drug addiction is characterized by high rates of relapse following prolonged periods of abstinence among habitual drug users. Factors that induce craving and relapse include exposure to the drug of abuse (Jaffe and Coalson, 1989; Ludwig *et al*, 1974), drug associated cues (Carter and Tiffany, 1999a, b; Ludwig *et al*, 1974) and exposure to stressors (Sinha, 2001; Sinha *et al*, 1999). Clinical data provide information about the pattern of drug

addiction but limited insight on the molecular processes that underlie the neural adaptations that drive relapse.

Thus, to better understand the pathophysiology of drug addiction, researchers have developed an animal model of drug addiction known as self-administration. Drug self-administration can be broken down into discrete phases that model different aspects of drug addiction: acquisition and maintenance (drug taking), extinction (withdrawal/abstinence from drug), and reinstatement (relapse) (Figure 1.1). During the maintenance phase, animals are placed into an operant conditioning chamber and learn to perform an operant behavior (e.g. lever pressing) that results in an intravenous drug delivery. An "inactive" lever is also present to gauge specificity. The frequency with which the animal presses the active lever is used as a proxy for drug seeking behavior. Visual and/or audio cues are also presented upon active lever press, which then become associated with drug delivery. During extinction, a placebo substitutes for the drug (e.g. saline instead of cocaine), and active lever pressing rapidly decreases because the drug is no longer reinforcing it.

The final phase of the self-administration paradigm, reinstatement, models relapse. Drug-seeking behavior can be reinstated by factors that have been shown to precipitate relapse in humans, such as a non-contingent administration of the drug, reintroduction of cues associated with drug-taking, or stressful stimuli (de Wit and Stewart, 1981; Gerber and Stretch, 1975; Ludwig *et al*, 1974; Sinha, 2001; Stretch *et al*, 1971). An important aspect of reinstatement is that it occurs under extinction conditions; presses on the formerly active lever are not reinforced, which is why reinstatement is considered "drug-seeking", as opposed to "drug-taking", behavior. The first studies that reported the drug-

priming effect showed that a non-contingent priming dose of cocaine or amphetamine could reinstate drug-seeking behavior following extinction (de Wit and Stewart, 1981; Gerber and Stretch, 1975; Stretch *et al*, 1971). The brain mechanisms that underlie drug-induced reinstatement include activation of the mesocorticolimbic system (see Fig. 1.2) (Shaham *et al*, 2003). Activation of dopamine (DA) neurons in the ventral tegmental area (VTA), which subsequently send projections to the nucleus accumbens (NAc) and prefrontal cortex (PFC), facilitates cocaine-primed reinstatement (McFarland and Kalivas, 2001). Similarly, direct infusion of cocaine, amphetamine or DA in the the NAc or the mPFC also reinstates drug seeking (Stewart and Vezina, 1988; Cornish and Kalivas, 2000; McFarland and Kalivas, 2001).

Similarly, studies that tested the effect of stressors also found that drug-seeking behavior could be reinstated following intermittent foot-shock (Brown and Erb, 2007; Erb *et al*, 2000a). Several types of stressors can reinstate drug-seeking behavior, including foot-shock and the anxiogenic drug yohimbine, (Shaham *et al*, 2000a; Shaham *et al*, 2003). The neurocircutiry that drives foot shock-induced reinstatement is distinct from drug-induced or cue-induced reinstatement. Studies have revealed that two neurotransmitters systems, corticotrophin release factor (CRF) and NE, and two brain regions, the central amygdala and the bed nucleus of the stria terminalis, are critically involved in foot shock-induced reinstatement (Erb *et al*, 2000a; Shaham *et al*, 2000a). Studies have also shown that foot shock stress releases NE into the ventrolateral BNST, an effect that can be blocked by infusion of the alpha2-adrenergic agonist into the A2 noradrenergic nuclei (Shaham *et al,* 2003). The NE projections from the LC do not appear to be involved in foot shock-induced reinstatement (Shaham *et al*, 2000b; Wang *et al*, 2001). Furthermore, blocking CRF

projections from the CeA to the BNST with a CRF antagonist and the sodium channel blocker tetradotoxin (TTX), which inhibits cell firing, significantly attenuates foot shock-induced reinstatement (Erb *et* al, 2001). Finally, the medial PFC and the orbital frontal cortex likely also play a role in stress-induced reinstatement. TTX-induced inactivation of the mPFC and the orbital cortex significantly attenuates foot shock-induced cocaine seeking (Capriles *et al*, 2003).

This animal model of self-administration and relapse provides predictive validity for several different classes of drugs including heroin, nicotine and alcohol. Clinically effective treatments developed for these addictions, such as methadone, varnicline, acomprosate, naltrexone, and methadone, reduce drug intake and/or reinstatement in animals and humans (Bachteler *et al*, 2005; Le *et al*, 1999; Leri *et al*, 2004; O'Connor *et al*, 2010). In contrast, because there are currently no effective pharmacotherapies for psychostimulant dependence, the predictive validity of the self-administration/reinstatement model has not yet been established for cocaine. However, preclinical studies with disulfiram, which has been reported to reduce cocaine use in several small clinical trials (Carroll *et al*, 2004b; Carroll *et al*, 1998; George *et al*, 2000a), show a reduction in drug-primed, cue-induced, and stress-induced reinstatement in rats, suggesting that this model is also valid for psychostimulants (Carroll *et al*, 2004b; Schroeder *et al*, 2010a; Schroeder *et al*, 2013).

Despite its face and predictive validity, the self-administration/reinstatement model has several limitations. For example, in the laboratory model of drug self-administration, animals go through a period in which operant drug-seeking responses are not reinforced. Humans do not typically undergo an extinction phase, but rather an abstinent phase in which drugs of abuse are not accessible either because of punishment, desire to quit, or

lack of access. Reinstatement in animals can be precipitated by an experimenter-administered dose of the drug. In humans, the drug-priming effect (e.g. a single drink) is typically self-administered (Ludwig *et al*, 1974). Furthermore, the drug seeking that characterizes relapse in humans typically results in drug consumption. This is markedly different than reinstatement, in which drug seeking is not reinforced. However, it can be argued that if given access to the drug following the trigger, animals would self-administer. Thus, the reinstatement model is thought to measure the motivation to seek a drug following a "trigger" that leads to relapse in humans.

1.4 EFFECT OF COCAINE ON THE MESOCORTICOLIMBIC CIRCUITRY

Natural rewards and drugs of abuse activate the mesocorticolimbic system (Di Chiara and Imperato, 1988; Wise and Rompre, 1989), which encompasses the dopaminergic cell bodies in the VTA and their projections to the PFC and the nucleus accumbens (NAc) (Swanson, 1982). In turn, the PFC sends glutamatergic projections to the NAc and the VTA (Kalivas and Volkow, 2011). Norepinephrine (NE) also plays a major role in modulating the mesocorticolimbic system. The VTA receives excitatory drive from noradrenegic neurons of the locus coeruleus (LC) that synapse onto midbrain DA neurons and glutamatergic afferents (Grenhoff *et al*, 1993a; Jones *et al*, 1977a; Liprando *et al*, 2004; Mitrano *et al*, 2012; Rommelfanger *et al*, 2009) (see figure 1.2). NE also provides excitatory influence on limbic and striatal DA release. Lesions of the LC reduced both basal DA levels and amphetamine-induced DA release in the NA and the caudate nucleus (Lategan *et al*, 1990). Additionally, blocking alpha-1 adrenergic receptors expressed in the NAc shell attenuates cocaine-induced dopamine overflow and locomotor activity (Mitrano et al

2012). In the PFC, noradrenergic input originates solely from NE neurons in the LC (Morrison *et al*, 1981; Swanson and Hartman, 1975). Alpha-1 adrenergic receptors are also expressed on glutamatergic dendrites and terminals in the PFC (Mitrano *et al*, 2012). Activation of alpha-1 adrenergic receptors increases glutamate release from presynaptic terminals and glutamate neuron firing (Luo *et al*, 2014). Activation of the alpha-1 receptors in the PFC also increases stimulant-induced DA release in the nucleus accumbens and locomotion (Darracq *et al*, 1998).

Cocaine dependency develops because of cocaine's ability to profoundly alter neurotransmission in limbic and cortical brain regions. The reuptake of monoamines is blocked by cocaine, resulting in an increase in extracellular levels of DA, NE, and serotonin (5-HT) in the brain (Heikkila *et al*, 1975; Reith *et al*, 1986; Ritz *et al*, 1990; Ritz *et al*, 1987; Roberts and Koob, 1982) (see figure 1.3). Later studies revealed that the potent reinforcing effects of cocaine are primarily mediated by its ability to inhibit DA reuptake via the DA transporter (DAT) (Ritz *et al*, 1987). Indeed, dopaminergic transmission is necessary and sufficient for maintaining drug-seeking behavior in the animal model of self-administration. For example, lesioning DA cell bodies located in the VTA decreases the rate of cocaine self-administration behavior in rodents (Roberts *et al*, 1977; Roberts *et al*, 1982). Furthermore, drug-seeking behavior during reinstatement is also dependent on dopamine. Inhibiting dopaminergic cells in the VTA with GABA agonists, blocks cocaine-primed reinstatement. Dopaminergic innervation of the PFC is also critical for expressing drug-seeking behavior in an animal model of relapse; infusing DA into the dorsal PFC is sufficient to restore drug-seeking behavior in animals with an inactivated VTA (McFarland and Kalivas, 2001). Additionally, dopaminergic transmission is also sensitive to modulation

by galanin. Pretreatment with a galanin receptor agonist prior to cocaine-primed reinstatement significantly reduces drug-seeking behavior. The effect of galanin receptor activation is also accompanied by significantly reduced cocaine-induced DA overflow in frontal cortex (Ogbonmwan *et al*, 2014b). The findings in preclinical models of addiction are also corroborated by similar findings in clinical studies. Human imaging studies demonstrated that the activation of mesolimbic dopaminergic regions, the orbitofrontal cortex and the prefrontal cortices, is linked with stimulant-induced high (Kufahl *et al*, 2005; Vollm *et al*, 2004). Cocaine-induced euphoria is also associated with DAT and DA D2 receptor binding (Volkow *et al*, 1999). Therefore, findings from clinical and preclinical studies provide strong support that the positive reinforcing effects of cocaine are a key element in cocaine addiction.

Similar to its actions on DA, cocaine also increases extracellular NE levels by inhibiting its uptake. Unlike DAT inhibition, NET inhibition does not influence the rate or amount of stimulant self-administration. However, NE does appear to influence the re-acquisition of drug-seeking behavior following a history of self-administration and extension training, a model of relapse that is commonly referred to as "reinstatement" (see above). An early study showed that blocking NE synthesis with a dopamine β-hydroxylase (DBH) inhibitor significantly reduces drug-seeking behavior during drug-primed reinstatement (Davis *et al*, 1975). More recently, it has been shown that NE activation of alpha-1 receptors is necessary for cocaine-primed reinstatement and that beta-adrenergic receptors are necessary for stress-induced reinstatement (Leri *et al*, 2002; Zhang and Kosten, 2005). Lastly, cue-induced reinstatement can be blocked by concurrently antagonizing alpha-1 and beta- adrenergic receptors or by reducing NE release via

stimulation alpha-2 adrenergic inhibitory autoreceptors (Smith and Aston-Jones, 2011d). Thus, different classes of adrenergic receptors mediate different forms of reinstatement.

We have also demonstrated the critical role of NE in mediating all modalities of reinstatement using disulfiram, a drug that inhibits DBH and NE synthesis and blocks cocaine-primed reinstatement (Schroeder *et al*, 2010a). Another, more selective DBH inhibitor, nepicastat, attenuates cocaine-primed, cue-induced and stress-induced reinstatement (Schroeder *et al*, 2013).

In addition to altering monoaminergic transmission, cocaine also changes glutamatergic transmission. Glutamate transmission in the NAc is necessary for drug-seeking behavior during reinstatement (Cornish *et al*, 1999; McFarland *et al*, 2003). Withdrawal from repeated cocaine exposure following self-administration results in dysregulated cysteine/glutamate exchange and reduced extracellular levels of glutamate in the NAc (Hotsenpiller *et al*, 2001) (Baker *et al*, 2003). Accumbal glutamate levels surge during cocaine-primed reinstatement, which drives drug-seeking behavior. Restoration of the cysteine/glutatmate exchange with systemic or locally administered N-acetyl-cysteine into the NAc elevates basal extracellular glutamate in animals with history of chronic cocaine exposure and prevents cocaine-primed increases in glutamate and reinstatement (Baker *et al*, 2003).

1.5 TREATMENTS

1.5.1 Pharmacotherapies

The quest for developing effective treatments has focused on pharmacotherpies that can restore normal brain function and prevent relapse in individuals with substance use

disorder. Dysregulation of neurotransmitters such as DA, NE, and glutamate is associated with various phases of the drug addiction cycle. Therefore, treatments currently under investigation have focused on modulating the neurotransmitters that are altered by cocaine abuse.

Dopaminergic drugs

Early findings about the pathology drug addiction found that DA was critical for maintaining cocaine self-administration in animals (Yokel and Wise, 1975, 1976). Thus, there was an intense focus on examining the effect of several different classes of drugs with dopaminergic activity as potential treatments for dependence to cocaine and other psychostimulants. Dopaminergic pharmacotherapies were investigated based on the "agonist substitution model", similar to the treatment of opiate dependence. The rationale for developing dopaminergic medications was based on the theory that mimicking some of the effects of drugs of abuse and partially restoring DA signaling could potentially mitigate withdrawal, decrease craving and prevent relapse (Kosten *et al*, 2002a).

The therapeutic potential of DAT inhibitors has been investigated for the treatment of cocaine dependence. Modafinil is a wake-promoting medication primarily indicated to treat narcolepsy that increases extracellular DA by blocking the DAT (Madras *et al*, 2006; Volkow *et al*, 2009). Early studies investigating the effects of modafinil on cocaine-dependent individuals demonstrated a reduction in cocaine use compared to placebo (confirmed via urine analyses) (Dackis *et al*, 2003; Malcolm *et al*, 2006). However, subsequent studies found no significant effects of modafinil (Anderson *et al*, 2009).

Another DAT inhibitor that was investigated for the treatment of cocaine dependence is methylphenidate, a medication used to treat ADHD. Some studies found that methylphenidate reduced the subjective effects of cocaine in cocaine-dependent individuals (Collins *et al*, 2006; Winhusen *et al*, 2006). However, an early clinical trial failed to demonstrate that methylphenidate could reduce cocaine use (Grabowski *et al*, 1997). Subsequent outpatient trials similarly found that methylphenidate does not reduce cocaine use in individuals without ADHD (Levin *et al*, 2007; Schubiner *et al*, 2002; Somoza *et al*, 2004). Buproprion is a dual DAT and NET inhibitor primarily used as an antidepressant and smoking cessation agent. A double blind clinical trial comparing buproprion to placebo found that it was not effective at reducing cocaine use in cocaine-dependent individuals (Margolin *et al*, 1995). Therefore, the DAT inhibitors largely show little to no clinical effectiveness in treating psychostimulant addiction.

DA agonists have also largely failed as pharmacotherapies for cocaine dependence (Amato *et al*, 2010). The self-administration paradigm can also be used to measure the interoceptive effects of a drug through a procedure called drug discrimination. The drug discrimination procedure provides an animal model of the subjective effects of drugs in humans (Schuster and Johanson, 1988). In the drug discrimination procedure the interecoptive effects of a training drug (e.g. cocaine) is used as a cue for performing an operant response (e.g. food-reinforced lever pressing). During the training for drug discrimination, an injection of the drug precedes a self-administration session during which pressing on a specific lever is reinforced with food. For example, in a drug discrimination procedure with cocaine as a training drug, an experimenter-administered injection of cocaine precedes a self-administration session during which the animal must

respond on a specific lever to receive a food pellet. Conversely, an experimenter-administered injection of vehicle precedes a self-administration session during which the animal responds on a different lever to receive a food pellet. The drug effect that leads to the correct response on the food-reinforced lever is called the discriminative stimulus (Manvich *et al*, 2013). Following training, rats are tested with an experimental drug to investigate whether the experimental drug can substitute for the cocaine. A drug that can substitute for cocaine indicates a potential for abuse and a shared pharmacological target. In discrimination studies, many DA antagonists have been investigated because it was believed that a drug that can interfere with the mechanism of action of cocaine could block cocaine's the discriminative effects.

Aripiprazole, a D2 DA receptor partial agonist that is indicated as an atypical antipsychotic, decreased the discriminative stimulus and reinforcing effects of cocaine and amphetamine in rodents (Lile *et al*, 2011). However, aripiprazole was found to lack therapeutic value as a treatment for cocaine dependence because it actually increased use of psychostimulants in humans, possibly to compensate for the blunted subjective effects of cocaine (Haney *et al*, 2011; Tiihonen *et al*, 2007). Similarly, a meta-analysis of twenty-three studies assessing the effectiveness of dopamine agonists as a treatment for cocaine abuse and dependence found no evidence supporting the efficacy of dopaminergic drugs for treating cocaine abuse (Amato *et al*, 2010).

Glutamatergic drugs

Changes in glutamate transmission also play a significant role in the pathophysiology of cocaine responses (McFarland *et al*, 2003; Olive *et al*, 2012; Pierce *et al*,

1996). Glutamatergic projections from the PFC to the NAc are critical for cue-, stress-, and cocaine-primed reinstatement of previously extinguished cocaine self-administration in animals (Backstrom and Hyytia, 2007; Cornish *et al*, 1999; Ping *et al*, 2008).

In the NAc, the glial cystine-glutamate exchanger maintains glutamate levels (Baker *et al*, 2003; Baker *et al*, 2002). Chronic cocaine use reduces the activity of the cystine-glutamate exchanger, resulting in lower basal levels of glutamate (Baker *et al*, 2003). The reduction in non-vesicular glutamate release from cystine-glutamate exchange following withdrawal of cocaine self-administration increases the susceptibility for drug-seeking behavior.

Thus, another active area of research in the treatment of cocaine addiction has focused on medications that reverse cocaine-induced changes in glutamate transmission. In particular, N-acetylcysteine, a prodrug for cystine, has been examined for alleviating relapse in preclinical studies. Systemic administration of N-acetylcysteine blocks cocaine-primed reinstatement of cocaine seeking a rodent model of relapse (Baker *et al*, 2003; Moran *et al*, 2005). Also, chronic N-acetylcysteine reduced cocaine reinstatement after drug self-administration was discontinued in rats (Reichel *et al*, 2011).

Fueled by promising preclinical data, several clinical studies have also investigated the effect of N-acetylcysteine in cocaine-dependent individuals. Consistent with animal studies, cocaine-dependent individuals exhibit reduced glutamate (as a ratio of creatine) (Schmaal *et al*, 2012; Yang *et al*, 2009). However, in human trials, N-acetylcysteine is associated with only modest reductions in cocaine cravings and withdrawal symptoms (LaRowe *et al*, 2006). An open-label pilot study found non-significant reductions in cocaine use (Mardikian *et al*, 2007), and a more recent study found that N-acetylcysteine did not

reduce cocaine use in cocaine dependent individuals who were actively using cocaine (LaRowe *et al*, 2013). However, in abstinent cocaine dependent individuals, N-acetylcysteine reduced the severity of withdrawal symptoms and prevented cocaine craving (LaRowe *et al*, 2013; Schmaal *et al*, 2012). Thus, despite promising preclinical findings, the results of clinical trials on the effects of N-acetylcysteine indicate only modest efficacy thus far.

Noradrenergic Drugs

Noradrenergic drugs have received renewed interest as potential candidates for psychostiumulant addiction. In particular, recent preclinical and clinical studies have generated some promising results for the treatment of cocaine dependence with disulfiram and nepicastat. Disulfiram, commonly marketed as Antabuse, is a medication indicated for alcohol dependence (Hald and Jacobsen, 1948). The mechanism of action for disulfiram's therapeutic effect on alcohol-dependent individuals is that it blocks the hepatic enzyme, aldehyde dehydrogenase, which is necessary for ethanol metabolism. Disulfiram inhibits aldehyde dehydrogenase by forming an intramolecular disulfide bridge at the active site of the enzyme (Shen *et al*, 2000). Inhibition of aldehyde dehydrogenase results in the accumulation of acetaldehyde which causes "Antabuse reaction" (flushing, nausea, and vomiting) deterring future alcohol consumption (Hald *et al*, 1948). Interestingly, disulfiram was also found to be effective in reducing cocaine use in alcoholics with a cocaine dependency (Carroll *et al*, 1998; Carroll *et al*, 1993). Furthermore, in a randomized clinical trial that assessed the efficacy of disulfiram therapy in cocaine abusers, disulfiram was more effective than the placebo in reducing cocaine use (Carroll *et al*, 2004a) even in

patients who did not abuse alcohol, suggesting the existence of an alternate therapeutic target.

One likely candidate enzyme that mediates the therapeutic effects of disulfiram against cocaine addiction is dopamine β-hydroxylase (DBH). The primary metabolite of disulfiram is a copper chelator and thus inhibits enzymes that require copper as a co-factor. The NE biosynthetic enzyme, DBH, is a copper-containing monooxygenase that is inhibited by copper chelation, and thus disulfiram (Goldstein *et al*, 1964). Disulfiram inhibition of DBH decreases NE and increases DA in central and peripheral tissues in animals (Musacchio *et al*, 1966). In humans, disulfiram decreases NE and its metabolites in the blood, urine and CSF (Hoeldtke and Stetson, 1980; Major *et al*, 1979; Paradisi *et al*, 1991; Rogers *et al*, 1979; Rosen and Lobo, 1987; Takahashi and Gjessing, 1972). The first trial that assessed the effect of disulfiram therapy in individuals with dual cocaine and alcohol dependence found that disulfiram reduced both alcohol and cocaine use. However, the reduction of cocaine use was initially attributed to the concurrent CBT treatment (Carroll *et al*, 1993). It was not until 2004, when it was demonstrated in a randomized placebo-controlled trial that disulfiram could reduce cocaine intake in cocaine-dependent individuals with no other comorbid addictions (Carroll *et al*, 2004b). These results provided evidence that concurrent reduction in alcohol use was not necessary for disulfiram's effectiveness against cocaine addiction. Further corroborating these initial findings, we showed that disulfiram inhibits DBH resulting in decreased brain levels of NE, increased DA and decreased NE/DA ratio in the frontal cortex. Furthermore, disulfiram blocked cocaine-primed reinstatement, but only at doses high enough to reduce brain NE levels (Schroeder *et al*, 2010a). Nepicastat, a drug that does not chelate copper and

selectively inhibits DBH by binding to the active site (Kapoor *et al*, 2011), also attenuated cocaine-primed, cue-induced and stress-induced reinstatement (Schroeder *et al*, 2013).

Initial human trials have supported the efficacy of disulfiram for treating cocaine-use disorder (Carroll *et al*, 2004a; George *et al*, 2000b). However, recent findings indicate that the effect of disulfiram may depend on DBH genotype (Carroll *et al*, 2012; Kosten *et al*, 2013; Oliveto *et al*, 2011; Schottenfeld *et al*, 2014). Furthermore, because it inhibits the activity of many enzymes that require copper, disulfiram has many non-specific targets that may cause unwanted side effects and/or interfere with its ability to reduce cocaine use. Thus, efforts to treat cocaine addiction with DBH inhibitors have recently focused on nepicastat because it has fewer off-target effects. A phase II, double blind, placebo-controlled clinical trial assessing the efficacy of nepicastat in cocaine-dependent individuals is currently underway. (http://clinicaltrials.gov/show/NCT01704196).

Galanin and drug responses

In general galanin attenuates responses to drugs of abuse (Picciotto, 2008; Picciotto *et al*, 2010). For example, intracerebroventricular (ICV) administration of galanin attenuates morphine conditioned place preference (Zachariou *et al*, 1999). Opiate withdrawal is decreased by galanin overexpression or by administration of the synthetic galanin receptor agonist, galnon, and conversely, opiate withdrawal is exacerbated by genetic knockout of galanin or GalR1 (Holmes *et al*, 2012; Zachariou *et al*, 2003). Galanin knockout mice are also hypersensitive to morphine and cocaine conditioned place preference, and these phenotypes are abolished by galnon administration (Narasimhaiah *et al*, 2009). By contrast, complete knockout of galanin has minimal effect on cocaine self-

administration in mice using several doses and schedules of reinforcement (Brabant *et al*, 2010a, b). Thus, the galanin system represents a promising new target for developing pharmacotherapies for cocaine abuse and dependence.

Immunotherapy

Conjugate vaccines for substance-use disorders are novel developments in the search for effective forms of treatments against addiction. The conjugate vaccine links the chemical derivative of the drug (hapten) to a large immunogenic protein such as inactivated tetanus or cholera toxin in order to produce an immunological response. The cocaine vaccine, TA-CD, is a cocaine hapten conjugated to inactivated cholera toxin B (Jertborn *et al*, 1992). When cocaine is taken in an immunized individual, the antibodies capture the drug before it crosses the blood-brain-barrier, preventing activation of the brain's reinforcement pathways. In animal studies, when sufficient antibodies were present in the plasma, cocaine uptake in the brain was significantly reduced (Fox *et al*, 1996). Immunization also blunted cocaine-induced locomotor activity and reinstatement in animal studies (Carrera *et al*, 1995; Carrera *et al*, 2000; Kantak *et al*, 2000). In clinical trials, individuals immunized with TA-CD generated an immunological response that was dose-dependent and lasted 3-6 months (Kosten *et al*, 2002b; Martell *et al*, 2005), and those with the highest titer of antibody were more likely to remain abstinent from cocaine use (Martell *et al*, 2005). Cocaine-dependent participants who produced high antibody levels had a significantly blunted response to the subjective effects of cocaine, while no significant effects were observed in individuals who generated a low antibody response (Haney *et al*, 2010). One consistent caveat of immunotherapy is the high variability in the antibody

response to the vaccine. About 25% of participants generate very low IgG (immunoglobulin G) responses (Orson *et al*, 2008; Shen and Kosten, 2011; Shen *et al*, 2012). Thus, individuals who do not produce sufficient antibodies to cocaine could override the effects of the vaccine with frequent use or high doses of cocaine. Furthermore, the initial immunization would require that the patient go to a treatment site for 5 vaccinations over the course of 2-3 months. Failure to continue and complete this course of treatment could lead to relapse (Shen *et al*, 2012). Although there are many areas of research focusing on neurotransmitter systems disrupted by cocaine dependence, effective pharmacologic treatments remain elusive.

1.5.2 Behavioral Therapies

The most common form of treatment for substance use disorders is behavioral intervention, including Cognitive Behavioral Therapy (CBT), Community Reinforcement Approach, contingency management or a combination of these therapies (Ciccarone, 2011). Behavioral treatments are moderately effective in promoting abstinence (Vocci *et al*, 2009). A meta-analysis found that 31.7% of substance users achieve abstinence following behavioral treatment (Dutra et al, 2008). However, the benefit of psychosocial treatment is tempered by the fact that studies examining cocaine-dependent people had a dropout rate of 42%. Thus, while psychosocial treatments do provide positive outcomes, they are at best moderately effective and hindered by high dropout rates.

Summary of treatments

The current pharmacotherapies and behavioral therapies for treating psychostimulant addiction provide some relief for patients but efficacy is highly varied. Most pharmacological treatments provide modest relief and the efficacy some treatments, such as disulfiram, may have adverse side effects and be dependent by the individual's genotype, limiting their widespread usage. Behavioral treatments are at least as effective as pharmacological treatments but are hindered by high drop out rates. The treatments investigated in this dissertation attempt to address some of the common caveats in the development of therapies for psychostimulant addiction. Exercise as an adjunct therapy has been shown to be associated with positive outcomes in individuals with a substance use disorder. Additionally, a recent study showed that exercise administered as an adjunct therapy for methamphetamine addicts had a 74% adherence rate and was associated with positive outcomes (Dolezal *et al*, 2013). Thus, exercise may prove to be more effective than current forms of treatment if incorporated into a treatment program for substance abuse. Research on the development of pharmacological treatments has largely focused on altering the neurotransmitters that are directly altered by psychostimulant abuse, such as dopamine. However, few of the developed treatments have shown to be effective and the FDA has approved none. The pharmacological treatment explored in this dissertation targets the galanin system. Galanin has been underexplored as a possible treatment for addiction. Unlike most previous pharmacotherapies, it is a neuromodulator that regulates monoamines. Thus, this treatment uses a novel mechanism to address relapse.

1.6 THE PROTECTIVE EFFECTS OF EXERCISE

1.6.1 Therapeutic effect of exercise on other psychiatric illnesses

In the quest to develop new therapies to prevent relapse; physical exercise has begun to be more thoroughly investigated as an adjunct therapy for drug addiction. The neuroprotective effects of exercise have convincingly been established for many other psychiatric illnesses. Physical activity can broadly be classified into one of two types of exercise, aerobic or anaerobic exercise. Aerobic exercise is exercise that is low to moderate intensity and requires high levels of oxygen intake. Aerobic exercise is typically performed for extended periods of time and increases heart rate. (Rooks *et al*, 2010). Anaerobic exercise is consists of brief strength-based activities that build muscle mass (Medbo *et al*, 1988). It should be noted that the early stages of all exercise is anaerobic. Most studies investigating the therapeutic effects of exercise on psychiatric illnesses employ aerobic exercise or combination of aerobic and anaerobic exercise. The molecular mechanisms that exercise activates have also begun to be characterized and could also provide some clues for how exercise can counteract drug-induced neural changes.

Chronic physical exercise improves mental and physical health. A few clinical trials have also demonstrated that the neuroprotective effects of exercise extend to substance use disorders. Exercise intervention, as an adjunct to alcohol dependency treatment, is associated with higher rates of abstinence among alcoholics (Brown *et al*, 2009a; Karoly *et al*, 2013; Sinyor *et al*, 1982). The therapeutic benefit of exercise on addiction has been demonstrated across different classes of drugs including cocaine, opiates and nicotine (Weinstock *et al*, 2008).

In the animal model of self-administration, exercise similarly reduces drug-seeking behavior. Rats trained to self-administer cocaine exhibited lower rates of active lever-presses during cocaine-primed or cue-primed reinstatement if they had a history of voluntary chronic aerobic exercise (Cosgrove *et al*, 2002; Sanchez *et al*, 2014; Smith *et al*, 2012; Smith *et al*, 2008; Smith *et al*, 2011b; Zlebnik *et al*, 2012; Zlebnik *et al*, 2010). However, most published studies administered the exercise regimen before and during the maintenance phase of self-administration exercise (Cosgrove *et al*, 2002; Smith *et al*, 2012; Smith *et al*, 2008; Smith *et al*, 2011b) and throughout extinction and reinstatement (Zlebnik *et al*, 2012). One confound of this design is that it is not clear if exercise is effective because the reinforcing effects of exercise are competing with the reinforcing effects of cocaine self-administration during maintenance. It has not been shown that exercise can provide therapeutic effect when only administered after the acquisition of self-administration but prior to reinstatement. Additionally, while there are studies that show that exercise improves resilience to stressors, the effect of exercise on stress-induced reinstatement has not been tested.

In addition to its effect on dependency, exercise is also beneficial in several other neuropsychiatric conditions. Reviews of several randomized clinical trials indicate that exercise improves depressive symptoms to a comparable extent of pharmacotherapy and psychotherapy (Blumenthal *et al*, 2012; Cooney *et al*, 2013; Mead *et al*, 2009). Exercise as an adjunct to the standard treatment of depression, resulted in decreased symptoms of depression and anxiety among depressed patients (Veale *et al*, 1992). Even as a monotherapy, exercise is effective as a moderate antidepressant (Dunn *et al*, 2005). For anxiety disorders, the efficacy of exercise treatment is comparable or better than the

standard treatment (Carek *et al*, 2011; Petruzzello *et al*, 1991; Wipfli *et al*, 2008). Chronic exercise reduces anxiety symptoms in people regardless of age, sex or medical condition (Herring *et al*, 2010) (US Department of Health and Human Services, 2008). Furthermore, the benefit of exercise for ameliorating anxiety symptoms increases in a dose dependent manner (Goodwin, 2003). Animal studies of anxiety disorders have also employed exercise to better understand how it affects the neuropathology of anxiety symptoms. Animals with history of chronic exercise are resilient to stress-evoked tests of anxiety (Sciolino *et al*, 2012a; Sciolino and Holmes, 2012c). There is also a growing body of evidence that exercise therapy improves the neurocognitive and behavioral function of individuals diagnosed with epilepsy. (Arida *et al*, 2013; Arida *et al*, 2012; Eom *et al*, 2014; Nakken *et al*, 1990). In animal models of epilepsy, chronic voluntary aerobic exercise reduces the susceptibility and severity of seizures (Epps *et al*, 2013; Reiss *et al*, 2009). Exercise is also well established as a therapy that ameliorates the physical and cognitive symptoms of Parkinson's Disease (Alonso-Frech *et al*, 2011; Loprinzi *et al*, 2013; Tillerson *et al*, 2001; Zigmond, 2006), and Alzheimers Disease (Arcoverde *et al*, 2008; Garcia-Mesa *et al*, 2011; Lautenschlager *et al*, 2008; Loprinzi *et al*, 2013)

1.6.2 Exercise-induced molecular changes

Several studies provide evidence for the positive influence of exercise on brain structure and function that may underlie its therapeutic effects on neurological disorders. Chronic exercise induces the expression of the brain derived neurotrophic (BDNF) gene (Murray and Holmes, 2011; Neeper *et al*, 1995). BDNF protein is a growth factor that promotes the formation, differentiation and maintenance of neurons (Acheson *et al*, 1995;

Huang and Reichardt, 2001; Murray *et al*, 2011; Neeper *et al*, 1995). It is distributed throughout the brain but is highly concentrated in the hippocampus (Murer *et al*, 1999). It is well documented that the beneficial effects of exercise on synaptogenesis, synaptic plasticity and learning and memory are mediated by BDNF (Christie *et al*, 2008). Cognitive deficits observed in Alzheimers disease and other dementias are also correlated with decreased levels of BDNF (Coelho *et al*, 2014; Nascimento *et al*, 2014).

In addition to BDNF, exercise induces the activity of a variety of growth factors including insulin-like growth factor, vascular endothelial growth factor, and inflammatory protein (Uysal *et al*, 2014; Voss *et al*, 2013). Six weeks of treadmill running (voluntary or involuntary) is correlated with improved memory and increased expression of vascular endothelial growth factor (VEGF) and BDNF in the hippocampus (Uysal *et al*, 2014). Exercise-related changes in the functional connectivity of the temporal cortex are associated with changes in BDNF, VEGF and insulin-like growth factor 1 (IFG-1). The bilateral parahippocampus and the bilateral middle temporal gyrus are part of the default mode network, which is adversely affected by age and mild cognitive impairment. In one study, serum BDNF (IGF-1) and VEGF levels were associated with exercise-induced changes in connectivity of the bilateral parahippocampus and the bilateral middle temporal gyrus in healthy older adults who engaged in an exercise program for one year compared to sedentary controls (Voss *et al*, 2013). Protection from radiation induced cognitive deficits is also mediated by exercise-induced pERK (Ji *et al*, 2014). The literature suggests that exercise increase the levels of growth factors that are correlated with positive mental health outcomes.

Epigenetics

Most clinical and preclinical studies assessing the effect of exercise on drug dependence are primarily behavioral. The mechanisms that underlie the therapeutic benefit of exercise have not been thoroughly explored but a few studies have begun to investigate what neurological substrate is likely involved. The persistence in the behavior seen in individuals that have a drug addiction indicate long lasting changes in gene expression. Thus, one locus that exercise may be altering is the epigenetic modification induced by chronic exposure to drugs of abuse. One study used c-fos expression to investigate how cocaine-induced activation of brain regions in the mesocorticolimbic system and frontal cortex is affected by exercise. Rats were allowed to run or remain sedentary for 21 days and subsequently challenged with a single dose of cocaine (15 mg/kg/ip) or saline, and cocaine-challenged rats with a history of exercise expressed significantly higher c-Fos positive cells in the NAc core, dorsomedial and dorsolateral CPu, prelimbic area, and the OFC than sedentary rats with cocaine exposure (Zlebnik *et al*, 2014). This study provides suggests some neurobiological substrates that may be modulated by chronic exercise in rats exposed to cocaine. However, to because this study did not test rats in a self-administration paradigm, it is not clear how exercise alters the neural substrates that are important in drug-taking behavior.

Exercise may influence the activity of gene transcription through the modulation of DNA methylation or acetylation (Denham *et al*, 2014). For example, global acetylation of histone 3 was increased in neurons of the dentate gyrus and the cerebellum in rats that engaged in aerobic exercise for 1 week (Abel and Rissman, 2013). Furthermore, exercise induced age-dependent changes on DNA methyltransferases and histone deacetylases,

enzymes involved in methylation and histone modification, respectively (Elsner *et al*, 2013). Thus, modifications of DNA methylation and histone acetylation in the brain are responsive to acute aerobic and resistance exercise (Denham *et al*, 2014).

Likewise, chronic exposure to cocaine and other drugs of abuse results in altered gene expression. (Nestler, 2014). Repeated exposure to drugs induces changes in histone modification and DNA methylation in brain reward-related regions. Chronic cocaine exposure can alter histone-modifying enzymes in the nucleus accumbens (Heller *et al*, 2014). DNA methyltransferase 3a, an enzyme that is involved in DNA methylation, is also differentially altered by acute and chronic exposure to cocaine (Anier *et al*, 2010; LaPlant *et al*, 2010). To date, there are no studies that directly investigated the effect of exercise on cocaine-induced epigenetic changes. However, because both of these stimuli can alter gene expression, future experiments should examine if exercise can counteract the cocaine-induced alterations of epigenetic modifications.

Galanin

Exercise also increases the mRNA expression of galanin in the LC. Galanin is a 29 amino acid peptide first discovered in the porcine gut (Tatemoto *et al*, 1983). In the brain it functions as a neuromodulator of neurotransmitters and has an effect on a wide array of physiological function including feeding behavior, nociception, mood regulation and responses to drugs of abuse (Branchek *et al*, 1998; Picciotto, 2008; Shen *et al*, 2003). Galanin and its receptors (GalR1, GalR2, GalR3) are widely distributed in the central nervous system (Lorimer and Benya, 1996; Melander *et al*, 1986a; Melander *et al*, 1986b; Mennicken *et al*, 2002; Nicholl *et al*, 1995; Skofitsch and Jacobowitz, 1985; Smith *et al*,

1997; Smith *et al*, 1998). What is intriguing about this peptide is that chronic exercise upregulates galanin mRNA in the LC but not tyrosine hydroxylase mRNA or galanin mRNA any other brain regions (Holmes and Picciotto, 2006a; Murray *et al*, 2011; O'Neal *et al*, 2001; Sciolino *et al*, 2012a; Van Hoomissen *et al*, 2004). Galanin extensively colocalizes with norepinephrine neurons in the LC (80% of neurons) (Holets *et al*, 1988; Levin *et al*, 1987). In the LC it is most likely released from the soma/dendrites and acts on autoreceptors and nearby neurons in an autocrine manner (Pieribone *et al*, 1995). During periods of high activity (burst firing) galanin is released and inhibits NE firing of LC neurons via the postsynaptic GAL1 receptor (Ma *et al*, 2001; Parker *et al*, 1995). Some of the neuroprotective effects of exercise have been shown to be mediated by galanin (Reiss *et al*, 2009). The anticonvulsant properties of exercise are significantly diminished with the galanin receptor antagonist M40 (Reiss *et al*, 2009).

Galanin binds to three G-protein coupled receptors: galanin receptor 1, 2 and 3 (GalR1, GalR2 and GalR3) (Picciotto 2010; Branchek et a 2000, Burgevin 1995). GALR1 distribution overlaps heavily with the expression of the galanin peptide mRNA. The highest concentrations of GALR1 are seen in prefrontal cortex, the hypothalamus (supraoptic nucleus), amygdala, ventral hippocampus, thalamus, brain stem (medulla oblongata, LC, and lateral parabrachial nucleus), and spinal cord (dorsal horn) (Burgevin *et al*, 1995; Gustafson *et al*, 1996; Mitsukawa *et al*, 2008; Parker *et al*, 1995). In contrast, GalR2 mRNA is mainly expressed in the dorsal root ganglia, hippocampus, the mammillary nuclei, the hypothalamus, the substantia nigra, LC and VTA (Depczynski *et al*, 1998; Hawes and Picciotto, 2004; Howard *et al*, 1997; O'Donnell *et al*, 1999; Wang *et al*, 1997). GalR3 mRNA expression is more restricted in the CNS with low levels observed the in the hypothalamus

and pituitary gland, BNST, the amygdala, periaqueductal gray, dorsal raphe, and the LC (Hawes *et al*, 2004; Mennicken *et al*, 2002; Smith *et al*, 1998).

The effect of galanin receptors activation on cellular physiology is largely inhibitory on the basis of its hyperpolarizing actions (Hokfelt 1987). GalR1 and GalR3 subtypes activate Gi and Go proteins (Lang *et al*, 2007), resulting in the inhibition of adenylate cyclase and the reduction of cAMP (Habert-Ortoli *et al*, 1994; Parker *et al*, 1995). In contrast, GalR2 subtypes activate the Gq protein, thereby increasing protein kinase C activity and intracellular calcium (Lang *et al*, 2007; Wang *et al*, 1998). The receptor pharmacology of GalR3 is less well defined but is similar to GalR1 (Hawes *et al*, 2004). In Xenoopus oocytes, GalR3 co-expressed with GIRK1 and GIRK4 resulted in inward K+ current characteristics of Gi/Go coupled receptors (Smith et al, 1998).

The broad distribution of galanin in the CNS allows it to modulate a wide variety of neurotransmitters including NE, DA, acetylcholine, GABA, glutamate (Melander *et al*, 1986b; Melander *et al*, 1985). The diverse neurotransmitters modulated by galanin mediates its role in many different physiological functions including learning and memory, cognition, nociception, and feeding (Counts et al, 2008; Lang et al, 2007; Ogren et al, 1998; Xu et al, 2010). More recently, the discovery of galaninergic pathways that alter neurotransmission of monoamines implicates galanin in drug addiction (Einstein et al, 2013; Picciotto, 2008).

1.7 EXPERIMENTAL DESIGN AND RATIONALE

Many questions still remain about how exercise can be used as a therapy for drug addiction. Most of the animal models have allowed access to wheel running at a time that

provides a preventive benefit. To determine if it would be effective as a treatment intervention, it is important to determine if restricting access to exercise after self-administration would still exert a therapeutic benefit.

Thus, one of the aims of this dissertation was to determine whether chronic voluntary aerobic exercise would attenuate cocaine-primed and stress-primed reinstatement in rats trained to self-administer cocaine. We tested the hypothesis that chronic voluntary wheel-running would be associated with reduced drug-seeking behavior. Previous exercise studies had revealed that chronic wheel-running is positively correlated with galanin mRNA expression in the LC in drug naïve rats. However, it is not clear if exercise-induced changes in galanin mRNA expression also occur in animals with a history of drug self-administration. Thus, a secondary aim of this study was to examine if chronic-wheel running in rats with a history of cocaine self-administration would be associated with a change galanin mRNA levels. We tested the hypothesis that chronic wheel-running would be associated with an increase in galanin mRNA expression.

Galanin is also neuroprotective against the reinforcing properties of cocaine and other drugs of abuse. However, several important aspects of drug responses have not been examined after galanin receptor activation, including relapse-like behavior and DA transmission. In this study, we examined the consequences of galnon administration on drug seeking during the maintenance and reinstatement phases of operant cocaine self-administration, as well as on cocaine-induced changes in DA overflow in the frontal cortex and the NAc.

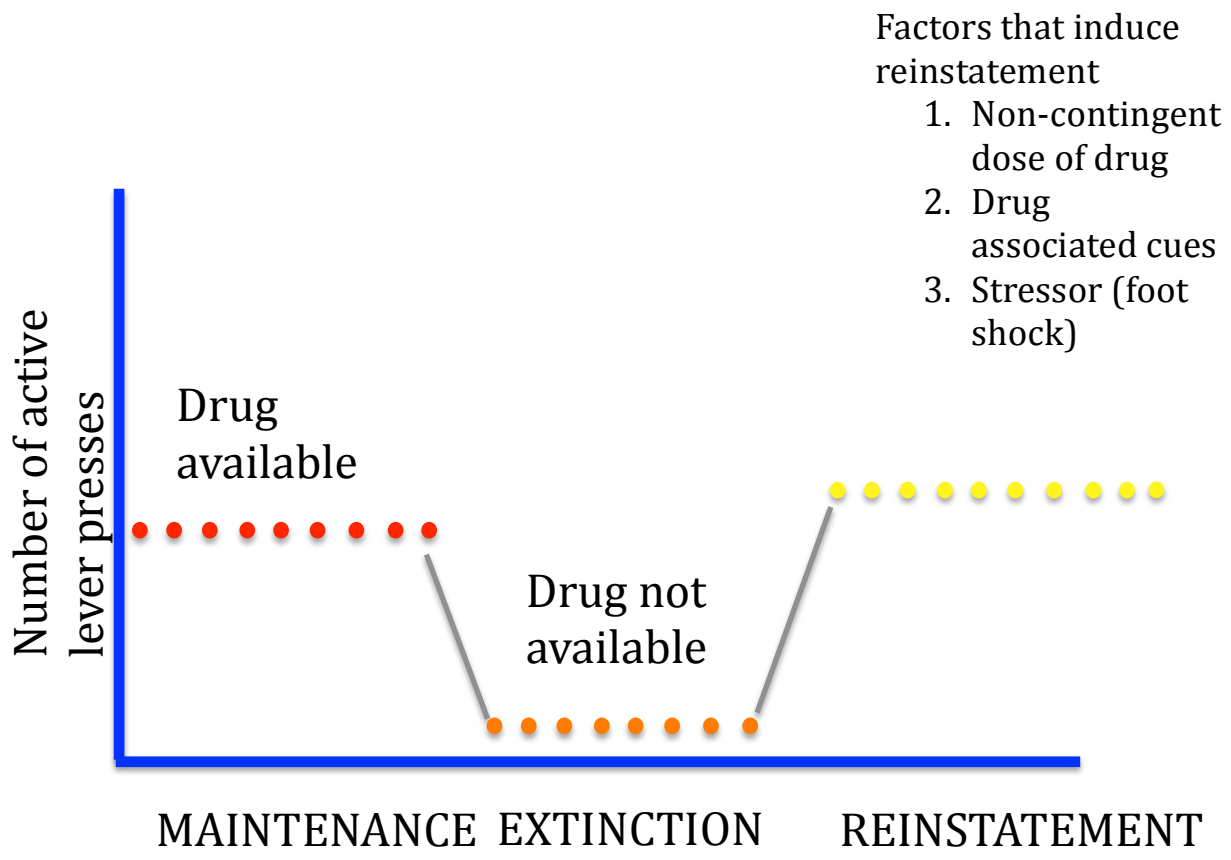

Figure 1.1. Self-administration model During maintenance, animals are placed in an operant conditioning chamber and given access to a drug of abuse (cocaine, methamphetamine, heroin, etc). They will self-administer the drug at a steady rate for as long as it is available. During extinction, the drug is taken away or replaced with saline; and active lever responses will decrease until the drug seeking behavior is extinguished. During reinstatement, drug-seeking behavior can be brought back by factors that have been shown to precipitate relapse in humans, such as a non-contingent administration of the drug, drug-associated cues, or stressors.

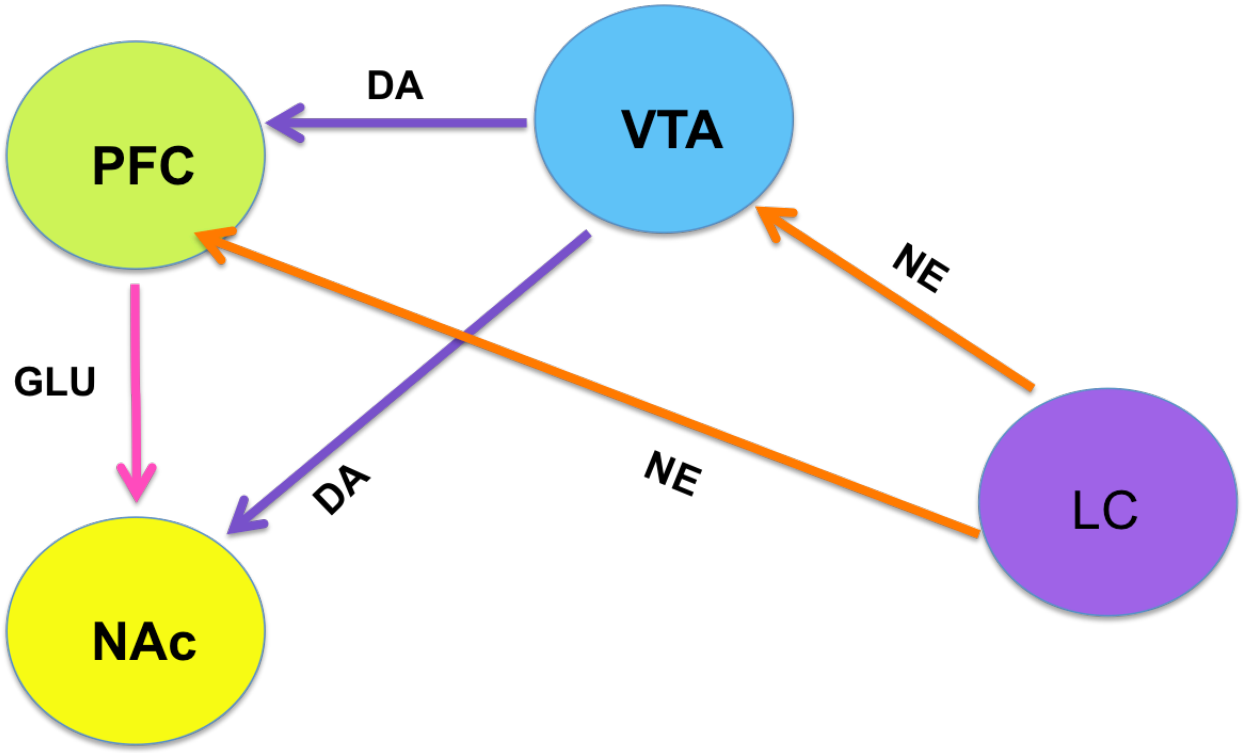

Figure 1.2 Mesocorticolimbic System The mesocorticolimbic system, encompasses the dopaminergic cell bodies in the ventral tegmental area (VTA) and their projections to the prefrontal cortex (PFC) and the nucleus accumbens (Nac). The PFC sends glutamatergic projections to the NAc and the VTA. Noradrenegic (NE) neurons of the locus coeruleus (LC) synapse onto VTA dopaminergic neurons and glutamatergic afferents.

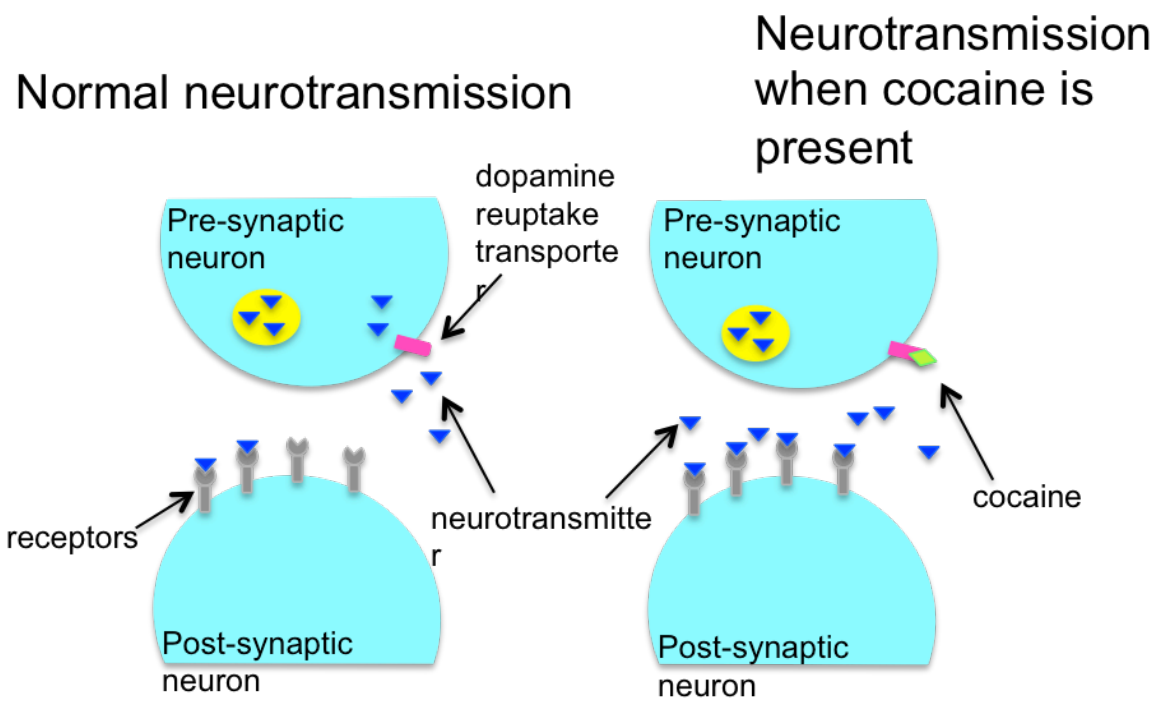

Figure 1.3 Effect of cocaine on monoamine synapse. Cocaine activates alters normal cell neurotransmission by blocking the monoamine transporters for DA, NE, and 5HT. During normal neurotransmission monoamines are released from a pre-synaptic cell when the cell is activated and bind to postsynaptic receptors. Excess neurotransmitters are shuttled back into the presynaptic cell though the monoamine transporter (DAT, NET, serotonin transporter). Once it is inside the cell, the neurotransmitters are repackaged into vesicles. When cocaine is present in the synapse, it binds to the transporter preventing the uptake of neurotransmitters into the pre-synaptic cell. Thus, excess neurotransmitter molecules remain in the synapse and continue to bind to the postsynaptic receptors.

CHAPTER 2: THE EFFECTS OF POST-EXTINCTION EXERCISE ON COCAINE-PRIMED AND STRESS-INDUCED REINSTATEMENT OF COCAINE SEEKING IN RATS

Adapted from:
Ogbonmwan YE, Schroeder JP, Holmes PV, Weinshenker D. (2014). The effects of post-extinction exercise on cocaine-primed and stress-induced reinstatement of cocaine seeking in rats. Psychopharmacology 2014.

2.1 Abstract

Rationale

Voluntary aerobic exercise has shown promise as a treatment for substance abuse, reducing relapse in cocaine-dependent people. Wheel running also attenuates drug-primed and cue-induced reinstatement of cocaine seeking in rats, an animal model of relapse. However, in most of these studies, wheel access was provided throughout cocaine self-administration and/or extinction and had effects on several parameters of drug seeking. Moreover, the effects of exercise on footshock stress-induced reinstatement have not been investigated.

Objectives

The purposes of this study were to isolate and specifically examine the protective effect of exercise on relapse-like behavior elicited by a drug prime or stress.

Methods

Rats were trained to self-administer cocaine at a stable level, followed by extinction training. Once extinction criteria were met, rats were split into exercise (24 h, continuous access to running wheel) and sedentary groups for three weeks, after which drug-seeking behavior was assessed following a cocaine prime or footshock. We also measured galanin mRNA in the locus coeruleus and A2 noradrenergic nucleus.

Results

Exercising rats ran ~4-6 km/d, comparable to levels previously reported for rats without a history of cocaine self-administration. Post-extinction exercise significantly attenuated cocaine-primed,

but not footshock stress-induced, reinstatement of cocaine seeking, and increased galanin mRNA expression in the LC but not A2.

Conclusion

These results indicate that chronic wheel running can attenuate some forms of reinstatement, even when initiated after the cessation of cocaine self-administration, supporting the idea that voluntary exercise programs may help maintain abstinence in clinical populations.

Keywords: cocaine, rat, reinstatement, exercise, stress, locus coeruleus

2.2 INTRODUCTION

The efficacy of exercise in maintaining physical and mental health is well established, and voluntary aerobic exercise is an effective intervention for a variety of stress-related neuropsychiatric and neurological disorders, including depression, anxiety, and epilepsy (Blumenthal *et al*, 2012; Cooney *et al*, 2013; Veale *et al*, 1992). Exercise has also shown promise as a treatment for drug addiction. The results from several small clinical studies indicate that individuals with a history of substance abuse who voluntarily participate in physical activity exhibit lower rates of relapse (Brown *et al*, 2010; Sinyor *et al*, 1982; Weinstock *et al*, 2008).

Likewise, several studies have demonstrated the potential therapeutic efficacy of voluntary, chronic aerobic exercise in animal models of psychiatric illnesses. We found that exercise confers resilience against seizures, stress, and depression-like behavior (Epps *et al*, 2013; Sciolino *et al*, 2012b), and several groups have reported reductions in various relapse paradigms. Rats trained to self-administer cocaine exhibit lower rates of drug-seeking behavior during cocaine-primed or cue-induced reinstatement if they had a history of voluntary chronic aerobic exercise (Lynch *et al*, 2010; Peterson *et al*, 2014; Smith *et al*, 2012; Zlebnik *et al*, 2010). However, in most of these studies, rats had access to running wheels before and during the acquisition and maintenance of cocaine self-administration (Smith *et al*, 2012), prior to extinction (Lynch *et al*, 2010; Peterson *et al*, 2014), or throughout extinction (Zlebnik *et al*, 2010). Because exercise also attenuated the acquisition, escalation, breakpoint, and extinction of cocaine self-administration under some conditions (Lynch *et al*, 2010; Smith and Pitts, 2011a; Smith *et al*, 2008; Smith *et al*, 2011b; Zlebnik *et al*, 2012; Zlebnik *et al*, 2010), it was not clear whether exercise was reducing reinstatement per se or whether its effects on relapse-like behavior were secondary to changes in self-administration or extinction. This is a key issue from

a therapeutic standpoint because interventions to curb addiction are most easily instituted to help prevent relapse after individuals have achieved abstinence. Moreover, the anxiolytic properties of exercise suggest that it would effectively inhibit stress-induced reinstatement, but this has yet to be rigorously tested; there is only a single report that exercise can attenuate reinstatement elicited by the pharmacological stressor yohimbine (Zlebnik *et al*, 2014), and none that examined a physiological stressor like footshock. Determining the ability of exercise to protect against different relapse trigger modalities may also help identify individuals who are most likely to benefit from this type of therapy.

The molecular mechanisms behind the therapeutic effects of exercise on relapse behavior are not well understood, but they may involve the neuropeptide galanin. We have previously shown that aerobic exercise increases galanin mRNA specifically in the noradrenergic locus coeruleus (LC) (Holmes *et al*, 2006b; Murray *et al*, 2010; O'Neal *et al*, 2001; Reiss *et al*, 2009). Galanin inhibits opiate- and psychostimulant-induced locomotor activity and place preference (Hawes *et al*, 2008b; Narasimhaiah *et al*, 2009; Picciotto, 2008; Zachariou *et al*, 2003) and suppresses NE transmission (Holmes and Picciotto, 2006a; Sciolino and Holmes, 2012c), which is required for multiple forms of relapse-like behavior (Leri *et al*, 2002; Schroeder *et al*, 2010a; Schroeder *et al*, 2013; Smith and Aston-Jones, 2011d; Weinshenker and Schroeder, 2007; Zhang and Kosten, 2005). Furthermore, we have recently shown that the galanin receptor agonist, galnon, attenuates cocaine-primed reinstatement (Ogbonmwan *et al*, 2014). Although these data indicate that galanin may contribute to the effects of exercise on drug seeking, the ability of chronic exercise to increase galanin expression in the LC has not been assessed in animals with a history of cocaine exposure.

This study had three main objectives. First, we determined whether chronic voluntary aerobic exercise, when provided only *after* cocaine self-administration and extinction, would attenuate cocaine-primed reinstatement. Second, we assessed the effects of exercise on footshock stress-induced reinstatement of cocaine seeking. Finally, we evaluated the ability of exercise to increase galanin in the LC in cocaine-experienced animals. Because NE derived from the A2 noradrenergic nucleus is specifically required for stress-induced reinstatement (Erb *et al*, 2000b; Leri *et al*, 2002; Shaham *et al*, 2000b), we also measured galanin mRNA in this brain region.

2.3 METHODS

Subjects

Male Sprague-Dawley rats (151-175 g, ~7-8 weeks of age upon arrival) (n=28) were used for cocaine-primed reinstatement experiments. Male Long-Evans rats (151-175 g) (n=14) were used for the stress-induced reinstatement experiments in accordance with the literature (Brown *et al*, 2009b; Erb *et al*, 2000b; Kupferschmidt *et al*, 2011; Leri *et al*, 2002). All rats were obtained from Charles River (Wilmington, MA). Rats were individually housed in clear polycarbonate cages (50 x 30 x 30 cm) and given ad libitum access to food and water unless otherwise specified. Rats were housed in a temperature- and humidity-controlled animal facility and maintained on a 12-h reverse light/dark cycle. Testing occurred during the dark phase with background noise emitted by a white noise generator. Animals were allowed to acclimate to the vivarium for one week prior to surgery. All experiments were conducted at Emory University in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Emory IACUC.

Food training

To facilitate the acquisition of drug self-administration, animals were first trained to lever press for food (45 mg pellets) in an operant conditioning chamber (Med Associates, St Albans, VT) prior to surgery. Each chamber was equipped with a house light and two retractable levers, with a stimulus light above each lever. Animals were trained on a fixed ratio 1 (FR1) schedule with a 20-s time out. Each active lever response resulted in the delivery of 1 food pellet. Presses on the inactive lever had no programmed consequences. Food training sessions lasted 8 h, or until the animal met criteria, defined as at least 70% active lever selection and at least 100 food pellets

obtained. Most rats met criteria on the first day of food training, but a few required 2–3 days. There were no significant differences between groups.

Jugular catheter surgery

All instruments and implants were sterilized prior to surgery. Rats were surgically implanted with a catheter into the right jugular vein after food training, as described (Schroeder *et al*, 2010a; Schroeder *et al*, 2013). Rats were anesthetized with isoflurane administered by vaporizer with oxygen delivered through a nose cone, and the surgical site was shaved and cleaned with Betadine. Catheter tubing was threaded subcutaneously from the back and guided over the shoulder into the right jugular vein, and tubing was sutured down. Rats received meloxicam (1 mg/kg, s.c.) immediately following surgery and were allowed to recover for 1 week prior to cocaine self-administration. Catheters were flushed daily with 0.05 ml gentamicin (3 mg/ml) and 0.1 ml heparinized saline (30 ml in sterile saline) to help maintain patency. Catheter patency was verified prior to cocaine self-administration by administering 0.08-0.12 ml of the short-acting barbiturate methohexital sodium (10 mg/ml, IV; Eli Lilly, Indianapolis, IN, USA), which rapidly produces moderate sedation.

Cocaine self-administration for cocaine-primed reinstatement experiment

Daily cocaine self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and each press of the active lever resulted in a cocaine infusion (0.5 mg/kg, 167 μl/kg, i.v., dissolved in 0.9% physiological saline) (NIDA Chemical Synthesis & Drug Supply Program, Bethesda, MD) accompanied by illumination of the stimulus light located above the lever. Following a 20-s timeout period

(during which time active lever presses were counted but did not result in drug infusion), the stimulus light was extinguished and responses were again reinforced. Responses on the inactive lever had no programmed consequences. The session was terminated early to prevent overdose if the number of cocaine infusions exceeded 40. Maintenance criteria were considered met when the number of active lever presses varied by < 20% of the mean, preference for the active lever exceeded 75% and at least 20 cocaine infusions were obtained for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration.

Extinction for cocaine-primed reinstatement experiment

The day after meeting maintenance criteria was reached, lever pressing was extinguished. Rats were placed in the self-administration chambers for daily 2-h sessions, during which responses on the previously active lever no longer resulted in cocaine delivery or presentation of cocaine-paired cues. Extinction criteria were met when active lever presses over 3 consecutive days were <30% of the average number of active lever presses during the last 3 days of maintenance.

Voluntary exercise for cocaine-primed reinstatement experiment

The day that rats reached extinction criteria, they were randomly assigned to either the sedentary group (n=13) or one of two exercise groups (n=7-8; see below). Exercise rats had continuous access to a running wheel placed in their home cage for 21 d, while running wheels were not introduced into the home cages of sedentary rats.

Cocaine-primed reinstatement

Reinstatement of cocaine-seeking behavior was tested after 21 days of wheel running in a cocaine-primed reinstatement session. Because we were interested in the chronic rather than the acute effects of exercise, running wheels were removed from the cages of a subset of exercise rats (n=8) on day 21, and cocaine-primed reinstatement was tested on day 22. We controlled for the potential stress of running wheel removal by leaving the wheels in the cages of the rest of the exercise rats (n=7). Notably, rats exercise almost exclusively during the dark cycle, so running was virtually nonexistent during the 12-h light cycle just prior to reinstatement testing, even when wheels were not removed. Rats were given a non-contingent priming injection of cocaine (10 mg/kg, i.p.) and placed in operant conditioning chambers under extinction conditions (i.e., presses on the formerly active lever had no programmed consequences) for 2 h. Immediately following the reinstatement session, rats were euthanized by CO2 asphyxiation, and brains were removed, blocked (a single mid-sagittal cut), frozen on dry ice, and stored at -80°C

Cocaine self-administration, extinction, and stress-induced reinstatement

Stress-induced reinstatement was conducted as previously described (Kupferschmidt *et al*, 2011; Schroeder *et al*, 2013). Briefly, subjects received a single 2-h long habituation session in the operant conditioning chambers. Rats were then trained in 3-h cocaine self-administration sessions on a FR1 schedule. Each self-administration session began with extension of the inactive lever for 5 min. The house light was then illuminated, and the active lever was extended. Responses on the active lever resulted in a cocaine infusion (0.5 mg/kg) accompanied by illumination of a discrete light cue located above the lever. Following a 20-s timeout period, the stimulus light was extinguished, and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, the session was terminated early

upon the attainment of 60 cocaine infusions. Following 10 d of cocaine self-administration, subjects were given 3-4 daily massed 1-h extinction trials, during which active lever responses resulted in the illumination of the stimulus light and activation of the infusion pump, but no delivery of cocaine. Subjects were extinguished until active lever presses over 3 consecutive extinction sessions were <25% of the average number of active lever presses during the last 3 days of maintenance.

The day that rats reached extinction criteria, they were randomly assigned to either an exercise or sedentary group. Exercise rats had ad libitum access to a stainless steel rodent activity wheel (Mini Mitter, Bend, OR) in their home cage for 21 days. Each wheel was connected to an electromagnetic counter that measured the number of wheel revolutions, which were recorded daily. The daily distance run was calculated for each rat by multiplying the number of wheel rotations by the wheel circumference (107.75 cm) and converting the result to km. Running wheels were not introduced into the home cages of sedentary rats. On day 22, subjects were placed in the operant conditioning chambers with both levers retracted. Following a 10-min habituation period, they were exposed to 15 min of intermittent footshock (0.6 mA; 0.5 s/shock, range of 4-80 s between shocks). The reinstatement session began with extension of the inactive lever for 5 min, followed by illumination of the house light and extension of the active lever. Responses on the active lever resulted in illumination of the cue light above the lever and activation of the infusion pump, which did not contain a syringe, so no cocaine was delivered. Following a 20-s timeout period, the stimulus light was extinguished, and responses were again accompanied by the cue light and infusion pump activation. Responses on the inactive lever had no programmed consequences.

In situ hybridization and densitometry

A subset of the brains collected from the rats in the cocaine-primed reinstatement experiment (6 sedentary, 4 exercise) were used for in situ hybridization for galanin mRNA. Only a subset was used because tissue from half of the animals was used for other analyses that are not included in the present manuscript, and some of the remaining samples were unusable due to experimenter error. There were no significant differences in reinstatement magnitude between the rats that were used for the in situ analysis and those that were not. Tissue from one hemisphere was cut in 12-μm sagittal sections on a cryostat and subsequently thaw-mounted onto gelatin-coated glass microscope slides (2 sections/slide), which were stored at −80°C until further processing. Anatomical location was also verified in adjacent sections using 0.1% thionin stain and a rat brain atlas (Paxinos and Watson, 1998a).

Tissue was processed as previously described (Murray *et al*, 2010; Sciolino *et al*, 2012b). For pretreatment, tissue was fixed in 4% formaldehyde in 0.12 M phosphate-buffered saline (PBS), rinsed in PBS, soaked in 0.25% acetic anhydride in 0.1 M triethanolamine HCl and 0.9% NaCl, dehydrated in a series of EtOH washes, delipidated in chloroform, and washed in EtOH. A oligonucleotide probe complementary to preprogalanin mRNA (5′-G AAG GTA GCC AGC GCT GTT CAG GGT CCA GCC TCT CTT CTC CTT T-3′; Oligos etc, Wilsonville, OR) was labeled at the 3' end with ^{35}S-dATP (1 mCi; Perkin Elmer, Boston, MA), tailing buffer, CoCl2, and terminal deoxynucleotransferase (Roche, Indianapolis, IN). Unbound radionucelotide was removed using column separation (Micro Bio-Spin P30 in Tris, Bio-Rad, Hercules, CA), and bound radionucelotide was stabilized using 1 M dithiothreitol. Sections were covered with radiolabeled probe in hybridization buffer (25% formamide, 72 mM NaCl, 3.2 mM Tris HCl, 0.0032 mM EDTA, 0.001% sodium pyrophosphate, 0.004% sodium dodecyl sulfate, 0.002

mg/ml heparin sulfate, and 2% dextran sulfate) and incubated for 24 h at 37°C. Sections underwent a series of washes in 1% SSC and 2% SSC-formamide (50:50) at 40°C and room temperature, as well as in distilled H2O and EtOH. Sections were allowed to dry and subsequently opposed to 35S-sensitive film (Kodak BioMax MR, Rochester, NY) for 14 d. Films were developed in Kodak GBX fixer and developer and air dried.

Film images were captured under optimized conditions using a light table (Northern Light D95, Imaging Research Inc., Piscataway, NJ) and digital camera equipped with a macro lens (Nikon D5000, Micro-NIKKOR 55mmf/2.8 lens, Melville, NY). Images were processed on a Macintosh computer (Apple, Inc., Cupertino, CA) using NIH Image (Bethesda, MD; http://rsb.info.nih.gov/nih-image/). Images of the LC and A2 were selected and measured using a uniform area. Mean grayscale brightness values were obtained from 2–4 sections per subject. Densitometry was performed on original images that were in no way digitally manipulated. Example photomicrographs were uniformly transformed across groups to a color scale using NIH Image.

Data analysis

Self-administration data were analyzed by t-test when comparing 2 groups and one-way ANOVA followed by Tukey's multiple comparisons post hoc tests and two-way ANOVA followed by Sidak's multiple comparisons post hoc tests when comparing more than 2 groups, wheel-running data were analyzed with linear regression, and in situ hybridization data were analyzed by *t*-test. All analyses were performed with Prism 6.0 for Macintosh.

2.4 RESULTS

Voluntary wheel running in rats with a history of cocaine self-administration

Twenty-one days of voluntary wheel running were recorded in rats following cocaine self-administration and extinction and prior to cocaine-primed and stress-induced reinstatement. Rats in both groups initially ran ~1-2 km/d, which increased to ~4-6 km/d by the end of the exercise phase (linear regression for cocaine-primed reinstatement group: $F_{1,313}$=35.30, p<0.0001; linear regression for stress-induced reinstatement group: $F_{1,145}$=30.98, p<0.0001) (Fig. 2.1). The line slopes for the cocaine-primed and stress-induced reinstatement groups were not significantly different from each other. These distances are comparable to those we have reported for drug-naïve rats (Epps *et al*, 2013; Reiss *et al*, 2009; Sciolino *et al*, 2012b).

Post-extinction exercise attenuates cocaine-primed reinstatement

Rats went through cocaine self-administration and extinction, and then were divided into a sedentary group ("SED"), an exercise group that had continuous (22 days) running wheel access ("WHEEL"), and an exercise group that had the wheels removed after 21days ("WHEEL-RM"). All rats were tested for cocaine-primed reinstatement 22 days after completing extinction training. No differences between the groups were observed for the number of days to reach maintenance criteria (SED 9±1.48, WHEEL 8.14±1.16, WHEEL RM 8.25±0.73), number of cocaine infusions obtained during the maintenance phase (SED 35.15±1.40, WHEEL 35.29±2.22, WHEEL RM 31.5±3.15) or active lever presses during the maintenance phase (SED 66.46±17.77, WHEEL 56.14±11.36, WHEEL RM 43±4.60), as assessed by one-way ANOVA. By contrast, we found that active lever responding during cocaine-primed reinstatement was attenuated in both exercise groups (Fig. 2.2). A two-way ANOVA (repeated measures by phase)

showed a main effect of experimental phase (i.e, extinction vs. reinstatement; $F_{1, 25}$=15.48, P<0.001) and treatment ($F_{2, 25}$=3.68, p<0.05), and a borderline significant interaction ($F_{2, 25}$=3.32, P=0.05). Post hoc tests revealed that sedentary rats significantly reinstated compared to extinction (t=5.12, p<0.0001), while the exercise rats did not. Furthermore, reinstatement responding was significantly lower in both exercise groups compared to the sedentary group (WHEEL, t=3.01, p<0.05; WHEEL-RM, t=3.12, p<0.05). Inactive lever responding was low during all phases and no significant differences were observed between groups (Maintenance: SED 1.08±0.42, WHEEL 2.38±0.80, WHEEL RM 0.57±0.30; Extinction: SED 5.54±3.02, WHEEL 3.71±1.29, WHEEL RM 6.50±1.62; Reinstatement: SED 10.38±5.22, WHEEL 2.29±0.42, WHEEL RM 6.13±2.23).

Exercise has no effect on footshock-induced reinstatement

Following cocaine self-administration, extinction, and 3 weeks under exercise or sedentary conditions, drug-seeking behavior was assessed after 15 min of intermittent footshock (0.6 mA, 0.5 s/shock, 4-80 s between shocks). No differences between the groups were observed for the number of cocaine infusions obtained during the maintenance phase (sedentary 45.81±3.95, exercise 47.86±6.16) or active lever presses during the maintenance phase (sedentary 50.04±3.23, exercise 59.86±7.10), as assessed by t-test. In contrast to the cocaine-primed reinstatement results, we found that exercise did not attenuate footshock-induced reinstatement of active lever pressing (Fig. 2.3). A two-way ANOVA (repeated measures by phase) showed a main effect only of experimental phase ($F_{1, 12}$=28.87, P<0.001). Post hoc tests revealed that both sedentary (t=4.42, p<0.05) and exercise (t=3.18, p<0.05) rats significantly reinstated compared to extinction, and there was no difference between sedentary and exercise groups. Inactive lever

responding was low during all phases and no significant differences were observed between groups (Maintenance: sedentary 3.29±1.00, exercise 4.05±1.27; Extinction: sedentary 3.43±1.39, exercise 6.14±1.59).

Exercise increases galanin mRNA in the LC but not A2

Galanin mRNA abundance in the LC was significantly higher in exercising rats compared to sedentary rats ($t=2.50$, $p<0.05$) (Fig. 2.4A, 2.4B, and 2.4C). The magnitude of the increase was comparable to what we have reported for drug-naïve rats (Reiss *et al*, 2009; Sciolino *et al*, 2012b; Van Hoomissen *et al*, 2004). Galanin mRNA levels in A2 were not above background in either the exercise or sedentary groups (Fig. 2.4A, 2.4B and data not shown).

2.5 DISCUSSION

Post-extinction exercise attenuates cocaine-primed reinstatement

Our results are consistent with several other reports that chronic voluntary aerobic exercise can attenuate cocaine-primed reinstatement (Smith *et al*, 2012; Zlebnik *et al*, 2010). However, in contrast to previously published paradigms, we provided access to the running wheel only after extinction had occurred. This allowed us to specifically test the ability of exercise to attenuate reinstatement and rule out potential secondary effects stemming from alterations in the reinforcing efficacy of cocaine during self-administration or facilitation of extinction learning. We found that even after drug self-administration ceased, chronic voluntary exercise significantly reduced drug-seeking behavior. Attenuation of cocaine-primed reinstatement was similar in rats that had access to running wheels until just prior to the reinstatement test and in rats that lost access to running wheels 24 hours before the reinstatement test, suggesting that the acute effects (i.e. < 24 hours) of the exercise were not required for its efficacy. Because we did not test a control group that has access to a locked wheel, we cannot distinguish between physical activity and environmental enrichment with certainty; indeed, these stimuli will always be confounded to varying degrees because rats will often spend a significant amount of time climbing on locked wheels. Thus, we used a sedentary group with no wheel access as our control group and consider voluntary wheel running an extreme form of environmental enrichment. Regardless, these results suggest that exercise therapy initiated after abstinence has been achieved will help prevent relapse in treatment-seeking individuals.

Exercise has no effect on footshock-induced reinstatement

While many studies have shown the ability of exercise to reduce drug-primed and cue-induced reinstatement of cocaine seeking, only one (Zlebnik *et al*, 2014) has assessed stress-induced reinstatement. In that set of experiments, rats self-administered cocaine for 10 days, were given access to running wheels during extinction training for 14 days, and then tested for reinstatement following administration of the anxiogenic drug yohimbine, with or without concurrent presentation of cocaine-associated cues. Exercise significantly attenuated yohimbine-induced reinstatement under both conditions. While yohimbine is considered a pharmacological stressor, its mechanism of action in the reinstatement of cocaine seeking is somewhat obscure and does not appear to require the canonical stress machinery in the brain, such as norepinephrine (NE) and corticotropin-releasing factor (CRF) (Brown *et al*, 2009b). By contrast, the neurochemical and neuroanatomical substrates underlying footshock stress-induced reinstatement are well defined and involve an A2-central nucleus of the amygdala-bed nucleus of the stria terminalis circuit driven by NE and CRF (Erb *et al*, 2000b; Leri *et al*, 2002; Shaham *et al*, 2000b). Unlike its ability to attenuate reinstatement elicited by other modalities, we found that exercise failed to block footshock-induced reinstatement. However, this result should be interpreted with caution, since overall responding was somewhat lower in exercise rats compared to sedentary rats. We do not think that the differences in the two procedures (e.g. Sprague-Dawley vs. Long Evans rats, single daily extinction trials without cues vs. massed extinction trials with cues, etc) can explain the ability of exercise to attenuate cocaine-primed, but not footshock-induced, reinstatement because there are many examples of interventions that work for both. For example, we have shown that the dopamine β-hydroxylase inhibitor, nepicastat, similarly attenuates both cocaine-primed and footshock-induced reinstatement of cocaine seeking using the distinct paradigms

(Schroeder *et al*, 2010a; Schroeder *et al*, 2013). It is also possible that the exercise regimen employed in this experiment was not sufficient to alter foot shock-induced reinstatement. In another study examining the effects of exercise on the behavioral consequences of stress, 6 weeks, but not 3 weeks, of wheel-running reduced behaviors of learned helplessness after exposure to uncontrollable tail-shocks (Greenwood *et al*, 2005). Thus, a longer period of wheel running is may be necessary to reduce stress-induced reinstatement. Nevertheless, our results combined with reports from the literature suggest that exercise might be more effective at preventing relapse caused by drug re-exposure or drug-associated cues than stress.

Possible mechanisms underlying reduction of relapse-like behavior by exercise.

We have shown that chronic exercise increases galanin mRNA in the LC of drug-naïve rats (Holmes *et al*, 2006b; Murray *et al*, 2010; O'Neal *et al*, 2001; Reiss *et al*, 2009), and our new finding that wheel running also increases galanin mRNA in the LC of rats with a history of cocaine self-administration suggests a potential mechanism to explain the ability of exercise to attenuate cocaine-primed reinstatement. We know that NE, most likely released from LC projections to the prefrontal cortex and/or ventral tegmental area, is required for the rewarding effects of cocaine and cocaine-primed reinstatement (Gaval-Cruz and Weinshenker, 2009; Mitrano *et al*, 2012; Schroeder *et al*, 2010a; Schroeder *et al*, 2013; Ventura *et al*, 2007; Weinshenker *et al*, 2007; Zhang *et al*, 2005). Because somatodendritically released galanin suppresses LC firing (Vila-Porcile *et al*, 2009; Xu *et al*, 2005), an increase in LC galanin could dampen the activation of this noradrenergic circuit and prevent drug-seeking behavior triggered by cocaine re-exposure. LC-derived galanin could also directly suppress the mesocortical

dopamine transmission that is necessary for cocaine-primed reinstatement. Galaninergic fibers of the LC project to and modulate the activity of ventral tegmental area (VTA) neurons (Jones and Moore, 1977b; Mejias-Aponte *et al*, 2009; Simon *et al*, 1979; Weiss *et al*, 2005), and we have shown that the galanin agonist, galnon, blocks cocaine-induced DA overflow in the frontal cortex and drug-primed cocaine seeking (Ogbonmwan *et al*, 2014). Other proposed molecules and mechanisms that may also contribute include phosphorylated extracellular signal-related kinase (pERK), brain-derived neurotrophic factor (BDNF), and epigenetic modifications (Lynch *et al*, 2013; Lynch *et al*, 2010; Peterson *et al*, 2014). Our failure to observe an upregulation of galanin mRNA in A2 could explain why exercise did not impact footshock stress-induced reinstatement in the present study. Although NE is also required for footshock-induced reinstatement, its origin is A2, not the LC, and involves the ventral noradrenergic bundle-central nucleus of the amygdala-bed nucleus of the stria terminalis circuit (Erb *et al*, 2000b; Leri *et al*, 2002; Shaham *et al*, 2000b). One limitation of this interpretation is that the galanin mRNA data came only from rats in the cocaine-primed reinstatement experiment. However, the increase of galanin mRNA in the LC but not A2 is a pattern that we have reported many times in naïve rats (Holmes *et al*, 2006b; Murray *et al*, 2010; O'Neal *et al*, 2001; Reiss *et al*, 2009). Because the rats in the cocaine-primed reinstatement experiment and the footshock-induced reinstatement experiment had very similar levels of cocaine exposure and identical amounts of exercise, it is very unlikely that the pattern of galanin expression would be different.

Conclusion

This study adds to the growing body of evidence that exercise may be beneficial in the treatment of substance abuse. Chronic voluntary aerobic exercise administered after cocaine self-

administration and extinction was sufficient to attenuate cocaine-primed but not stress-induced reinstatement. Future studies will help identify the neurobiological mechanisms behind this, as well as test the contribution made by LC-derived galanin.

2.6 Acknowledgements

We thank Cheryl Strauss for helpful editing of the manuscript. This work was supported by the National Institute of Drug Abuse (DA027535 to DW and PVH, DA033091 and DA015040 to YEO).

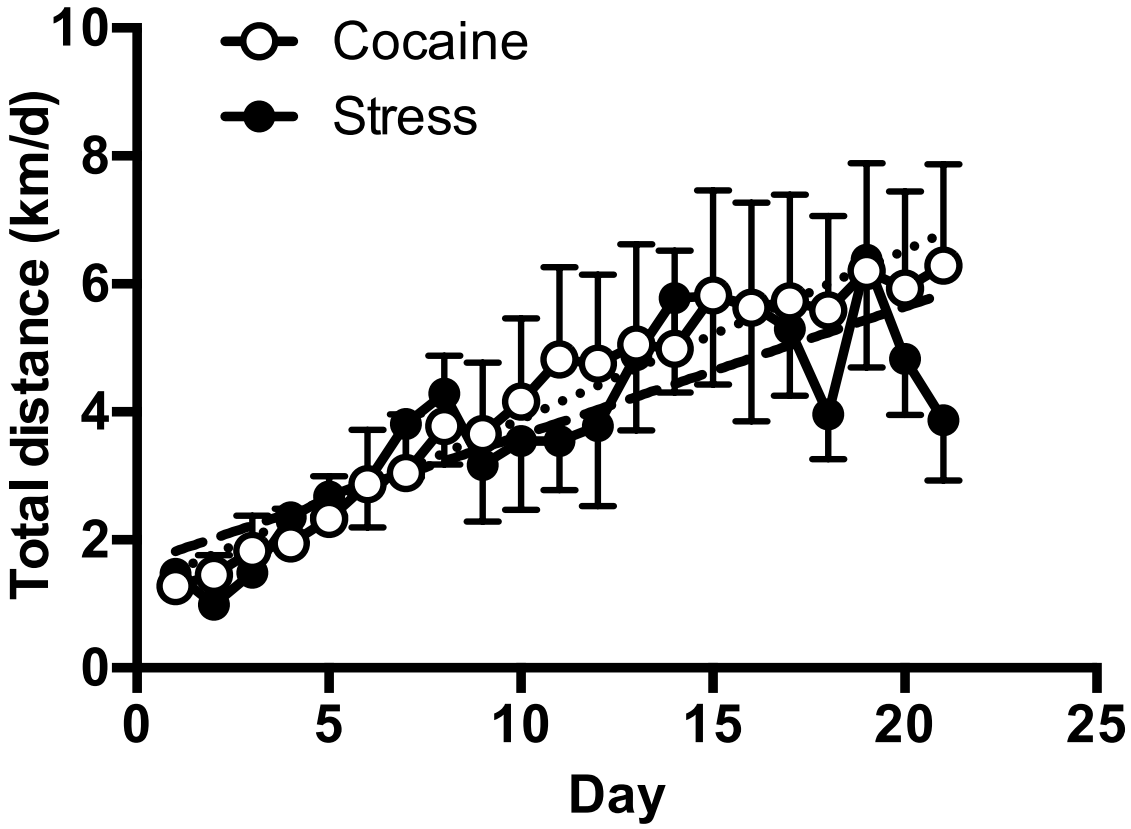

Figure 2.1 Voluntary wheel running after cocaine self-administration and extinction. Rats destined for cocaine-primed ("Cocaine") and stress-induced ("Stress") reinstatement were given continuous access to running wheels in their home cages for 3 weeks following cocaine self-administration and extinction. Mean ± SEM distance run per day in kilometers is shown. Line of best fit is also shown (dotted line for Cocaine rats, dashed line for Stress rats).

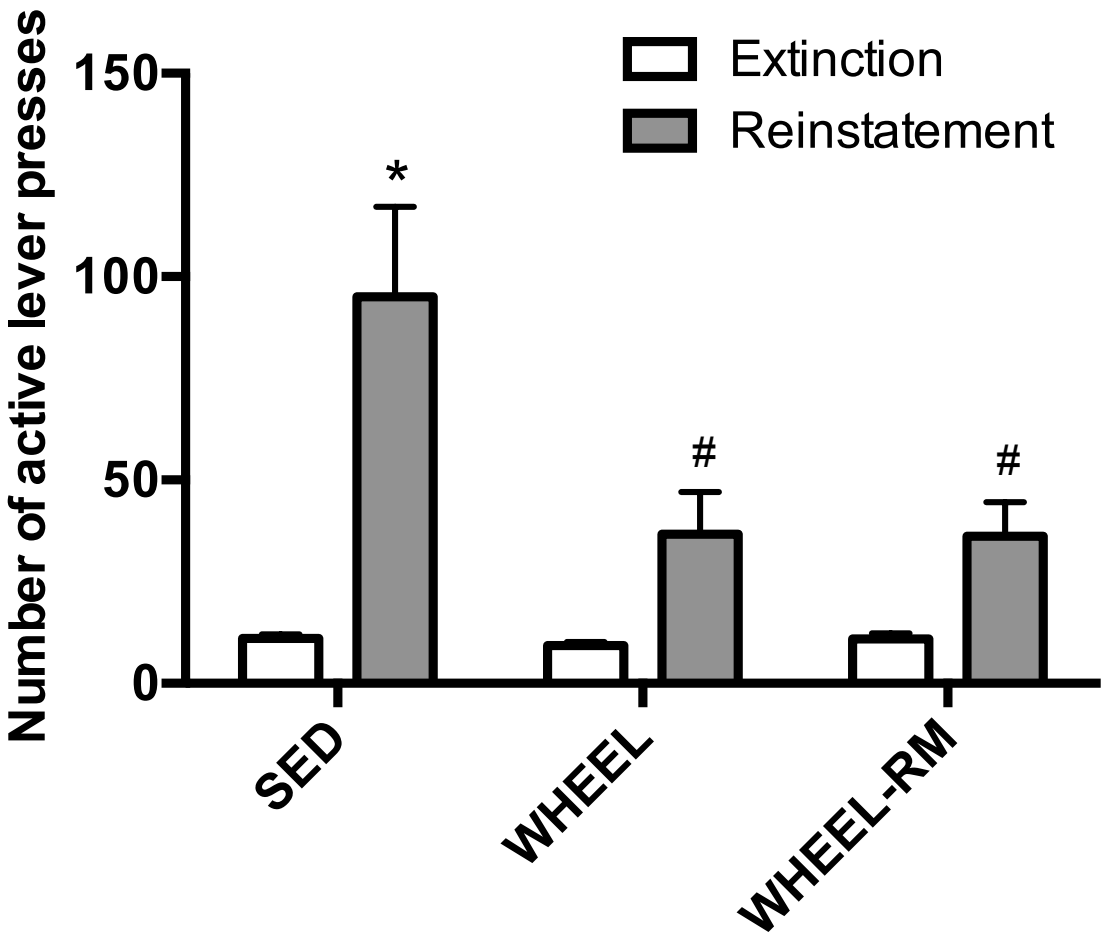

Fig. 2.2 Post-extinction exercise attenuates cocaine-primed reinstatement. Following cocaine self-administration and extinction, rats were divided into 2 exercise groups ("WHEEL", running wheel in home cage; "WHEEL-RM", running wheel removed from home cage the day before the reinstatement test) or sedentary ("SED", home cage with no running wheel) groups for 3 weeks, and then given a cocaine-primed reinstatement test. Shown is the mean ± SEM number of active lever presses during the last 3 days of extinction and the single reinstatement test. *$p<0.0001$ compared to Ext, $^{\#}p<0.05$ compared to SED

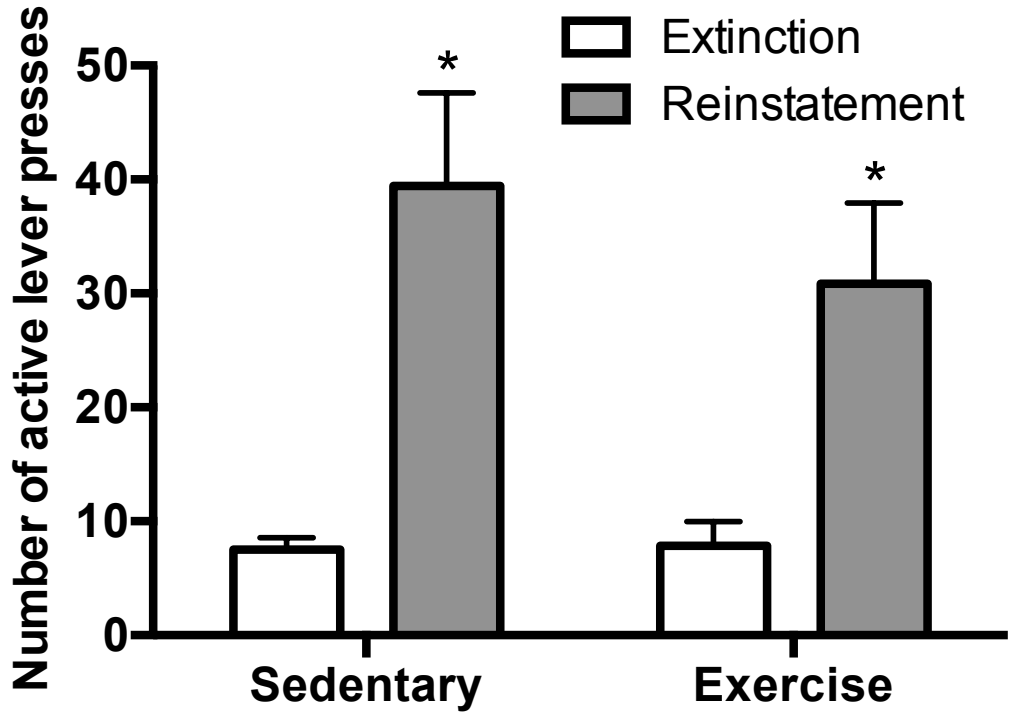

Fig. 2.3 Exercise has no effect on footshock-induced reinstatement. Following cocaine self-administration and extinction, rats were divided into exercise (running wheel in home cage) and sedentary (home cage with no running wheel) groups for 3 weeks, and then given a footshock-induced (15 min, 0.6 mA, 0.5 s/shock, 4–80 s between shocks) reinstatement test. Shown is the mean ± SEM number of active lever presses during the last 3 extinction sessions and during the single reinstatement test. *$p<0.0001$ compared to Ext, $^{\#}p<0.05$ compared to SED. *$p<0.05$ compared to extinction.

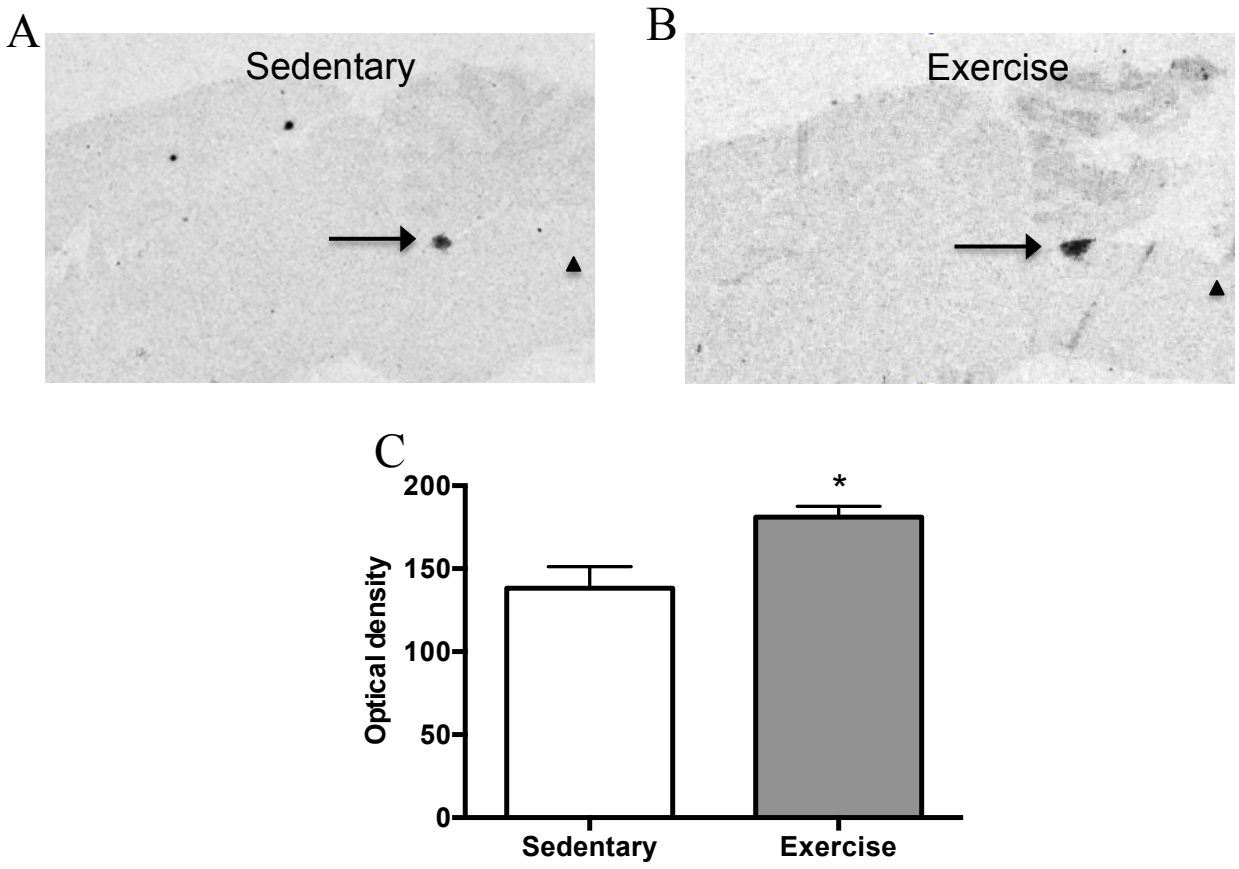

Figure 2.4 Exercise increases galanin mRNA in the LC but not A2. Representative in situ hybridization micrographs from an exercise rat (A) and a sedentary rat (B) showing galanin mRNA expression in the brain. Arrows indicate the galanin mRNA signal in the LC, arrowheads indicate the lack of galanin mRNA signal in the approximate location of A2. Shown in (C) is the mean ± SEM optical density in the LC. *$p<0.05$ compared to Sedentary.

CHAPTER 3: THE GALANIN RECEPTOR AGONIST, GALNON, ATTENUATES COCAINE-INDUCED REINSTATEMENT AND DOPAMINE OVERFLOW IN THE FRONTAL CORTEX.

Adapted from:

Ogbonmwan YE, Sciolino NR, Groves-Chapman JL, Freeman KG, Schroeder JP, Edwards GL, Holmes PV, Weinshenker D. (2014). The galanin receptor agonist, galnon, attenuates cocaine-induced reinstatement and dopamine overflow in the frontal cortex. Addiction Biology. 2014 Jul 23. doi: 10.1111/adb.12166.

3.1 Abstract

Relapse represents one of the most significant problems in the long-term treatment of drug addiction. Cocaine blocks plasma membrane monoamine transporters and increases dopamine (DA) overflow in the brain, and DA is critical for the motivational and primary reinforcing effects of the drug as well as cocaine-primed reinstatement of cocaine seeking in rats, a model of relapse. Thus, modulators of the DA system may be effective for the treatment of cocaine dependence. The endogenous neuropeptide galanin inhibits DA transmission, and both galanin and the synthetic galanin receptor agonist, galnon, interfere with some reinforcing properties of cocaine. The purpose of this study was to further assess the effects of galnon on cocaine-induced behaviors and neurochemistry in rats. We found that galnon attenuated cocaine-induced motor activity, reinstatement, and DA overflow in the frontal cortex at a dose that did not reduce baseline motor activity, stable self-administration of cocaine, baseline extracellular DA levels, or cocaine-induced DA overflow in the nucleus accumbens (NAc). Similar to cocaine, galnon had no effect on stable food self-administration but reduced food-primed reinstatement. These results indicate that galnon can diminish cocaine-induced hyperactivity and relapse-like behavior, possibly in part by modulating DA transmission in the frontal cortex.

Key Words: cocaine, cortex, dopamine, galanin, galnon, reinstatement

3.2 Introduction

Because relapse is a major obstacle in the treatment of drug addiction, one promising strategy is to develop therapies that block the ability of triggers such as the drug itself, drug-associated cues, or stress to precipitate relapse (Bossert *et al*, 2013; Sinha, 2009). Cocaine blocks plasma membrane monoamine transporters, which in turn increases extracellular levels of monoamines in the brain. It is well established that DA is critical for mediating the motivational and reinforcing effects of cocaine, and blocking its transmission attenuates drug-seeking behavior during reinstatement, a model of relapse (Bossert *et al*, 2013; Schmidt *et al*, 2005).

One intriguing molecule that modulates DA transmission and behavioral responses to addictive drugs is the neuropeptide galanin. Galanin and its G protein-coupled receptors (GalR1-3) are expressed within the mesocorticolimbic circuit implicated in drug addiction (Hawes *et al*, 2004; Melander *et al*, 1986b). Galanin receptors can also be activated by galnon, a synthetic non-peptide agonist that crosses the blood-brain barrier and binds to GalR1 and GalR2 (Saar *et al*, 2002). In general, galanin reduces DA release (Jansson *et al*, 1989; Melander *et al*, 1987; Nordstrom *et al*, 1987; Tsuda *et al*, 1998), and both galanin and galnon attenuate responses to drugs of abuse (Picciotto, 2008)Narasimhaiah *et al*, 2009; Holmes *et al*, 2012). By contrast, complete knockout of galanin has minimal effect on cocaine self-administration in mice using several doses and schedules of reinforcement (Brabant *et al*, 2010; Narasimhaiah *et al*, 2009). However, several important aspects of drug responses have not been examined after galanin receptor activation, including relapse-like behavior and DA transmission. In this study, we examined the consequences of

galnon administration on drug seeking during the maintenance and reinstatement phases of operant cocaine self-administration, as well as on cocaine-induced changes in DA overflow in the frontal cortex and the nucleus accumbens (NAc).

3.3 Materials and Methods

Subjects

Male Sprague-Dawley rats (151-175 g) were used for all experiments. Self-administration experiments were conducted at Emory University (*N*=54) rats purchased from Charles River, Wilmington, MA) and motor activity and microdialysis experiments were conducted at the University of Georgia (*N*=107 rats purchased from Harlan, Prattville, AL). Rats were individually housed in clear polycarbonate cages (50 x 30 x 30 cm) and given *ad libitum* access to food and water unless otherwise specified in a temperature and humidity controlled animal facility and maintained on a 12-hour reverse light/dark cycle. Testing occurred during the dark phase with background noise emitted by a white noise generator. Animals were allowed to acclimate to the vivarium for one week prior to surgery. Rats were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Experiments were approved by Emory and University of Georgia IACUCs.

Drugs

Cocaine was obtained from NIDA and dissolved in 0.9% physiological saline. Galnon (Bachem, Torrance, CA, USA) was sonicated in 1% DMSO. Galnon is a non-selective galanin receptor agonist (K_d =1 μM) with a half-life of approximately 60 min (Bartfai and Wang,

2013). Galnon was injected 20-30 min before cocaine based on effective pretreatment schedules in rodents (Jackson *et al*, 2011; Lu *et al*, 2005a; Narasimhaiah *et al*, 2009; Rajarao *et al*, 2007). All injections were performed in a volume of 1 ml/kg.

Stereotaxic cannulation surgery

Rats were anesthetized with isoflurane administered by vaporizer with oxygen delivered through a nose cone, and the surgical site was shaved and cleaned with Betadine. Rats were positioned in a stereotaxic apparatus, a longitudinal incision was made along the scalp, and 3 screws were anchored to the skull. Unilateral guide cannulae (MAB 6.6.IC; SciPro, Sanborn, NY) were implanted targeting the frontal cortex (3.2 mm anterior, 2.2 mm lateral, -1.5 mm ventral, relative to bregma) or NAc shell (1.7 mm anterior, 0.6 mm lateral, -6.5 mm ventral) according to the atlas of Paxinos and Watson (1998b). Cannulae and a plastic guard were fixed to the skull using fast-drying epoxy. Rats received banamine (2.5 mg/kg, s.c.) immediately and 24 h after surgery. At the end of experiments, 4 µl of dye (2 mg/ml India ink) was injected to verify cannulae placement, determined by inspection of dye and termination of the cannulae track in 12 µm Nissl-stained coronal cryosections.

Motor activity

Rats were placed in the center of an open arena (44.5 x 44 x 30 cm; ENV 515-16, Med Associates, St. Albans, VT) that contained fresh bedding as described (Sciolino *et al*, 2012a). Infrared beam breaks were used to track the coordinate position and movement of the rat (ENV-520, Med Associates) every 50 ms using default settings (SOF-810). Behavior was recorded continuously during the 140 min test, pausing in 20 min intervals for all rats to

collect a dialysis sample or to inject drug. Behavior was sampled for 40 min before treatment with vehicle or galnon (2, 5, or 10 mg/kg i.p.) (Phase I, "habituation"), collected for another 20 min before cocaine (10 mg/kg i.p.) (Phase II, "pretreatment"), and recorded for 60 min post-cocaine (Phase III, "cocaine"). Ambulation was initiated after 3 beam breaks and measured as ambulatory distance traveled in continuous (< 500 ms without rest) movement outside a 2x2 area of x-y beams. Non-ambulatory movement was defined as movements that did not achieve criteria for ambulation, and thus was the continuous movement within a 2x2 area of x-y beams. Rats were not habituated to the open field before testing because pilot experiments showed that novelty-induced increases in ambulation diminished steadily across baseline testing, after which time locomotor exploration was nearly zero (i.e., see Fig. 1A).

In vivo microdialysis

Microdialysis was performed in all rats during the motor activity test (see above), as we described previously (Soares *et al*, 1999a). A microdialysis probe was inserted into cannulae targeting the frontal cortex (MAB 9.6.2, 0.6 mm OD, 6 kDa cutoff PES membrane; SciPro, Sanborn, NY) or NAc (S-8020, 0.36 mm OD, 20 kDA cutoff PAN membrane; Synaptech, Marquette, MI) the night before testing and extended 2 mm beyond the guide cannulae. Probes were connected to tubing (PE50, VWR, Westchester, PA) before experimentation. Rats were allowed to freely explore an open field while artificial CSF (pH 7.4; 0.13 M NaCl, 3.1 mM $KHCO_3$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 2.5 mM dextrose, 1.2 mM $CaCl_2$; Sigma Chemicals, St. Louis, MO) was delivered through the probe at 2 µl/min. Dialysate was collected every 20 min during the 140 min open field test, including before

galnon (2, 5, or 10 mg/kg i.p.) or vehicle injection at time -40, before cocaine at time 0, and for 60 min post-cocaine. These procedures were employed to allow DA levels to equilibrate and become Ca^{++}-dependent (i.e., probes inserted 24 hours before testing, baseline sampling lasted 40 min before drug manipulation), as shown previously (Santiago and Westerink, 1990). Dialysate was transferred to sterile microcentrifuge vials (Fischer Scientific, Suwanee, GA) pre-filled with 0.1% phosphoric acid and DHBA (10% sample volume, 0.08 ng/ul) or 0.1 M perchloric acid in EDTA (1 mM of final solution volume). Different preservatives were used in a counterbalanced manner to determine if analyte detection could be improved, but preservatives did not alter DA content in our study (data not shown). Samples were transported in an opaque container on ice and stored at -80° C. Tubing was flushed with 70% EtOH, distilled H_20, air, and aCSF between rats.

High performance liquid chromatography

Samples were thawed and injected into the HPLC system that consisted of two ESA 584 pumps (ESA, Chelmsfor, MA) with a pre-column filter (Synergi Max-RP 4u Security Guard, 150 x 4.6 mm, Phenomenex Inc., Torrance, CA) and Max-RP cartridges (Phenomenex). Mobile phase, containing 100 mM sodium phosphate monobasic (Fisher), 0.1 mM EDTA (Sigma), 0.25 mM octanesulfonic acid (Sigma), and 5% acetonitrile (JT Baker) was delivered at 1 ml/min. Samples and standards were housed and sampled using an ESA 542 autosampler maintained at 4º C. Dialysate injection was 20 µl, and a duplicate set of standards was run every 12 samples. Peaks were detected over 30 min using an ESA CoulArray electrochemical detector (-150 mV on initial electrode, 200 mV on a subsequent electrode). The position and height of peaks for DA were compared with reference

standard solutions (Sigma; diluted in aCSF). Peak areas from chromatograms were integrated and analyzed by CoulArray Data Station Software 3.05.

Food training

To facilitate acquisition of drug self-administration, rats were first trained to lever press for food (45 mg pellets) in an operant conditioning chamber (Med Associates, St Albans, VT) prior to surgery. Each chamber was equipped with a house light and two retractable levers with a stimulus light above each lever. Animals were trained on a fixed ratio 1 (FR1) schedule with a 20-s time out. One lever press on the active lever resulted in the delivery of one food pellet. Presses on the inactive lever had no programmed consequences. Food training sessions lasted 8 h, or until the animal met criteria, defined as at least 70% active lever selection and at least 100 food pellets obtained. Most rats met criteria on the first day of food training, but a few required 2–3 days.

Jugular catheter surgery

All instruments and implants were sterilized prior to surgery. Rats were surgically implanted with a catheter into the right jugular vein after food training, as described (Schroeder *et al*, 2010b; Schroeder *et al*, 2013). Rats were anesthetized with isoflurane administered by vaporizer with oxygen delivered through a nose cone, and the surgical site was shaved and cleaned with Betadine. Catheter tubing was threaded subcutaneously from the back and guided over the shoulder into the right jugular vein, and tubing was sutured down. Rats received meloxicam (1 mg/kg, s.c.) immediately following surgery, and allowed to recover for 1 week prior to cocaine self-administration. Catheters were flushed daily

with 0.05 ml gentamicin (3 mg/ml) and 0.1 ml heparinized saline (30 ml in sterile saline) to help maintain patency. Catheter patency was verified prior to cocaine self-administration by administering 0.08-0.12 ml of the short-acting barbiturate methohexital sodium (10 mg/ml, IV; Eli Lilly, Indianapolis, IN, USA), which rapidly produces moderate sedation.

Cocaine self-administration

Daily cocaine self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and rats received a non-contingent infusion of cocaine (0.5 mg/kg). During training, each press of the active lever resulted in a cocaine infusion (0.5 mg/kg, 167 μl/kg) accompanied by a discrete flashing light above the lever. Following a 20-s timeout period (during which time active lever presses were recorded but did not result in drug infusion), the stimulus light was extinguished and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, the session was terminated early if the number of cocaine infusions exceeded 40. The effects of galnon were assessed once rats reached a stable level of responding (number of drug infusions varied by <20% of the mean and preference for the active lever was at least 75% for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration). Rats received an injection of vehicle or galnon (2 mg/kg, i.p.) 30 min prior to the self-administration session. Each rat received both pretreatments in a counterbalanced fashion.

Extinction

Following completion of the maintenance phase of cocaine self-administration, lever pressing was extinguished in daily 2-h sessions during which presses on the previously active lever no longer resulted in delivery of cocaine or presentation of cocaine-paired cues. Behavior was considered extinguished when active lever presses over 3 consecutive days was <30% of the average number of active lever presses during the last 3 days of maintenance.

Reinstatement

Rats were pretreated with vehicle or galnon (2 mg/kg, i.p.) the day after extinction criteria were met. Thirty min later, they were given a non-contingent priming injection of saline or cocaine (10 mg/kg, i.p.) and placed in operant conditioning chambers under extinction conditions (i.e., presses on the "active" lever had no programmed consequences) for 2 h. Each rat received both pretreatments in a counterbalanced fashion, separated by extinction sessions until they met criteria (described above).

Food self-administration

Separate groups of rats were used for the food self-administration and reinstatement experiments. Rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening at least 1 h after self-administration sessions ended. Parameters of food self-administration were identical to the cocaine self-administration experiments, except that rats received a food pellet instead of a cocaine infusion for each active lever press, and sessions lasted 1 h and were terminated if the number of reinforcers obtained

exceeded 60. Once rats reached maintenance criteria, rats received an injection of vehicle or galnon (2 mg/kg, i.p.) 30 min prior to the self-administration session. Each rat received both pretreatments in a counterbalanced fashion.

Extinction and food-primed reinstatement

After extinction training was completed (extinction criteria were identical to those used for cocaine-primed reinstatement), one group of rats was pretreated with vehicle or galnon (2 mg/kg, i.p.). Thirty min later, they were placed in the operant conditioning chambers for either another extinction session or a food-primed reinstatement session. For the "food-primed reinstatement" group of rats, three food pellets were delivered non-contingently in the first 10 sec of the session and levers were presented. Responses on either of the levers had no programmed consequence. Throughout the 60 min food reinstatement session, a food pellet was delivered every 3 min non-contingently, and responses upon the formerly active and inactive levers were recorded. Each rat received both pretreatments in a counterbalanced fashion, separated by extinction sessions as described above.

Statistics

Self-administration data were analyzed by ANOVA followed by Newman-Keuls post hoc tests or by Chi-square (for the fraction of rats that obtained the maximum number of reinforcers). Motor activity was combined from rats implanted with cannulae in the cortex and NAc because there were no differences between these groups. Motor activity were analyzed using repeated measures ANOVA, and area under the curve (AUC) was performed as follow-up tests for significant interaction effects to detect the source of group differences

during relevant experimental phases, specifically during habituation (Phase I: -60 to -20 min), pretreatment (Phase II: -20 to 0 min), and cocaine phases (Phase III: 0 to 60 min). Analyte levels are reported in nmol/ml for descriptive purposes. Repeated measures ANOVA was used to analyze % DA overflow (post-cocaine analyte at 0, 20, 40, or 60 min / lowest baseline analyte at -40 or -20 min X 100) to reduce within-subject variability. To test *a priori* hypotheses and minimize a type II error, AUC for % DA was performed to assess the effects of galnon during the cocaine phase and *t*-tests were performed to assess the effects of galnon at baseline (time 0). Based on standard criteria (2 standard deviations ± mean), occasional outliers (e.g., <1% of values) were removed and missing values (e.g., ~6% of values due to loss of the microdialysis sample during transfer or collection) were replaced by the group mean to prevent loss of statistical power. In addition, one rat in the galnon 10 mg/kg group was removed entirely from locomotor activity analyses because this subject achieved outlier criteria. The number of subjects per group differed in the behavioral and dialysis studies because HPLC data were not available for all subjects and we focused our analysis on the vehicle and 2 mg/kg galnon dose. Data were analyzed using SPSS (IBM PAWS Statistical Software, Chicago, IL) and graphed using Prism 5.0 (GraphPad, La Jolla, CA).

3.4 Results

Galnon reduces cocaine-induced motor activity.

The effect of galnon (2, 5, or 10 mg/kg) on baseline and cocaine-induced motor activity were assessed in an open field. Ambulatory distance changed across time in a cubic fashion ($F_{1,100}$=130.97, p<0.01; Cubic) (Fig. 3.1A). Distance traveled reduced across time

compared to initial exploration (-40 vs. 0 min; $p<0.01$), was stimulated immediately after cocaine administration (10 min; $p<0.01$), and then steadily declined at 40 min ($p<0.01$), 50 min ($p<0.01$), and 60 min post-cocaine ($p<0.01$) (Fig. 3.1A) compared to the initial effects of cocaine at 10 min. Although the main effect of galnon was not significant for ambulatory distance ($F_{3,102}=1.08$, $p=0.36$), there was a significant drug x time interaction ($F_{3,102}=3.94$, $p<0.01$) (Fig. 1A). Follow-up AUC analyses reveled that ambulatory distance was no different across groups during habituation in phase I (-60 to -20 min; $F_{3,102}=0.39$, $p=0.76$), after pretreatment with vehicle or galnon in phase II (-20 to 0 min; $F_{3,102}=0.92$, $p=0.44$), overall post-cocaine in phase III (1 to 60 min; $F_{3,102}=1.56$, $p=0.21$), or initially after cocaine administration in phase IIIa (1 to 30 min; $F_{3,102}=0.43$, $p=0.73$). However galnon reduced cocaine-induced ambulatory distance later in phase IIIb compared to vehicle (31 to 60 min; $F_{3,102}=3.08$, $p<0.05$). Posthoc tests revealed a significant effect of the 2 mg/kg ($p<0.05$) and 5 mg/kg ($p<0.05$) doses. The 10 mg/kg dose also tended to reduce the locomotor effects of cocaine during phase IIIb, but it was not significant ($p=0.13$) (Fig. 3.1B).

Non-ambulatory movement was also different across time ($F_{1,102}=150.30$, $p<0.01$; Cubic) (Fig. 3.1C). Non-ambulation was less frequent across time compared to initial ambulation (-40 vs. 0 min; $p<0.01$), more frequent immediately after cocaine administration (10 vs. 0 min; $p<0.01$), and then became less frequent at 40 min ($p<0.01$), 50 min ($p=0.01$), and 60 min post-cocaine ($p<0.01$) (Fig. 3.1C) compared to initial effects of cocaine at 10 min. Although the main effect of galnon was not significant for non-ambulatory movement ($F_{3,102}=0.74$, $p=0.53$), there was a significant drug x time interaction ($F_{3,102}=4.02$, $p<0.01$) (Fig. 3.1C). Follow-up AUC analyses revealed that non-ambulatory movement was no different across groups during habituation in phase I (-60 to -20 min;

$F_{3,102}$=0.94, p=0.43), after pretreatment with vehicle or galnon in phase II (-20 to 0 min; $F_{3,102}$=0.07, p=0.97), overall post-cocaine in phase III (1 to 60 min; $F_{3,102}$=1.36, p=0.26), or initially after cocaine administration in phase IIIa (1 to 30 min; $F_{3,102}$=0.34, p=0.80). However, galnon reduced cocaine-induced ambulatory distance later in phase IIIb compared to vehicle (31 to 60 min; $F_{3,102}$=2.90, p<0.05). Posthoc tests showed a significant effect of the 5 mg/kg dose (p<0.01), but not at the 2 mg/kg (p=0.26) or 10 mg/kg doses (p=0.15) (Fig. 3.1D). Based on these results and other data showing galnon suppresses general motor activity and consummatory behavior at higher doses (Abramov et al., 2004; our unpublished data), we chose the 2 mg/kg dose for the remainder of the experiments.

Galnon attenuates cocaine-induced dopamine overflow in the frontal cortex but not the nucleus accumbens.

DA in the frontal cortex was measured in rats treated with vehicle and galnon (2 mg/kg) during motor activity testing, as shown in the timeline (Fig. 3.2A). Extracellular DA levels were not different between groups at baseline (vehicle 77.81±18.87 nmol/ml, galnon 73.39±22.33 nmol/ml, p>0.05) or following galnon pretreatment relative to vehicle (Time 0; t_{22}=1.59, p=0.11) (Fig. 3.2B). DA overflow increased following cocaine administration, and was attenuated by galnon (Fig. 3.2B). Two-way repeated measures ANOVA revealed a significant effect of time ($F_{1,22}$=5.09, p<0.05; Quadratic) and galnon ($F_{1,22}$=7.27, p=0.01), but not a galnon x time interaction ($F_{1,22}$=0.76, p=0.39) (Fig. 3.2B). AUC analyses revealed that galnon significantly reduced post-cocaine DA overflow relative to vehicle (t_{22}=3.03, p<0.01) (Fig. 3.2C).

DA in the NAc was measured in a separate group of rats treated with vehicle or galnon (2 mg/kg) during motor activity testing (Fig. 3.3). Extracellular DA levels were not different between groups at baseline (vehicle 30.60±3.31 nm/ml, galnon 28.12±3.54 nmol/ml, $p>0.05$) or following galnon pretreatment relative to vehicle (Time 0; $t_{13}=-1.13$, $p=0.28$) (Fig. 3B). Cocaine increased DA overflow ($F_{1,13}=15.26$, $p<0.01$; Quadratic), but there was no main effect of galnon ($F_{1,13}=1.85$, $p=0.20$). There was a significant galnon x time interaction ($F_{1,13}=10.36$, $p<0.01$; Cubic) (Fig. 3B). Post hoc tests revealed that extracellular DA levels were not different 20 min post-cocaine ($p=0.29$), but were significantly different 40 ($p<0.05$) and 60 min post-cocaine ($p<0.05$) (Fig. 3.3B). Total post-cocaine DA overflow, as assessed by AUC, was not significantly different in the vehicle and galnon groups ($t_{13}=-1.65$, $p=0.12$) (Fig. 3.3C).

Galnon has minimal effect on cocaine self-administration but blocks cocaine-primed reinstatement of cocaine seeking.

After reaching maintenance criteria for cocaine or food self-administration, rats were pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session, and we found that galnon had no effect on operant responding for drug over the 2-hour session (one way repeated measures ANOVA: active lever, $F_{2,14}=0.24$, $p=0.79$; reinforcers earned, $F_{2,14}=0.01$, $p=0.99$; inactive lever, $F_{2,14}=0.03$, $p=0.97$; Chi-square test for fraction of rats that obtained the maximum number of reinforcers = 1.07, $p=0.59$) (Fig. 3.4A, 3.4B). However, breaking down the 2-hour session into 30-min bins revealed a modest but significant difference in the pattern of cocaine infusions. Vehicle-treated rats tended to obtain most of their infusions during the first 30 min and then tapered off, while

infusions were more stable in galnon-treated rats, resulting in fewer infusions during the first 30 min and more infusions during the final 30 min compared to vehicle (Fig. 3.7). A two-way repeated measures ANOVA showed a main effect of time ($F_{3,21}$=10.08, p<0.001) and a time x treatment interaction ($F_{3,21}$=3.38, p<0.05), although post hoc tests did not reveal significant pairwise differences for any individual time bin.

Rats were next subjected to non-reinforced sessions until meeting extinction criteria, and were then pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a reinstatement test following a non-contingent priming injection of saline or cocaine (10 mg/kg, i.p.). A saline prime did not reinstate cocaine-seeking behavior, and no differences were seen between pretreatment groups (active lever, $F_{2,8}$=1.77, p=0.23; inactive lever, $F_{2,8}$=0.31, p=0.74) (Fig. 3.5A, 3.5B). In contrast to its inability to alter cocaine self-administration, we found that galnon attenuated cocaine-primed reinstatement of cocaine seeking (Fig. 3.5C). One-way repeated measures ANOVA showed a main effect of treatment on active lever presses ($F_{2,12}$=16.07, p<0.001). Post hoc tests revealed that vehicle-pretreated rats robustly reinstated following cocaine prime compared to extinction (p<0.001), while galnon-pretreated rats did not significantly reinstate (p>0.05). Galnon-pretreated rats also displayed significantly fewer active lever presses than vehicle-pretreated animals (p<0.01). Inactive lever presses were low and did not differ between groups ($F_{2,12}$=1.35, p=0.30) (Fig. 3.5D).

Galnon attenuates food-primed reinstatement of food seeking.

To determine whether the effects of galnon on cocaine-primed reinstatement were specific to drug-induced relapse-like behavior, we also evaluated the consequences of galnon on

food self-administration and reinstatement. Similar to what we found with cocaine, galnon (2 mg/kg, i.p.) did not significantly affect reinforced food self-administration (one way repeated measures ANOVA: active lever, $F_{2,18}$=0.12, p=0.88; all rats obtained the maximum number of reinforcers except for one animal on one day that obtained 57 instead of the 61 possible; inactive lever, $F_{2,18}$=3.45, p=0.054) (Fig. 3.6A, 3.6B). In contrast to our cocaine self-administration data, breaking down the 1-h session into 15-min bins did not reveal a difference in the pattern of food reinforcement; all animals obtained a majority of their food reinforcers during the first 15 min, and all rats except for one obtained the maximum number of food reinforcers in the first 30 min (Fig. 3.8). Galnon also had no effect on extinction responding (active lever, $F_{2,10}$=0.1, p=0.91; inactive lever, $F_{2,10}$=4.28, p=0.05) (Fig. 3.9A, 3.9B). However, galnon did partially attenuate food-primed reinstatement of food seeking (active lever: $F_{2,18}$=12, p<0.001) (Fig. 3.9C). Posthoc tests revealed that, while both vehicle- (p<0.001) and galnon- (p<0.05) pretreated rats significantly reinstated following a food prime compared to extinction, galnon-pretreated animals displayed significantly less active lever pressing than vehicle-pretreated animals (p<0.05). Inactive lever presses were low and did not differ between groups ($F_{2,18}$=0.93, p=0.41) (Fig. 3.9D).

3.5 Discussion

Our study is the first to report that galanin receptor activation decreases relapse-like behavior at a dose that had no impact on locomotion following habituation to a novel environment or reinforced cocaine/food self-administration. The behavioral effects of galnon were accompanied by a complete suppression of cocaine-evoked DA overflow in the frontal cortex, but not the NAc. These results are consistent with, and extend, previous

reports showing that galanin receptor signaling diminishes the reinforcing properties of cocaine and other drugs of abuse, and support targeting the galanin system as a potential treatment for addiction.

The effects of galnon on motor activity

Galanin signaling has little impact on basal motor activity and attenuates drug-induced ambulation under some conditions. For instance, galnon fails to alter general locomotion at doses below 5 mg/kg (Abramov *et al*, 2004; Brabant *et al*, 2010; Hawes *et al*, 2008a), but at high doses (e.g. 10 mg/kg and above) it impairs motor activity and food consumption under some conditions (Abramov *et al*, 2004) (our unpublished data). Consistent with these data, we found that doses of galnon ranging from 2-10 mg/kg had no effect on motor activity before cocaine administration. We also found that galnon decreased cocaine-induced motor activity during the second half of the time course examined, consistent its ability to suppresses morphine-induced locomotion in galanin knockout mice (Hawes *et al*, 2008a). In contrast to our present findings in rats, locomotor activity following cocaine was not altered in mice pretreated with galnon (1-4 mg/kg) or in galanin knockout mice (Brabant *et al*, 2010), suggesting the existence of species differences. Combined, these data indicate that, at low doses that do not reliably alter baseline motor activity, galnon modestly attenuates hyperlocomotion following acute administration of cocaine or morphine. To avoid a general locomotor effect of galnon, we chose the lowest dose (2 mg/kg) for the remaining experiments in this study, which aimed to examine the effects of galnon on reward-related behaviors and neurochemistry.

The effects of galnon on cocaine and food self-administration and reinstatement

In general, galanin receptor signaling opposes the reinforcing properties of cocaine and other drugs of abuse. For example, conditioned place preference to cocaine and morphine is facilitated in galanin knockout mice, while galanin or galnon suppresses the reinforcing effects of these drugs (Brabant *et al*, 2010; Hawes *et al*, 2008a; Narasimhaiah *et al*, 2009; Picciotto, 2008; Zachariou *et al*, 1999). However, nicotine conditioned place preference is *attenuated* in galanin knockout mice (Neugebauer *et al*, 2011), suggesting the nature of the drug influences the valence of galanin on reward. The effect of galnon on conditioned place preference likely does not extend to the reinforcing properties of cocaine as measured by operant self-administration; neither galanin knockout nor galnon altered cocaine self-administration in mice (Brabant *et al*, 2010; Narasimhaiah *et al*, 2009). Our data showing that galnon did not profoundly alter stable operant responding for cocaine during the maintenance phase are consistent with these results, although we only examined FR1 responding, and it has been reported that galanin or a GalR1 agonist can alter operant responding for a reinforcer under higher contingency schedules of reinforcement (Anderson *et al*, 2013; McNamara and Robinson, 2010). There was modest but significant change in the pattern of cocaine infusions between groups. The significance of this result is not clear, but it may be related to the delay in peak accumbal DA overflow in response to cocaine we observed in galnon-treated rats.

The effect of galanin receptor activation on relapse-like behavior has not been studied, and we employed the reinstatement paradigm to address this gap in the literature. It is important to note that reinstatement responding differs from the maintenance phase of self-administration sessions because it is run under extinction conditions (i.e., active

lever presses have no programmed consequences), and represents non-reinforced drug-seeking behavior thought to model aspects of relapse (Bossert *et al*, 2013). We found that galnon abolished cocaine-primed reinstatement. There was no significant difference between extinction and reinstatement responding following galnon pretreatment, while robust reinstatement was observed following vehicle pretreatment. This reduction in drug seeking appears to be specific for reinstatement as opposed to a general suppression of motor activity or operant behavior because the dose of galnon used had no effect on baseline motor activity, cocaine self-administration, food self-administration, or inactive lever presses during any phase. Moreover, galnon only modestly attenuated cocaine-induced motor activity, which unlikely accounts for the robust loss of reinstatement behavior because reinstatement was also reduced for a non-drug reinforcer (food) that does not produce hyperactivity.

Interestingly, galnon pretreatment also reduced food-primed reinstatement of food seeking. Like cocaine, dopaminergic agonists mimic the reinforcing stimuli produced by food (Duarte *et al*, 2003). Specifically, activation of the D2 receptors appears to be necessary for the priming effects of food during reinstatement. Similarly, D1 and D2 receptors are also involved in cocaine-primed reward seeking behavior (Sun and Rebec, 2005). Thus, the similar results observed by galanin receptor activation on cocaine- and food-primed reinstatement may be driven by galanin's ability to alter dopaminergic transmission.

The effects of galnon on cocaine-induced changes in dopamine overflow

Cocaine-primed reinstatement of cocaine seeking requires DA transmission in the prefrontal cortex, but not the NAc (Cornish and Kalivas, 2000; McFarland and Kalivas,

2001; McFarland *et al*, 2003; Schmidt *et al*, 2005; Sun *et al*, 2005). Galanin can reduce DA release in some brain regions (Jansson *et al*, 1989; Melander *et al*, 1987; Nordstrom *et al*, 1987; Tsuda *et al*, 1998), but the consequences of galanin receptor activation on cocaine-induced DA overflow have not been investigated. We employed *in vivo* microdialysis in the frontal cortex and NAc to determine whether changes in extracellular DA accompanied galnon's ability to attenuate the behavioral effects of cocaine. The region of frontal cortex selected for microdialysis sampling primarily represents motor cortex. This area receives dense dopaminergic innervation from ventral tegmental area neurons (Lindvall *et al*, 1978b) and was selected because it is a reliable target for catecholamine recovery based on our previous microdialysis experiments (Soares *et al*, 1999a). The region is also highly sensitive to acute cocaine challenge as indicated by magnetic resonance imaging of regional cerebral blood volume in rats (Chen *et al*, 2011). In these studies, cocaine elicited equivalent increases in peak blood volume in motor cortex, dorsal striatum, and nucleus accumbens. DA overflow in the motor cortex thus serves as a proxy for cocaine-induced activation of the mesocortical system. We found that galnon had no immediate impact (e.g., 20 min later) on baseline DA levels following habituation to a novel environment. We also found that galnon prevented cocaine-induced increases in locomotor activity and DA overflow in the frontal cortex. These data suggest that attenuated cocaine-induced DA overflow in the frontal cortex may contribute to the ability of galnon to reduce cocaine-induced locomotor activity and possibly aspects of relapse-like behavior, although the latter hypothesis needs to be directly evaluated by collecting dialysate in the prefrontal cortex during reinstatement testing. Our results are consistent with data from slice preparations showing that galanin reduces DA release (Melander *et al*, 1987; Nordstrom *et*

al, 1987; Tsuda *et al*, 1998), although one study found increased DA utilization in the striatum following galanin administration (Jansson *et al*, 1989).

By contrast, we show that galnon does not reduce DA overflow in the NAc following cocaine administration. A simple explanation is that mesocortical DA neurons are more sensitive to inhibition by galanin receptor activation than mesolimbic DA neurons. Relative to other areas (e.g., NAc, ventral tegmental area), all three galanin receptors are highly expressed in the prefrontal cortex (Hawes and Picciotto, 2004), and this region is changed substantially in both structure and function by cocaine administration (Kalivas, 2007; Morales and Pickel, 2012; Tritsch and Sabatini, 2012). Alternatively, the failure of galnon to alter DA overflow in the NAc may result from complex interactions within mesolimbic circuitry. For example, because cortical DA transmission opposes subcortical DA transmission (Doherty and Gratton, 1996; King *et al*, 1997b; Pycock *et al*, 1980; Ventura *et al*, 2004), suppression of cortical DA overflow by galnon could help preserve normal accumbal DA levels. In support of this idea, our finding that the peak increase in cocaine-induced DA overflow in the NAc following galnon pretreatment is delayed (~40 min post-cocaine) compared with vehicle-pretreated animals (~20 min post-cocaine) implicates a secondary circuit rather than a direct excitatory influence of galnon on mesolimbic DA neurons.

The exact mechanisms underlying the changes in cortical DA transmission in the present study are not clear. One possibility is that galnon modulates cocaine-induced DA release by suppressing the activity of ventral tegmental area DA neurons that project to the frontal cortex or by acting directly on mesocortical DA terminals. Indeed, galanin typically inhibits neuronal activity (Xu *et al*, 2005), and galanin receptors are present in both brain

regions (Hawes *et al*, 2004). Alternatively, galnon may act indirectly by altering the activity of other brain nuclei (i.e., locus coeruleus) that, in turn, project to and control the activity of mesocortical DA neurons (Picciotto, 2008). In support of this hypothesis, galnon suppresses morphine-induced neuronal activity in the locus coeruleus, and transgenic overexpression of galanin in noradrenergic neurons reduces behavior associated with morphine withdrawal (Zachariou *et al*, 2003). Although future experiments are needed to identify the loci and mechanisms of action that mediate the benefit of galnon on cocaine-induced behavior, these data collectively suggest that galnon is well positioned to alter catecholamine transmission across circuits targeted by drugs of abuse and support a model in which galnon primarily affects mesocoritcal DA neurons that drive cocaine-primed reinstatement (Fig. 3.10).

Conclusions

In summary, we report that galnon reduces reward-seeking behavior during reinstatement and cocaine-induced DA overflow in the frontal cortex. However, the results should be interpreted with caution, and further studies are required to define the underlying mechanisms. Galnon is a synthetic non-peptide galanin receptor agonist that crosses the blood brain barrier and has equal binding affinity for GalR1 and GalR2 (Lu *et al*, 2005b; Saar *et al*, 2002). It is important to note that high concentrations of galnon (e.g., 10 uM) *in vitro* also produce agonist activity at other GPCRs and activate intracellular G-proteins independent of receptor activation (Floren *et al*, 2005). However, at doses comparable to those used in the present study, the behavioral effects of galnon are similar to galanin (Sollenberg *et al*, 2005) and blocked by co-administration of a galanin receptor antagonist

(Abramov *et al*, 2004; Saar *et al*, 2002; Wu *et al*, 2003). Thus, the effects of galnon in the present study are in all likelihood mediated, at least in part, by galanin receptor signaling. The modulatory action of galanin on other drug-induced behaviors appears to involve GalR1 and GalR2 receptor subtypes (Einstein *et al*, 2013; Holmes *et al*, 2012). Future experiments using selective antagonists for GalR1 and GalR2 will be necessary to assess the contribution of each receptor subtype. In addition, although all existing evidence points to a central effect of galanin and galnon (Zachariou *et al*, 2003; Zachariou *et al*, 1999), peripheral galanin receptors cannot be ruled out due to our systemic route of galnon administration. Although further experiments are required to identify the galanin receptor subtype and neuroanatomical substrates involved, the data presented here suggest that the galaninergic system is a candidate target for anti-relapse therapies.

3.6 Acknowledgements

These studies were funded by the National Institutes of Health (NIDA Grants DA027535 to DW and PVH, and DA033091 and DA015040 to YEO). DW is co-inventor on a patent concerning the use of selective dopamine β-hydroxylase inhibitors for the treatment of cocaine dependence (US-2010-0105748-A1; "Methods and Compositions for Treatment of Drug Addiction"). The other authors declare no conflicts of interest.

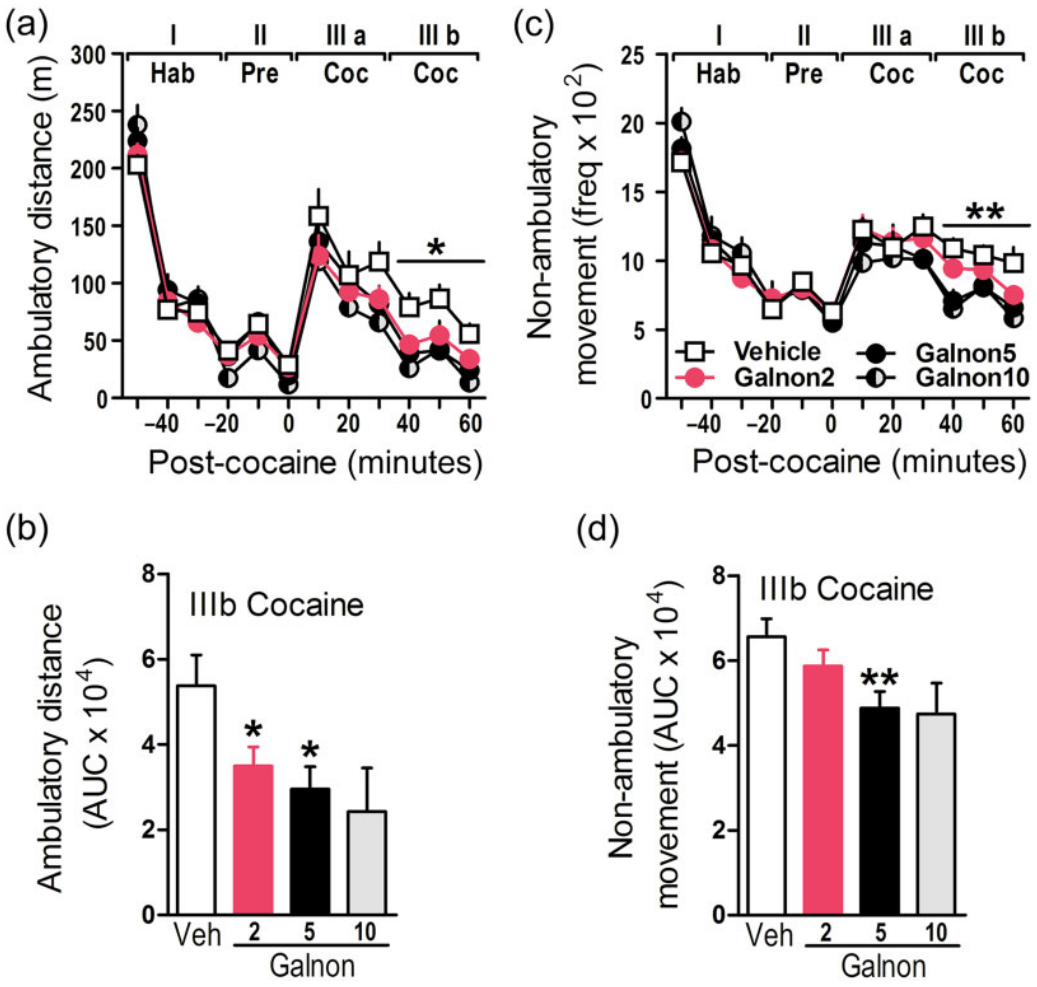

Figure 3.1 Galnon reduces cocaine-induced hyperactivity. Rats were placed in an open field for 40 minutes, administered vehicle or galnon (2, 5 or 10 mg/kg, i.p.), and injected with cocaine (10 mg/kg, i.p.) 20 minutes later. Shown are the mean SEM for (a) ambulatory distance across all time points, (b) AUC for ambulatory distance during the second half of post-cocaine time, (c) frequency of non-ambulatory movement across all time points and (d) AUC for non-ambulatory movement during the second half of post-cocaine time. n = 46 (vehicle), 32 (galnon 2 mg/kg), 23 (galnon 5 mg/kg) and 5 (galnon 10 mg/kg). **P < 0.01, *P < 0.05 compared with vehicle. Hab = habituation (phase I); Pre = pretreatment (phase II); Coc = cocaine (phase III);Veh = vehicle.

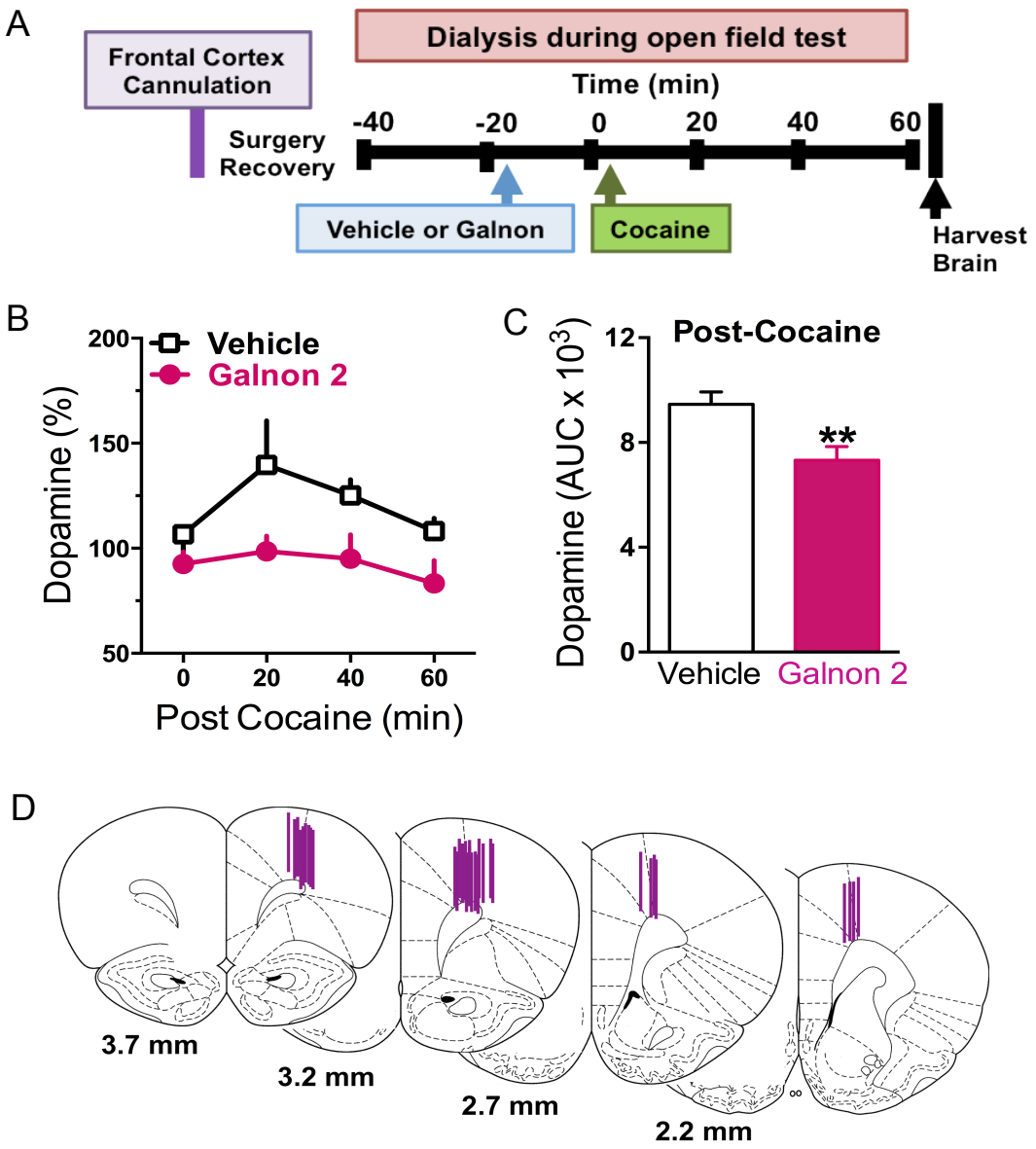

Figure 3.2 Galnon attenuates cocaine-induced increases in dopamine overflow in the frontal cortex. Microdialysis samples were collected from rats during behavioral testing (see Fig. 1) through probes targeting the frontal cortex. Shown are (A) experimental timeline, (B) mean ± SEM extracellular DA levels (% baseline) for vehicle (n=7) and galnon 2 mg/kg (n=7), (C) mean + SEM AUC for total post-cocaine extracellular DA levels and (D) probe placements. **p<0.01, *p<0.05 compared to vehicle.

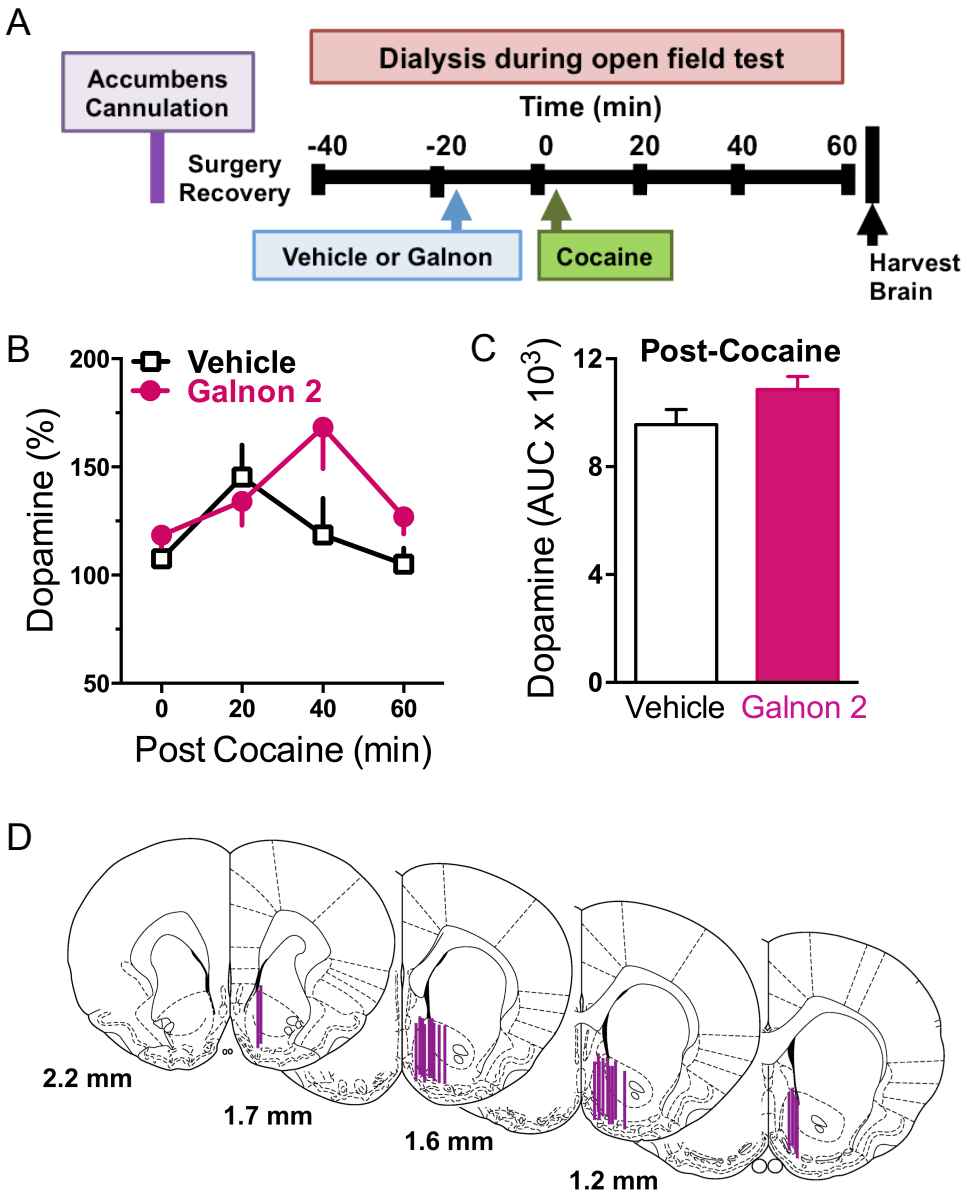

Figure 3.3 Galnon does not alter cocaine-induced increases in dopamine overflow in the nucleus accumbens. Microdialysis samples were collected from rats during behavioral testing (see Fig. 1) through probes targeting the NAc shell. Shown are (A) experimental timeline, (B) mean ± SEM extracellular DA levels (% baseline) for vehicle (n=14) and galnon 2 mg/kg (n=10), (C) mean + SEM AUC for total post-cocaine extracellular DA levels and (D) probe placements.

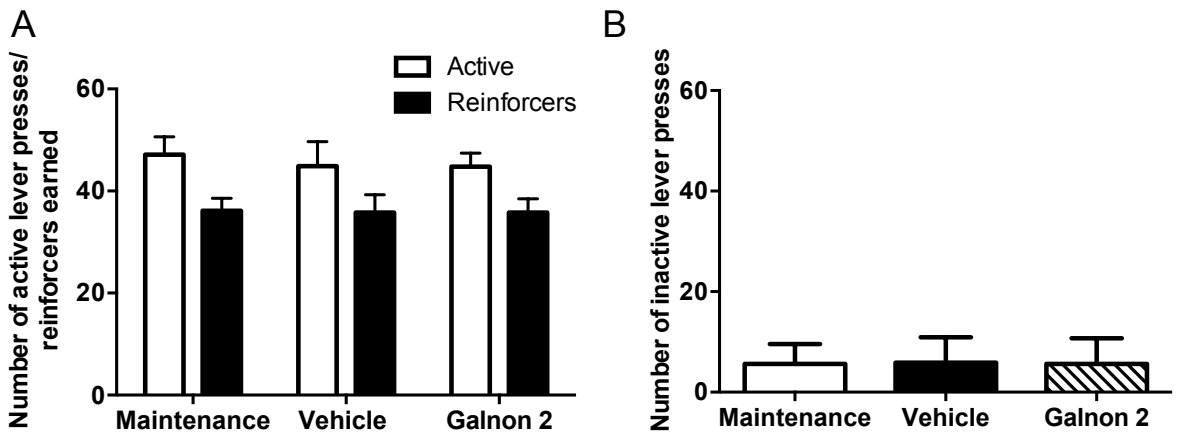

Figure 3.4 Galnon has no effect on cocaine self-administration. Following the establishment of stable maintenance responding for cocaine on a FR1 schedule (maintenance values reflect an average of the last 3 days of maintenance sessions), subjects (n=8) were treated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session. Shown are the mean ± SEM of (A) active and (B) inactive lever presses during the 2 h test session.

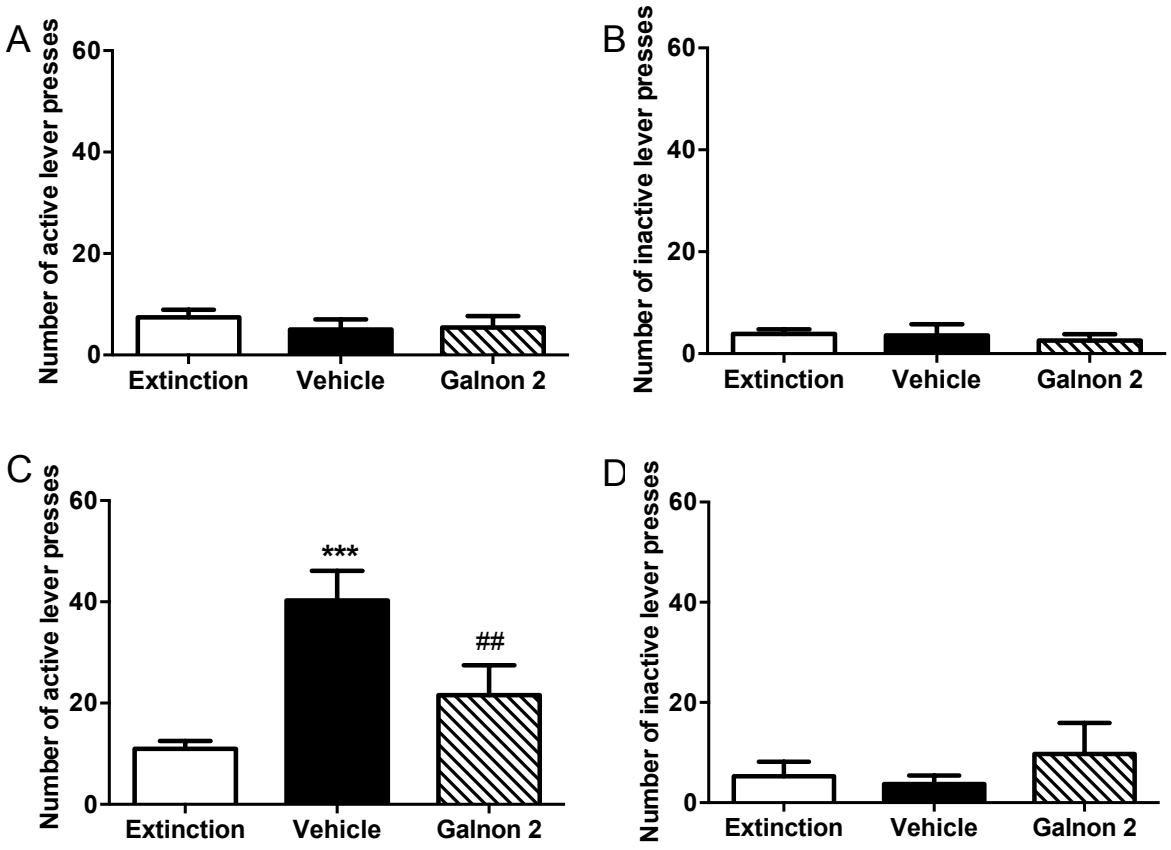

Figure 3.5 Galnon attenuates cocaine-primed reinstatement. Lever pressing was then extinguished (extinction values reflect an average of the last 3 days of extinction), and rats were pre-treated with vehicle or galnon (2 mg/kg, i.p.) 30 minutes prior to a cocaine primed (10 mg/kg, i.p., n = 7) or saline-primed (n = 5) reinstatement test. Shown are the mean + SEM active and inactive lever responses during the 2-hour saline-primed (a and b) and cocaine-primed (c and d) sessions. ***P < 0.001 compared with extinction; ##P < 0.01 compared with vehicle

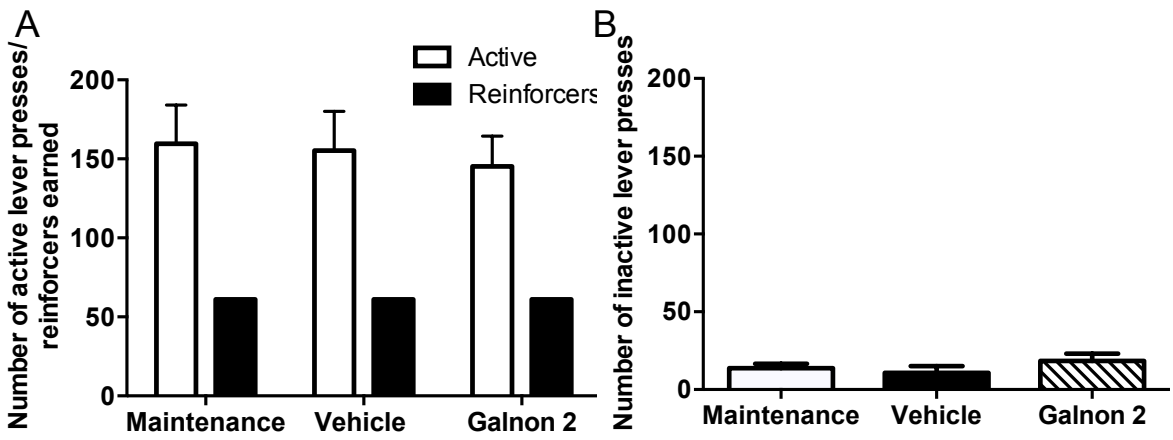

Figure 3.6 Galnon has no effect on food self-administration. Following the establishment of stable maintenance responding for food pellets on a FR1 schedule (maintenance values reflect an average of the last 3 days of maintenance sessions), subjects (n=10) were treated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session. Shown are the mean ± SEM of (A) active and (B) inactive lever presses during the 2 h test session.

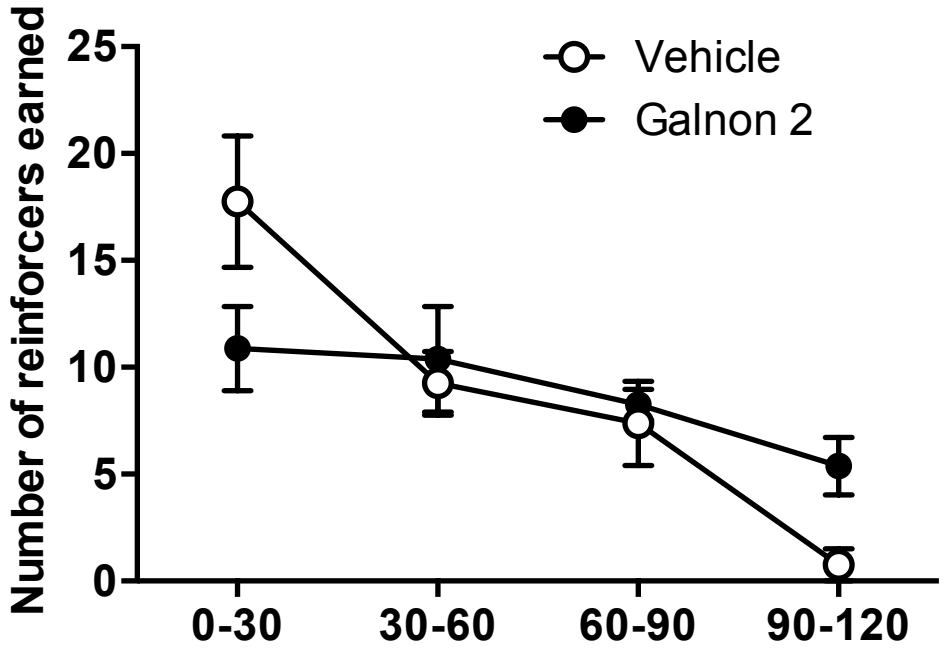

Figure 3.7 Effects of galnon on the pattern of cocaine self-administration.

Shown are the mean + SEM of cocaine reinforcers earned during the 2-hour test session (see Fig. 3.4a) broken down into 30-minute bins.

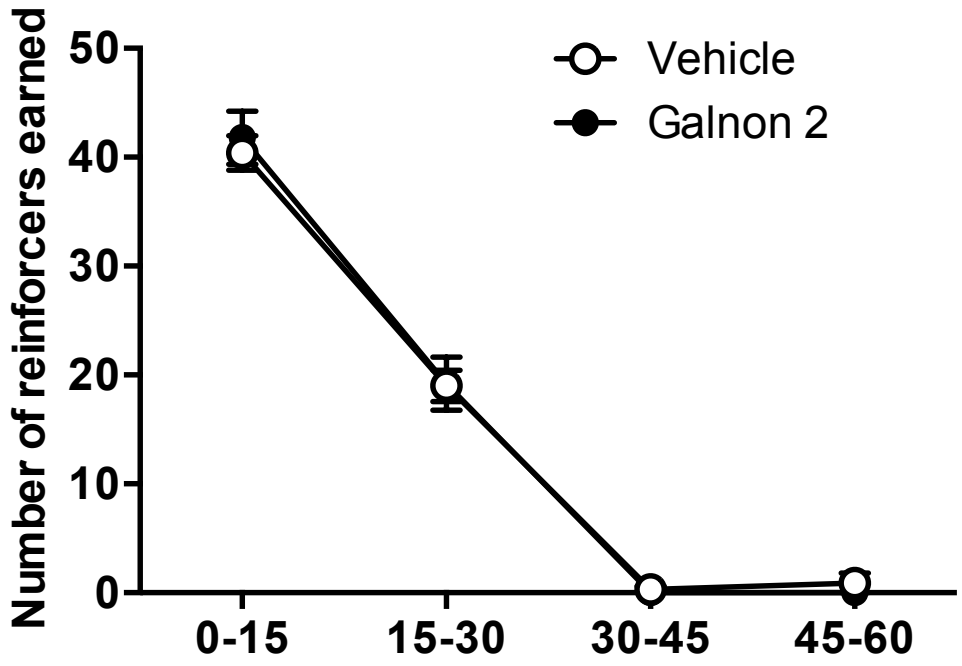

Figure 3.8 Effects of galnon on the pattern of food self-administration.

Shown are the mean + SEM of food reinforcers earned during the 2-hour test session (see Fig. 3.5a) broken down into 15-minute bins.

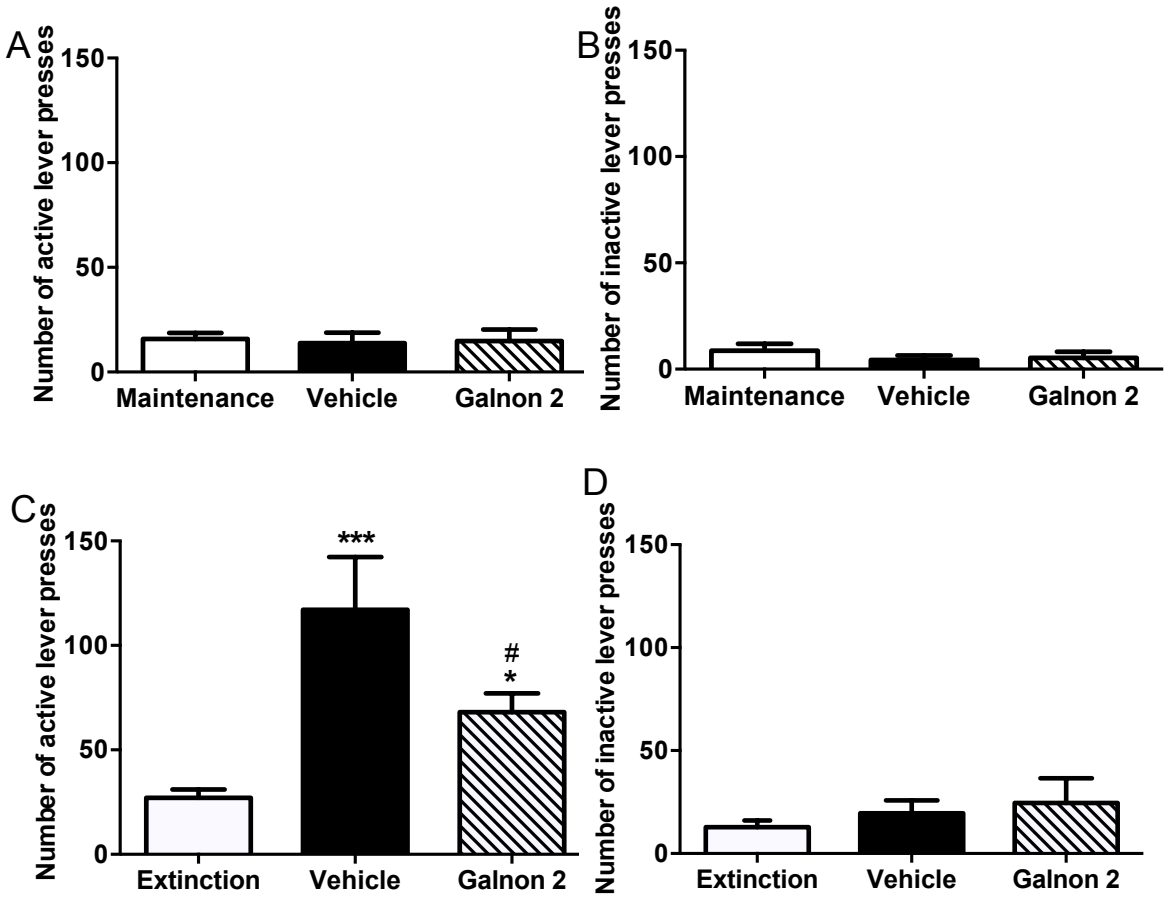

Figure 3.9 Galnon attenuates food-primed reinstatement. Lever pressing was then extinguished (extinction values reflect an average of the last 3 days of extinction), and rats were pre-treated with vehicle or galnon (2 mg/kg, i.p.) 30 minutes prior to another extinction session (n = 6) or a food-primed reinstatement test (n = 10). Shown are the mean $\pm$ SEM active and inactive lever responses during the 1-hour extinction (a and b) and food-primed reinstatement (c and d) sessions. *P < 0.05 compared with extinction; ***P < 0.001 compared with extinction; #P < 0.05 compared with vehicle

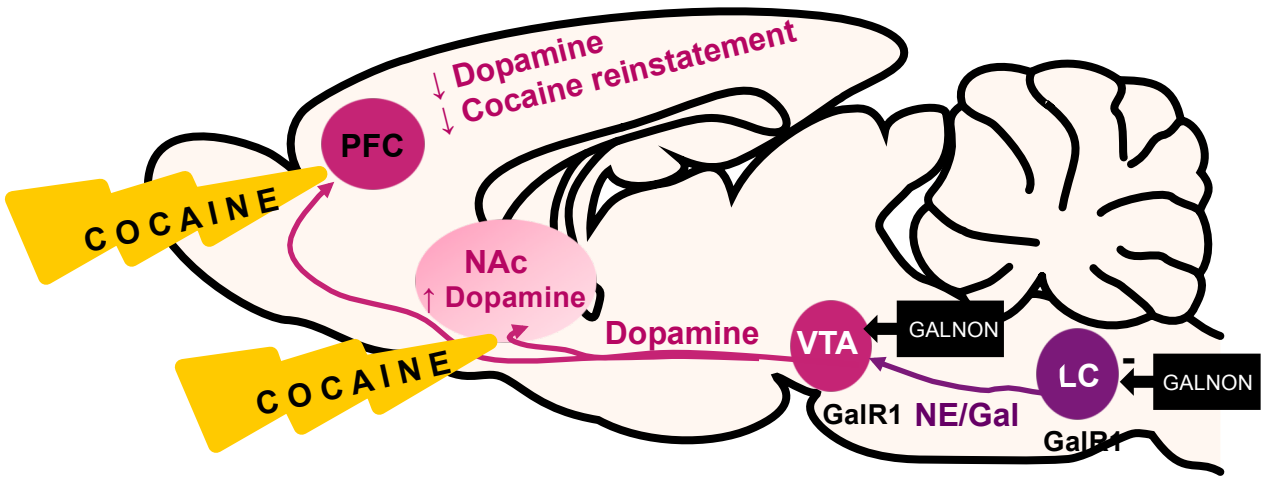

Figure 3.10 How galnon may be reducing cocaine-induced behaviors.

Galnon attenuates the reinforcing properties of cocaine. Although the mechanism is not clear, it is likely that galnon reduces the reinforcing properties of cocaine by altering dopamine transmission in the mesocorticolimbic circuit. One way that galnon modulates cocaine-induced DA release is by suppressing the activity of ventral tegmental area DA neurons that project to the frontal cortex or by acting directly on mesocortical DA terminals. Alternatively, galnon may act indirectly by altering the activity of other brain nuclei (i.e., locus coeruleus) that, in turn, project to and control the activity of mesocortical DA neurons

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 SUMMARY

The results from our studies provide a better understanding of the role of exercise on relapse neurobiology. Three weeks of running after the extinction phase but prior to cocaine-primed reinstatement significantly reduced drug-seeking behavior. Our results are consistent with several other reports that chronic wheel running attenuates cocaine-primed reinstatement (Cosgrove *et al*, 2002; Smith *et al*, 2012; Zlebnik *et al*, 2012; Zlebnik *et al*, 2010). In contrast to previously published paradigms, our study specifically assessed the effect of exercise as a therapeutic intervention on reinstatement behavior without the potential secondary effects stemming from alterations in the reinforcing efficacy of cocaine during self-administration or facilitation of extinction learning. Furthermore, our design more accurately models the way exercise might actually be used for treatment in an inpatient or outpatient facility in which patients typically enter with an extensive history of drug use. One caveat, however, is that exercising animals had *ad libitum* access to a wheel. Animals ran for several hours and averaging 4-6 km per day. This level of running is far above the amount of exercise most people would engage in a treatment facility or in their daily lives. According to the Center for Disease Control, the recommended federal guidelines for vigorous intensive aerobic activity are 75 minutes per week (Schoenborn *et al*, 2013). Furthermore, most clinical studies that have incorporated exercise as a treatment intervention for substance abuse provide exercise for a 1-2 hours a day ranging from 2-5 days a week (Brown *et al*, 2009a; Brown *et al*, 2010; Roessler, 2010; Sinyor *et al*, 1982; Weinstock *et al*, 2008;

Zschucke *et al*, 2012). In rats, 40 minutes of running per day 6 days a week (about 1000 meters) is sufficient to induce an increase in galanin mRNA expression in the LC (O'Neal 2001). Therefore, one way to refine this animal model would be to limit access to 1-2 hours per day for 3-6 weeks and determine whether exercise still exerts a therapeutic benefit on reinstatement.

Finally, the future experiments assessing the effect of exercise on drug self-administration should investigate potential sex differences. In clinical and preclinical studies, female subjects are more sensitive to the reinforcing properties of drugs of abuse (Anker and Carroll, 2011), and the female gonadal hormone estrogen facilitates the acquisition, escalation and reinstatement of cocaine seeking (Anker *et al*, 2011; Carroll and Anker, 2010). While female rats run significantly more than male rats, the effect of exercise on cocaine self-administration and reinstatement does not differ (Smith 2011, 2012). It is possible that hormonal fluctuations across the estrogen cycle could impact various facets of drug self-administration. Thus future experiments should include female subjects to directly assess the effect of sex hormones on exercise and drug self-administration.

4.2 EXERCISE AS AN INTERVENTIONAL THERAPY FOR COCAINE DEPENDENCE

The cognitive/behavioral mechanism underlying the ability of exercise to reduce drug-seeking behavior was not explored directly in this study; however, previously published randomized clinical studies provide some clues. For example, individuals in treatment for drug dependence who participated in an exercise program reported a reduction in craving and withdrawal symptoms (Roessler,

2010; Zschucke *et al*, 2012). Additionally, exercise reduced comorbid risk factors, such as mood disorders, that are associated with and are thought to drive substance abuse (Pettinati et al, 2013). Antidepressant medication had a modest but beneficial effect on depressive symptoms and substance use among patients with comorbid depression and drug addiction (Nunes and Levin, 2004). Thus, one potential mechanism for the beneficial effects of exercise on drug abuse is its ability to alleviate depressive/anxiety symptoms and reduce the need for "self medication" (Cooney *et al*, 2013; Mead *et al*, 2009, Arida *et al*, 2013; Nakken *et al*, 1990).

It has been proposed that exercise reduces cocaine reinstatement because it provides environmental enrichment. However, in studies that directly compared rats with access to an unlocked running wheel versus rats with access to a locked wheel, only rats with the unlocked running wheel showed significantly lower rates of responding during reinstatement (Lynch *et al*, 2010; Zlebnik *et al*, 2010). The presence of a wheel, even a locked one, can be considered a novel object and a form of environmental enrichment. This result suggests that the somatosensory effects and neurobiological changes induced by chronic exercise, rather than simply the presence of an enriched environment, play an important role. Some preclinical studies have further examined the effects of environmental enrichment by introducing rats to a larger living space, novel objects, running wheels, and social interaction (Chauvet *et al*, 2009; Chauvet *et al*, 2011; Kelly *et al*, 2009; Thiel *et al*, 2009). These studies found that rats exposed to an enriched environment exhibited reduced drug-seeking behavior during reinstatement (Chauvet *et al*, 2009; Chauvet *et al*, 2011; Kelly *et al*, 2009; Ranaldi *et al*, 2011; Solinas *et al*, 2008; Thiel *et al*,

2009). However, because of the multifactorial design of this environmental enrichment, it is difficult to determine which facet was responsible for the observed effect. Using an alternative environmental design that isolated the specific components of environmental enrichment, it was found that each component of environmental enrichment provided a specific therapeutic benefit (Gregoire *et al*, 2014). Voluntary wheel running was associated with increased hippocampal neurogenesis (a result not observed in rats given a complex living environment or social housing with no running wheel), social housing was associated with depolarization-associated c-fos expression within the granule cell layer of the hippocampus, and complex environment was associated with decreased plasma corticosterone concentration (Gregoire *et al*, 2014). Thus, the interaction with a stimulating environment alone does not confer the same neuroprotective effects and cognitive benefits of voluntary exercise (Kobilo *et al*, 2011; Mustroph *et al*, 2012). However, a direct comparison of rats exposed to a novel environmental enrichment and rats with a history of chronic exercise would be necessary to determine what differential effects these two behavioral interventions may have on reinstatement.

Another mechanism by which exercise may exert its protective effects on addiction is by activating the endogenous brain reward system because running is naturally rewarding. For example, rats will voluntarily run an average of 4-6 kilometers a day when given free access to a wheel (Murray *et al*, 2010; Sciolino *et al*, 2012b). The reinforcing properties of running can be measured by training animals in an operant conditioning paradigm in which lever-pressing provides an

opportunity to run on a wheel, and wheel running indeed serves as a positive reinforcer (Belke, 1997; Belke and Wagner, 2005; Kagan and Berkun, 1954). Furthermore, pairing wheel running with a specific environmental context establishes a conditioned place preference (CPP) for that context. Specifically, repeated placement in a distinctive chamber after 2 hours of wheel running results in a preference for that chamber compared to one that was not paired with wheel running (Lett *et al*, 2000), suggesting that the reinforcing properties of exercise persist after the physical activity has ended (Belke *et al*, 2005; Greenwood *et al*, 2011; Lett *et al*, 2000). Chronic voluntary wheel running also blunted the increase in corticosterone induced by social exploration and prevented the reduction in social exploratory behavior, shock-elicited fear, and deficits in escape learning produced by uncontrollable stress for up to 15 days following the forced cessation of exercise (Greenwood *et al*, 2012). Long-term repeated voluntary wheel running produces neuroplastic changes within the mesocoricolimbic system which include increased delta Fos b/Fos b immunoreactivity, delta opioid receptors, and kappa opioid receptor in various subregions of the NAc and prevented stress-induced loss of dendritic spines in the medial PFC (Greenwood *et al*, 2011; Greenwood *et al*, 2013). Exercise-induced activation of the reward system also suppresses natural reward-seeking behaviors, such as feeding (Lett *et al*, 2000). Thus, the reinforcing effects associated with wheel running could be reducing or replacing the reinforcing properties of drugs of abuse. These data suggest that running activates the reward system, resulting in positive affective state that lasts beyond the bout of physical

activity and renders the brain less sensitive to other reinforcing stimuli such as food or drugs.

Addiction research has consistently shown that agents that are rewarding and reinforcing to people have a potential for abuse. Like substance abuse, the reinforcing properties of exercise make it a candidate for addiction in some individuals that are susceptible to compulsive behaviors. Exercise addiction can be defined as a compulsive desire to engage in exercise despite negative consequences (Freimuth et al, 2011; Szabo et al, 2013). Although exercise addiction is not recognized in the DSM-V as a behavioral disorder, there is evidence that compulsive exercise engagement shares many characteristics with the symptoms outlined in the DSM-IV for substance abuse. Like substance abuse disorder, exercise addiction may include tolerance, withdrawal, loss of control, and persistence to engage in the behavior despite exercise exacerbating negative consequences (injuries, social disruption, interpersonal conflict) (Freimuth et al, 2011; Hausenblas and Fallon, 2002). Treatment for exercise addiction primarily relies on cognitive behavioral therapy that is also used to treat substance use disorders. Because patients in treatment for substance use disorders would be receiving concurrent behavioral therapy along with exercise therapy, the risk is low that they would develop an exercise addiction while in treatment. Although rare, clinicians should become aware of the symptoms associated with exercise addiction so as to properly address it. When exercise is administered as a treatment, clinicians should remain vigilant that the recommended regimen is not exceeded and that exercise has not become problematic (Freimuth et al, 2011).

Clinical studies assessing the efficacy of various therapies on cocaine addiction, both pharmacological and behavioral, have focused on the reduction of drug use rather than relapse from abstinence. While the reinstatement model is predictive for alcohol, nicotine and opiates it has not yet shown to be predictive for psychostimulants because no FDA-approved treatments currently exist. The reinstatement model may not be sufficient for developing effective therapies against psychostimulants.

The maintenance-extinction-reinstatement animal model of drug addiction only examines one aspect of the complex behaviors that are involved in human drug relapse. The reinstatement phase tests rats' propensity to seek drug after drug priming (de Wit and Stewart, 1981), drug-associated cues (Crombag and Shaham, 2002; Meil and See, 1996; Weiss *et al*, 2000) or stress (Erb *et al*, 1996; Leri *et al*, 2002; Shepard *et al*, 2004). Other aspects of drug addiction could be altered by chronic exercise, such as the motivation to seek the drug. In a previously published study, rats were tested in a self-administration paradigm in which animals were trained to self-administer cocaine on a fixed ratio (FR) schedule before being tested on a progressive ratio (PR) schedule (Smith *et al*, 2008), in which the number of lever presses necessary for receiving a single reinforcer increases until a point is reached at which the subject is no longer willing to respond. The point in which responding ceases is defined as the breakpoint and is considered a measure of motivation to seek drug. In this study, the exercise group was given access to a wheel before and throughout self-administration training (both FR and PR) and found that concurrent access to a wheel during self-administration attenuated the

positive reinforcing effects of cocaine and the motivation to work for a cocaine reward during PR testing (Smith *et al*, 2008). However, it is not clear if exercise as a therapy in rats with a prior history of chronic self-administration would still have the same effect on their motivation to work for a drug reward. This could be tested by first training the rats to self-administer under a fixed ratio 1 schedule until responding stabilizes. Rats would then be assigned to an exercise condition in which they have *ad libitum* access to running wheel in their homecage or a sedentary condition for 3 weeks. Following the abstinence period, animals could then be tested on a PR schedule to determine whether an exercise intervention alters their motivation to seek a drug reward. I hypothesize that rats in the exercise condition would achieve a lower breakpoint than animals in the sedentary condition.

This exercise study provides evidence that exercise administered after maintenance but prior to reinstatement attenuates drug-seeking behavior during cocaine-primed reinstatement. However, there are several other facets of drug addiction that this model did not directly test. The extinction phase in standard reinstatement paradigms is a laboratory experimental design that facilitates the study of relapse-like behavior by forcing the animal to reduce their operant responding before being tested during reinstatement. However, extinction is an active learning process that does not accurately model what human addicts experience. Drug addiction is often marked by long periods of voluntary or involuntary abstinence that precedes relapse, thus future experiments should take this into account to directly assess the effect of exercise on relapse-like behavior after abstinence, not extinction. In this study, rats went through both extinction and

forced abstinence (i.e. the 3-week period of wheel running or sedentary condition following extinction but preceding reinstatement. Time-dependent increases in cocaine seeking following withdrawal, referred to in the literature as "incubation of craving" have been previously reported in preclinical (Grimm *et al*, 2001; Pickens *et al*, 2011) and clinical studies (Bedi *et al*, 2011). To directly test the effects of exercise on this phenomenon, animals would self-administer cocaine until stable intake is reached, followed by several weeks of abstinence (but no extinction training) during which animals are assigned to an exercise condition or sedentary condition.

Another aspect of drug addiction that was not tested in the current study is "binge-like" behavior, which is modeled in animals by the escalation of drug intake observed during extended drug access (long access, or "LgA" paradigms). The LgA self-administration paradigm is thought to reflect a change in hedonic set point that occurs during the transition from recreational to uncontrollable drug use. The progressive elevation of the reward threshold drives the escalated consumption of cocaine during prolonged access, a process not observed during short access (Ahmed *et al*, 2002; Koob and Kreek, 2007). Elevation of brain reward threshold following prolonged access to cocaine fails to return to baseline between repeated prolonged self-administration sessions (Ahmed *et al*, 2002; Koob *et al*, 2007). Long-access to self-administration also results in persistent molecular adaptations. It is associated with the recruitment of the CREB/dynorphin axis in the NAc and an increase in excitatory signaling in the mesolimbic circuitry (Edwards and Koob, 2013). Long access to cocaine self-administration is associated with excessive drug

intake and the potentiation of the stress and brain reward circuitry. Thus, the neuroadaptations that occur as a result of extended access to drugs of abuse are distinct from the molecular changes that occur during short access.

When adolescent rats are given concurrent access to wheel during 6-hour daily sessions (as opposed to the conventional 1-2 h/d sessions) of cocaine self-administration, rats with access to an unlocked wheel exhibited lower rates of cocaine self-administration than rats given a locked wheel. In contrast, wheel access did not alter cocaine self-administration in adult rats (Zlebnik *et al*, 2012). This study showed that concurrent access to a non-drug reinforcer, wheel–running, could lower rates of cocaine self-administration and prevent escalation intake in adolescent rats, but not in adult rats. It is not surprising that concurrent access to wheel running during the LgA phase did not alter drug intake in adult rats because several previous studies had shown that concurrent access to wheel-running does not alter drug responding during maintenance (Smith *et al*, 2012; Smith *et al*, 2011c). However, the main implication from this study is that exercise may be an effective preventive measure against the escalation of drug intake and that this effect may be age-dependent. It is not clear why exercise had a greater effect on adolescents because wheel-running behavior was similar between both age groups. However, the ability of exercise to attenuate drug seeking behavior after escalation to drug intake has already occurred remains unexplored. To test this, it would be necessary to train rats to self-administer cocaine in a 6-hour (or longer) daily sessions until rats escalate their intake, along with a control group of rats that receive 2-hour daily access. Following extinction, rats would be assigned to a 3-

week exercise or sedentary condition prior to a reinstatement session. This procedure would test if an exercise intervention is sufficient for reducing drug-seeking behavior in a relapse like model in rats with a history of escalated drug intake.

4.3 Potential Design for Future Clinical Studies Assessing Exercise as a Treatment for Cocaine Addiction

Based on previously published clinical studies that assessed the effect of exercise on drug dependence and the results from our preclinical study on the effect of an exercise intervention in a drug addiction animal model, I propose the following study design for a randomized controlled trial for the treatment of cocaine abuse (Dolezal *et al*, 2013). Patients that meet the DSM-IV criteria for cocaine dependence seeking treatment in an inpatient rehabilitation facility should be recruited within two weeks of admission. After receiving informed consent for participation in the study, a baseline assessment of the participants' medical history and physical examination should be attained to screen for eligibility. Eligible participants should be randomly assigned to one of two treatment groups, an exercise-training group (ET) and a health education group (HE). The ET should be aerobic exercise sessions consisting of running, cycling or a step class. The ET should be administered 40-60 minutes a day, three times a week for 8 weeks. The health education sessions would follow the same schedule and would be educational seminars about health. A previously published feasibility study found that methamphetamine dependent patients given an exercise intervention 3 times a week for 8 weeks were able to

maintain a 74% adherence rate and found that exercise trained patients exhibited improved fitness (Dolezal *et al*, 2013). Thus, a similar exercise-training program should also be feasible in cocaine dependent individuals. Both groups would continue to participate the standard form of care provided at the facility, which should include behavioral therapy (ex CBT), a 12-step interventional treatment and family therapy with incentives for participation. Follow-ups should be conducted 2 months, 4 months, and 6 months following discharge from the facility. The primary outcome for the effect of exercise on drug use would be gathered by self-report and confirmed with a cocaine urine test. Secondary outcomes that should be measured would include how exercise affects craving, anxiety, fitness, behaviors assessed by the Addiction Severity Index (Moura *et al*, 2014) and adherence to treatment. In previous studies, the outlined outcomes have been associated with higher abstinence rates (Dolezal *et al*, 2013; Taylor *et al*, 2007; Zschucke *et al*, 2012). Additionally, blood could be collected after each exercise session to assess biomarkers associated with chronic exercise such as BDNF or galanin. In previous human studies, acute bouts of exercise were positively correlated with increases in BDNF and galanin hormones (Legakis *et al*, 2000; Seifert *et al*, 2010). Analysis of the biomarkers could then be correlated with subsequent behavioral outcomes at the end of the study. To date, the results from a randomized controlled study assessing the efficacy of exercise adjunct therapy for the treatment of illicit substances such as cocaine have not yet been published. An adequately powered study following the preceding criteria would provide the evidence to determine whether an exercise adjunct therapy is effective in reducing cocaine relapse.

4.4 MOLECULAR MECHANISMS UNDERLYING THE ABILITY OF EXERCISE TO ATTENUATE RELAPSE-LIKE BEHAVIOR: FOCUS ON GALANIN

As noted in the Introduction, chronic exercise produces numerous changes in the brain that could impact drug-seeking behavior. For my dissertation, I focused on the potential contribution of the neuropeptide galanin. The broad distribution of galanin in the CNS allows it to modulate a wide variety of neurotransmitters including norepinephrine, dopamine, acetylcholine, GABA, glutamate (Melander *et al*, 1986; Melander *et al*, 1985). The diverse neurotransmitters modulated by galanin mediates its role in many different physiological functions including, learning and memory, cognition, nociception, and feeding (Counts et al, 2008; Lang et al, 2007; Ogren et al, 1998; Xu et al, 2010). More recently, galaninergic pathways that alter neurotransmission of monoamines implicate a modulatory role for galanin on the development of drug addiction (Einstein et al, 2013; Picciotto, 2008).

4.4.1 Drug responses

Galanin plays a protective role against drug dependence and withdrawal. Galanin receptor activation with the synthetic galanin agonist, galnon, attenuates morphine place preference (Zachariou et al, 1999). Additionally, opiate withdrawal symptoms are significantly exacerbated in mice with a genetic knock out of galanin compared to wildtype mice (Holmes et al, 2012; Zachariou et al, 2003). Systemic administration of galnon reverses the increased withdrawal signs in galanin knockout mice (Zachariou et al, 2003). Likewise, galanin overexpression under a

DBH promoter significantly reduces the severity of opiate withdrawal in mice (Picciotto et al, 2005). Transgenic mice that overexpress galanin in the brain are less sensitive to amphetamine-induced increases in locomotor activity (Kuteeva et al, 2005). Conversely, galanin knockout mice are more sensitive to morphine or cocaine place preference and this effect is reversed by systemic galnon treatment (Narasimhaiah *et al*, 2009). However, the effects of a complete galanin knockout do not extend to cocaine self-administration (Brabant *et al*, 2010). Until this study, the impact of galanin signaling on reinstatement of operant drug self-administration had never been tested.

4.4.2 Exercise increases galanin mRNA in the LC

Previous reports showed chronic voluntary wheel running increases galanin mRNA expression in the LC of drug-naïve rats (Holmes *et al*, 2006; O'Neal *et al*, 2001; Van Hoomissen *et al*, 2004), and we found that this molecular change persisted in rats with history of cocaine self-administration. It is unclear exactly how chronic exercise induces an increase in galanin mRNA expression in the LC. Because many genes are upregulated by neuronal activity, it is possible that exercise increases LC firing, which then facilitates galanin gene expression. I hypothesize that somatosensory activation of the LC neurons could occur via increased activity of the vagal nerve. 80% of the fibers of the vagal nerve carry afferent sensory information to the CNS (Nemeroff *et al*, 2006). The cell bodies of the vagal nerve originate from the nodose ganglia and project to the nucleus of the solitary tract (NTS). In turn, the nucleus of the solitary tract sends direct projections to many brain regions including

the LC (Groves and Brown, 2005; Nemeroff *et al*, 2006). The NTS relays excitatory input the nucleus paragigantocellularis, which sends gluatamatergic afferents to the LC (Mello-Carpes and Izquierdo, 2013). Vagal nerve stimulation activates noradrenergic neurons and increases their firing activity (Cunningham *et al*, 2008; Dorr and Debonnel, 2006; Manta *et al*, 2009). Furthermore, exercise can also increase vagal nerve activity. 30 days of treadmill running results in a 31% increase in vagal nerve activity (Ranaldi *et al*, 2011). Therefore, there is evidence to suggest the existence of an exercise-vagal nerve activity-LC firing-galanin mRNA loop, which should be explored in future experiments. It should be noted that the NTS also contains the A2 nuclei (Ritchie *et al*, 1982). However, as reported earlier exercise did not increase galanin mRNA in the A2. It is not clear why the NTS activation did not increase galanin mRNA in the A2 population of noradrenergic cells.

4.5 GALNON AND COCAINE-PRIMED REINSTATEMENT

Due to the effects of galanin on other aspects of drug addiction noted above, I suspected an increase in galanin mRNA in the LC might underlie the reduction in reinstatement afforded by exercise. However, my exercise experiments established only an association, not a causal link, between LC galanin and cocaine-seeking behavior. As an initial test of my hypothesis, I determined whether galanin signaling, alone, could recapitulate the effects of exercise on cocaine-primed reinstatement using the galanin receptor agonist, galnon. My results showing that galnon pretreatment reduced cocaine-primed reinstatement indicate that activation of galanin signaling is *sufficient* to reduce relapse-like behavior. The next step will be

to determine whether an increase in galanin transmission in *necessary* for exercise-induced decreases in cocaine-primed reinstatement. It is possible to block some therapeutic effects of exercise on maladaptive brain function with the galanin receptor antagonist M40. For example, animals with a history of chronic voluntary exercise exhibit significantly fewer kainate-induced seizures compared to their sedentary counterparts (Reiss *et al*, 2009). The therapeutic effect of exercise on kainite-induced seizures can be blocked by M40, thus establishing a causal link between exercise-induced galanin transmission and changes in seizure susceptibility. To test if galanin receptor activation is required for exercise-induced decreases in reinstatement, it would be necessary to determine the effects of M40 pretreatment on drug-seeking behavior in animals that have a history of chronic voluntary wheel running. If chronic exercise failed to attenuate cocaine seeking in rats treated with M40, then a causal link between the therapeutic benefits of exercise via galanin receptor activation could be established.

4.6 NEUROANATOMICAL AND NEUROCHEMICAL SUBSTRATES UNDERLYING THE EFFECT OF GALANIN SIGNALING ON COCAINE-PRIMED REINSTATEMENT

Galanin's effect on cocaine-induced changes in neurotransmission is likely related to its inhibitory function on catecholamine neurotransmitter release. Galanin can reduce DA release in some brain regions (Jansson et al, 1989; Melander et al, 1987; Nordstrom et al, 1987; Tsuda et al, 1998), but the consequences of galanin receptor activation on cocaine-induced DA overflow have not been investigated previously. Using in vivo microdialysis in the frontal cortex and NAc,

this study demonstrated whether changes in extracellular DA accompanied galnon's ability to attenuate the behavioral effects of cocaine. The NAc was chosen because the reinforcing effects of acute psychostimulant administration are mediated by limbic circuitry including dopaminergic projections from the VTA to the NAc in both human and animal studies (McFarland *et al*, 2001; Robbins and Everitt, 1996). The motor cortex is the region primarily represented in microdialysis sampling collected from the frontal cortex. This area was chosen because it receives dense dopaminergic innervation from ventral tegmental area neurons (Chen *et al*, 2011; Lindvall and Bjorklund, 1978a) and is a reliable target for catecholamine recovery based on our previous microdialysis experiments (Soares *et al*, 1999b). The region is also highly sensitive to acute cocaine challenge as indicated by magnetic resonance imaging of regional cerebral blood volume in rats (Chen et al, 2011).

In this study, galnon had no impact on baseline DA levels in the frontal cortex but prevented cocaine-induced increases in extracellular DA. Because DA transmission in the PFC is required for cocaine-primed reinstatement (McFarland *et al*, 2001), this seems like a likely mechanism to explain the behavioral effects of galnon. One limitation of sampling from the motor cortex is that there is no evidence that DA in this region is important for operant drug-seeking behavior. Future experiment should examine other parts of the cortex, such as the orbitofrontal cortex and dorsal prefrontal cortex, where DA signaling is required for cocaine-primed reinstatement (Volkow *et al*, 2010). Our results are consistent with data from slice preparations showing that galanin reduces DA release (Melander *et al*. 1987; Nordstrom *et al*. 1987; Tsuda *et al.* 1998), although one study

found increased DA utilization in the striatum following galanin administration (Jansson et al. 1989). Unlike the frontal cortex, cocaine-induced increases in DA overflow in the NAc was slightly delayed but otherwise not affected by galnon pretreatment. One interpretation of the differences between frontal cortex and NAc is that mesolimbic dopaminergic cells are less sensitive to galanin receptor activation compared to mesocortical dopaminergic cells. This finding could also be a result of the complex interactions between mesocortical dopaminergic neurons and mesolimbic dopaminergic neurons. Cortical DA transmission opposes subcortical DA transmission (Doherty *et al*, 1996; King and Finlay, 1997a; King *et al*, 1997b; Pycock *et al*, 1980). Thus, suppression of cortical DA overflow could disinhibit subcortical DA transmission.

It is unclear exactly how galnon attenuates cocaine-induced DA release in the frontal cortex. Because inhibitory galanin receptors are highly expressed in VTA neurons (Einstein *et al*, 2013; Hawes *et al*, 2004), one possibility is that galnon alters cocaine-induced DA overflow by inhibiting the activity of VTA DA neurons that project to the frontal cortex or by acting directly on mesocortical DA terminals. To confirm that the actions of systemically administered galnon are primarily acting in selected brain sites, future experiments should test intracranial infusion of galnon or galanin on cocaine-induced dopamine overflow in the frontal cortex. Some candidate infusion sites are the VTA and the frontal cortex itself. It would also be informative to assess the behavioral effects of these infusions to determine where galanin signaling is important for attenuating cocaine-primed reinstatement.

The behavioral effects of galnon could also be mediated downstream of the DA neurons themselves, independent of DA release. The dorsal striatum and the NAc are critical for the integration of reward signals from cortical and limbic inputs, and galanin receptors are expressed in these brain regions as well (Carlezon and Thomas, 2009; Einstein *et al*, 2013; Hawes *et al*, 2004; Stuber *et al*, 2010). Galanin decreases the amplitude of EPSPs in the dorsal striatum and the NAc, and this effect was lacking in GalR1 and GalR2 KO mice. Galanin KO mice also show significantly greater sensitivity to cocaine conditioned place preference, and this effect is reversed by galnon administration (Narasimhaiah *et al*, 2009). Cocaine CPP coincided with an increase in phosphylarated ERK (pERK) in the VTA and NAc of Galanin -/- mice following cocaine injection. Galnon normalized pERK levels in the NAc, but not the VTA, following treatment with cocaine in Gal -/- mice. Thus, the results from that study imply that galanin receptor signaling in DA responsive neurons can prevent ERK activation by cocaine and may be involved in the behavioral effects of galnon.

4.7 LINKING EXERCISE-INDUCED INCREASES IN GALANIN MRNA IN THE LC TO ATTENUATION OF COCAINE-PRIMED REINSTATEMENT

As discussed, the increase in galanin mRNA in the LC following chronic exercise formed the rationale for our studies using galnon. Again, the galnon studies were designed to determine whether galanin signaling could recapitulate the effects of exercise. Because we administered galnon systemically, its effects on reinstatement could be mediated by any number of brain regions and mechanisms,

some of which are listed above. However, the exercise-induced increase in galanin mRNA was restricted to the LC. Thus, if we believe that galanin is mediating the ability of exercise to attenuate cocaine-primed reinstatement, by definition that galanin must be LC-derived.

Although the experiments in this dissertation did not directly test how exercise-induced galanin attenuates drug-seeking responses, the results collected from the exercise and the galnon experiments provide some evidence for a potential mechanism that may underlie the therapeutic benefit of exercise. The LC projects to nuclei in the mesocorticolimbic system and provides excitatory drive on dopaminergic neurons in the VTA and PFC (Jones *et al*, 1977a; Liprando *et al*, 2004; Pan *et al*, 2004; Simon *et al*, 1979). For example, stimulation of noradrenergic LC neurons elicits firing of dopaminergic neurons in the VTA (Grenhoff *et al*, 1993a). Alternatively, inhibition of LC noradrenergic transmission decreases burst firing of dopaminergic VTA neurons (Grenhoff and Svensson, 1993b). Additionally, prefrontal NE depletion or a genetic knockout of DBH (resulting in mice that lack NE) blocks amphetamine-induced dopamine release into the NAc (Schank *et al*, 2006; Ventura *et al*, 2003). Thus, inhibition of norepinephrine release can reduce cortical and midbrain dopaminergic activity. Furthermore, somatodendritic release of galanin in the LC suppresses noradrenergic transmission (Pieribone *et al*, 1995; Xu *et al*, 2001). There is also evidence that galanin interacts with norepinephrine in the frontal cortex (Holets *et al*, 1988; Skofitsch *et al*, 1986). In cortical slices, galanin has been shown to inhibit NE release implying that the peptide interacts with NE terminals (Tsuda *et al*, 1989). We hypothesize that galanin-induced inhibition of NE

release could attenuate dopaminergic activity in the VTA and in the PFC. NE transmission is critical for mediating reinstatement (Schroeder *et al*, 2010b; Zhang *et al*, 2005). Thus, this galanin-norepinephrine-dopamine molecular interaction is one pathway that could underlie the ability of exercise to reduce drug-seeking responses during reinstatement.

4.7.1 Stress-Induced Reinstatement

The selective increase of galanin mRNA in the LC also provides a potential explanation for why exercise had no effect on stress-induced reinstatement of cocaine seeking. Although chronic exercise is known to decrease stress-reactivity, 3 weeks of wheel running was not effective in reducing stress-induced reinstatement. It was previously shown that 3 weeks of wheel running could significantly blunt foot shock-induced increases in extracellular levels of NE in the frontal cortex (Soares et al, 2000). This finding is significant because NE plays an important role in mediating reinstatement (Gaval-Cruz *et al*, 2009; Schroeder *et al*, 2010b; Schroeder *et al*, 2013; Weinshenker *et al*, 2007; Zhang *et al*, 2005). However, it is possible that a longer period of wheel running could have decreased levels of lever responding after a foot shock stressor. In another study, 6 weeks but not 3 weeks of wheel running was effective in reducing learned helplessness behaviors induced by uncontrollable tail shocks compared to sedentary controls (Greenwood et al, 2005; Greenwood et al, 2003; Greenwood et al, 2013).

Furthermore, the differential effects of chronic exercise on cocaine-primed reinstatement and stress-induced reinstatement may be due to key differences in

the circuitry and neurochemistry that underlie these different models of relapse. While both stress-induced and cocaine-primed reinstatement require the activation of similar limbic and cortical brain regions that include the VTA, dorsal PFC, NAc core and ventral pallidum (McFarland and Kalivas 2001, McFarland and Kalivas 2004), stress-induced reinstatement also incorporates the central amygdala, the bed nucleus of the stria terminalis (BNsT) and the NAc shell (Leri et al, 2002; McFarland et al, 2004). In addition, foot shock-induced reinstatement can be blocked by intra-BNST administration of a β-adrenergic receptor antagonist (Leri et al 2002), but this treatment has no effect on cocaine-primed reinstatement, which is mediated instead by α1-adrenergic receptors in the PFC (Zhang *et al*, 2005). As previously reported, galanin peptide can inhibit noradrenergic release (Ma et al, 2001; Nishibori et al, 1988; Tsuda et al, 1992). The BNsT receives noradrenergic input from the A1/A2 nuclei in the brain stem, while the LC supplies NE to the PFC (Moore, 1978; Phelix et al, 1992), and we observed an increase in galanin mRNA only in the LC, not A2 following chronic exercise. Therefore, we hypothesize that exercise did not impair stress-induced reinstatement because it did not engage a mechanism that dampens neurotransmission in the ventral noradrenergic circuit.

4.8 GALANIN RECEPTORS CONTRIBUTING TO REINSTATEMENT

Published reports indicate that galanin's ability to modulate responses of drugs of abuse is primarily mediated through GalR1 activation. GalR1 activation with a receptor subtype specific agonist, M617, decreases operant reward responding under a high contingency schedule (Anderson et al, 2013). When GalR1

is knocked out in mice, opiate withdrawal symptoms are exacerbated compared to the wild type, and the administration of galnon attenuates this severity (Holmes et al, 2012). Expression of galanin and GalR1 are also associated with nicotine dependence and the somatic signs of withdrawal, with decreased galanin signaling linked to more severe somatic withdrawal symptoms. Galnon treatment reduced dependence and withdrawal symptoms, suggesting a protective role for GalR1 (Jackson et al, 2011). Galanin has been shown to reduce midbrain dopaminergic activity by antagonizing expression of the DA biosynthetic enzyme, tyrosine hydroxylase. In primary cultures of rat embryonic (E14) ventral mesencephalon, dibutyryl cAMP (dbcAMP) treatment increased tyrosine hydroxylase-immunoreactive neurons by about 100%. Galanin treatment of dbcAMP-treated ventral mesencephalon neurons reduced this increase by 50%. This result suggests that galanin's ability to block dbcAMP-mediated induction of tyrosine hydroxylase in cultured VM neurons may be activity dependent. Moreover, the galanin-induced reduction of tyrosine hydroxylase-immunoreactive neurons can be antagonized by M40 treatment, indicating that this effect is mediated via galanin receptors. Additionally, dbcAMP treatment of primary cultures of rat embryonic (E14) ventral mesencephalon was associated with a ~200% increase of GalR1 mRNA but no change in GalR2 and GalR3 mRNA expression. Thus, galanin's inhibition of midbrain dopaminergic activity may involve GalR1 mediated reduction of tyrosine hydroxylase (Counts *et al*, 2002). The dorsal striatum and the nucleus accumbens are critical for the integration of reward signals from cortical and limbic inputs (Carlezon *et al*, 2009; Stuber *et al*, 2010). Therefore, GALR1- mediated reduction DA

transmission (Counts et al, 2002), would be expected to alter responses to cocaine and other drugs of abuse.

The VTA also receives projections from the LC, a brain region in which galanin receptor activation also similarly attenuates neurotransmitter release. The LC expresses some of the highest concentrations of GalR1 (Burgevin *et al*, 1995; Gustafson *et al*, 1996; Mitsukawa *et al*, 2008; Parker *et al*, 1995). The somotadendritic release of galanin in the LC acts on GalR1 receptors in a paracrine manner. GalR1 activation hyperpolarizes LC neurons and decreases NE release (Pieribone *et al*, 1995; Xu *et al*, 2001). Decreasing the activity of LC neurons also reduces the excitatory drive provided from LC afferents to VTA DA neurons.

Combined, these studies suggest that the ability of galanin signaling to attenuate cocaine-primed reinstatement may be mediated by GalR1 activation. However, it is also possible that GalR2 signaling may be involved. Galanin decreases the amplitude of EPSPs in the dorsal striatum and the NAc, and this effect was lacking in GalR1 and GalR2 KO mice. Additionally, GalR1 and GalR2 -/- mice were less sensitive to morphine CPP (Einstein *et al*, 2013). To test the contributions of different galanin receptor subtypes on relapse like behavior, I propose that future reinstatement experiments should determine the effect of selective galanin receptor activation on reinstatement using the GalR1-specific agonist, M617 (Mazarati *et al*, 2006), and the GalR2-specific agonist, M1145 (Runesson *et al*, 2009). Concurrent activation of both GalR1 and GalR2 may be necessary for attenuating cocaine-primed reinstatement, which could be tested using a cocktail of M617 and M1145.

4.9 BDNF AND NEUROGENESIS AS POSSIBLE MECHANISMS THAT MAY ACCOUNT FOR ATTENUATION OF REINSTATEMENT BY EXERCISE

Exercise also induces a number of other trophic factors and cellular changes that could alter responses to drugs of abuse. It is well established that exercise induces expression of BDNF mRNA in several brain regions most notably in the hippocampus and caudal cortex (Groves-Chapman *et al*, 2011; Neeper *et al*, 1995; Neeper *et al*, 1996; Stranahan *et al*, 2009; Vaynman *et al*, 2003). Interestingly, BDNF can also reduce responses to drugs of abuse. BDNF infused into the medial PFC suppresses cocaine-seeking behavior elicited by a response-contingent conditioned stimulus presentation or an i.p. injection of cocaine (10 g/kg) after extinction training (Berglind *et al*, 2007; Berglind *et al*, 2009). It is likely that the therapeutic effect of BDNF is mediated by its ability to inhibit cocaine-induced increases in extracellular levels of glutamate in the NAc (Berglind *et al*, 2009).

Additionally, exercise induces neurogenesis in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Fabel *et al*, 2003; van Praag *et al*, 1999a; van Praag *et al*, 1999b; Yau *et al*, 2014). Several studies have demonstrated an interaction between hippocampal neurogenesis and exposure to psychostimulants or opiates. Inhibition of neurogenesis in the dentate gyrus during extinction training potentiates drug-seeking behavior to a cocaine prime (Deschaux *et al*, 2014). Likewise, suppression of adult hippocampal neurogenesis increases cocaine self-administration (Noonan *et al*, 2010). Chronic voluntary exercise can restores alcohol-induced cellular degeneration in the hippocampus by enhancing survival of cells during abstinence (Maynard and Leasure, 2013). These findings imply that

exercise-induced neurogenesis can attenuate cellular damaged caused by excessive alcohol intake. However a recent study found that exercise did not alter cell proliferation in the SGZ of rats trained to self-administer methamphetamine on an extended-access schedule (Sobieraj *et al*, 2014). Thus, there are several other molecular pathways that are induced by chronic voluntary exercise that may also underlie its ability to reduce reinstatement.

4.10 POTENTIAL SIDE EFFECTS OF PHARMACOTHERAPIES THAT TARGET GALANIN

My data indicates that galanin transmission can prevent some forms of reinstatement and suggests that galanergic drugs might be effective pharmacotherapies for the treatment of cocaine dependence. The wide distribution of galanin and its receptors is also reflected in the variety of brain functions that galanin influences. Thus, treatment with a pharmacotherapy that targets the galanin system might risk effecting appetite, alcohol consumption, nicotine dependence, metabolism, seizure susceptibility and nociception.

In contrast to its ability to reduce pyschostimulant- and opiate-induced behaviors, galanin actually promotes the rewarding and reinforcing properties of alcohol. Mice overexpressing the galanin gene consume significantly more ethanol than wild-type mice (Karatayev *et al*, 2009), and galanin KO mice show decreased ethanol intake and preference compared to controls (Karatayev *et al*, 2010). Interestingly, galanin's ability to promote consumption of ethanol is likely mediated via the GalR3, not the more brain-abundant GalR1 or GalR2 (Ash *et al*, 2014; Ash *et*

al, 2011). Certain GalR3 haplotypes are associated with an increased risk for alcoholism (Belfer *et al*, 2007). The combination of the GalR3 single nucleotide polymorphism (SNP) (rs3091367) risk allele and the galanin gene risk haplotypes led to a significant association with alcoholism as compared with the effect of the galanin gene or GalR3 gene alone (Belfer *et al*, 2007). Galanin agonist treatment could have the adverse effect of increasing alcohol consumption, which is problematic given the high incidence of comorbidity between cocaine and alcohol dependence. Thus, treatment for illicit drug abuse may be safest to use in individuals who do not have coinciding alcohol dependence.

In preclinical studies, galanin's interaction with nicotine consumption is more similar to that of alcohol than psychostimulants and opiates. In one study, GAL -/- mice showed decreased sensitivity to nicotine, indicating that galanin promotes the reinforcing properties of nicotine (Neugebauer *et al*, 2011). In a nicotine conditioned place preference test, wild-type mice established CPP on the post-conditioning test day at a dose of .18 mg/kg. However, galanin KO mice did not establish CPP at the 0.18 mg/kg dose but did establish CPP at the 0.36 mg/kg dose. Thus, galanin KO mice were less sensitive to the reinforcing effects of nicotine (Neugebauer *et al*, 2011). However, because there are no studies assessing the role of galanin on nicotine self-administration or reinstatement, it is not clear if galanin promotes nicotine consumption. In clinical trials, the effect of galaninergic system on smoking cessation and craving is more mixed. Several studies have found associations between SNPs in galanin-related genes and nicotine consumption. In a retrospective study, the GALR1 variants rs2717162 and rs2717164 were associated

with heavy smoking (Jackson *et al*, 2011). In a prospective study, individuals with the rs2717162 gene variant were found to be less likely to quit smoking while on buproprion treatment and exhibited a faster rate of relapse with the presence of at least 1 minor C allele (Gold *et al*, 2012). The study also assessed nicotine craving with rs2717162, but did not find an association with this gene variant (Gold *et al*, 2012). By contrast, others found that rs2717162 was associated with self-reported craving at the T allele for the rs2717162 GALR1 SNP, but not the C allele (Lori *et al*, 2011). Differences in the results from these clinical trials likely reflect the different methodology used to assess the association between nicotine craving and consumption and galanin gene interactions. Moreover, the functional consequences of these variants on receptor expression, distribution, and signaling are unknown. While it is possible that a galanin-based treatment for cocaine dependence might increase the risk for heavy smoking in individuals exhibiting particular gene variants, more research is needed.

Galanin also has broad regulatory effects on appetite, obesity, insulin resistance and metabolism (Fang *et al*, 2012). Galanin injected directly in the central amygdala (CeA) or the paraventricular nucleus of the hypothalamus increases feeding behavior in rats (Corwin *et al*, 1993; Kyrkouli *et al*, 2006). Of note, there is a preferential increase in the consumption of a high-fat diet (Tempel *et al*, 1988). Centrally infused galanin-induced increases in food consumption are likely regulated via GalR1 (Fang *et al*, 2012a), and antagonizing galanin receptors with M40 blocks the orexigenic effects of galanin in rats (Corwin *et al*, 1993). A review of the literature on galanin reveals that it is closely related to metabolic syndrome

(Fang *et al*, 2012b). In contrast, we found that galnon had no effect on food self-administration and significantly *decreased* food-primed reinstatement. One reason for this result is that reinstatement to a reinforcing stimuli is dependent on cellular activity in the mesolimbic circuit. Dopaminergic projections from the VTA to the NAc support reinforcement-related behaviors for natural rewards such as food and water (Hernandez and Hoebel, 1988; Kiyatkin and Gratton, 1994; McCullough and Salamone, 1992). Shared neural substrate of food and cocaine-primed reinstatement could explain why galnon had similar effects for both rewards. Our reinstatement results are supported by a previous study showing that activation of peripheral galanin receptors does not increase daily food intake. Stimulation of peripheral galanin receptors was examined in a study that used transgenic galanin mice that exhibit elevated levels of circulating serum galanin (more than 10-fold higher than wildtype mice). Activation of peripheral galanin did not affect daily food intake in galanin transgenic mice (Poritsanos *et al*, 2009). In fact, in one study,, i.c.v. or i.p. administration of galnon reduces food intake in rats and mice (Abramov *et al*, 2004). It is possible that galnon metabolites may have differential effects on galanin receptors. These results indicate that galanin and galnon may have differential effects on food intake. Thus, further study is required to determine what possible side effects a galanin agonist treatment for cocaine dependence might have on food intake and body weight.

Galanin receptor activation reduces the susceptibility to seizures (Clynen *et al*, 2014; Kovac and Walker, 2013), and GalR1 and GalR2 likely mediate the anticonvulsant effect of galanin. GalR1 -/- mice are more seizure susceptible than

wildtype (Mazarati *et al*, 2004b). Similarly, knockdown of GalR2 increases severity and susceptibility to seizures (Mazarati *et al*, 2004a). Systemic administration galnon also has anticonvulsant effects on pentylenetetrazole-induced seizures and suppresses status epilepticus (Saar *et al*, 2002). Cocaine intoxication is also associated with an increased risk of seizures (Koppel *et al*, 1996; Macedo *et al*, 2010; Zimmerman, 2012). Thus, a pharmacotherapy that targets the galanin system may attenuate this risk.

Targeting the galanin system could provide a novel pharmacotherapy for the treatment of psychostimulant abuse. Galanin's modulatory effect on monoamines altered by chronic drug use reduces the rewarding and reinforcing properties of psychostimulants. However, because of it's broad regulatory effects on monoamines careful attention should be paid to possible side effects or risks of a galanin pharmacotherapy. Formulating a receptor a GalR1 specific agonist treatment could reduce the incidence of target effects. Additionally, this medication would have to be formulated as a synthetic galanin receptor agonist that can cross the blood brain barrier. This pharmacotherapy could be administered intravenously or orally. Although ease of administration could influence adherence to treatment, both oral and intravenous delivery of a medication are well tolerated in people (McCune *et al*, 2001; Reginster, 2005).

4.11 CONCLUSION

The outlined experiments provide a broader understanding of the pathophysiology of cocaine dependence and highlights novel ways to suppress relapse-like behavior. The exercise study established that an interventional exercise therapy was effective in suppressing cocaine-primed reinstatement. This study also demonstrated an association with chronic voluntary exercise in cocaine-exposed rats and expression of galanin mRNA in the LC, a peptide that opposes the reinforcing properties of psychostimulants and opiates. Galanin's antagonism against reinforcing effects of psychostimulants and opiates makes it an intriguing target for the treatment of psychostimulant abuse. Thus, we investigated if galanin receptor activation would have a similar effect on cocaine-primed reinstatement. In the galnon study, we demonstrated that galanin receptor activation could also reduce relapse like behavior in rats trained to self-administer cocaine.

Linking the results of these two studies provide a potential mechanism for exercise-induced suppression of cocaine-primed reinstatement via galanin (Fig 4.11). The somatosensory stimulation provided by chronic exercise activates the vagal nerve. This vagal nerve stimulation is transduced to the nucleus of the solitary tract, which projects to many brain regions including the LC, inducing burst firing of the noradrenergic neurons. Burst neural firing of noradrenergic cells induces galanin mRNA expression. Much like immediate early genes such as cFos, galanin is likely to be an activity-dependent peptide; neuronal burst firing results in an increase in galanin mRNA. The galanin peptide, which is translated from galanin mRNA, is released from the soma or dendrites and acts extra-synaptically on LC

neurons via GalR1, which is abundantly expressed in the LC. GalR1 activation hyperpolarizes and reduces the spike discharge of burst firing LC neurons (Pieribone *et al*, 1995). This galanin-induced inhibition is mediated via an increase in potassium conductance (Pieribone *et al*, 1995). Because the LC provides excitatory drive to dopaminergic VTA and PFC neurons, noradrenergic inhibition also results in decreased dopaminergic activity. Additionally, galanin could directly reduce dopaminergic activity. Galanin inhibits stimulation-induced DA release in rat striatal slices, indicating a direct role for galanin's activity dependent effects on the mesolocorticolimbic system (Tsuda *et al*, 1998).

Reduced dopaminergic transmission in the VTA or the PFC abolishes reinstatement. Our experiments highlight one molecular mechanism that likely contributes to the suppressive actions of exercise on drug-seeking behavior. The results also provide a better understanding of how to integrate exercise as a behavioral therapy for addiction. Furthermore, focus on the galanin system draws attention to a novel target for the development of a pharmacotherapy that in conjunction with behavioral treatments (exercise, CBT, etc) could more effectively alleviate cocaine addiction.

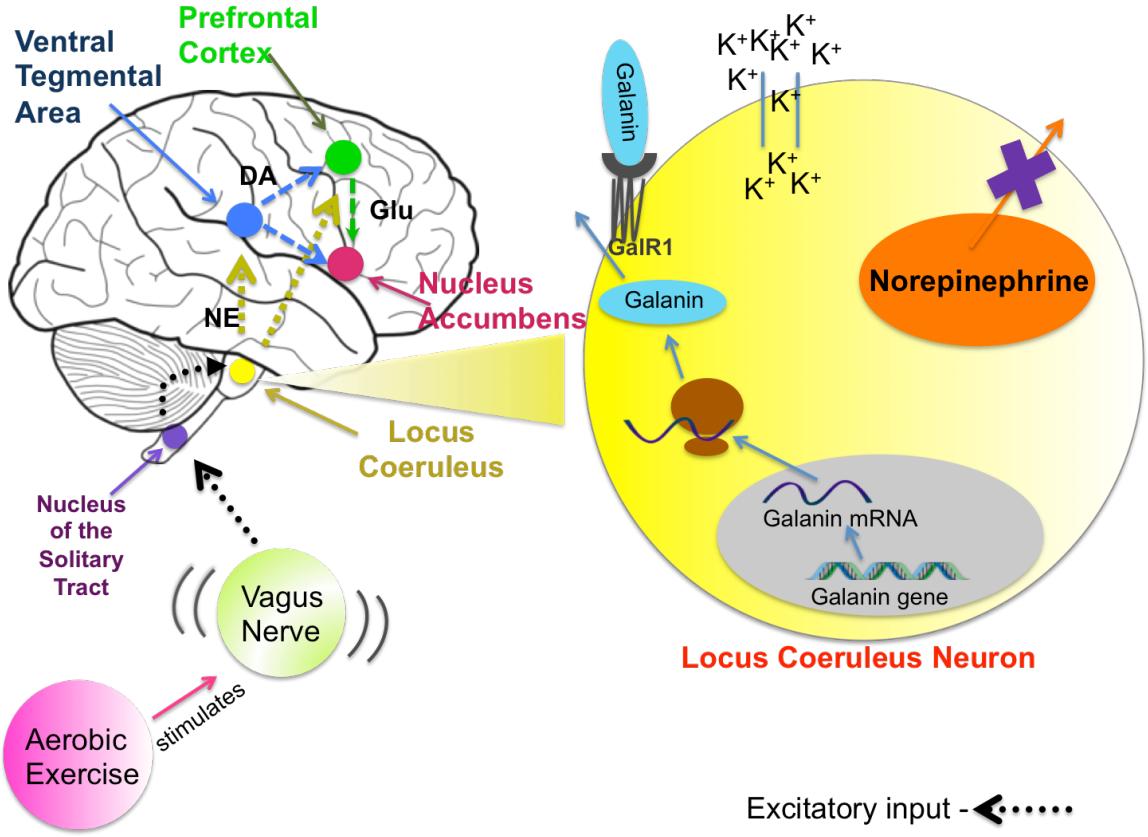

Figure 4.1 Proposed exercise-induced reduction of norepinephrine transmission via galanin. The somatosensory stimulation provided by chronic exercise activates the vagal nerve. This vagal nerve stimulation is transduced to the nucleus of the solitary tract, which sends excitatory afferents to the LC, inducing burst firing of the noradrenergic neurons. Burst neural firing of noradrenergic cells induces galanin mRNA expression. The galanin peptide, which is translated from galanin mRNA, can inhibit norepinephrine release. In the LC, galanin from soma or dendrites released from noradrenergic neurons in response to burst firing where it binds to the galanin receptor 1 (GalR1) in a paracrine manner. Activation of GalR1 hyperpolarizes the LC neuron via an increase in potassium conductance which in

turn reduces the spike discharge. Galanin receptor activation ultimately leads to a reduction in norepinephrine release. Without the noradrenergic drive provided by the LC, dopaminergic activity in the VTA and PFC is decreased. Reduced dopaminergic transmission in the VTA or the PFC ultimately results in the blockade of cocaine-primed reinstatement.

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APPENDIX

DISULFIRAM ATTENUATES DRUG-PRIMED REINSTATEMENT OF COCAINE SEEKING VIA INHIBITION OF DOPAMINE B-HYDROXYLASE.

Adapted from:
Schroeder JP, Cooper DA, Schank JR, Lyle MA, Gaval-Cruz M, Ogbonmwan YE, Pozdeyev N, Freeman KG, Iuvone PM, Edwards GL, Holmes PV, Weinshenker D. (2010). Neuropsychopharmacology. 2010 Nov;35(12):2440-9. doi: 10.1038/npp.2010.127.

A.1 ABSTRACT

The anti-alcoholism medication disulfiram (Antabuse) inhibits aldehyde dehydrogenase (ALDH), which results in the accumulation of acetaldehyde upon ethanol ingestion and produces the aversive "Antabuse reaction" that deters alcohol consumption. Disulfiram has also been shown to deter cocaine use, even in the absence of an interaction with alcohol, indicating the existence of an ALDH-independent therapeutic mechanism. We hypothesized that disulfiram's inhibition of dopamine β-hydroxylase (DBH), the catecholamine biosynthetic enzyme that converts dopamine (DA) to norepinephrine (NE) in noradrenergic neurons, underlies the drug's ability to treat cocaine dependence. We tested the effects of disulfiram on cocaine and food self-administration behavior and drug-primed reinstatement of cocaine seeking in rats. We then compared the effects of disulfiram with those of the selective DBH inhibitor, nepicastat. Disulfiram, at a dose (100 mg/kg, i.p.) that reduced brain NE by ~40%, did not alter responding for food or cocaine on a fixed ratio 1 (FR1) schedule, whereas it completely blocked cocaine-primed (10 mg/kg, i.p.) reinstatement of drug seeking following extinction. A lower dose of disulfiram (10 mg/kg) that did not reduce NE had no effect on cocaine-primed reinstatement. Nepicastat recapitulated the behavioral effects of disulfiram (100 mg/kg) at a dose (50 mg/kg, i.p.) that produced a similar reduction in brain NE. Food-primed reinstatement of food seeking was not impaired by DBH inhibition. Our results suggest that disulfiram's efficacy in the treatment of cocaine addiction is associated with the inhibition of DBH and interference with the ability of environmental stimuli to trigger relapse.

A.2 INTRODUCTION

Disulfiram (Antabuse) has been used for more than 50 years in the treatment of alcoholism (Fuller et al., 1986). Disulfiram inhibits ALDH, which results in the accumulation of acetaldehyde upon ethanol ingestion. This toxic metabolite produces aversive symptoms, such as flushing, nausea, and vomiting, and a desire to avoid this reaction encourages abstinence. Because 50-90% of patients who abuse cocaine also abuse alcohol (Weiss et al., 1988; Grant and Harford, 1990; Closser and Kosten, 1992; Khalsa et al., 1992), the belief was that discouraging alcohol consumption in cocaine- and alcohol-dependent individuals might lower cocaine use. Indeed, disulfiram was found to reduce alcohol and cocaine intake in this patient population (Carroll et al., 1993; 1998; 2000). Surprisingly, further studies went on to reveal that disulfiram is at least as effective at treating cocaine addicts who do not consume alcohol, and may even be more effective (George et al., 2000; Petrakis et al., 2000; Carroll et al., 2004). Therefore, an aldehyde dehydrogenase-independent mechanism must be responsible for the ability of disulfiram to promote cocaine abstinence (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009).

Cocaine increases extracellular levels of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) in the brain by blocking plasma membrane monoamine transporters. Thus, pathways critical for the production or transmission of these neurotransmitters are a reasonable place to look for targets underlying the efficacy of disulfiram in the treatment of cocaine dependence. Because the primary metabolite of disulfiram, diethyldithiocarbamate (DDC), is a copper chelator (Hald

and Jacobsen, 1948; Johnston, 1953), disulfiram impairs the function of many copper-containing enzymes, including ALDH, carboxylesterase, cholinesterase, and dopamine β-hydroxylase (DBH). Of particular interest, the inhibition of DBH by disulfiram reduces production of NE, with a concomitant increase in tissue levels of DA in rodents (Goldstein, 1966; Musacchio et al., 1966; Bourdelat-Parks et al., 2005). Disulfiram also decreases NE and its metabolites in the urine, blood, and CSF of humans (Takahashi and Gjessing, 1972; Major et al., 1979; Rogers et al., 1979; Hoeldtke and Stetson, 1980; Rosen and Lobo, 1987; Paradisi et al., 1991). We have shown that disulfiram has no effect on catecholamine levels in DBH knockout (*Dbh* -/-) mice, which lack NE, indicating that disulfiram's effects on NE and DA are mediated solely by DBH inhibition (Bourdelat-Parks et al., 2005). Disulfiram also inhibits cocaine-metabolizing enzymes and increases peak plasma cocaine levels under some conditions in humans (McCance-Katz et al., 1998a, 1998b; Baker et al., 2007) but not rodents (Gaval-Cruz et al., 2008).

The efficacy of disulfiram in treating cocaine dependence has been attributed to several different mechanisms, including a decrease in cocaine reward, an increase in cocaine aversion, and as a "DA replacement therapy" that elevates DA levels and restores normal reward function in hypodopaminergic addicts (Weinshenker and Schroeder, 2007; Sofuoglu et al., 2008; Gaval-Cruz and Weinshenker, 2009); however, the data have been ambiguous. Different human laboratory studies report that genetic or pharmacological DBH inhibition increases cocaine-induced paranoia and decreases, increases, or has no effect on psychostimulant-induced euphoria (Hameedi et al., 1995; McCance-Katz et al., 1998a, 1998b; Cubells et al., 2000;

Petrakis et al., 2000; Baker et al., 2007; Kalayasiri et al., 2007; Sofuoglu et al., 2008). In rodents, disulfiram decreases the locomotor-activating effects of acute cocaine administration, but facilitates cocaine sensitization (Maj et al., 1968; Haile et al., 2003).

The available human and animal data give us a hazy picture of how disulfiram discourages cocaine use. The influence of disulfiram on the reinforcing properties of cocaine have yet to be investigated in an animal model, and while DBH inhibition has been suggested to underlie disulfiram's efficacy, this hypothesis has not been tested directly. In an effort to resolve these issues, we assessed the effects of disulfiram in operant rat paradigms of drug taking (cocaine self-administration) and relapse (cocaine-primed reinstatement) at doses that inhibit DBH in the brain. To determine whether the effects of disulfiram were mediated by inhibition of DBH, we employed the selective DBH inhibitor, nepicastat. Nepicastat is a direct, competitive inhibitor of DBH with greater potency than disulfiram (IC_{50} = 9 nM for nepicastat versus $IC_{50} \cong 1$ μM for disulfiram; Green, 1964; Goldstein, 1966; Stanley et al., 1997), as well as better selectivity (does not chelate copper, no significant interaction with a panel of other enzymes and receptors tested, including aldehyde dehydrogenase and tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis) (Stanley et al., 1997; K. Walker, Roche Biosciences, personal communication).

A.3 MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (175-200 g) were purchased from Charles River (Wilmington, MA, USA). All subjects were maintained in a temperature-controlled environment on a 12-h reverse light/dark cycle with the lights on from 7 pm to 7 am with ad libitum access to food and water. Rats were acclimated to the vivarium for 1 week prior to catheter implantation surgery. All self-administration sessions occurred during the dark cycle and were performed using standard methods with minor modifications (McFarland and Kalivas, 2001; Fuchs et al., 2004). All animals were treated in accordance with NIH policy, and experiments were approved by the Emory IACUC committee.

Drug Doses

In initial pilot experiments, we tested the effects of disulfiram (10, 25, 50, 75, 100, or 200 mg/kg, i.p.) and nepicastat (50 or 100 mg/kg, i.p.) on brain catecholamine levels and operant responding for food. Disulfiram was obtained from Sigma-Aldrich (St. Louis, MO), sonicated in sterile saline, and injected as a suspension. Nepicastat was obtained from Synosia Therapeutics (South San Francisco, CA), sonicated in sterile saline containing 1.5% DMSO and 1.5% Cremaphor EL (Sigma), and injected as a suspension. We chose the 100 mg/kg dose of disulfiram based on 3 criteria. First, 100 mg/kg was the maximum dose that significantly inhibited dopamine β-hydroxylase but did not impair the ability of rats to perform operant responses. Second, the 100 mg/kg dose has been shown by others to alter other behavioral

effects of cocaine in rats, such as locomotor activity and sensitization (e.g. Haile et al., 2003). Third, the 100 mg/kg dose inhibits aldehyde dehydrogenase in rats and is in the range typically used for alcohol studies (e.g. Deitrich and Erwin, 1971; Yourick and Faiman, 1991; Karamanakos et al., 2001). Fourth, the 100 mg/kg dose is therapeutically relevant. The typical therapeutic dose for the cocaine studies performed in humans is 250-500 mg/day (e.g. Carroll et al., 1998; McCance-Katz et al., 1998), which translates to ~ 3-7 mg/kg for a 70 kg human, or ~ 10-fold lower than we used in our study. Because of their higher metabolic rate, rodents require much larger doses of psychoactive drugs to produce behavioral and neurochemical effects compared to humans, and the 3-7 mg/kg dose has been shown to inhibit DBH in humans with a magnitude similar to the 100 mg/kg dose in rats (e.g. compare Vesell et al., 1971; Major et al., 1979; Rogers et al., 1979; Paradisi et al., 1991 human studies to our current rat study). Thus, use of the 100 mg/kg dose in rats is a close functional match to therapeutic doses in humans. We chose the 10 mg/kg dose of disulfiram for an additional experiment because it was the maximum dose in our pilot studies that did not significantly reduce brain NE levels. The 50 mg/kg dose of nepicastat was chosen to match the level of DBH inhibition observed with the 100 mg/kg dose of disulfiram.

Quantification of Catecholamine Levels

Rats were injected with disulfiram (10 or 100 mg/kg, i.p.), nepicastat (50 mg/kg, i.p.), or vehicle (saline for disulfiram, 1.5% DMSO + 1.5% Cremaphor EL in saline for nepicastat; 1 ml/kg, i.p.). Two hours later, rats were euthanized by CO_2, brains were

removed, and the prefrontal cortex was dissected on ice and frozen. The prefrontal cortex was chosen because it contains comparable amounts of NE and DA, and thus can be used to accurately assess DBH inhibition. NE and DA levels were determined using HPLC followed by coulometric detection. DA and NE concentrations were normalized to wet tissue weight for each sample.

Analytical samples from saline- and disulfiram-treated rats were prepared by adding 10 volumes of ice-cold mobile phase [0.1 mM NaHSO4, monohydrate 0.1 mM EDTA, 0.2 mM octane sulfonic acid, 6.5% acetonitrile (pH 3.1)], and sonicated until completely homogenized. Samples were centrifuged at 13.2 rpm x 1000 for 30 min at 4°C, and the supernatant removed from the tubes. The supernatant was centrifuged again at 13.2 rpm x 1000 for 30 min at 4°C using a 22-micron filter column. The resulting eluant was injected using an ESA 542 Autosampler (ESA Biosciences Inc., Chelmsford, MA) onto a Synergi Max-RP 4u (150 x 4.6mm) with Security Guard precolumn filter with Max-RP cartridges (Phenomenex, Inc., Torrance, CA) at a constant rate of 1 ml/min maintained by ESA 584 pumps. An ESA CoulArray 5600A detector with a potential set at -150 mV, 200 mV was used to visualize the peaks. The retention time and height of NE and DA peaks were compared with reference standard solutions (Sigma). Peak heights were quantified by CoulArray software (ESA Biosciences Inc.).

Analytical samples of vehicle and nepicastat-treated rats were prepared by adding 70 µL of ice-cold 0.1 N perchloric acid and 0.04% sodium metabisulfite to the tissue, and then sonicating until completely homogenized. Samples were centrifuged at 15 rpm x 1000 for 10 min at 4°C. This supernatant was injected at a constant flow

rate of 1 mL/min onto an Ultrasphere ODS 250 × 4.6 mm column, 5 μm (Beckman Coulter, Fullerton, CA, USA) with mobile phase (0.1 mM EDTA; 0.35mM sodium octyl sulfate; 0.6% phosphoric acid; 5% acetonitrile (pH 2.7). A coulometric electrochemical array detector (Agilent Technologies; guard cell set at 600 mV and analytical cell at 300 mV) was used to visualize the peaks. The retention time, height, and area of NE and DA peaks were compared with reference standard solutions (Sigma) and quantified by ChemStation chromatography software (Agilent Technologies).

Food Training

Rats were trained to lever-press for food in standard rat operant conditioning chambers (Med Associates, St. Albans, VT) prior to drug exposure to facilitate acquisition of drug self-administration, as described (Fuchs et al., 2004). Each chamber was equipped with a house light, two levers (active and inactive), and stimulus lights above both levers. Fan motors provided ventilation and masked noise for each chamber. A microcomputer with Logic '1' interface and MED-PC software (MED Associates) controlled schedule contingencies and recorded data. Animals had access to a water bottle and received 45-mg food pellets following active lever presses on a fixed ratio 1 (FR1) schedule, meaning the rat received a reinforcer following each active lever press. The food training sessions lasted for 8 h, or until the animal met criteria, defined as at least 70% selection of the active lever and at least 100 food pellets obtained. Most rats met criteria on the first day of food training, but a few rats required 2-3 days.

Surgery

Following food training, rats were anesthetized with isoflurane and implanted with indwelling jugular catheters using standard methods. Briefly, catheters were inserted into the jugular vein and anchored with suture material and tissue adhesive. The catheter was then threaded subcutaneously through the skin between the shoulder blades, and the catheter was anchored. Catheters were flushed daily with 0.05 mL gentamicin (4 mg/mL) and 0.1 mL heparin solution (30 U/mL in sterile saline). Catheter patency was verified periodically by infusing 0.08-0.12 ml of methohexital sodium (10 mg/ml, IV; Eli Lilly and Co., Indianapolis, Ind., USA), which produces a rapid loss of muscle tone only when administered intravenously.

Cocaine Self-Administration

Daily self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and rats received a non-contingent infusion of cocaine (0.5 mg/kg). During training, each press of the active lever resulted in a cocaine infusion (0.5 mg/kg in a volume of 167 μl/kg) accompanied by a discrete flashing light above the lever. Following a 20-s timeout period (during which time active lever presses did not result in drug infusion), the stimulus light was extinguished, and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, the session was terminated early if the number of cocaine infusions exceeded 40.

Once rats reached a stable level of responding (number of drug infusions varied by <20% of the mean, and preference for the active lever was at least 75% for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration), the effects of disulfiram were assessed. Rats received an injection of saline (2 ml/kg, i.p.) or disulfiram (100 mg/kg, i.p.) 2 h prior to the self-administration session. The rats were then allowed 1-2 days of self-administration sessions with no pretreatment. The following day, rats received the opposite pretreatment (saline or disulfiram) 2 h prior to the self-administration session in a counterbalanced fashion.

Extinction

Following the completion of the maintenance phase of cocaine self-administration, lever pressing was extinguished in daily 2-h sessions during which presses on the previously active lever no longer resulted in delivery of cocaine or presentation of cocaine-paired cues. Behavior was considered extinguished when active lever presses over 3 consecutive days was <25% of the average number of active lever presses during the last 3 days of maintenance.

Cocaine-Primed Reinstatement

The day after extinction criteria were met, rats were pretreated with saline (2 ml/kg, i.p.) or disulfiram (10 or 100 mg/kg, i.p.). Two hours later, they were given a noncontingent priming injection of cocaine (10 mg/kg, i.p.) and placed in the operant conditioning chambers under extinction conditions (i.e., presses on the "active" lever had no programmed consequences) for 2 h. Rats then underwent a

second round of extinction, as described above. When extinction criteria were met, rats were again tested for cocaine-primed reinstatement, but received the opposite pretreatment (saline or disulfiram) in a counterbalanced fashion (order was randomized). Some of the rats used for the reinstatement tests were the same ones that received disulfiram at the end of the maintenance phase of cocaine self-administration, while others were from a separate group that did not receive any pretreatments during maintenance. We found no differences in reinstatement, and these groups were combined. To determine whether the effects of disulfiram on reinstatement were mediated by DBH inhibition, separate groups of rats went through cocaine self-administration and extinction, then were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicastat (50 mg/kg, i.p.) prior to counterbalanced reinstatement sessions, as described for disulfiram.

Food Self-Administration

Separate groups of rats were used for the food self-administration and reinstatement experiments. Rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening at least 1 h after self-administration sessions had ended. Parameters of food self-administration were identical to the cocaine self-administration experiments, except that rats received a food pellet instead of a cocaine infusion for each active lever press, and sessions lasted 1 h and were terminated if the reinforcers obtained exceeded 60.

Food-Primed Reinstatement

Food-primed reinstatement of food seeking was performed using a modified version of published protocols (e.g. Sun and Rebec, 2005; Peters and Kalivas, 2006). Once maintenance criteria for operant food self-administration were met (maintenance criteria and extinction criteria were identical to those used for cocaine-primed reinstatement), rats were pretreated with vehicle (1.5% DMSO, 1.5% Cremaphor EL in saline, 1 ml/kg, i.p.) or nepicistat (50 mg/kg, i.p.). 2 h later, they were placed in the operant conditioning chambers and the reinstatement session was started. Three food pellets were delivered non-contingently in the first ten seconds of the session and the levers were presented to the subjects. As during extinction, responses on either of the levers had no programmed consequence. Throughout the 60 min food reinstatement session, a food pellet was delivered every 3 min non-contingently, and responses upon the formerly active and inactive levers were recorded. Rats then underwent a second round of maintenance and extinction training for operant food self-administration, as described above, then were tested for food-primed reinstatement following the opposite pretreatment (vehicle or nepicistat) in a counterbalanced fashion (order was randomized).

Data Analyses

Catecholamine level data were analyzed by Student's t-test, and self-administration data were analyzed by ANOVA followed by Bonferroni post hoc tests using Prism 4.0 for Macintosh.

A.4 RESULTS

Disulfiram Inhibits DBH and Decreases Brain NE Levels

DBH is the enzyme in the catecholamine biosynthetic pathway that converts DA to NE in noradrenergic neurons. Thus, inhibition of DBH has the unique effect of simultaneously decreasing NE production and increasing DA (Figure 1). To confirm previous reports that systemic disulfiram administration inhibits DBH in the rat brain, we measured NE, DA, and the NE/DA ratio in the frontal cortex following administration of saline or disulfiram (100 mg/kg, i.p.). We chose the frontal cortex because it contains NE and DA in similar concentrations; thereby allowing the detection of both decreases and increases in these neurotransmitters. As expected, disulfiram was a bona fide DBH inhibitor, as it decreased NE, increased DA, and decreased the NE/DA ratio (Figure 2). Inhibition of other catecholamine biosynthetic enzymes would have had different patterns, such as decreases in both NE and DA following tyrosine hydroxylase (TH) inhibition.

Disulfiram Has No Effect on Self-Administration of Food or Cocaine

To ensure that we were using a dose of disulfiram that did not impair the ability of rats to perform an operant task, we assessed responding for food pellets following saline or disulfiram (100 mg/kg, i.p.) administration. Disulfiram had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment (n = 4 per group). To determine whether disulfiram altered the reinforcing or aversive effects of cocaine, we assessed maintenance levels of responding for cocaine infusions (0.5

mg/kg/infusion) following saline or disulfiram (100 mg/kg, i.p.). Disulfiram had no effect on cocaine self-administration (Figure 3). Repeated measures ANOVA revealed no significant effects for active lever presses ($F_{23,2} = 0.77$, $p = 0.48$) or reinforcers obtained ($F_{23,2} = 0.97$, $p = 0.4$). Inactive lever presses were negligible (0-2 presses per animal) and did not differ between groups.

Disulfiram Blocks Cocaine-Primed Reinstatement of Cocaine Seeking

We next tested the effects of disulfiram on drug-primed reinstatement of cocaine seeking. Following the attainment of stable self-administration and extinction, rats were treated with saline or disulfiram (100 mg/kg, i.p.) prior to a noncontingent priming injection of cocaine (10 mg/kg, i.p.). Rats that were pretreated with saline showed a robust reinstatement of responding on the previously active lever following cocaine prime. In contrast, disulfiram pretreatment completely blocked cocaine-primed reinstatement (Figure 4). ANOVA revealed a significant effect of treatment phase ($F_{4,51} = 8.17$, $p < 0.0001$), and Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 3.62$, $p < 0.05$), but not between extinction responding and disulfiram pretreatment ($t = 0.22$, $p > 0.05$). In addition, there was a significant difference between reinstatement responding with saline pretreatment and disulfiram pretreatment ($t = 2.81$, $p < 0.05$). There was no effect of pretreatment on inactive lever responding.

We next tested the ability of a lower dose of disulfiram (10 mg/kg, i.p.) to attenuate cocaine-primed reinstatement. This dose of disulfiram, which we found in

pilot studies to be the highest one that does not significantly reduce NE levels in the PFC (vehicle = 0.32 ± 0.04 ng/mg tissue, disulfiram = 0.29 ± 0.08, $p > 0.05$, n = 4 per group), did not impair cocaine-primed reinstatement (Figure 4). Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following low dose disulfiram pretreatment ($t = 2.69$, $p < 0.05$, but not between saline and low dose disulfiram pretreatment ($t = 0.18$, $p > 0.05$).

Nepicastat Blocks Cocaine-Primed Reinstatement of Cocaine Seeking

The previous experiments indicated that a dose high enough to inhibit DBH is required for the efficacy of disulfiram in blocking cocaine-primed reinstatement. However, because DBH has many other targets, it was unclear whether DBH inhibition alone was sufficient to block reinstatement. Thus, we repeated the self-administration experiments with the selective DBH inhibitor, nepicastat, at a dose (50 mg/kg, i.p.) that inhibited DBH to a similar extent as the effective dose of disulfiram (100 mg/kg, i.p.) (Figure 5), and found that nepicastat pretreatment mimicked the effects of disulfiram in several ways. First, nepicastat had no affect on the maintenance phase of cocaine self-administration (Figure 6). Repeated measures ANOVA revealed a non-significant trend for active lever presses ($F_{26,2} = 3.36$, $p = 0.06$) and no effect on reinforcers obtained ($F_{26,2} = 0.38$, $p = 0.69$). Inactive lever presses were negligible and did not differ between groups. Second, nepicastat blocked cocaine-primed reinstatement (Figure 7). Repeated measures ANOVA revealed a significant effect of treatment phase ($F_{3,23} = 18.14$, $p < 0.0001$), and

Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following saline pretreatment ($t = 5.17$, $p < 0.001$) and between vehicle pretreatment and nepicastat pretreatment ($t = 4.67$, $p < 0.01$), but not between extinction responding and cocaine-primed reinstatement following nepicastat pretreatment ($t = 0.5$, $p > 0.05$). Pretreatment had no effect on inactive lever responding. Third, nepicastat (50 mg/kg, i.p.) had no effect on food responding; all rats obtained the maximum number of reinforcers possible during the session (61), regardless of pretreatment (n = 8 per group).

Because the neural and molecular pathways underlying reinstatement of cocaine and food seeking are partially overlapping (Nair et al., 2009), we tested whether the attenuation of reinstatement by DBH inhibition was specific to cocaine, and found that nepicastat did not significantly reduce food-primed reinstatement of food seeking (Figure 8). Repeated measures ANOVA revealed a significant effect of treatment phase ($F_{3,27} = 29.49$, $p < 0.0001$), and Bonferroni post hoc analysis showed a significant difference between extinction responding and cocaine-primed reinstatement following vehicle or nepicastat pretreatment (vehicle $t = 4.27$, $p < 0.05$; nepicastat $t = 2.57$, $p < 0.05$), but not between cocaine-primed reinstatement following vehicle and nepicastat pretreatment ($t = 1.70$, $p > 0.05$). These results indicate that the blockade of cocaine-primed reinstatement by nepicastat cannot be attributed to an inability to perform the operant task and that DBH inhibition does not impair reinstatement of responding for a natural reward.

A.5 DISCUSSION

Disulfiram has shown promise as a treatment for cocaine dependence in several clinical trials (Carroll et al., 1993; 1998; 2000; 2004; Petrakis et al., 2000; George et al., 2000; Grassi et al., 2007; Pettinati et al., 2008). Because concurrent alcohol use is not necessary for disulfiram to have beneficial effects on cocaine addiction, an ALDH-independent mechanism is likely. Furthermore, whatever the underlying molecular mechanism, why disulfiram treatment reduces cocaine use remains unclear; several human laboratory studies have produced conflicting results over how DBH inhibition influences the reinforcing and aversive effects of cocaine. The purpose of our study was therefore two-fold. First, to gain insight into which aspects of addiction were being altered in the clinic, we determined which "phase" of cocaine self-administration (i.e., maintenance vs. reinstatement) was affected by disulfiram in rats. Second, to test the hypothesis that disulfiram was acting via DBH inhibition, we used a lower dose of disulfiram that does not inhibit DBH and the selective DBH inhibitor, nepicastat.

Treatments that alter the reinforcing effects of cocaine, such as dopaminergic manipulations, typically change cocaine self-administration behavior (Koob et al., 1994). Given the history of NE manipulations and cocaine self-administration, it is not surprising that disulfiram had no effect on maintenance responding for cocaine. NE transporter (NET) inhibitors themselves do not support self-administration, and neither NET inhibitors nor adrenergic receptor antagonists alter cocaine self-administration (Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker,

2009 Yokel and Wise, 1976; Roberts et al., 1977; Woolverton, 1987; Wee et al., 2006; Howell and Byrd, 1991; Skjoldager et al., 1993; Tella, 1995).

Drug addiction is a chronic relapsing disorder (Hunt et al., 1971; Leshner, 1997), as patients in treatment often slip back into drug taking after periods of sobriety. Several types of stimuli can trigger drug craving and lead to relapse, including re-exposure to the drug, stress, and drug-associated cues; these stimuli also trigger reinstatement in the rat model. The reliability, species generality, as well as face and construct validity of the reinstatement model are high, because it recapitulates many of the features of human addiction (Panlilio and Goldberg, 2007). In contrast to the lack of data to support an influence on the maintenance phase of psychostimulant self-administration, the role of NE in the reinstatement of drug seeking is clear (Erb et al., 2000; Weinshenker and Schroeder, 2007; Gaval-Cruz and Weinshenker, 2009). Central infusion of NE itself, or the facilitation of NE transmission with reuptake inhibitors or inhibitory autoreceptor antagonists, induces reinstatement in rats and non-human primates (Lee et al., 2004; Platt et al., 2007; Brown et al., 2009). Conversely, blockade of β-adrenergic receptors prevents stress-induced reinstatement, whereas blockade of α1-adrenergic receptors prevents drug-primed reinstatement (Leri et al., 2002; Zhang and Kosten, 2005). Because we examined cocaine-primed reinstatement, it is likely that reinstatement was blunted following disulfiram or nepicastat pretreatment due to reduced NE production and a failure to engage α1-adrenergic receptors. The ability of DBH inhibition to block cocaine-primed reinstatement provides further support for the critical role of NE in this paradigm, and we propose that the clinical efficacy of

disulfiram, via DBH inhibition and reduction of NE, reduces the risk for relapse. Most disulfiram clinical trials to date have not been designed to examine cocaine relapse specifically. It will be important to build measures into future trials that can distinguish between abstinence due to altered subjective drug effects vs. healthier responses to environmental triggers.

The evidence available suggests that blockade of cocaine-primed reinstatement by disulfiram involves the impairment of neurotransmission in the nucleus accumbens (NAc). DA and glutamate release in the NAc are both essential for cocaine-primed reinstatement (Schmidt et al., 2005; Kalivas et al., 2009). Noradrenergic neurons project to the mesocorticolimbic DA system, and NE promotes DA transmission, primarily via activation of α1-adrenergic receptors. For example, depletion of NE, or attenuation of α1-adrenergic receptor signaling via genetic, pharmacological, or neurotoxic means impairs psychostimulant-induced DA release in the NAc (Darracq et al., 1998; Drouin et al, 2002; Ventura et al., 2003). It is important to note that while DBH inhibition increases tissue levels of DA, it decreases DA release because NE-mediated excitation of DA neurons is reduced (Schank et al., 2006; Weinshenker and Schroeder, 2007; Weinshenker et al., 2008). Thus the failure of a cocaine prime to provoke DA release in the NAc may underlie the efficacy of disulfiram in this paradigm. While proof of a direct role for NE in regulating cocaine-induced glutamate release in the NAc is lacking, we have recently found that α1-adrenergic receptors are enriched in presumptive glutamatergic terminals throughout the mesocorticolimbic system (Rommelfanger et al., 2009),

and we predict that a loss of noradrenergic tone may also attenuate the glutamate release essential for cocaine-primed reinstatement.

Although the blockade of cocaine-primed reinstatement by disulfiram could involve several targets, our results strongly suggest that it is mediated primarily by DBH inhibition, NE reduction, and a decrease in α1AR signaling, as the effects of disulfiram require a dose that significantly inhibits DBH and are mimicked by the selective DBH inhibitor, nepicastat (present study), and the α1AR antagonist, prazosin (Zhang and Kosten, 2005). What remains unclear is why a reduction of NE/α1AR signaling hampers drug-primed reinstatement, but not the maintenance phase of cocaine self-administration. Earlier findings revealed that blockade of α1ARs does not affect "conventional" operant responding for cocaine, but does attenuate the escalation of cocaine self-administration elicited by long-access "binge" paradigms or prior drug sensitization (Zhang and Kosten, 2007; Wee et al., 2008). Combined, these results suggest that while NE does not play a critical role in the primary reinforcing effects of cocaine, as measured by standard operant self-administration, it does have significant effects under conditions that escalate or reinstate drug-seeking behavior. Furthermore, medications that impair NE production, such as disulfiram or nepicastat, may short circuit the ability of environmental triggers to promote relapse, and therefore make promising pharmacotherapies for the treatment of dependence on cocaine and other stimulants.

A.6 DISCLOSURE/CONFLICT OF INTEREST

The authors declare that JPS, DAC, JRS, MAL, MG, YEO, NP, KGF, PMI, GLE, and PVH, except for income received from their primary employers, have received no financial support or compensation from any individual or company entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a conflict of interest. The authors declare that DW, over the past 3 years, has received research funds from Cephalon Pharmaceuticals, the developer of therapeutics for central nervous system disorders, and is co-inventor on a patent concerning the use of selective DBH inhibitors for the treatment of cocaine dependence (US-2010-0105748-A1; "Methods and Compositions for Treatment of Drug Addiction").

A.7 ACKNOWLEDGEMENTS

We thank Synosia Therapeutics, Inc. for providing the nepicastat, K. Frantz and lab members for advice and technical assistance, and C. Strauss for helpful editing of the manuscript. This work was supported by the National Institute of Drug Abuse (DA017963 and DA027535 to DW, DA019746 to JRS, DA25040 and DA015040 to MGC) and the National Eye Institute (EY004864 and P30 EY06360 to PMI).

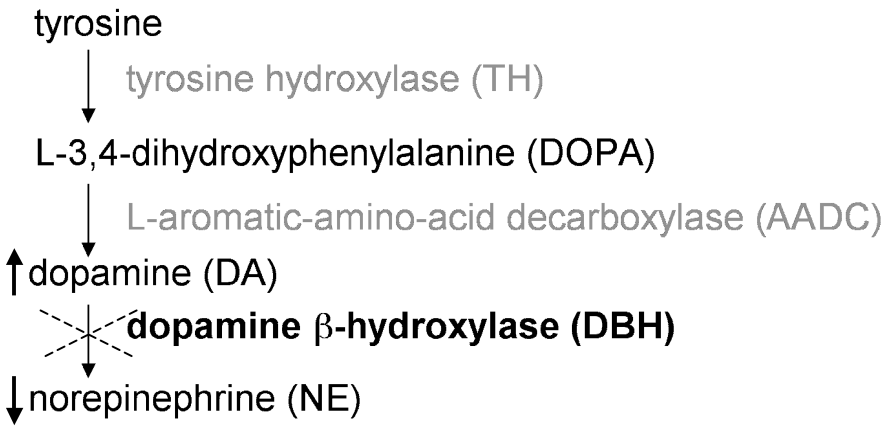

Figure A1.1 Catecholamine biosynthetic pathway. Because DBH converts DA to NE in noradrenergic neurons, inhibition of DBH is unique in its ability to decrease NE while increasing DA.

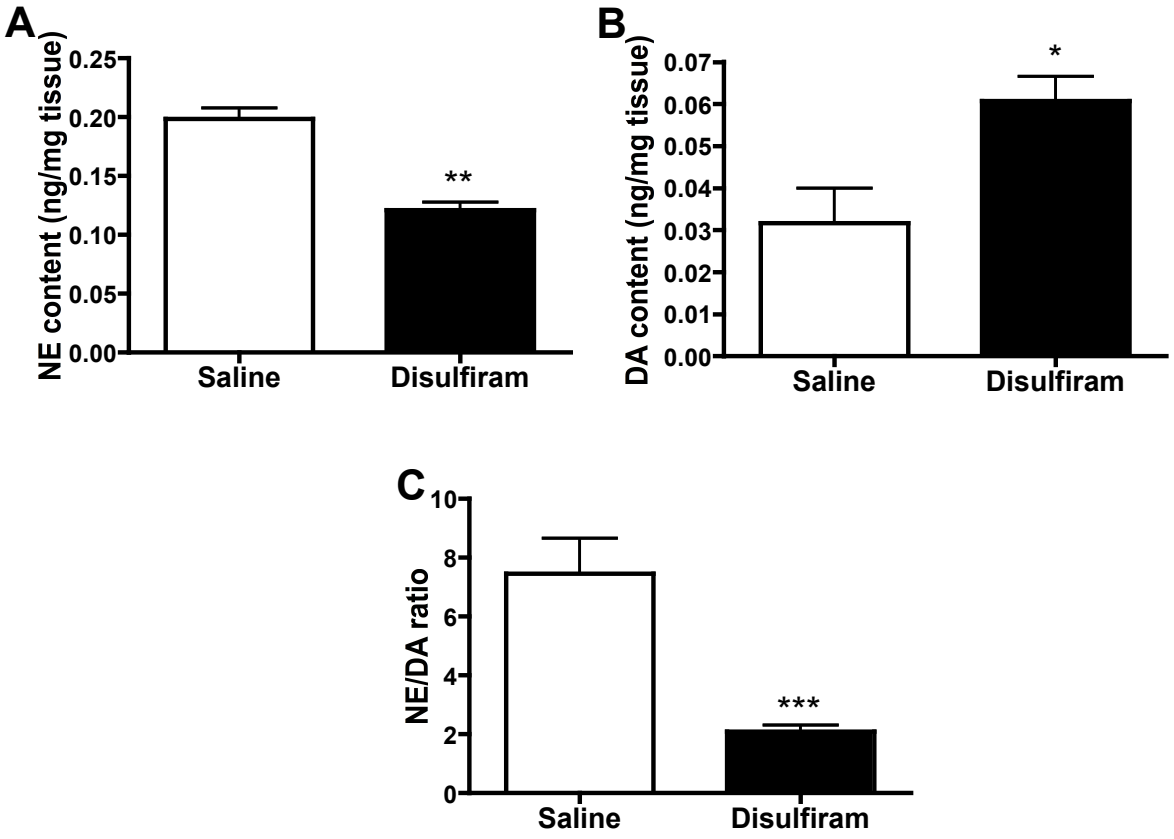

Figure A1.2 Effect of disulfiram on catecholamine levels in the rat prefrontal cortex. Shown is the mean ± SEM for (A) NE levels, (B) DA levels, and (C) the NE/DA ratio in the prefrontal cortex of rats after treatment with saline or disulfiram (single injection of 100 mg/kg, i.p., catecholamines measured 2 hours after disulfiram administration by HPLC followed by electrochemical detection; N = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle.

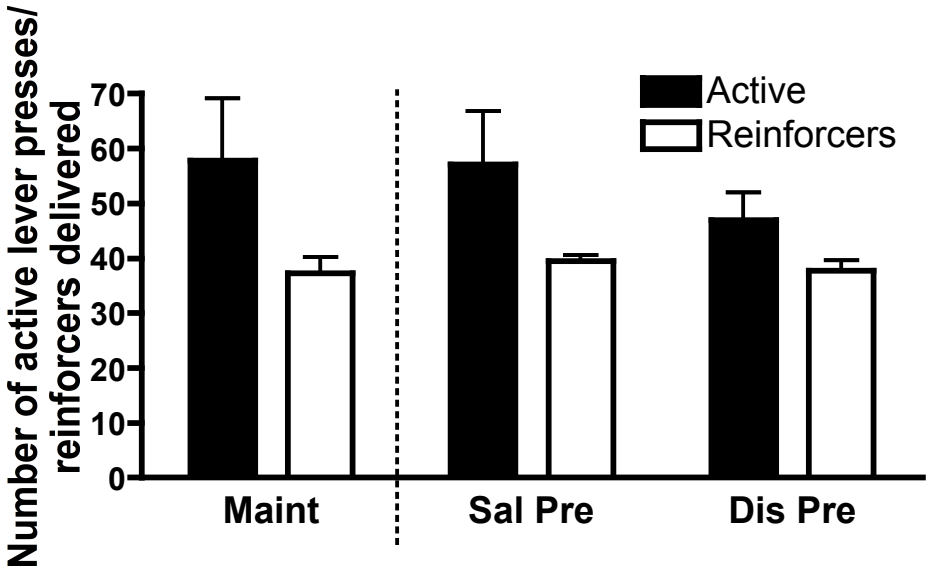

Figure A1.3 Disulfiram does not affect maintenance of cocaine self-administration. After reaching maintenance levels for operant cocaine self-administration ("Maint"), rats were pretreated with saline ("Sal Pre") or disulfiram (100 mg/kg, i.p.; "Dis Pre") 2 hours prior to cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-hour session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-second timeout periods result in more active lever presses than reinforcers received. N = 8 per group.

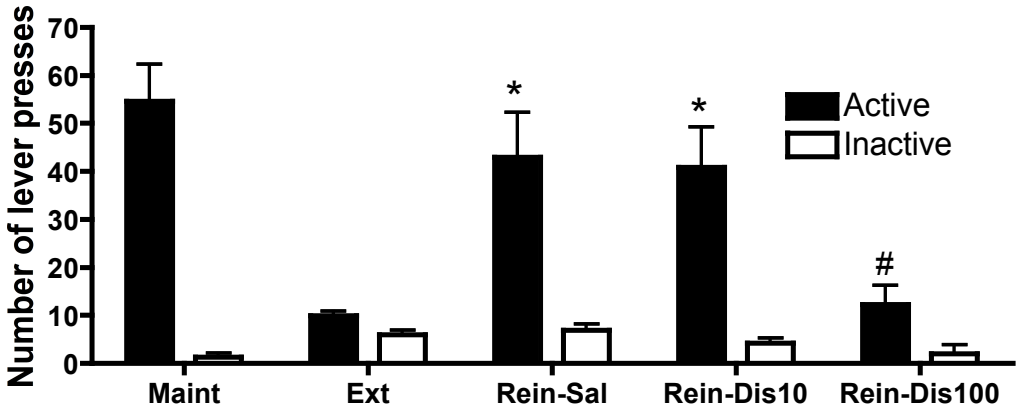

Figure A1.4 Disulfiram blocks cocaine-primed reinstatement. Once maintenance ("Maint") and extinction ("Ext") criteria for operant cocaine self-administration were met, rats were pretreated with saline ("Rein-Sal", N = 13) or disulfiram (10 or 100 mg/kg, i.p.) ("Rein-Dis10", N = 6 and "Rein-Dis100", N = 7) 2 hours prior to cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. *$P < 0.05$ compared with active lever responses during extinction, $^{\#}P < 0.05$ compared with active lever responses during cocaine-induced reinstatement tests with saline pretreatment (N = 7 per group).

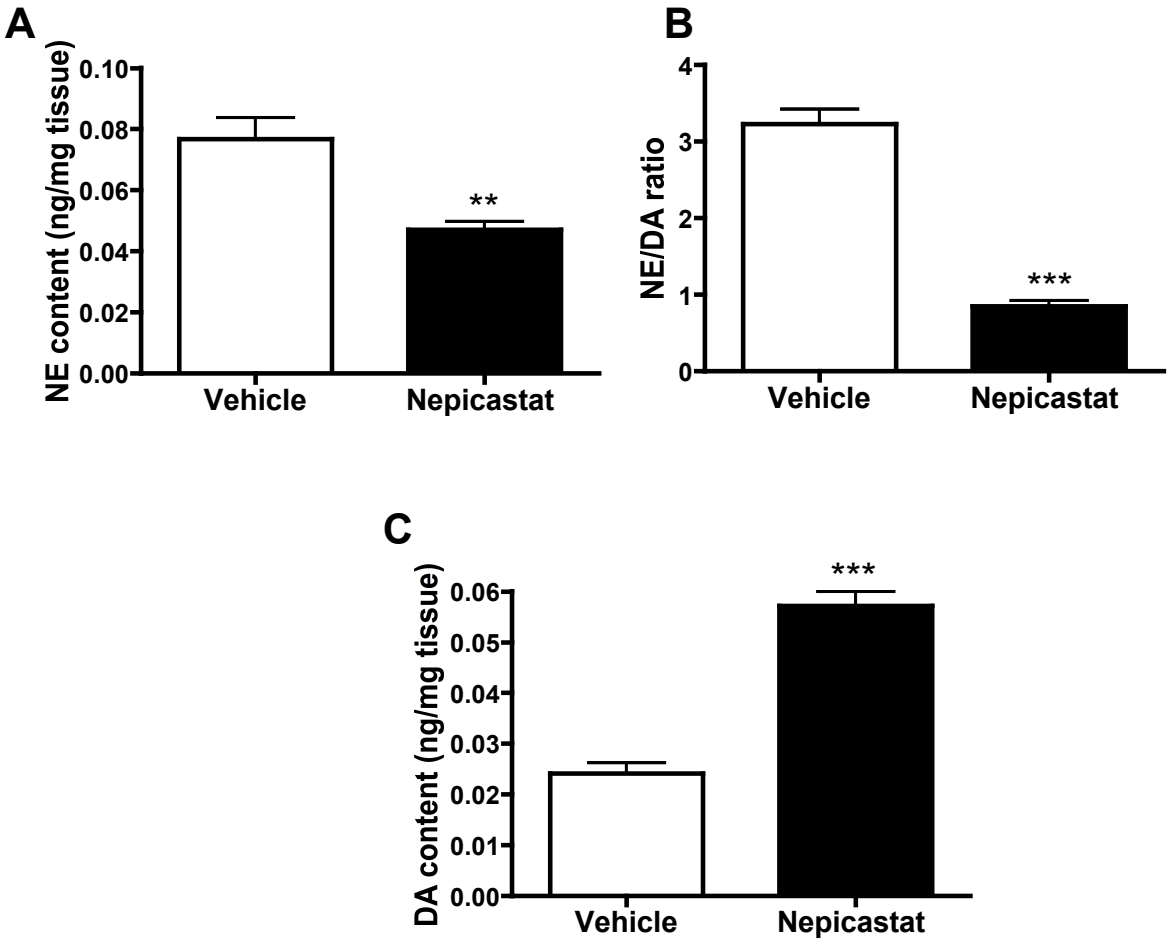

Figure A1.5 Effect of nepicastat on catecholamine levels in the rat prefrontal cortex. Shown is the mean ± SEM for (A) NE levels, (B) DA levels, and (C) the NE/DA ratio in the prefrontal cortex of rats after treatment with vehicle or nepicastat (single injection of 50 mg/kg, i.p., catecholamines measured 2 hours after nepicastat administration by HPLC followed by electrochemical detection; N = 8 per group). **P < 0.01, ***P < 0.001 compared with vehicle.

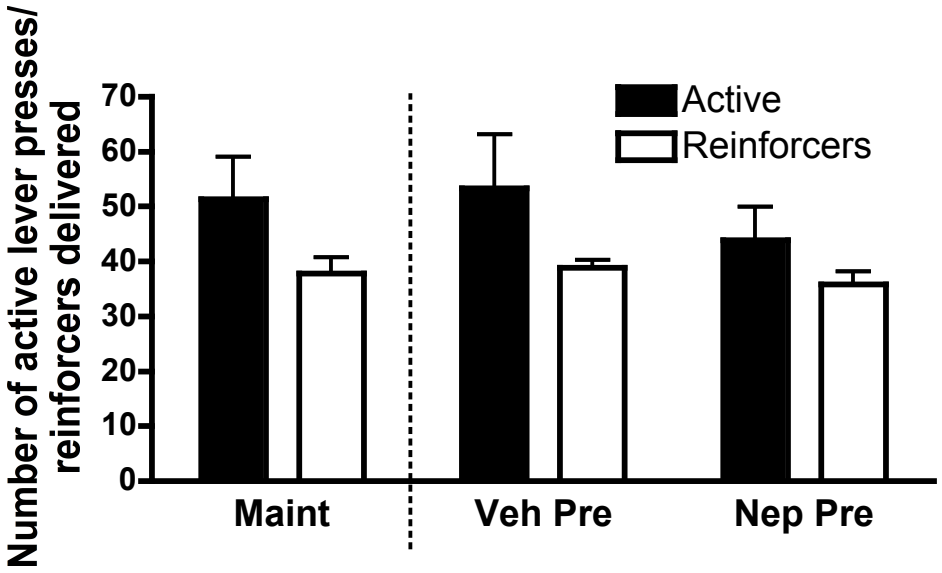

Figure A1.6 Nepicastat does not affect maintenance of cocaine self-administration. After reaching maintenance levels of operant cocaine self-administration ("Maint"), rats were pretreated with vehicle ("Veh Pre") or nepicastat (50 mg/kg, i.p.; "Nep Pre") 2 hours prior to cocaine self-administration sessions. Shown are mean ± SEM active lever responses and number of reinforcers obtained over a 2-hour session. Maintenance values reflect an average number of responses and reinforcers obtained over the last 3 days of maintenance. Occasional active lever pressing during the 20-second timeout periods result in more active lever presses than reinforcers received. N = 6 per group.

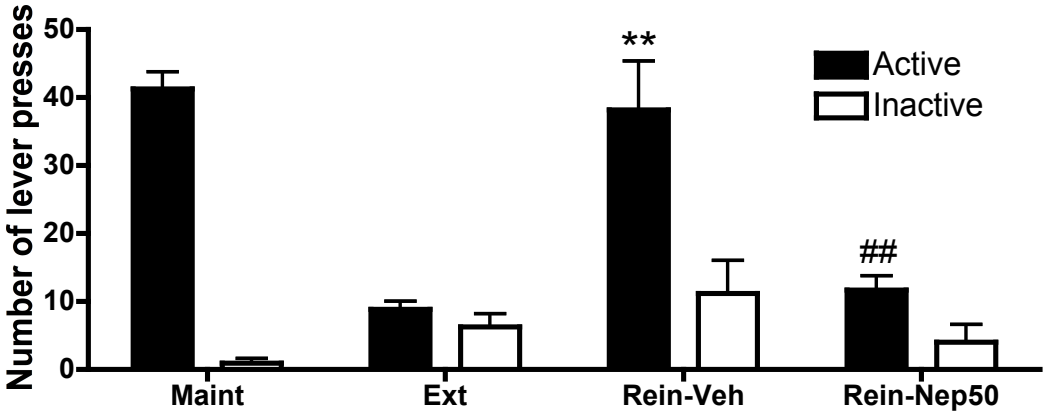

Figure A1.7 Nepicastat blocks cocaine-primed reinstatement. Once maintenance ("Maint") and extinction ("Ext") criteria for operant cocaine self-administration were met, rats were pretreated with vehicle ("Rein-Veh") or nepicastat (50 mg/kg, i.p.; "Rein-Nep50") 2 hours prior to cocaine prime (10 mg/kg, i.p.) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. $^{**}P < 0.01$ compared with active lever responses during extinction, $^{\#\#}P < 0.01$ compared with active lever responses during cocaine-induced reinstatement tests with saline pretreatment (N = 6 per group).

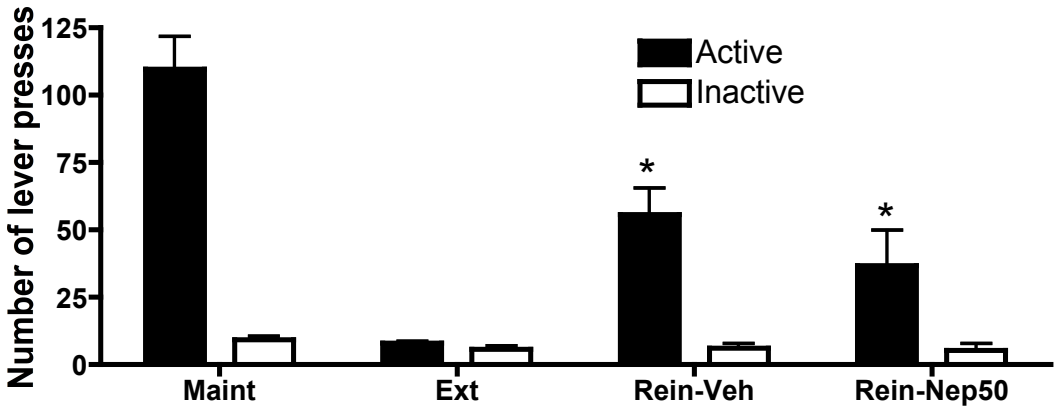

Figure A1.8 Nepicastat does not affect food-primed reinstatement of food seeking. Once maintenance ("Maint") and extinction ("Ext") criteria for operant food self-administration were met, rats were pretreated with vehicle ("Rein-Veh") or nepicastat (50 mg/kg, i.p.; "Rein-Nep50") 2 hours prior to food prime (3 pellets at beginning of session, then 1 pellet every 3 min over the 60 min session) and placement into the self-administration chambers. Shown are mean ± SEM active and inactive lever responses. Maintenance values reflect an average of the last 3 days of maintenance sessions, and extinction values reflect an average of the last 3 days of extinction. *$P < 0.05$ compared with active lever responses during extinction ($N = 7$ per group).

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