The efficacy of exercise to attenuate cocaine relapse is associated with changes in *Bdnf* and altered by sex and estrous cycle phase

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Abstract

Exercise is a potential intervention for cocaine addiction that has shown promise in both human and animal studies. However, the conditions that produce the most efficacious response have yet to be determined. Thus, the goal of this dissertation is to determine the exercise conditions that produce the most efficacious response in a preclinical model of cocaine addiction. The overall hypothesis is that exercise will be an effective intervention that reduces cocaine relapse vulnerability and blocks subsequent neuroadaptations that develop over an abstinence period. Although exercise is known to induce widespread effects in the brain and act on many different signaling pathways (e.g., norepinephrine, opioid, serotonin, endocannabinoid, cortisol); this dissertation will focus on its effects on brain derived neurotrophic factor (Bdnf) exon IV expression in the prefrontal cortex (PFC) as potential mechanism given that it is a marker of epigenetic regulation implicated in cocaine relapse and known to be regulated by exercise.

Chapter I of this dissertation provides background on the behavior and underlying neurobiology (i.e. epigenetic regulation of *Bdnf*) of cocaine addiction. This chapter also provides evidence to support the hypothesis that the efficacy of exercise for preventing relapse vulnerability is associated with alterations in Bdnf expression. Chapter II demonstrates that the efficacy of wheel running, an animal model of exercise, is dose-dependent, with greatest effects following longer access, and is associated with epigenetic regulation of *Bdnf exon IV* expression in the PFC. Chapter III demonstrates that early, but not late, access to a running wheel during abstinence attenuates subsequent cocaine-seeking. Chapter IV demonstrates that the efficacy of wheel running is

influenced by sex, estrous cycle phase, and dose conditions. In this chapter I demonstrate that males are more sensitive to the beneficial effects of wheel running, while in females longer access conditions is required to override estrus-induced vulnerability. Overall, the data presented in this dissertation establishes exercise as a promising intervention that has the ability to reduce cocaine relapse vulnerability likely by blocking cocaine-induced neuroadaptations (e.g. *Bdnf*) that develop over an abstinence period.

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Chapter I

General Introduction

Cocaine Addiction: Behavior and Underlying Neurobiology

Dissertation Rationale and Outline

There are currently no approved pharmacotherapies for treating cocaine addiction. We believe that exercise is an attractive potential non-pharmacological intervention that may have secondary health benefits. Although exercise has not been well studied for cocaine addiction, initial findings in illicit drug-dependent individuals, including those with cocaine dependency have been promising (Brown et al. 2010). Thus, using animal models, the overall goal of this dissertation is to examine the efficacy of exercise as a potential treatment for cocaine addiction. Exercise is known to induce widespread effects in the brain and act on many different signaling pathways (e.g., norepinephrine, opioid, serotonin, endocannabinoid, cortisol; Smith and Lynch 2013); however, this dissertation will focus on its effects on brain derived neurotrophic factor (Bdnf) expression, particularly at the exon IV region, in the prefrontal cortex (PFC) as a potential mechanism given that is an important marker of epigenetic regulation implicated in cocaine relapse and known to be regulated by exercise. Overall this dissertation demonstrates that the efficacy of exercise is mediated by epigenetic changes in *Bdnf* and altered by sex, hormones, and dose. Specifically, in this chapter the behavior (from both human and animal studies) and underlying neurobiology of cocaine addiction will be discussed. Chapter II of this dissertation will provide evidence that the efficacy of exercise is dose-dependent and associated with epigenetic regulation of the *Bdnf* gene in the PFC. Chapter III will provide evidence for time-dependent effects of exercise on cocaine-seeking, while chapter IV will discuss sex, dose, and estrous cycle phase-

dependent effects on the efficacy of exercise to reduce cocaine-seeking. Chapter V will provide a general discussion and future directions.

Stages of Cocaine Addiction and Relapse

Human literature

In 1980, cocaine was considered a safe non-addicting euphoriant, and historical descriptions of cocaine dependence were dismissed as exaggerations causing a vast increase in cocaine use (Gawin, 1991). Thirty years later, the probability of US adult illicit drug users transitioning from casual use to dependence is second highest among cocaine users (Lopez- Quintero, 2011) and approximately 1.1 million individuals in the US are cocaine abusers (SAMHSA 2009). Cocaine abuse is characterized by a maladaptive pattern of use resulting in substantial recurrent adverse effects including inability to fulfill family, home, or work obligations, legal problems, and difficulty maintaining interpersonal relationships (Penberthy et al.2010). Despite significant substance-related problems, cocaine abusers continue use which quickly leads to dependence and cycles of relapse.

Cocaine addiction starts with use that is often recreational in nature; however the drug euphoria experienced is highly reinforcing and users often transition quickly to an impulsive use state. As addiction manifests there is a transition to high dose, long duration binges where drug use is uncontrolled and compulsive. High cocaine intake is followed by periods of abstinence and subsequent relapse. After the first year of cocaine use onset the probability of transitioning to dependence is 7.1% and increases to 20.9% a

decade after initial use (Lopez- Quintero, 2011). This statistic highlights the difficulty cocaine dependent individuals often have maintaining abstinence. Embedded within relapse susceptibility in cocaine addiction are long lasting cravings that are prevalent even after years of discontinued use. Cocaine craving is a complicated phenomenon that is vastly intensified by social and environmental stimuli (i.e. people, places, or paraphernalia) associated with drug use that ultimately promote relapse. To further understand the biological basis for cocaine addiction and relapse researchers have looked to mimic the characteristics seen in the human condition through the use of animal models.

Animal models of cocaine addiction and relapse

Much of the progress in understanding the mechanisms of addiction has derived from the development of animal models (Koob et al., 1998). While no animal model fully recapitulates the human condition, animal models permit investigation into the neurobiological adaptations involved in the different stages of addiction. In laboratory animals, researchers model the major phases of addiction seen in the human condition through drug self-administration paradigms. Traditionally, animal models of addiction begin with an acquisition stage in which there is a transition from initial sampling to continued regular use (Carroll et al. 2004). Following acquisition is the maintenance stage in which drug self-administration is well regulated under short access conditions with no increasing or decreasing trends in intake (Carroll et al. 2004). Although these models have been extremely useful at determining the neurobiology of drug-

reinforcement under controlled and stable drug intake conditions, such models do not recapitulate the compulsive binge drug use seen in human cocaine addiction.

Animal models of addiction have employed multiple methods to model the shift from controllable stable intake to dysregulated uncontrollable drug use. These methods have used paradigms that increase access to the drug either by increasing drug dose, session length or the number of injections that are available/hr (Lynch, 1998). Amhed and Koob (1999) showed that cocaine intake escalates over time under extended access conditions (6-hr session length) and remains elevated even after 2 months of reduced drug availability. Our lab has employed the discrete trial 4 procedure to achieve high and dysregulated patterns of intake (Dobrin and Roberts 2012; Lynch and Roberts, 2004). With this procedure rats are given 24-hr extended access to cocaine infsuions in discrete 10-min trials (4 trials/hr) and self-administer in 'binge/abstinent' patterns taking nearly every infusion available for the first 1-2 days, followed by periods of abstinence that are interspersed with periods of active drug use (Roberts et al. 2002; Morgan et al. 2005; Lynch and Roberts, 2004). Based on previous literature it appears that these high access conditions (e.g. 6-24 hr/day) are necessary to induce an addicted phenotype which is characterized by an enhanced subsequent motivation to self-administer cocaine (Ramoa et al. 2013; Lynch and Taylor, 2004; for review see Roberts et al. 2007) and increased relapse vulnerability following prolonged abstinence (Peterson et al. 2013; for review see Lynch et al. 2013). Overall, the use of extended access paradigms help capture critical components of human cocaine addiction, such as the shift from stable intake to dysregulated drug use and enhanced subsequent motivation to obtain cocaine.

Vulnerability to relapse is a hallmark characteristic of cocaine addiction. Notably, the same cues that elicit drug-seeking in humans, also elicit drug-seeking in animals (Episten et al. 2006). In laboratory animals, relapse is modeled through the use of reinstatement procedures in which cues used to elicit cocaine-seeking can be a priming injection of the previously self-administered drug, exposure to a stressor, or the presentation of environmental cues that were formerly associated with the drug. During the reinstatement session the usual measure is drug-seeking (i.e. responses on levers previously associated with the drug) that is assessed following chronic self-administration and abstinence. Like the human situation where craving for cocaine is reported to increase over the first several weeks of abstinence (Gawin and Kleber 1986), reinstatement responding for cocaine also increases over an abstinence period in animals (Grimm et al. 2001; Lu 2004). The increase in reinstatement responding that occurs over an abstinence period has been termed the "incubation effect" (Grimm et al. 2001) and has been proposed as a critical contributing factor for the enduring drive to seek cocaine. Recent evidence also indicates that factors such as sex and hormones may predict relapse vulnerability and alter cocaine-seeking behavior.

Gender and hormonal influences in cocaine addiction and relapse in humans

Women have historically had lower rates of cocaine use and abuse compared to men (SAMHSA 2007) leading to the impression that they are less vulnerable to cocaine addiction. However, there is increasing evidence from clinical and preclinical studies suggesting that females are not less vulnerable to cocaine addiction than males, and with regard to certain aspects of drug abuse, have an augmented biological vulnerability

(Lynch 2006). Specifically, women have been observed to take less time to become dependent following initial use (Kosten et al.1993) and to enter treatment programs sooner than men (Griffin et al. 1989) suggesting that women have an escalated course to addiction (Brady and Randall 1999). In conjunction, during abstinence women also have shorter abstinence periods although both sexes have similar patterns of cocaine intake (Kosten 1993), suggesting the presence of gender differences during cocaine relapse. Available evidence also suggests that men and women relapse for different reasons. Previous studies have shown that women are more likely than men to attribute relapse to drug use to depression, distress, and a lack of positive experiences; whereas, men are more likely than women to attribute relapse to self-justification (i.e. they were entitled to use more cocaine; McKay et al. 1996). Cocaine-dependent women are also more likely to report cocaine craving in response to cocaine associated cues than cocaine dependent men (Robbins et al. 1999) and this difference may possibly be attributed to increased cue reactivity in women (Lynch et al. 2002). Further investigation into distinguishing gender differences in relapse susceptibility can possibly lead to better treatment outcomes for both men and women.

Evidence suggests an influence of hormones on addiction vulnerability in cocaine-dependent individuals. In humans, results have revealed that the subjective effects of stimulants vary across the menstrual cycle (Terner and de Wit 2006) with greatest subjective effects observed when estrogen levels are high and relatively unopposed by progesterone (Justice and de Wit 1999) suggesting that it is the ratio of the two hormones that underlie sensitivity to the reinforcing effects of drugs of abuse. Similar findings have been reported in studies focusing on hormonal influences on

cocaine craving with results showing that women with high levels of progesterone had significantly lower stress-induced and drug cue-induced cocaine craving and reduced drug cue-induced anxiety levels when compared with the low progesterone group (Sinha, 2007). Recently, Fox et al. (2013) demonstrated that administration of exogenous progesterone in early abstinent cocaine-dependent individuals reduced cue-induced cocaine craving and measures of stress (i.e. cortisol levels) to a greater extent in women than men. This finding suggests that hormone levels contribute to relapse vulnerability and highlight the need for sex–specific treatments.

Sex and hormonal influences in animal models of cocaine addiction and relapse

Similar to clinical studies, most preclinical studies reveal a greater biological vulnerability in females as compared to males with results showing that they exceed males during the initiation, escalation (binge), extinction (drug-seeking in the absence of drug associated cues), and reinstatement (relapse) phases of the addiction process (for reviews see Anker and Carroll 2010; Carroll and Anker 2009; Carroll et al. 2004; Lynch 2006; Lynch et al. 2002). During early phases of the addiction process, sex differences in cocaine self-administration are best revealed under low self-administration dose conditions (for review see Lynch 2006). For example, under low dose conditions a greater percentage of drug naive female rats meet criteria for acquisition of cocaine self-administration and they acquire cocaine self-administration at a faster rate than drug naïve male rats (Lynch and Carroll, 1999). Females have also been reported to self-administer greater levels of cocaine under extended access conditions (Lynch and Taylor 2004; Roth and Carroll 2004) and show greater increases in motivation for cocaine

following extended access self-administration (Lynch and Taylor 2004). Sex differences under relapse testing conditions appear to vary by the cues used to trigger drug seeking (Carroll and Anker 2009). For example, female rats reinstated more than male rats following the administration of the pharmacological stressor yohimbine (Carroll and Anker 2009). However the opposite effect (males>females) was found with cue-primed reinstatement (Fuchs et al. 2005) suggesting that the direction of sex differences depends on the reinstatement stimulus (i.e. drug, stressor, drug-prime, or cue). Overall, studies using animal models of relapse coincide with clinical data of a sex difference in the escalated course to addiction in females and sex differences in triggers for relapse.

Consistent with findings in humans, the enhanced biological vulnerability seen in female laboratory animals is believed to be mediated by ovarian hormones, particularly estrogen which has been shown to increase vulnerability at each of the phases of the addiction process (Kerstetter et al. 2008; Lynch 2006; Roth and Carroll 2004; Larson et al. 2007). Although females in all phases of their estrous cycle exhibit incubation of cocaine craving (Kerstetter et al. 2008), females tested during estrus (defined as high levels of estrogen relative to progresterone; Feltenstein et al. 2009) maintain a potentiation in cocaine-seeking longer than those in other phases (Kippin et al. 2005; Feltenstein and See 2007; Kersetter et al. 2008). These findings are consistent with reports in ovariectomized rats supplemented with estradiol that displayed increased vulnerability during reinstatement testing conditions (for reviews see Anker and Carroll 2010; Carroll and Anker 2009, and Evans 2007) and adds further support to the idea that hormonal status influences relapse vulnerability. It is not known whether estrous cycle phase also influences treatment outcomes.

Neurobiology of Cocaine Addiction

Brain derived neurotrophic factor transcription and expression following normal neuronal activity

One of the hallmarks of cocaine addiction is the progressive increase in cocaine craving that occurs over an abstinence period (i.e. incubation effect), a phenomenon observed in both humans (Gawin and Kleber 1986) and laboratory animals (Grimm et al. 2001). Although the underlying neurobiology of the incubation effect remains unclear, available evidence indicates that epigenetic regulation of *Bdnf* may be involved. In order to better understand cocaine-induced alterations in Bdnf expression, this section will first discuss *Bdnf* transcription and protein synthesis promoted by normal neuronal activity and later focuses on cocaine-induced chromatin remodeling of the Bdnf gene as a possible mechanism underlying the incubation effect.

Bdnf is a member of the neurotrophin family that includes nerve growth factor, neurotrophin 3, and neurotrophin 4 and is a critical regulator of neural circuit function and synaptic plasticity. The transcriptional regulation, expression, and secretion of Bdnf are directly controlled by neural activity (Park and Poo 2013). *Bdnf* transcription begins when neuronal activity induces membrane depolarization that causes an influx of calcium (Ca^{2+}) through voltage gated Ca^{2+} channels and Ca^{2+} -permeable NMDA type glutamate receptors (Tao et al. 1998). This action triggers binding of transcription factors, Ca^{2+}-response factor (CaRF) and cyclic AMP (cAMP) response element (CRE)-binding protein (CREB), to specific regulatory elements of the *Bdnf* gene (Tao et al. 2002). Recent evidence has demonstrated that there are nine exons on the *Bdnf* gene in both

humans and rodents with exon IV as highly responsive to neuronal activity. Notably, exon IV is also regulated by various stimuli encoded by Ca^{2+} and cAMP signaling (Metsis et al. 1993; Tao et al. 2002), and due to this promoter region containing multiple Ca^{2+} -responsive sequences and CREs that bind CaRF and CREB, respectively, transcription readily occurs (Park and Poo 2013). In vitro studies have demonstrated that stimuli-dependent neuronal activity induces membrane depolarization of Ca^{2+} and calmodulin kinase II (CaMKII) phosphorylates methyl-CpG-binding protein 2 (MeCP2), a transcriptional repressor that binds to methylated DNA in the *Bdnf* promoter IV region (Zhou et al. 2006), and causes the release of both MeCP2 and co-repressor histone deacetylase 1 (HDAC) from this promoter region. The removal of both MeCP2 and HDAC 1 from the promoter IV region collectively increases *Bdnf* transcription (Chen et al. 2003; Martinowich et al. 2003).

Although the rodent Bdnf gene has multiple untranslated exons on the 5' end (exons I through VIII) and one principle protein coding exon (exon IX) which can result in many possible Bdnf transcript variants, the resulting protein product of these splice variants show minimal variation (with some exceptions; for review see Karpova 2013). Specifically, each exon (I- VIII) produces a different 5' untranslated region and only exon IX contains protein coding DNA. Most of the different splice variants have the same amino acid composition with only the untranslated regions differing. Based on the literature, it appears that there is a combination of two exons, either exons I-VIII coupled to exon IX (Liu et al. 2005). Notably, there are small changes in amino acid sequence when exon 1 versus 4 are used and induce different protein isoforms that resulted from either the addition or deletion of amino acids (Timmusk et al. 1995). Specifically,

evidence indicates that exon I transcript contains an additional translation codon which adds amino acids (Aid et al. 2007), while exon IV transcript undergoes alternative splicing leading to a mature Bdnf protein isoform that lacks 48 amino acids (Liu et al. 2005). It is not yet known whether these protein isoforms function differently. However, it can be surmised that altering the number of amino acids can possibly trigger changes in intracellular trafficking of Bdnf and play a role in its translation in different subcellular compartments.

Following transcription Bdnf transcripts accumulate locally in dendrites (An et al. 2008), although direct evidence for translation of *Bdnf* mRNA in this area has yet to be demonstrated. However, on the circuitry level, *Bdnf* mRNA has been reported to be robustly expressed in cortical and midbrain dopamine (DA) neurons (Schmidt et al. 2013; Lipska et al. 2001) and thought to supply approximately 80% and 20%, respectively, of Bdnf protein within the striatum (Altar et al. 1997). This finding is interesting as *Bdnf* mRNA is low in the striatum (Alter et al. 1997; Hofer et al. 1990; Maisonpierre et al. 1990) and supports the theory that Bdnf is anterogradely transported by axons to the terminals of Bdnf-expressing neurons (Alter et al. 1997). Bdnf protein is synthesized in the endoplasmic reticulum as a precursor protein, pre-pro-Bdnf, which is converted to Pro-Bdnf that is cleaved to generate mature Bdnf (Park and Poo 2013). Mature Bdnf binds to and activates tropomyosin receptor kinase B (TrkB) docked on dendrites on GABAergic medium spiny neurons in the striatum and is expressed in glutamatergic pyramidal neurons from the PFC (McGinty et al. 2010), in which both regions are critical for cocaine addiction and relapse. It is important to note that both *Bdnf* mRNA and

protein levels are extremely low in the brain (Barde et al. 1982) and studies involving exogenous Bdnf may alter how endogenous Bdnf is "normally" processed in the neuron.

Role of Brain derived neurotrophic factor Expression in Cocaine Relapse (Incubation Effect)

Drugs of abuse, like cocaine, function primarily through the mesolimbic reward pathway that is comprised of DAergic projections from the ventral tegmental area (VTA), to the NAc, amygdala, and PFC (Kalivas 2007). Throughout this reward pathway DA transmission is tightly regulated by DA transporters (DAT) that bind to DA and transport it out of the synaptic cleft and back into the presynaptic terminal. However, when cocaine is onboard it binds to DAT and prevents removal of DA. Thus, DA concentrations are allowed to reach non-physiological levels, promoting a learned association between cocaine's subjective and physiological effects, environmental stimuli, and behaviors that result in cocaine delivery (Kalivas 2007). As addiction research has progressed researchers now propose that DA is critical during the initial stages of cocaine addiction. However, as abstinence is prolonged and subsequent relapse occurs there is emerging evidence implicating epigenetic modifications in chromatin containing the *Bdnf* gene in PFC as potential mechanism involved in the incubation effect (Grimm et al. 2001; Lu et al. 2004; Pickens et al. 2011). In support of this idea, available evidence indicates that in recovering cocaine-dependent individuals, elevated BDNF serum levels predicted shorter subsequent time to relapse, higher total cocaine use, and cocaine craving (D'Sa et al. 2011; Corominas-Roso et al. 2012). In conjunction, laboratory animals display a progressive increase in Bdnf protein levels in mesolimbic areas, including the PFC

(Grimm et al. 2003; McGinty et al. 2010; Pickens et al. 2011) following prolonged abstinence.

Further work has demonstrated time-dependent alterations in *Bdnf* mRNA expression and protein levels during abstinence from chronic cocaine exposure. Specifically, during early abstinence from cocaine self-administration, levels of cocaine-seeking are low and accompanied by a decrease in both *Bdnf* mRNA expression and protein levels in mesolimbic areas, including the PFC (Angelucci et al. 2007; Whitfield et al. 2011; Grimm et al. 2003; McGinty et al. 2010; Berglind et al. 2007; 2009). However, as abstinence is prolonged Bdnf protein levels rebound and are associated with a progressive increase in cocaine-seeking (Whitfield et al. 2011; Grimm et al. 2003; McGinty et al. 2010; Sadri-Vakili et al. 2011). Additionally, site-specific infusion of Bdnf into the PFC immediately following cocaine self-administration has been reported to suppress cocaine-seeking but to have no effect on elevated cocaine-seeking when infused during later abstinence (e.g. 6-7 days) (Berglind et al. 2007; 2009; Whitfield et al. 2011). Together, these findings suggest that interventions that may prevent cocaine-induced time-dependent changes in Bdnf expression may provide long term protection against cocaine relapse vulnerability.

Epigenetic Regulation of Brain derived neurotrophic factor in the Prefrontal Cortex Associated with Relapse

Epigenetics is defined as heritable/ stable changes in gene expression within mature postmitotic neurons that do not include changes in DNA sequence (Bird 2007; Tsankova et al. 2007). One example of epigenetic regulation is environmental stimuli,

such as cocaine self-administration and abstinence, promoting stable alterations in chromatin structure that will activate or repress gene transcription. Chromatin remodeling, including histone modifications and DNA methylation, and its influence on gene regulation, specifically regulation of the *Bdnf* gene, has become an increasing focus in drug addiction research (Schmidt et al. 2013; Maze and Nestler, 2011; McQuown and Wood, 2011). Recent evidence implicates the exon IV region of the Bdnf gene in particular as a marker of enhanced relapse vulnerability (Sadri-Vakili et al. 2011). Specifically, following protracted abstinence from cocaine self-administration MeCP2 has decreased association with the promoter IV region on the *Bdnf* gene and histone 3 becomes hyperacetylated leading to a removal of transcriptional repression and an open chromatin confirmation (Sadri-Vakili et al. 2011). Hyperacetylation of histone 3 associated with the *Bdnf* gene has been reported to induce low rates of acquisition to cocaine self-administration in male offspring that were fathered by males with a history of cocaine self-administration (Vassoler et al. 2013). Thus, changes in acetylation of chromatin containing the *Bdnf* gene are functionally relevant in cocaine addiction because like infusion of Bdnf into the PFC, inhibition of HDAC that results in a general increase in histone 3 acetylation, including acetylation of histones associated with the promoter IV region of the Bdnf gene (Yasuda et al. 2009), decreases motivation for cocaine, cocaine-seeking, and cocaine-induced locomotor sensitization (Malvaez et al. 2010; 2013; Romieu et al. 2011; Kennedy et al. 2013). Further evidence supporting the hypothesis that altering histone acetylation may provide beneficial behavioral and molecular effects on cocaine-seeking will be presented in Chapter II of this dissertation. Together, the data presented in this section indicate that cocaine has the ability to induce

substantial changes in chromatin within the PFC which in turn causally effects the expression of *Bdnf* and subsequent cocaine-seeking.

Section Summary

Cocaine addiction is a debilitating psychiatric disorder that starts with use that is often recreational in nature, however the euphoria experienced is so great users often transition to dependence and cycles of abstinence and relapse. Researchers model the hallmark characteristics of cocaine addiction in animal models by employing self-administration paradigms. Sex differences in relapse, demonstrated in both humans and laboratory animals, demonstrate the need to develop treatments that can be tailored by sex in order to reduce the incidence of relapse. Embedded within relapse susceptibility are persistent changes in epigenetic regulation of gene expression, specifically, the *Bdnf* gene, that likely mediates the enduring drive to crave cocaine. Key to developing possible treatments that attenuate drug craving and subsequent relapse is to focus on potential treatments that can be tailored by sex and induce epigenetic modifications in brain function that can attenuate repeated uncontrollable drug use.

Exercise as Novel Intervention for Cocaine Addiction and Relapse

Evidence from Human Studies

When cocaine-dependent individuals abstain from use, DAergic activity is vastly reduced causing withdrawal symptoms including anhedonia and craving. Thus, many pharmacotherapies for relapse prevention have focused on DA agonists that mimic cocaine-induced increases of DA transmission. Clinical evaluations of this type of pharmacotherapy have shown variable efficacy with most reports concluding it's ineffectiveness at treating cocaine addiction. Additionally, following the administration of DAergic agents some individuals have reported adverse side effects including nausea, headaches, hypertension, tachycardia, and psychosis (Hart and Lynch 2005). To this end, it is becoming apparent that pharmacologically augmenting DA transmission during abstinence does not attenuate relapse. Thus, there is a need for other treatments outside of pharmacotherapies to be investigated for cocaine addiction. One such non-pharmacological treatment that has shown promise for cocaine addiction is exercise. Researchers initially examined the efficacy of exercise as a potential intervention because it activates the same reward pathway as cocaine, through increases in DA (Dishman et al. 2006) and DA binding protein concentrations (MacRae et al. 1987). Recently, exercise has also been reported to induce epigenetic modifications in chromatin at specific regions implicated in cocaine addiction (Gomez-Pinilla et al. 2011; Sadri-Vakili et al. 2010). Therefore, exercise may protect against cocaine-induced neuroadaptations following chronic exposure.

Exercise is an attractive intervention for cocaine addiction in men and women for two reasons. The first reason is that exercise has been shown to be an efficacious intervention in smokers that induced an increase in abstinence, alleviation of withdrawal symptoms, and a decrease in smoking (for review see Ussher et al. 2008). There is also evidence demonstrating that both moderate and high intensity exercise, such as walking and running respectively, reduces levels of nicotine craving (Scerbo et al. 2010). Second, exercise has been shown to alleviate depression, reduce measures of anxiety, and enhance cognition (for reviews see Brisswalter et al. 2002; Martinsen 2008), and each of these factors is known to contribute to cocaine relapse in men and women (Aharonovich et al. 2006; Poling et al. 2007).

Although clinical studies have examined the efficacy of exercise at different stages of the addiction process for illicit, nicotine, alcohol, and cannabis dependent individuals (for review see Lynch et al. 2013) this dissertation will focus primarily on evidence for preventing relapse in illicit drug-dependent individuals. While few studies have examined the efficacy of exercise in this population, epidemiological data have demonstrated lower levels of illicit drug use among more physically active individuals as compared to more sedentary individuals (Ströhle et al. 2007; Kirkcaldy et al. 2002; Kulig et al. 2003). Controlled studies in drug-dependent individuals, including cocaine dependent individuals, have shown that regular physical activity or exercise provided either alone or in conjunction with behavioral therapy, improves treatment outcomes (Berg et al. 2012; Brown et al. 2010). However, the efficacy of exercise is somewhat variable between studies and the long-term benefits are controversial possibly due to variability between studies in terms of the conditions used for exercise (Roberts et al.

2012; i.e. type, amount, and intensity). The exercise conditions that will produce the greatest treatment outcome have yet to be determined.

Evidence from Animal Studies

Animal studies can be used to determine the exercise conditions that produce the most efficacious response on measures of drug abuse vulnerability and underlying mechanisms mediating its efficacy. To-date most studies have focused on the effects of unlimited access to a running wheel, a form of aerobic exercise that is reinforcing in rats (Greenwood et al. 2011), on concurrent drug self-administration (for review see Smith and Lynch, 2011). Results from these studies have yielded promising findings showing that chronic exercise reduces the reinforcing effects of cocaine (Smith et al. 2008; Cosgrove et al. 2002) and decreases cocaine-seeking when exercise is concurrently available (Zlebnik et al. 2010). This dissertation will focus on examining the dose of wheel running that produces the most efficacious response on cocaine-seeking and associated neuroadaptative changes in the PFC (see Chapters II and III). Subsequent chapters will also provide an in-depth analysis of current evidence from animal studies examining the efficacy of unlimited wheel running in attenuating cocaine-seeking.

Gender and Sex differences in the efficacy of exercise

The effects of gender and sex in the efficacy of exercise for attenuating cocaine relapse will be discussed in greater detail in subsequent chapters. Briefly, beneficial

effects of exercise have been observed in illicit drug-dependent males and females (for review see Lynch et al. 2013); however, few studies have included a large enough sample size to examine sex and hormonal differences in its efficacy. In laboratory animals, sex differences in the efficacy of exercise on cocaine-seeking are largely unknown, with only one study demonstrating an effect of unlimited wheel running on cocaine-primed reinstatement responding in both males and females (Smith et al. 2012). Given findings indicating a critical role of hormone levels influencing relapse vulnerability and treatment outcome (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007; Feltenstein et al. 2011), hormonal status may possibly affect the efficacy of wheel running to reduce cocaine-seeking. Further work is needed to determine whether the efficacy of wheel running to attenuate relapse vulnerability differs by sex and hormonal status (see Chapter IV).

Exercise Induced Epigenetic Regulation of Prefrontal Cortex *Bdnf*

Exercise, like cocaine, also affects protein levels and epigenetic regulation of the Bdnf. Thus, exercise may induce long-term neuroadaptive changes that may provide protection during relapse. Studies have shown that exercise has the ability to increase Bdnf serum and plasma in humans (Rasmussen et al. 2009) and in animals' hippocampal Bdnf protein and mRNA levels (Sartori et al. 2011). Preclinical studies have demonstrated that the cortex is the most responsive region to wheel running, an animal model of aerobic exercise, and results in a robust increase in mature Bdnf protein (Quirie et al. 2012). Wheel running has also been reported to induce epigenetic modification of

chromatin containing the *Bdnf* gene, specifically at the exon IV region that has also been implicated in the incubation effect (Gomez-Pinilla et al. 2011). Furthermore, Gomez-Pinilla and colleagues (2011) also reported that following chronic wheel running there is an increase in histone 3acetylation and DNA demethylation at the promoter IV region and disassociation of MeCP2 all of which enabled *Bdnf* transcription. Taken together, these findings indicate that exercise has the ability to induce epigenetic modifications in chromatin containing the *Bdnf* gene, similar to cocaine, and thus may provide potential benefits during relapse.

Section Summary

In both clinical and preclinical studies exercise has been shown to provide beneficial effects in reducing cocaine craving and relapse vulnerability. Gender differences in the efficacy of exercise to prevent relapse are largely unknown due to small sample sizes and inconsistent exercise measures. Further work is needed to ascertain possible influences of sex and hormonal status on the beneficial effects of exercise to attenuate relapse vulnerability (see Chapter IV). Preclinical studies have made strides in understanding the underlying neurobiology mediating the effects of exercise on cocaine-seeking. We believe a highly possible mechanism underlying the efficacy of exercise involves epigenetic modifications in chromatin containing the *Bdnf* gene. Thus, this dissertation will provide evidence to support the idea that the beneficial effects of exercise provided during abstinence is likely mediated by epigenetic regulation of *Bdnf exon IV* in the PFC (see Chapters II and III).

Chapter II

Dose-Dependent Effects of Wheel Running on Cocaine-Seeking and Prefrontal Cortex Bdnf exon IV Expression

Introduction

Neuroadaptive changes that occur in the brain following chronic drug use are thought to underlie long-lasting behavioral changes that lead to relapse. Extensive research has resulted in approved pharmacotherapies to prevent relapse for opiate, nicotine, and alcohol addiction; however, none are currently approved for cocaine (Kampman, 2010). Physical activity, and specifically exercise, has been proposed as a non-pharmacological intervention for cocaine addiction due in part to its ability to activate the DAergic reward pathway through a similar mechanism to cocaine (Dishman et al. 2006; MacRae et al. 1987). Exercise also has widespread effects in the brain and acts on many of the different signaling pathways/processes that are known to be involved in cocaine addiction (e.g., norepinephrine, opioid, serotonin, endocannabinoid, cortisol; Smith and Lynch 2011). Recent evidence also suggests that epigenetic mechanisms, particularly remodeling of chromatin containing *Bdnf* gene, may underlie long-term changes in relapse vulnerability (Schmidt et al. 2013; Maze and Nestler, 2011; McQuown and Wood, 2011). In rodents, both wheel running, a proposed model of exercise, and cocaine induce epigenetic modifications in chromatin containing the *Bdnf* gene (Gomez-Pinilla et al. 2011). Therefore, exercise may prevent cocaine relapse by altering epigenetic changes in chromatin containing the *Bdnf* gene that develop following chronic cocaine exposure.

Although few studies have examined the efficacy of exercise for preventing relapse, epidemiological data have demonstrated lower levels of illicit drug use among more physically active individuals as compared to more sedentary individuals (Ströhle et al. 2007; Kirkcaldy et al. 2002; Kulig et al. 2003). Controlled studies in drug-dependent

individuals, including cocaine dependent individuals, have shown that regular physical activity or exercise provided either alone or in conjunction with behavioral therapy, improves treatment outcomes (Berg et al. 2012; Brown et al. 2010). However, the efficacy of exercise is somewhat variable between studies and the long-term benefits are controversial possibly due to variability between studies in terms of the conditions used for exercise (Roberts et al. 2012; i.e. type, amount, and intensity).

Animal studies can be used to determine the exercise conditions that produce the most efficacious response on measures of drug abuse vulnerability. However, to-date, most studies have focused on the effects of unlimited access to a running wheel on concurrent drug self-administration (Smith and Lynch, 2011). Results from these studies have yielded promising findings showing that wheel running reduces the reinforcing effects of cocaine (Smith et al. 2008; Cosgrove et al. 2002) and decreases cocaine-seeking (Zlebnik et al. 2010). We recently demonstrated that 2 hr/day of access to a running wheel during abstinence was sufficient to suppress subsequent cocaine-seeking, suggesting that a modest level of wheel running is effective and even when not concurrently available with cocaine (Lynch et al. 2010). Importantly, we also observed that extracellular signal-regulated kinase phosphorylation (pERK) levels in the PFC, a downstream target of Bdnf, which was positively associated with cocaine-seeking, were significantly decreased by wheel running during abstinence (Lynch et al. 2010). These findings suggest that wheel running, when available during abstinence, may provide long-term beneficial effects by preventing neuroadaptive changes that occur following chronic cocaine exposure and abstinence. The exercise conditions that lead to the most efficacious response are not yet known.

In this study we manipulated the amount of wheel running access that was available during abstinence and examined its effects on subsequent cocaine-seeking as well as associated neuroadaptive changes in the PFC, a region critical for relapse vulnerability. In humans, persistent relapse vulnerability has been attributed in part to a progressive increase in cocaine craving seen during the first several weeks of abstinence. In conjunction, elevated BDNF serum levels predicted shorter subsequent time to relapse, higher total cocaine use and cocaine craving in abstinent cocaine-dependent individuals (D'Sa et al. 2011; Corominas-Roso et al. 2012). A similar phenomenon is observed in animal models in which cocaine-seeking incubates over an abstinence period (Lu et al. 2004). As in humans, this incubation effect is associated with a progressive increase in Bdnf protein levels in mesolimbic areas, including the PFC (Grimm et al. 2003; McGinty et al. 2010; Pickens et al. 2011), with recent evidence implicating the exon IV region of the *Bdnf* gene in particular as a marker of enhanced relapse vulnerability (Sadri-Vakili et al. 2011). Importantly, this region of the Bdnf gene is also known to be regulated by wheel running (Gomez-Pinilla et al. 2011). A goal of this study was to examine the possibility that the epigenetic regulation of *Bdnf* in the PFC is associated with the efficacy of exercise. Thus, in addition to examining the dose-dependent effects of wheel running on levels *Bdnf exon IV* expression, we also examined the effects of sodium butyrate (NaBu), a HDAC inhibitor, that like exercise, upregulates gene transcription, including *Bdnf exon IV* expression (Yasuda et al. 2009). We hypothesized that wheel running would dose-dependently decrease subsequent cocaine-seeking, that its efficacy would be associated with changes in PFC *Bdnf exon IV* expression, and that the behavioral and molecular effects of exercise would be mimicked by NaBu.

Methods and Materials

Animals

Adult male (n = 94) Sprague Dawley rats (Charles River, Portage, ME, USA), weighing 320-380g at the start of the study, were used in this experiment. Animals were housed in individual operant conditioning chambers (Med Associates, St. Albans, VT) maintained on a 12-h light/dark cycle (lights on at 0700) with free access to food and water. Animals were trained to lever press for sucrose pellets during habituation to the operant conditioning chambers to facilitate later acquisition of cocaine self-administration using methods previously described (Lynch et al. 2010). Animals were then implanted with a jugular catheter using methods previously described (Abdolahi et al. 2010) and placed back into individual operant conditioning chambers where they resided for the duration of the self-administration procedure. All procedures were approved by the University of Virginia Animal Care and Use Committee.

Drugs

Cocaine hydrochloride was obtained from National Institute of Drug Abuse with the same concentration (g/ml) maintained throughout the study. Cocaine hydrochloride dose (mg/kg) was adjusted three times per week based on animal body weight (2 seconds/100g body weight). Sodium butyrate was obtained from Sigma Aldrich with a dose of 200 mg/kg used in this study.

Cocaine Self-Administration

Methods for cocaine self-administration were conducted using procedures previously described (Lynch et al. 2010). Briefly, rats were trained to self-administer cocaine (1.5 mg/kg/infusion) during daily sessions under a fixed ratio 1 schedule with a maximum of 20 infusions/session available. This relatively high cocaine dose was selected to produce maximal and rapid rates of acquisition. Once the acquisition criterion was met (defined as two consecutive sessions in which all 20 infusions were obtained), rats were given 24 hr access to cocaine (1.5 mg/kg/infusion) for a total of ten consecutive days under a discrete trial procedure (four trials/hr). In order to prevent overdose and achieve high and dysregulated patterns of intake, the number of infusions available each hour was limited to four (Dorbin and Roberts, 2012; Lynch and Roberts, 2004). With this procedure, rats self-administer high levels of cocaine in 'binge/abstinent' patterns taking nearly every infusion available for the first 1-2 days, followed by periods of abstinence that are interspersed with periods of active drug use (Roberts et al. 2002; Morgan et al. 2005; Lynch and Roberts, 2004). Importantly, increased cocaine-seeking, as well as increased motivation for cocaine, critical features for cocaine addiction in humans, are observed following extended-access self-administration when examined after a 14 day abstinence period (Lynch et al. 2010; Ramôa et al. 2013). Following the last discrete trial session, cocaine infusions were again available under a fixed-ratio 1 schedule with a maximum of 20 infusions for 2 consecutive sessions. These fixed-ratio sessions served 2 purposes: 1) to equate all groups on intake prior to abstinence and 2) to confirm catheter patency. Initially an n of 103 rats started the study; however, 9 animals were removed

due to patency issues or death and these animals were not included in any of the statistical analyses.

Wheel Running Access Conditions

Rats were randomly assigned to 1, 2, or 6 hour unlocked (n = 33) or locked (n = 30) running wheel groups, following the last cocaine self-administration session. Each animal was transferred to a polycarbonate cage with an attached 35.6cm diameter running wheel (Med Associates). Rats began a 14 day forced abstinence period in which they remained housed in individual polycarbonate cages. Rats assigned to the unlocked wheel condition were given voluntary access to an unlocked running wheel for 1, 2, or 6 hrs each day starting at 0900 for all groups and ending between 1000-1500 depending on access condition. Rats assigned to the locked wheel condition were placed in similar boxes and at same time of day as the rats in the unlocked wheel conditions; however the wheel was locked at all times for rats in this group. Wheel rotations were recorded daily during 1000-1500 depending on session length condition. Additional groups of saline controls received 2 hr/day access to either an unlocked wheel (n = 9) or locked wheel (n = 9). To test the effects of HDAC inhibition during abstinence on subsequent cocaine-seeking, an additional cohort of rats received NaBu (200 mg/kg) or vehicle treatment (n = 9, n = 4, respectively) intraperitoneally (IP) administered before access to a locked running wheel for 2 hr/day. Following the last unlocked or locked wheel session, rats were returned to the same operant chambers they previously self-administered overnight for habituation.

Reinstatement of Cocaine-Seeking

Methods for cue-induced reinstatement were conducted using procedures previously described (Lynch et al. 2010). Briefly, following the 14th day of abstinence, when cocaine-seeking is known to be high (Grimm et al. 2001), rats were tested under an extinction/reinstatement paradigm consisting of a minimum of 6 one hour extinction sessions, and following extinction (fewer than 15 responses per hour on the previously active left lever; Lynch, Mangini, and Taylor, 2005), a one-hour cue-induced reinstatement session. Each extinction session was separated by 5 minutes and began with the introduction of the formerly active lever into the operant conditioning chamber. Responses during extinction were recorded, but did not produce any consequence. The cue-induced reinstatement session began five minutes after the last extinction session with the introduction of the formerly active lever as well as a single 5 second presentation of cues formerly associated with cocaine (illumination of stimulus light above the previously active lever and the sound of the infusion pump). Subsequent responses during this session on the previously active lever presented these stimuli under a fixed ratio 1 schedule. Following the cue-induced reinstatement session rats were sacrificed by rapid decapitation with brains quickly removed. The medial prefrontal cortex was dissected from 2-mm-thick coronal brain slices based on coordinates from Paxinos and Watson (1998) (Figure 2). Brain tissue was rapidly frozen on dry ice and stored in a freezer at -80˚C until further processing.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

An RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA) was used to isolate total RNA according to the manufacturer's protocol. The quantity and quality of the RNA were determined using a NanoVue™ Spectrophotometer. cDNA templates were prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. The ABI StepOnePlus real-time PCR system was used to perform qRT-PCR. Rat BDNF Exon IV and GAPDH primers, described by (Schmidt et al. 2012), were synthesized by Invitrogen (Carlsbad, CA) for SYBR® Green-Based Detection (Applied Biosystems; Carlsbad, CA). Target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid inter-sample variance. A no-reverse transcriptase reaction was run in parallel to cDNA synthesis samples to control for contamination and genomic amplification. Each of these qRT-PCR reactions was verified for a single PCR product of expected size with the disassociation melting curve stage. All genes of interest were analyzed with StepOne™ software using the comparative cycle thresholds method (CT) method.

Statistical Analysis

The repeated measures ANOVA was used to examine the effects of wheel condition on levels of cocaine self-administration, wheel running, and subsequent cocaine-seeking. The main dependent measures were the number of cocaine infusions self-administered over the 10 day extended access period, the number of wheel rotations

over the entire 14-day wheel running period, as well as during the last three days, the number of responses made during each of the six 1-hour extinction sessions, the number of responses made during the last extinction session, and the number of responses made during the reinstatement session. Wheel rotations from the cocaine and saline groups that received access to an unlocked running wheel for 2 hrs/day were collapsed since no significant differences were observed between these groups. Data on extinction and reinstatement responding from the rats in the locked conditions were collapsed between groups for all subsequent comparisons since no significant differences were observed between these groups. For the NaBu study, data from the rats in the 2 hr/day locked wheel condition that received vehicle pretreatment during abstinence were combined with the data from the rats in 2 hr/day locked wheel condition without pretreatment as no differences were observed between these groups on any measure. Separate analyses were performed to examine behavioral differences within the saline control groups. The Univariate ANOVA was used to examine the effects of wheel running on *Bdnf* promoter IV mRNA expression in the PFC. Data from saline controls were collapsed across groups since levels of *Bdnf-IV* expression in the PFC did not differ significantly between unlocked and locked running wheel groups. The Dunnett t-test was used to examine differences as compared to saline. The one–tailed t test was used for all a priori predicted hypotheses and the Bonferroni correction was used to control for multiple comparisons. The relationship between cocaine-seeking (defined as total number of responses made during the cue-induced reinstatement session) and levels of *Bdnf* promoter IV mRNA expression was examined by calculating the Pearson correlation co-efficient. Statistical analyses were performed with IBM SPSS Statistics, version 20 with alpha set at 0.05.

Results

Prior to abstinence and wheel and treatment assignment (unlocked or locked) all rats had similar cocaine intake over the 10 day extended access period (data not shown, $p > 0.05$). Although each of the unlocked groups ran at a similar level during the initial wheel running sessions (data not shown; $p > 0.05$), by the end of the wheel running period significant group differences in running were observed where rats given longer access to the unlocked wheel ran more than rats given shorter access (i.e. day of abstinence 12-14) (Figure 3a, group effect, $F_{2, 31} = 5.22$, $p < 0.05$). However, levels of running were highly variable, and although a significant difference was observed between the 6 hr versus the 1 hr and the 2 hr (ps' < 0.05 for both comparisons), no differences were observed between the 1 and 2 hr conditions ($p > 0.05$).

Although wheel running did not significantly affect levels of responding during extinction (data not shown, $p > 0.05$), a robust effect was observed during reinstatement. Specifically, levels of cocaine-seeking, in the presence of cocaine-associated cues, were increased from last extinction, and dose-dependently decreased by wheel running (Figure 3b, group effect, $F_{3,40} = 9.30$, $p < 0.01$; group by session, $F_{3, 40} = 8.43$, $p < 0.01$). Although responding was similar between groups prior to reinstatement testing, subsequent comparison within the reinstatement session revealed that rats given 2 and 6 hr/day access to an unlocked running wheel responded significantly less than rats given 1 hr/day access (ps' < 0.05 for both comparisons) and compared to rats given access to a locked wheel ($p < 0.05$ and $p < 0.01$; respectively). Rats in the 6 hr/day unlocked wheel group also responded significantly less than those in the 2 hr/day unlocked wheel group ($p < 0.05$). Saline control groups responded at minimal levels during both the extended

access period and the extinction/reinstatement testing period and levels did not differ significantly between wheel conditions (data not shown; $p > 0.05$). Levels of wheel running also did not differ significantly between cocaine and saline groups (data not shown; $p > 0.05$) that received limited daily access to an unlocked running wheel. In summary, wheel running during abstinence dose-dependently attenuated subsequent cocaine-seeking under cue-induced reinstatement testing, but not extinction testing, where longer access to an unlocked running wheel resulted in greater suppression of reinstatement responding.

Levels of *Bdnf exon IV* expression were increased following abstinence from extended access cocaine self-administration (saline versus cocaine locked wheel, $t_{(24)} = 5.28$, $p < 0.05$), and wheel running dose-dependently attenuated this increase (Figure 4a, group effect, $F_{1,35} = 41.06$, $p < 0.01$). Subsequent comparison within the cocaine groups, revealed that levels of *Bdnf exon IV* were significantly lower in all unlocked wheel groups as compared to the locked wheel group (1, 2, and 6 hr/day versus locked wheel; ps' < 0.05 for all comparisons). Although the 1 and 2 hr/day groups did not differ from each other, the 6 hr group was significantly lower than both the 1 hr and the 2 hr groups ($p < 0.05$ for both comparisons). Interestingly, the 6 hr group also did not differ from saline controls ($p > 0.05$) suggesting that access to 6 hr/day of wheel running during abstinence blocked cocaine-induced changes in *Bdnf exon IV* expression. The association between levels of *Bdnf exon IV* expression and reinstatement responding was not significant within the overall sample. However, in conditions where wheel running significantly reduced cocaine-seeking (2 and 6 hr/day conditions), this association was significant (Figure 4b, $r = .48$, $p < 0.05$) and levels of reinstatement responding were

inversely associated with levels of *Bdnf exon IV* expression. Data from saline controls were collapsed across groups since levels of Bdnf exon IV expression in the PFC did not differ significantly between unlocked and locked running wheel groups (data not shown; $p > 0.05$). These data indicate that wheel running access during abstinence dose-dependently attenuates cocaine-induced increases in PFC *Bdnf exon IV* expression.

Consistent with our results for the effects of wheel running, NaBu treatment attenuated cocaine-seeking under reinstatement testing conditions (Figure 5a, group effect, $F_{2, 26} = 4.79$, $p < 0.05$; group by session, $F_{2, 26} = 5.53$, $p < 0.05$), but not extinction conditions (data not shown). Also like wheel running, NaBu treatment during abstinence significantly reduced levels of *Bdnf exon IV* expression in the PFC as compared to vehicle treatment (Figure 5b, group effect, $F_{2, 28} = 8.92$, $p < 0.05$). Interestingly, the NaBu group did not differ from the 2 hr/day unlocked wheel group on levels of either cocaine-seeking ($p > 0.05$) or *Bdnf exon IV* expression ($p > 0.05$). No difference was observed between the NaBu and vehicle groups on cocaine intake over the 10-day extended access period prior to abstinence (mean number of infusions 60 ± 7; 63 ± 5; respectively).

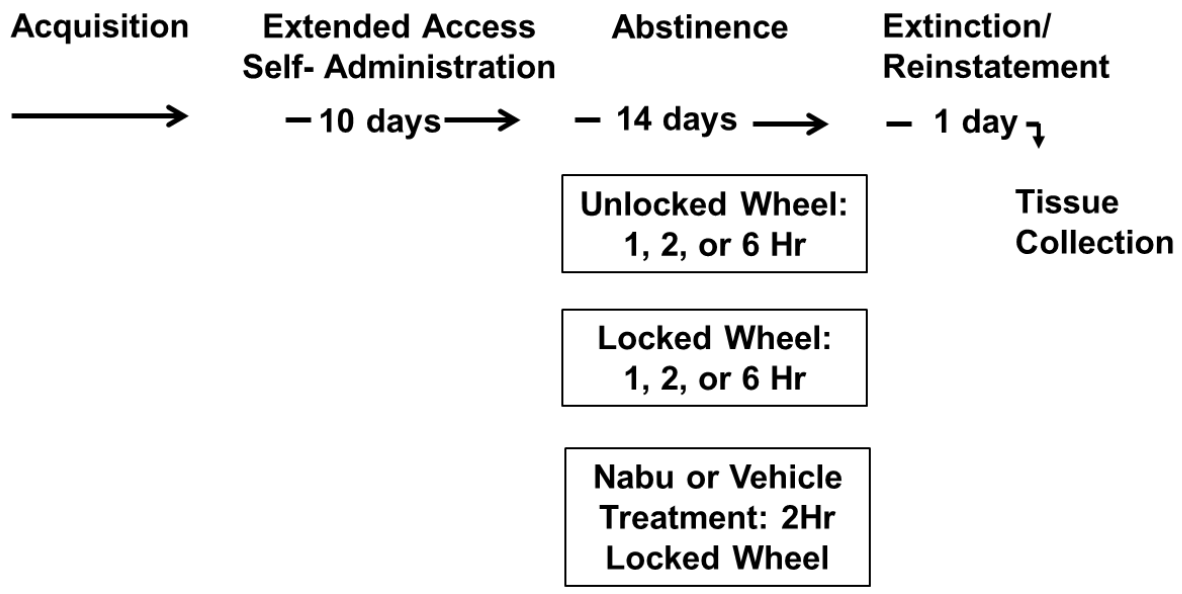

Figure 1- Time-line of experimental events. Following acquisition, rats were given 24 hr extended access to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure (4 trials/hr) for a total of 10 days. Following extended access self-administration rats were given 1, 2, or 6 hr/day unlocked (n = 33) or locked (n = 30) wheel access during a 14-day abstinence period. Additional groups of saline controls received 2 hr/day access to either an unlocked wheel (n = 9) or locked wheel (n = 9). The effects of HDAC inhibition during abstinence on subsequent cocaine-seeking were examined under sedentary (locked wheel) conditions in an additional cohort of rats. These rats received daily sodium butyrate (200 mg/kg, IP) or vehicle treatments (n = 9, n = 4, respectively) 30 minutes before 2 hr/day session. Following the 14th day of abstinence rats were tested under a within-session extinction/reinstatement procedure. Tissue was collected immediately following the reinstatement test.

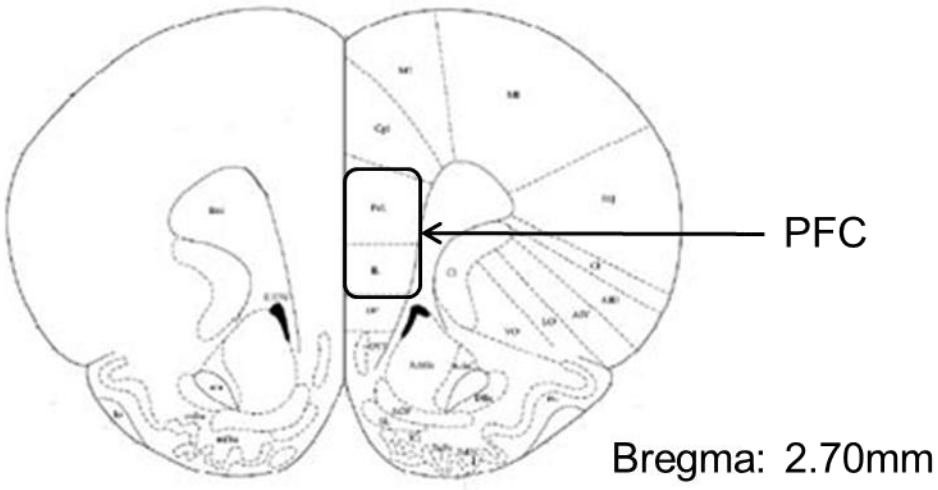

Figure 2- Schematic illustration of the brain region dissected for PFC.

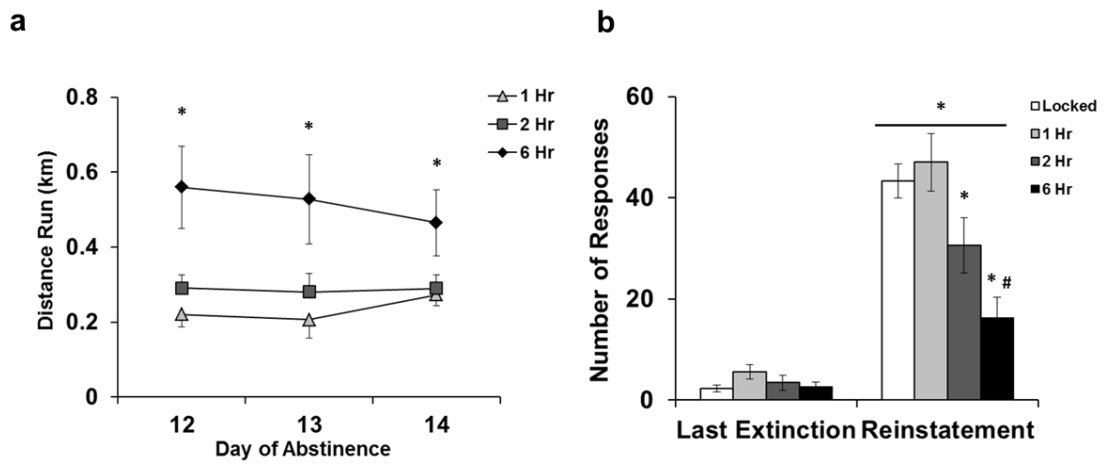

Figure 3- Wheel running dose-dependently affects distance run and subsequent cocaine-seeking. (a) Levels of wheel running plotted for the last three days of abstinence (i.e. days 12- 14). The filled triangles represent data points for the 1-hr unlocked wheel group, filled squares represent data points for the 2-hr unlocked wheel group, and filled diamonds represent data points for the 6-hr unlocked wheel group. (b) Number of responses (mean ± SEM) plotted during the last extinction and cue-induced reinstatement sessions. The white bar represents responding from the locked wheel group, the light grey bar represents responding from the 1-hr unlocked wheel group, the dark grey bar represents responding from the 2-hr unlocked wheel, and the black bar represents responding from the 6-hr unlocked wheel group. An asterisk indicates a significant group difference ($p < 0.05$). A bar and * indicates a significant time effect in responding from last extinction. The # symbol indicates a significant difference in responding from the 2-hr unlocked wheel group.

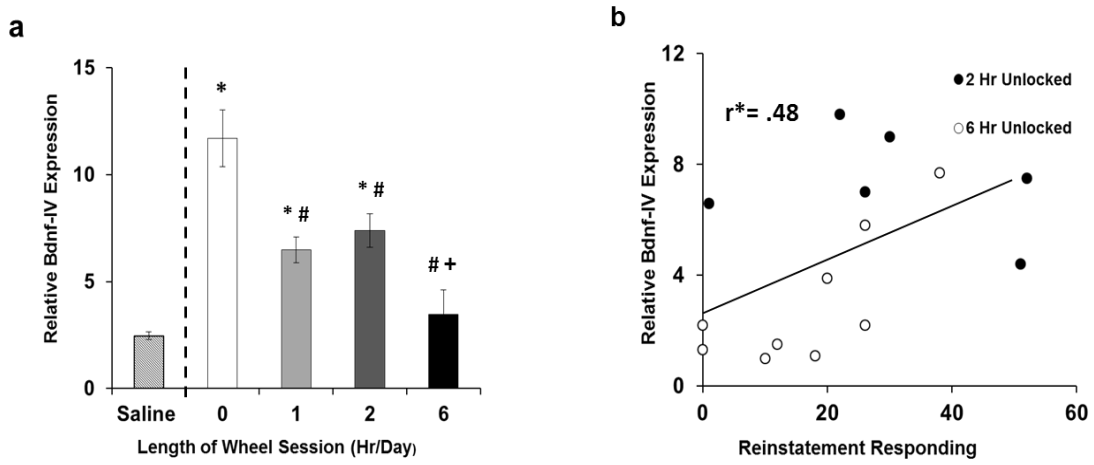

Figure 4- Wheel running dose-dependently reduced cocaine induced upregulation of Bdnf exon IV expression in the PFC of rats following cue-induced reinstatement and was inversely associated. (a) Relative Bdnf exon IV expression plotted by length of wheel session compared to saline and locked wheel controls. The grey patterned bar represents Bdnf exon IV expression in the saline group, the white bar represents expression in the locked wheel group, the light grey bar represents expression in the 1-hr unlocked wheel group, the dark grey bar represents expression in the 2-hr unlocked wheel group, and the black bar represents expression in the 6-hr unlocked wheel group. An * indicates a significant difference from saline, # symbol indicates a significant difference from locked wheel, + symbol indicates a significant difference from 1 and 2-hr unlocked wheel groups. Error bars represent SEM. (b) Data represent relative Bdnf exon IV expression as a function of number of responses observed during the 1 hr cue-induced reinstatement test for the 2 and 6 hr/day unlocked (filled black and white symbols, respectively) wheel groups. The Pearson Correlation Coefficient (r-value), as well as a regression line, is

shown for the overall association. An asterisk indicates a significant correlation of Bdnf exon IV expression levels and reinstatement responses.

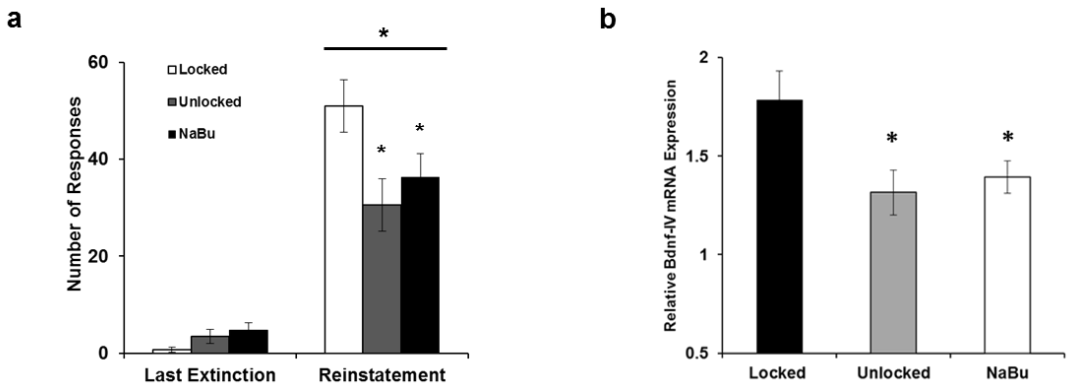

Figure 5- Sodium butyrate (NaBu, a HDAC inhibitor) mimics the beneficial behavioral and molecular effects of exercise. (a) Number of responses (mean ± SEM) plotted during the last extinction and cue-induced reinstatement sessions in vehicle and NaBu treated groups. The white bar represents responding from the vehicle treated 2-hr locked wheel group, the dark grey bar represents responding from the vehicle treated 2-hr unlocked wheel, and the black bar represents responding from the NaBu treated 2-hr locked wheel group. An asterisk indicates a significant group difference ($p < 0.05$). A bar and * indicates a significant time effect in responding from last extinction. (b) Relative Bdnf exon IV expression plotted by treatment group and wheel access condition. The black bar represents Bdnf exon IV expression in the vehicle treated 2-hr locked wheel group, the light grey bar represents expression in the vehicle treated 2-hr unlocked wheel group, and the white bar represents expression in the NaBu treated 2-hr locked wheel group. An * indicates a significant difference from the vehicle treated 2-hr locked wheel group and significant group difference ($p < 0.05$).

Discussion

Despite data from both human and animal models suggesting the efficacy of exercise in preventing cocaine relapse, there is a lack of evidence directly addressing the amount of exercise needed to be efficacious, and little information on the mechanisms mediating its efficacy. Our study is the first to demonstrate that the efficacy of wheel running, an animal model of exercise, at decreasing cocaine-seeking is dose-dependent and likely mediated via epigenetic regulation of *Bdnf exon IV* expression in the PFC. Specifically, levels of cocaine-seeking under reinstatement testing conditions were highest under the locked wheel condition, and wheel running during abstinence dose-dependently attenuated this effect with the greatest attenuation observed under the longest access condition (i.e. 6 hr/day). Cocaine also increased *Bdnf exon IV* expression, and wheel running during abstinence dose-dependently attenuated this increase, with complete blockade to saline control levels in rats given the longest access. Notably, the efficacy of exercise was inversely associated with *Bdnf exon IV* expression, and both its efficacy and its effects on *Bdnf exon IV* expression were mimicked by treatment during abstinence with the HDAC inhibitor, NaBu. These effects appear to be due to wheel running, and not to environmental enrichment or exploratory behaviors, given that rats in the locked wheel control group were exposed to the same environmental conditions as the rats in the unlocked wheel group. Taken together, these results demonstrate that wheel running effectively reduces cocaine-seeking, that higher access conditions produces a more efficacious response, and that its efficacy is likely mediated via epigenetic regulation of *Bdnf exon IV* expression in the PFC.

Levels of cocaine-seeking in the presence of cocaine-associated cues progressively decreased with increasing levels of access to an unlocked running wheel (i.e. 2 and 6 hr/day groups). This finding is consistent with other reports showing that wheel running has the ability to decrease cocaine's reinforcing effects (Smith et al. 2008; Cosgrove et al. 2002) and cocaine-seeking when the wheel is concurrently available (Zlebnik et al. 2010). These findings are also consistent with data from our group demonstrating that 2 hr/day of wheel running during abstinence attenuates subsequent cocaine-seeking (Lynch et al. 2010). Although, the present study did not observe a significant effect of wheel running on extinction responding, previous reports for the effects of wheel running during extinction have been modest with the greatest attenuation observed under cue-induced reinstatement testing conditions (Lynch et al. 2010).

Consistent with other reports, we observed an increase in *Bdnf exon IV* expression following cocaine self-administration and protracted abstinence (McGinty et al. 2010; Sadri-Vakili et al. 2010), and notably, this increase was dose-dependently decreased by wheel running. These findings parallel our current behavioral findings and suggest epigenetic regulation of *Bdnf exon IV* expression as a potential mechanism. In support of this idea, we also found an inverse association between the efficacy of exercise and levels of *Bdnf exon IV* expression in the PFC. One caveat to this idea, however, is that while wheel running reduced *Bdnf exon IV* expression at all access conditions including the 1 hr/day condition, a minimum of 2 hr/day access was required to suppress cocaine-seeking. Although the reason for this discrepancy is not clear, one possibility is that both suppression in *Bdnf exon IV* and a normalization of withdrawal circuitry through activation of DAergic neurons (Goggi et al. 2003) may be required to attenuate cocaine-

seeking. Thus, low access wheel running may produce a decrease in *Bdnf exon IV* expression in the PFC, but may not adequately affect DA in the nucleus accumbens, whereas, longer wheel access produces both a decrease in *Bdnf exon IV* expression and an increase DAergic signaling in the mesolimbic system. Further work is needed to examine this possibility, as well as the potential involvement of other signaling pathways, and to determine the effects of exercise in combination with direct manipulation of *Bdnf exon IV* in the PFC in order to establish a causal relationship.

Our finding showing that wheel running during abstinence decreased *Bdnf exon IV* expression is consistent with the idea that lower levels of *Bdnf* is protective against cocaine relapse (D'Sa et al. 2011; Corominas-Roso et al. 2012). While these findings suggest that the efficacy of wheel running may be related to its ability to decrease *Bdnf exon IV* expression, this potential mechanism is unlikely given a large body of work showing that wheel running increases rather than decreases Bdnf expression (Ding et al. 2011; Gomez-Pinilla et al. 2011).While its effects in the brain have focused mainly on the hippocampus, where it is known to robustly upregulate Bdnf (Ding et al. 2011; Gomez-Pinilla et al. 2011), similar effects of exercise have also been observed in other brain regions including the PFC (Chen et al. 2012; Quire et al 2012). An alternative possibility is that the efficacy of exercise is mediated via upregulation of *Bdnf* during early abstinence which results in normalization of expression, and as observed here, lower levels as compared to those observed in locked wheel controls. This possibility is consistent with not only the larger body of work showing that exercise increases *Bdnf* expression, but also an emerging literature demonstrating time-dependent alterations in cocaine-induced *Bdnf exon IV* expression that have different behavioral consequences.

Specifically, although Bdnf protein levels are elevated in the PFC following protracted abstinence from cocaine (when levels of cocaine-seeking are high; Grimm et al. 2001), during early abstinence (when levels of cocaine-seeking are low, Koya et al. 2009), markers of Bdnf activity are decreased (McGinty et al. 2010). Work has also shown that site-specific infusion of Bdnf into the PFC during early abstinence, but not following protracted abstinence, attenuates subsequent cue-induced reinstatement responding (Berglind et al. 2007) and normalizes neuroadaptations that are associated with the high levels of cocaine-seeking (Whitfield et al. 2011). This idea of time-dependent upregulation of Bdnf is also supported by our current findings showing that when provided beginning during early abstinence, HDAC inhibition, which is known to increase Bdnf exon IV expression (Yasuda et al. 2009), mimicked both the behavioral and molecular effects of wheel running. A caveat to this idea, however, is that wheel running did not increase Bdnf exon IV expression among saline controls. However, based on findings suggesting that the efficacy of exercise at upregulating BDNF is enhanced in individuals with lower levels and in disease states characterized by low levels of BDNF (e.g., Chen et al. 2012; Hopkins et al. 2011; Laske, 2010), it is possible that wheel running upregulated Bdnf in these cocaine self-administering animals during early abstinence. However, this possibility is speculative and requires further research.

Our findings showing the NaBu treatment reduced both cocaine-seeking and Bdnf exon IV expression are consistent with other reports showing beneficial effects of HDAC inhibition in reducing cocaine-seeking and extinction of conditioned place preference for cocaine (Malvaez et al. 2010; 2013). Although NaBu is a non-specific HDAC inhibitor, and in this study its effects were examined following systemic administration, evidence

suggests that inhibition of HDAC activity affects the expression of only approximately 2% of genes (Van Lint et al. 1996; Davie et al. 2003). Evidence also shows that *Bdnf exon IV* is activated in cortical regions including the PFC following systemic NaBu administration (Yasuda et al. 2009), and that in the cortex, exon IV-dependent *Bdnf* transcription accounts for the majority of the neuronal activity-induced Bdnf expression (Tao et al. 2002; Timmusk et al. 1994). While these results provide support for the idea that epigenetic regulation of Bdnf likely mediates the efficacy of NaBu treatment, further work is needed to rule out other mechanisms (e.g., DAergic signaling).

Taken together, these results suggest that exercise may dose-dependently protect against cocaine relapse by blocking epigenetic modifications in *Bdnf exon IV* in the PFC that occur following chronic cocaine exposure and abstinence. One caveat to these findings, however, is that it may be difficult in cocaine-dependent individuals to regularly attain even a modest level of exercise. It is also not yet known whether the beneficial effects of exercise can be sustained for long intervals. Despite these challenges, the initial findings in humans have been promising (for review see Lynch et al. 2013), and our findings further suggest neuroadaptive benefits of exercise. Our findings also suggest that varying conditions of exercise (i.e. type, amount, and intensity), and perhaps HDAC inhibitors, be examined as a potential treatment for cocaine relapse either alone, or as a supplement to pharmacotherapy.

Chapter III

Wheel Running during Early, but not Late, Abstinence Attenuates Subsequent Cocaine-Seeking

Brief Report

Introduction

Craving for drugs of abuse is a crucial component of addiction that ultimately leads to heightened relapse vulnerability. The goal of preventing drug relapse is to develop pharmacotherapies aimed at reducing craving; however there are none approved for cocaine addiction (Kampman 2010). Numerous pharmacotherapies have focused on DA agonists that restore the decrease in DAergic signaling in the mesolimbic reward pathway that is characteristic of early abstinence from chronic cocaine exposure. However, such approaches have proved to have variable efficacy and significant side effects that often limit patient compliance (Hart and Lynch 2005). One non-pharmacological intervention that similarly upregulates DAergic signaling in the reward pathway, but presumably without inducing negative side-effects, is exercise.

Exercise as an intervention for drug addiction, including cocaine, has shown promise in both human and animal studies (for review see Lynch et al. 2013). Although, the underlying neurobiology mediating the efficacy of exercise has not been definitively shown, available evidence indicates DA as a potential mechanism. Another possible mechanism is through epigenetic regulation of *Bdnf* in the PFC. In the previous chapter we showed that wheel running had the ability to block the increase in *Bdnf exon IV* expression normally observed following prolonged abstinence from chronic cocaine exposure. These data in combination with previous work demonstrating that an increase in Bdnf expression in the PFC during early, but not late abstinence effectively attenuates cocaine-seeking (Berglind et al. 2007) suggests that wheel running provided during early, but not late abstinence, may also be effective. To examine this possibility, we investigated time-dependent effects of wheel running, an animal model of exercise,

during early versus late abstinence from chronic cocaine exposure on subsequent cocaine-seeking. Based on our recent results (Chapter 2), and findings from others on time-dependent functional effects of Bdnf, we hypothesized that early, but not late, access to a running wheel during abstinence would attenuate subsequent cocaine-seeking.

Methods and Materials

The procedures used for cocaine self-administration, wheel running, and cue-induced reinstatement have been described previously (Lynch et al. 2010; Peterson et al. 2013). Briefly, adult male (n = 12) Sprague Dawley rats (350-400g) were trained to self-administer cocaine (1.5 mg/kg/infusion) under a fixed-ratio 1 schedule with a maximum of 20 infusions available each day. Once the acquisition criterion was met (first two consecutive sessions where all 20 infusions available were obtained), rats were given extended (24-hr) access to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure (four trials/hr) for a total of ten consecutive days. Following the last cocaine self-administration session, rats were moved to polycarbonate cages with wheel attachments and given voluntary access to an unlocked wheel for 2-hr/day during early (i.e. day 1-7) or late (i.e. day 8-14) abstinence (n = 6; for both groups) during the 14-day abstinence period. When wheel running was unavailable (i.e., days 7-14 for the early group and days 1-7 for the late group) a metal partition separated the polycarbonate cage from the wheel attachment. Following the last wheel session, rats were placed back into their previous self-administration chambers to allow for habituation overnight. The following day cocaine-seeking was assessed under a within-session extinction/cue-induced

reinstatement paradigm. This paradigm consisted of six 1-hr extinction sessions and following extinction, (when fewer than 15 responses/hour were made on the cocaine associated lever) a 1-hr cue-induced reinstatement session. Data for cocaine intake over the 10-day extended access period, wheel running per day during abstinence, responding during the six, 1-hour extinction sessions, and responding during the last extinction as compared to the 1-hr reinstatement session were analyzed by repeated measures ANOVA. The bonferroni corrected t-test was used for post hoc comparison for extinction and reinstatement responding with alpha set at 0.05. Greater details for all methods are provided in Chapter II of this dissertation.

Results

Prior to abstinence total daily cocaine intake did not significantly differ between the early (i.e. days 1-7) and late (i.e. days 8-14) wheel running groups during the 10-day extended access cocaine self-administration period (60 ± 2 versus 64 ± 2 (1.5 mg/kg) infusions/day, respectively; $p > 0.05$). However, wheel running during early abstinence, but not late abstinence, significantly reduced the number of responses during extinction testing (Figure 6a, group effect, $F_{1, 10} = 4.89$, $p < 0.05$), particularly during the first 1-hr session ($t_{10} = 2.33$, $p < 0.05$). Notably, the effects of early wheel running on extinction responding were similar to our previous findings for the effects of wheel running throughout abstinence (Lynch et al. 2010; Peterson et al. 2013; also see Figure 1 for behavioral comparison).

We also observed a robust effect of timing of wheel running availability on subsequent reinstatement responding where early, but not late, running effectively reduced cue-induced responding (Figure 6b). Specifically, a repeated measures ANOVA comparing the last extinction session to the reinstatement session revealed a significant effect of group ($F_{1, 10} = 7.72$, $p < 0.03$) and session by group interaction ($F_{1, 10} = 10.45$, $p < 0.01$). Subsequent comparison within each group revealed a significant increase in responding during cue-induced reinstatement from the last extinction session in the late, but not early, access group ($p < 0.01$ and $p > 0.05$, respectively). Although a group comparison did not reveal significant differences during the last extinction session ($t_{10} = 0.48$, $p > 0.05$), a significant effect during reinstatement was observed. Specifically, access to an unlocked running wheel for 2 hr/day during early abstinence significantly reduced responding during cue-induced reinstatement ($p < 0.03$) as compared to levels observed in the late access group. Notably, levels of wheel running did not differ between both early versus late access groups (0.43 ± 0.15 versus 0.46 ± 0.11, (km) average distance run/day, respectively; $p > 0.05$) and there was no correlation, in either early or late access groups, between levels of wheel running and total extinction ($r = 0.29$, $r = .22$; respectively) or cue-induced reinstatement ($r = 0. 33$, $r = .12$; respectively) responding. The effects of early access to a running wheel on cue-induced responding were also similar to our previous findings for the effects of wheel running throughout abstinence (Lynch et al. 2010; Peterson et al. 2013; also see Figure 1 for behavioral comparison). Specifically, our previous findings (Lynch et al. 2010; Peterson et al. 2013) demonstrated that access to a running wheel throughout abstinence reduced cue-induced responding, but failed to block the time-dependent increase in responding during cue-induced

reinstatement from the last extinction session. Together, these data indicate that timing of wheel running availability during abstinence differentially impacts cocaine-seeking during both extinction and cue-induced reinstatement testing.

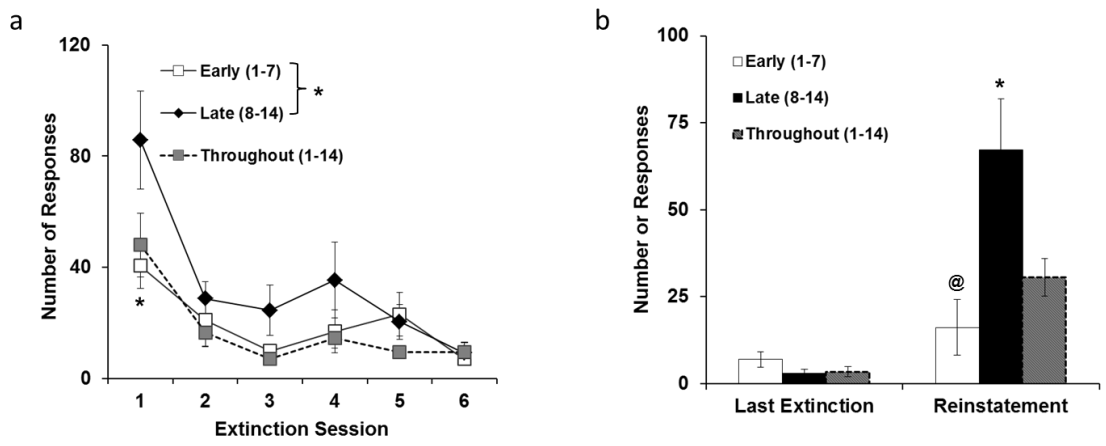

Figure 6- Time-dependent effects of wheel running on cocaine-seeking during extinction and cue-induced reinstatement. (a) Early, but not late, access to an unlocked running wheel significantly reduced responding during the first hour of extinction. Black filled squares represent the early access group (n = 6), while white filled squares represent the late access group (n = 6). An asterisk indicates a significant group effect. (b) Following the last extinction session, early, but not late, access to an unlocked running wheel significantly reduced responding during subsequent cue-induced reinstatement. Black filled bars represent the early access group (n = 6), while white filled bars represent the late access group (n = 6). An asterisk indicates a significant effect of time in responding from last extinction session to cue-induced reinstatement. The @ symbol indicates a significant difference in responding from the late access group. Error bars represent SEM.

Discussion

Exercise as a potential intervention for cocaine addiction is gaining momentum as an area of investigation in both human and animal studies (for review see Lynch et al. 2013). However, the conditions that lead to the most efficacious response have yet to be determined. Here, we demonstrated for the first time that 2 hr/day of access to an unlocked running wheel during early, but not late, abstinence from extended access cocaine self-administration attenuated subsequent cocaine-seeking during both extinction and cue-induced reinstatement testing. Notably, the protective effects of wheel running appear to be due to timing of when the wheel is provided and not environmental enrichment, given that both groups were housed in the same environmental conditions throughout abstinence. We also demonstrated that early access to a running wheel also blocked reinstatement of cocaine-seeking as evidenced by a lack of increase in responding during cue-induced reinstatement from last extinction. Together, these data indicate that the efficacy of wheel running is time-dependent and that wheel running provided during early, but not late, abstinence may provide long-term protection against cocaine relapse.

Levels of cocaine-seeking in both the absence and presence of cocaine-associated cues were altered by timing of wheel availability during abstinence. Only one other study (Smith and Witte 2012) has examined the temporal relationship between wheel running and cocaine-induced behaviors. Specifically, Smith and Witte (2012) demonstrated that access to a running wheel differentially affected cocaine self-administration when provided only before (no effect) or after (reduction) initial cocaine exposure. This finding suggests that the timing of wheel running availability differentially impacts behavioral

outcomes. In conjunction, the effects of early wheel running on extinction and cue-induced responding were similar to our previous findings for the effects of wheel running throughout abstinence (Lynch et al. 2010; Peterson et al. 2013; see Figure 1for behavioral comparison). However in this study early, but not late access, to a running wheel blocked the time-dependent increase in responding during cue-induced reinstatement from the last extinction session. Thus, our findings are quite striking given that the time from the last exercise session to extinction and reinstatement testing was much longer in the early access group. Taken together, these findings indicate the presence of a critical time period when wheel running is most efficacious in reducing cocaine-induced behaviors.

Although the exact mechanism mediating the time-dependent efficacy of wheel running on cocaine-seeking observed here remains to be determined, a possible mechanism may lay in DAergic signaling within the reward pathway. Wheel running has the ability to upregulate DA under normal conditions (Greenwood et al. 2011) and this ability may possibly be critical for cocaine self-administering animals during abstinence. Specifically, during early abstinence DAergic activity is decreased and this reduction in activity has been linked to withdrawal symptoms and craving (for review see Koob and Volkow 2010). However, as abstinence progresses there appear to be a switch to other signaling pathways (e.g. glutamatergic) outside of DA (for review see Koob and Volkow 2010). Thus, wheel running may induce time-dependent effects on cocaine-seeking through its ability to upregulate DA during early, but not late, abstinence. Another possible mechanism for the time-dependent effects of wheel running is through epigenetic regulation of brain derived neurotrophic factor (Bdnf). Evidence from both human and animal studies suggests that cocaine may induce long-lasting epigenetic

changes in Bdnf that may mediate persistent vulnerability to relapse (for review see Lynch et al. 2013). Wheel running is known to upregulate Bdnf under normal conditions with recent work showing that it does so through epigenetic mechanisms (Gomez-Pinilla et al. 2011). In support of this idea, we recently demonstrated that the efficacy of wheel running on cocaine-seeking was associated with epigenetic regulation of Bdnf exon IV expression in the prefrontal cortex (PFC) (Peterson et al. 2103; Chapter 2). Although we previously demonstrated that wheel running during abstinence decreased Bdnf exon IV expression in the PFC, we speculated that the opposite was true in which wheel running upregulated Bdnf in a time-dependent manner. Specifically, during early abstinence from chronic cocaine exposure Bdnf expression is decreased (McGinty et al. 2010) and later rebound following protracted abstinence (Sadri-Vakili et al. 2011). Thus, it is possible that wheel running during early abstinence may induced protective effects by upregulating Bdnf expression in the PFC, at a time when expression is known to be low (McGinty et al. 2010), and in doing so normalized the abnormally high expression following prolonged abstinence. This idea is supported by evidence indicating that site-specific infusion of Bdnf into the PFC during early, but not late (day 6), abstinence induced suppressive effects on cocaine-seeking (Berglind et al. 2007). Our current findings support this idea and suggest upregulation of Bdnf during early, but not late abstinence as a potential mechanism. Further work is needed to examine these possibilities and to determine the effects of wheel running in combination with direct manipulation of DA and/or Bdnf in order to establish a causal relationship. Taken together, our findings suggest that the time of exercise availability should be considered when developing exercise-based interventions for drug-dependent populations.

Chapter IV

Dose-Dependent Efficacy of Exercise to Attenuate Cocaine-Seeking: Impact of Sex and Estrous Cycle

Introduction

Despite lower rates of drug use and abuse in women than men, evidence from both clinical and preclinical studies suggests that women are not less vulnerable to cocaine addiction than men, and with regard to certain aspects of cocaine abuse, may have an augmented biological vulnerability (Lynch et al. 2002; for review see Lynch 2006). In humans, levels of cocaine craving vary across the menstrual cycle (Fox et al. 2008) with evidence to suggest that these levels are influenced by the ratio of estradiol to progesterone (Sinha et al. 2007). Similar findings have been observed in animal models in which levels of cocaine-seeking vary across the estrous cycle with the highest levels observed during estrus (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007; Feltenstein et al. 2011), a period when there are higher levels of estradiol compared to progesterone (Feltenstein et al. 2009). Results have also shown that the incubation effect, the progressive increase in cocaine-seeking over protracted abstinence (Grimm et al. 2001), is heightened and prolonged in estrus females compared to males and to females in non-estrus phases (Kersetter et al. 2008). These findings suggest sex and estrous cycle as key factors that contribute to cocaine craving and seeking in both human and animal studies, and highlight the need to examine sex-specific interventions for cocaine relapse.

Exercise may be an ideal intervention for cocaine addiction that can be tailored by sex. Although controlled studies focusing on its efficacy in preventing drug relapse are few, initial findings in both male and female illicit drug-dependent individuals, including those with cocaine dependency, are promising (Brown et al. 2010; for review see Lynch et al. 2013). Specifically, Brown and colleagues (2010) demonstrated that exercise

provided alone or as a supplement to behavioral therapy produced a better treatment outcome than behavioral therapy alone in male and female substance abusers. While beneficial effects of exercise have been observed in both illicit drug-dependent males and females, few studies have included a large enough sample size to examine sex and hormonal differences in its efficacy. Results from several studies in smokers have examined the protective effects of exercise solely in females with results suggesting limited effectiveness due to poor exercise adherence during treatment (Marcus et al. 2005; Marcus et al. 1995) and inconsistent exercise conditions measured (Bock et al. 2012; Roberts et al. 2012; Ussher et al. 2012; i.e. type, amount, and intensity). Although preliminary, results from meta-analyses on the effects of exercise in smokers suggest that its efficacy at reducing smoking and preventing relapse may be more pronounced in males than females. However, it is not clear whether these differences reflect a biological sex difference in the efficacy of exercise as a potential treatment for addiction or whether they reflect other environmental or social differences (e.g., levels of exercise, adherence).

Animal models can be used to determine the exercise conditions that most effectively reduce relapse vulnerability and determine whether these conditions differ by sex. Most preclinical studies have focused on the effects of unlimited access to a running wheel, a form of aerobic exercise that is reinforcing in rats (Greenwood et al. 2011), on concurrent cocaine self-administration (for review see Smith and Lynch, 2011). The results from these studies generally report beneficial effects of wheel running in both males and females at reducing the reinforcing effects of cocaine (Smith et al. 2008; Cosgrove et al. 2002) and decreasing cocaine-seeking (Zlebnik et al. 2010). Recently, our lab demonstrated that even short bouts of wheel running (2 hrs/day) during abstinence

effectively reduced subsequent cocaine-seeking and associated neuroadaptations (Lynch et al. 2010). This finding is important because shorter bouts of wheel running in animals may more appropriately model the limited exercise conditions engaged in by humans. Our lab has also examined wheel running under different dose conditions with evidence to suggest that the efficacy of wheel running is dose-dependent. Specifically, we demonstrated that although 1 hr/day of access to a running wheel during abstinence did not affect subsequent cocaine-seeking, 2 hr/day was effective, and 6 hr/day almost completely suppressed subsequent cocaine-seeking (Peterson et al. 2013) in males. Sex differences in the exercise conditions that produce the most efficacious response on cocaine-seeking are largely unknown, with only one study demonstrating an effect of unlimited wheel running on cocaine-primed reinstatement responding in both males and females (Smith et al. 2012). Further work is needed to determine whether the efficacy of wheel running to attenuate relapse vulnerability differs by sex and dose condition.

Here we examined sex differences in the dose-dependent effects of wheel running during abstinence on subsequent cocaine-seeking. Given findings indicating a critical role of estrous cycle phase on relapse vulnerability and treatment outcome (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007; Feltenstein et al. 2011), we also examined whether the efficacy of exercise was influenced by estrous cycle phase in freely cycling females. We hypothesized that wheel running would dose-dependently decrease subsequent cocaine-seeking in both males and females, and that its efficacy would be mediated by estrous cycle in females.

Materials and Methods

Subjects

Adult intact male (n = 89) and female (n = 82) Sprague Dawley rats weighing 380-410g and 280-310g, respectively, at the start of the study, were used in this experiment. Results from a subset of the male rats (n = 63) were published as part of another study (Peterson et al. 2013) with an additional 26 males added here that were run contemporaneously with the females in order to examine sex differences in the efficacy of exercise to reduce cocaine-seeking. Animals were housed in individual self-administration chambers (Med Associates, St. Albans, VT) with ad libitum access to food and water and maintained on a 12-h light/dark cycle (lights on at 0700). To facilitate later acquisition of cocaine self-administration, animals were trained to lever press for sucrose pellets during habituation to the operant chambers. Animals were then implanted with a jugular catheter using methods previously described (Abdolahi et al. 2010; Sanchez et al. 2013; Lynch et al. 2010) and placed back into individual operant chambers where they resided for the duration of the self-administration procedure. General health was monitored daily and animals were weighed every other day. All procedures were approved by the Animal Care and Use Committee at the University of Virginia and were conducted within the guidelines set by the NIH.

Drugs

Cocaine hydrochloride was obtained from National Institute of Drug Abuse with the same concentration (g/ml) maintained throughout the study. The infusion duration was adjusted three times per week based on animal body weight (2 seconds/100g body weight) to maintain a constant cocaine dose (1.5 mg/kg/infusion).

Cocaine Self-Administration

Detailed methods for cocaine self-administration were conducted using methods previously described in Chapter II of this dissertation. Briefly, Rats were trained to acquire cocaine self-administration under limited access conditions (i.e. fixed ratio 1 schedule of reinforcement; limit of 20 infusions per day). Once rats acquired this behavior (defined as two consecutive days in which all 20 infusions were obtained), they were given 24-hr extended access to cocaine (1.5 mg/kg/infusion) for 10 consecutive days under a discrete trial procedure (four trials/hour). Following the last discrete trial session, cocaine infusions were again available under a fixed-ratio 1 schedule with a maximum of 20 infusions for 2 consecutive sessions. These fixed-ratio sessions served 2 purposes: 1) to equate all groups on intake prior to abstinence and 2) to confirm catheter patency.

Experiment 1: Sex and estrous cycle-dependent effects of 2 hr/day wheel running during abstinence on subsequent cocaine-seeking

Following the last self-administration session, rats began a 14-day abstinence period with voluntary access to a running wheel that was either unlocked (males: n = 9; females: n = 12) or locked (males: n = 9; females: n = 12) for 2-hr/day. Given findings indicating a critical role of estrous cycle phase on relapse vulnerability (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007; Feltenstein et al. 2011), we monitored the estrous cycle in freely cycling females to determine whether phase influenced the efficacy of wheel running to attenuate subsequent cocaine-seeking. Estrous cycle phase was ascertained by vaginal swabbing during the last four days of abstinence and on the extinction/cue-induced reinstatement testing day. Males underwent a similar procedure to control for handling. Due to a lack of apparent behavioral difference between females in diestrus, metestrus, and proestrus phases, these females were combined and are hereafter referred to as non-estrus and were compared to females in estrus. This type of strategy of examining estrus versus non-estrus cycle phases has also been used by other groups and is based on numerous findings showing that while estrus females have higher levels of cocaine-seeking and motivation for cocaine, females during other estrous phases (i.e. metestrus, diestrus, and proestrus), do not differ from each other (Fuchs et al. 2005; Kippin et al. 2005; Feltenstein and See 2007). Estrus was categorized by the presence of predominantly non-nucleated cornified epithelial cells while non-estrus was categorized by predominantly nucleated epithelial cells, leukocytes, and necrotic epithelia (Lynch and Taylor 2004). Females verified as estrus (E) or non-

estrus (NE) had an n of 5 to 8 animals for each of the experimental groups (i.e. locked, unlocked, and polycarbonate cage).

Extinction/reinstatement testing

Detailed methods for cocaine self-administration were conducted using methods previously described in Chapter II of this dissertation. Briefly, rats were placed back into their previous self-administration chambers following the last wheel session on the 14th day of abstinence to allow for habituation. During the following day cocaine-seeking was assessed under a within-session extinction/cue-induced reinstatement paradigm. Specifically, extinction testing consisted of a minimum of 6 one hour extinction sessions and lever pressing behavior was deemed extinguished once a minimum of six extinction sessions and fewer than 15 responses/session on the cocaine-associated lever occurred. Extinction testing was then followed by a one hour reinstatement session (Peterson et al. 2013; Lynch et al 2010; Lynch et al. 2005) that began with a single presentation of cues formerly associated with the delivery of cocaine (illumination of stimulus light above the previously left (cocaine-associated) lever and sound of infusion pump).

Experiment 2: Dose, sex, and estrous cycle-dependent effects of wheel running on cocaine-seeking behavior

To determine which wheel running dose conditions produced the most efficacious response and whether there were sex differences its effectiveness, rats were given

voluntary access during the 14-day abstinence period to a running wheel for 1, 2, 6, or 24-hr each day that was either unlocked (males: n = 8, n = 9, n = 9, n = 8, respectively; females: n = 9, n = 12, n = 8, n = 8) or locked (males: n = 10, n = 9, n = 8, n = 8, respectively; females: n = 9, n = 12, n = 9, n = 8) starting at 0900 for all groups. While the locked wheel condition served as a control for potential environmental enrichment, rats under this condition were still quite active within the locked wheel (Koteja et al. 1999); thus, we added additional groups of males (n = 7) and females (n = 9) that were housed in individual polycarbonate cages without wheel attachments during the 14-day abstinence period. Estrous cycle phase was monitored using the same procedures as described above. Cocaine-seeking was assessed under the same within-session extinction/reinstatement paradigm as described above. Male and female data on wheel running, extinction and reinstatement responding from the 2-hr/day locked and unlocked wheel condition from Experiment 1 were also included in this experiment to examine dose-effect relationships.

Data analysis

The main dependent measures included daily number of infusions self-administered during the extended access cocaine self-administration period, daily distance run during abstinence, and total responding during the extinction and reinstatement sessions and were analyzing using repeated measures analysis of variance (ANOVA). The dose-effect relationship of wheel running on cocaine-seeking, as well as the effects of locked versus unlocked wheel access were different between males and females (Experiment 2) and

thus separate analyses were necessary for males versus females. In males, no significant differences on extinction and reinstatement responding were observed under the two control conditions (locked wheel at each level and polycarbonate housing) and thus we were able to collapse these data into one control condition. However, in females, the control conditions were different and thus, had to be consolidated differently. Specifically, although 1 and 2-hr/day access to a locked wheel did not differ from polycarbonate housing, both 6 and 24-hr/day condition produced a different effect from those seen under polycarbonate housing conditions. Based on these data and evidence demonstrating duration-dependent effects of locked and unlocked wheel access (i.e. short versus extended access; Greenwood et al. 2011), we collapsed the limited access locked wheel conditions (1 and 2-hr/day) and the polycarbonate condition, which did not differ, into one control group and compared these data to an extended access locked wheel condition (6 and 24-hr/day), which also did not differ, for graphical presentation and statistical analyses. These consolidations were necessary to allow for adequate power for detecting estrous cycle phase effects under control versus experimental conditions. Specifically, data from estrus females were compared to non-estrus females under 4 conditions: control, limited unlocked, extended unlocked, and extended locked. Post-hoc comparisons were made using the one-tailed t-test for all a priori predicted hypotheses using the Bonferroni correction for multiple comparisons. All data are presented as mean ± standard error of mean (SEM). Statistical analyses were performed using IBM SPSS Statistics (version 20) and alpha was set to 0.05 for all tests.

Results

Experiment 1: Sex and estrous cycle-dependent effects of 2-hr/day wheel running during abstinence on subsequent cocaine-seeking

Under extended access cocaine self-administration conditions, both males and females self-administered high levels of cocaine; however females self-administered significantly higher levels of cocaine as compared to males during the 10-day self-administration period (Figure 7a, group effect, $F_{1, 39} = 4.23$, $p < 0.05$). Cocaine intake during the 10-day extended access period did not differ between the locked and unlocked wheel groups in either males or females prior to abstinence (p's > 0.05; data not shown). During the 14-day abstinence period females given 2-hr/day access to an unlocked running wheel ran significantly greater distances than males (Figure 7b, group effect, $F_{1, 28} = 16.89$, $p < 0.01$). In females, but not males, running distances increased over the 14-day abstinence period (day by sex interaction, $F_{1, 28} = 5.38$, $p < 0.01$; females' day effect, $F_{5, 45} = 4.22$, $p < 0.001$; males' day effect, $p > 0.05$).

Although wheel running did not significantly affect total levels of responding during extinction (group effect, $F_{2, 40} = 1.98$, $p = 0.18$; data not shown), wheel running significantly attenuated reinstatement responding and this effect was more pronounced in males than in females (Figure 8a). Specifically, following the last extinction session, where responding was minimal, the presence of cocaine-associated cues induced a robust increase in responding that was significantly attenuated by 2-hr/day of wheel running (wheel condition, $F_{1, 38} = 4.96$, $p < 0.05$; wheel condition by session, $F_{1, 40} = 4.216$, $p = 0.04$). Although the overall effect of sex was not significant, planned comparison of the

effects of wheel condition within each sex revealed a significant effect within males ($F_{1, 16} = 5.05$, $p < 0.05$), but only a trend for an effect in females ($p = 0.08$). To account for the limited efficacy of wheel running on subsequent cocaine-seeking in females, we examined the influence of estrous cycle phase during reinstatement (Figure 8b). Responding during the last extinction session was similar between the estrus and non-estrus locked and unlocked wheel groups (effect of group, $p > 0.05$). Comparison during the cue-induced reinstatement session, revealed that while responding was reinstated by cocaine-associated cues in the locked wheel condition, it was reduced by unlocked wheel access in non-estrus, but not estrus females (wheel condition, $F_{1, 14} = 5.76$, $p < 0.05$; wheel condition by session, $F_{1, 14} = 8.94$, $p < 0.05$; estrous phase by wheel condition, $F_{1, 18} = 7.64$, $p < 0.05$). These findings suggest that while even modest exercise in males has protective effects, in females, its beneficial effects are negated when testing occurs during estrus. In the next experiment, we examined dose-dependent effects of exercise in both sexes and sought to determine whether higher 'doses' of exercise could surmount the effects of estrus on cocaine-seeking in females.

Experiment 2: Dose, sex, and estrous cycle-dependent effects of wheel running on cocaine-seeking behavior

As in Experiment 1, cocaine intake under extended access conditions was higher in females across all groups as compared to their male counterparts (58 ± 6 versus 66 ± 8 infusions/day, respectively, $t\ 18 = 3.43$, $p < 0.05$). Intake during the 10-day extended access period was similar within males and females for all experimental groups (1, 2, 6,

and 24-hr/day locked and unlocked wheel, and polycarbonate cage; p's>0.05; data not shown). Although levels of running were variable in both sexes, significant dose-dependent differences in levels of running were observed in both males (Figure 9a, effect of group, $F_{2, 34} = 5.48$, $p < 0.05$) and females (Figure 9b, effect of group, $F_{2, 34} = 7.71$, $p < 0.05$). Specifically, in males significant differences were observed between the 24-hr versus 6-hr groups ($p < 0.05$), 24 hr versus 1 hr and 2 hr groups ($p < 0.05$, both comparisons), and 6-hr versus the 1-hr and the 2-hr ($p < 0.05$ for both comparisons). No differences were observed between the 1 and 2-hr conditions ($p > 0.05$) in males. In females, significant differences were observed between the 24-hr versus 6-hr groups ($p < 0.05$), 24-hr versus 1 hr and 2-hr groups ($p = 0.04$ both comparisons), and 6-hr versus the 1 hr and the 2-hr groups ($p < 0.05$, both comparisons). No significant differences between the 1 and 2-hr groups ($p > 0.05$) in females.

Similar to Experiment 1 there was no effect of wheel running, at any dose condition, on subsequent responding during extinction (group effect, $F_{3, 42} = 1.98$, $p = 0.15$; data not shown). However, during cue-induced reinstatement testing, differences in the efficacy of wheel running were observed that were modulated by sex, estrous cycle, and dose of wheel access (Figures 10a and 10b). In males, responding was significantly higher during the cue-induced reinstatement session as compared to the last extinction session (Figure 10a, effect of session, $F_{1, 55} = 151.30$, $p < 0.001$) and wheel running dose-dependently attenuated this effect (effect of group, $F_{4, 55} = 13.20$, $p < 0.001$; interaction of session by group, $F_{4, 55} = 17.71$, $p < 0.001$). Post-hoc comparison within each group revealed that responding was significantly higher than the last extinction session in each group (control, $p < 0.001$; 1-hr unlocked, $p < 0.001$; 2-hr unlocked, $p = 0.001$; 6-hr, $p <$

0.05) except under the 24-hr/day unlocked wheel condition ($p > 0.05$). Although no effect of group was observed within the last extinction session ($p > 0.05$), a significant effect of group was observed within the reinstatement session ($F_{4, 55} = 16.58$, $p < 0.001$) with post-hoc comparison revealing that each of the unlocked wheel conditions, except the 1-hr-day condition ($p > 0.05$), reduced reinstatement responding as compared to control (2-hr, $p < 0.05$; 6-hr, $p < 0.001$; 24-hr, $p < 0.001$). Further comparison between each of the unlocked wheel groups revealed a significant difference between the 1-hr/day group and all other groups (p's < 0.05), and between the 2-hr/day group as compared to both the 6-hr/day and 24-hr/day groups (p's < 0.05).

Females also responded significantly more during cue-induced reinstatement as compared to the last extinction session (Figure 10b left, effect of session, $F_{1, 70} = 146.79$, $p < 0.001$), but in contrast to males, effects in females were observed under both the locked and unlocked wheel conditions, and these effects depended on estrous cycle phase. Repeated measures ANOVA revealed overall effects of housing ($F_{3, 70} = 5.52$, $p < 0.01$) and estrous phase ($F_{1, 70} = 18.67$, $p < 0.001$), as well as interactions of session by housing ($F_{3, 70} = 6.60$, $p < 0.001$), session by estrous phase ($F_{1, 70} = 19.62$, $p < 0.001$), session by estrous phase by housing ($F 3, 70 = 3.65$, $p < 0.05$), and a trend for an interaction of estrous phase by housing ($p=0.054$). Subsequent analysis within the locked wheel/control condition revealed that levels of responding were higher during the reinstatement session as compared to the last extinction session (Figure 10b left; effect of session, $F_{1, 40} = 62.48$, $p < 0.001$), particularly during estrus and under the control conditions (effect of estrous phase, $F_{1, 40} = 5.70$, $p < 0.05$; effect of housing, $F_{1, 40} = 11.64$, $p < 0.001$; interaction of estrous phase by session, $F_{1, 40} = 5.05$, $p < 0.05$;

interaction of session by housing, $F_{1, 40} = 17.80$, $p < 0.001$). Post-hoc comparison revealed significantly higher levels of responding during the reinstatement session as compared to the last extinction session for each group (control estrus, $p < 0.001$; control non-estrus, $p < 0.001$; extended locked estrus, $p = 0.001$; extended locked non-estrus, $p < 0.05$). Although no significant effects were observed within the last extinction session ($p > 0.05$), significant effects of both group and estrous phase were observed within the reinstatement session ($F_{1, 55} = 15.34$, $p < 0.001$; $F_{1, 40} = 5.68$, $p < 0.05$, respectively). Specifically, higher levels of reinstatement responding were observed in estrus females as compared to non-estrus females ($p < 0.05$), and these levels were reduced by extended access exposure to a locked wheel (compared to control estrus, $p < 0.05$). Reinstatement responding was also decreased in non-estrus females given extended access to a locked wheel as compared to their control counterparts ($p < 0.05$).

Within the unlocked wheel condition we also observed a significant increase in responding during cue-induced reinstatement testing as compared to responding during the last extinction session (Figure 10b right, effect of session, $F_{1, 30} = 79.73$, $p < 0.001$). Repeated measures ANOVA revealed an overall effect of estrous phase ($F_{1, 30} = 12.76$, $p = 0.01$), as well as interactions of estrous phase by housing, ($F_{1, 30} = 5.59$, $p < 0.05$), session by estrous phase ($F_{1, 30} = 14.24$, $p = 0.01$), and session by estrous phase by housing ($F_{1, 30} = 6.66$, $p < 0.05$). Post-hoc comparison revealed significantly higher levels of responding during the reinstatement session as compared to the last extinction session for each group (limited unlock estrus, $p < 0.001$; limited unlock non-estrus, $p = 0.05$; extended unlocked estrus, $p=0.01$; extended unlocked non-estrus, $p < 0.05$). Although no significant effects were observed within the last extinction session ($p >$

0.05), significant effects of estrous phase and an interaction of housing by estrous phase were observed within the reinstatement session ($F_{1, 30} = 14.08$, $p = 0.01$; $F_{1, 30} = 6.38$, $p < 0.05$, respectively). Specifically, reinstatement responding was significantly attenuated in non-estrus females given either limited or extended access to an unlocked wheel as compared to those in estrus who received only limited access to an unlocked wheel ($p < 0.001$ and $p < 0.01$; respectively). Notably, we also observed a significant attenuation in cocaine-seeking in estrus females given prior extended unlocked wheel access as compared to estrus females given prior limited unlocked wheel access ($p < 0.05$).

Together, these results suggest that in males longer access to an unlocked wheel (2, 6, and 24-hr/day) results in a greater attenuation of cocaine-seeking than short (1-hr/day) or no running wheel access. In females, the dose-effect relationship was not as straightforward with results showing beneficial effects of both unlocked and locked wheel access, particularly under extended access conditions. The beneficial effect of limited wheel running was only observed in non-estrus females, while extended access to an unlocked wheel was necessary to surmount estrus-induced increases in cocaine-seeking.

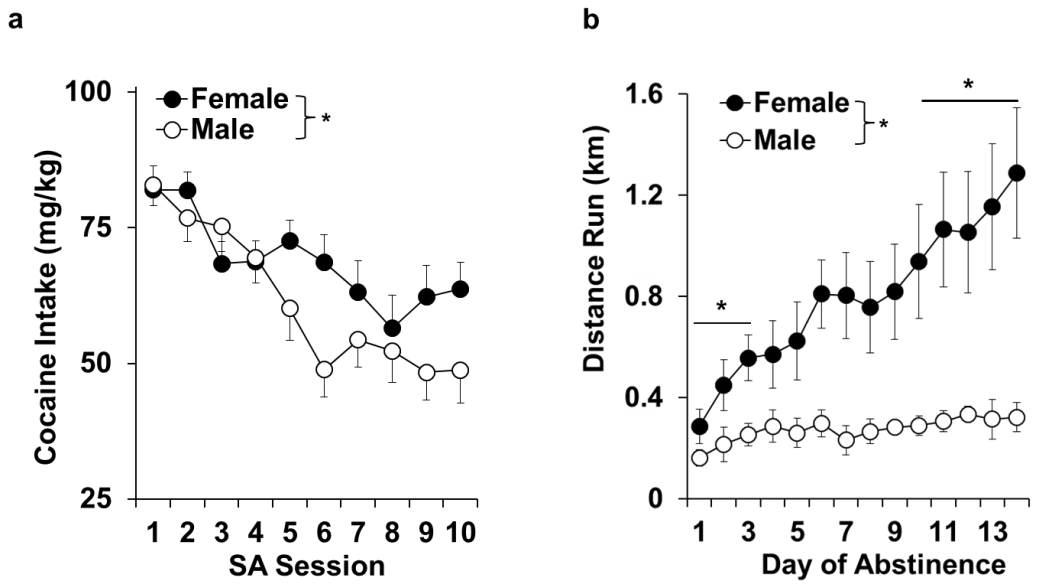

Figure 7- Sex differences in extended access cocaine self-administration and distance run over a 14-day abstinence period (Experiment 1). (a) Number of cocaine infusions self-administered (mean ± SEM) is plotted for each of the 10 days of the extended access period. The open circles represent male data points (n = 18) and filled circles represent female data points (n = 24). (b) Levels of wheel running are plotted for each of the 14 days during the abstinence period. The open circles represent male data points (n = 18) and filled circles represent female data points (n = 24). An asterisk indicates a significant group difference ($p < 0.05$). A bar and * indicates a significant effect of session. Error bars represent SEM.

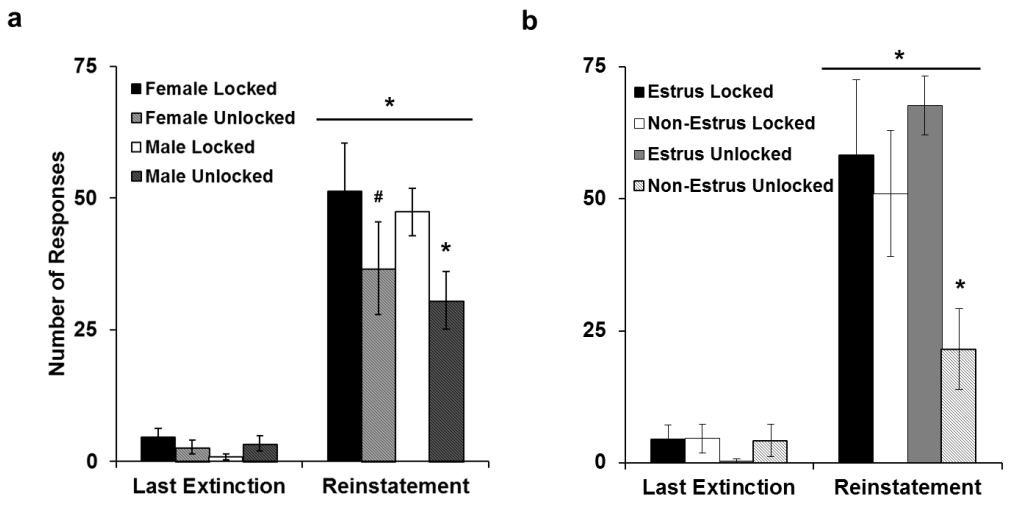

Figure 8- Sex and estrous cycle mediate the protective effects of wheel running on subsequent cocaine-seeking. (a) Number of responses (mean ± SEM) plotted during last extinction and cue-induced reinstatement sessions in males and females. Black bar represents the female locked group, dark grey bar represents female unlocked group, white bar represents male locked group, and light grey bar represents male unlocked group. (b) Number of responses (mean ± SEM) plotted during last extinction and cue-induced reinstatement sessions in estrus versus non-estrus females. Black bar represents the estrus locked group (n = 5), white bar represents non-estrus locked group (n = 6), dark grey bar represents estrus unlocked group (n = 7), and light grey bar represents non-estrus unlocked group (n = 6). An asterisk indicates a significant group difference ($p < 0.05$) and # symbol indicates a trend for an effect ($p < .10$). A bar and * indicates a significant effect of session. Error bars represent SEM.

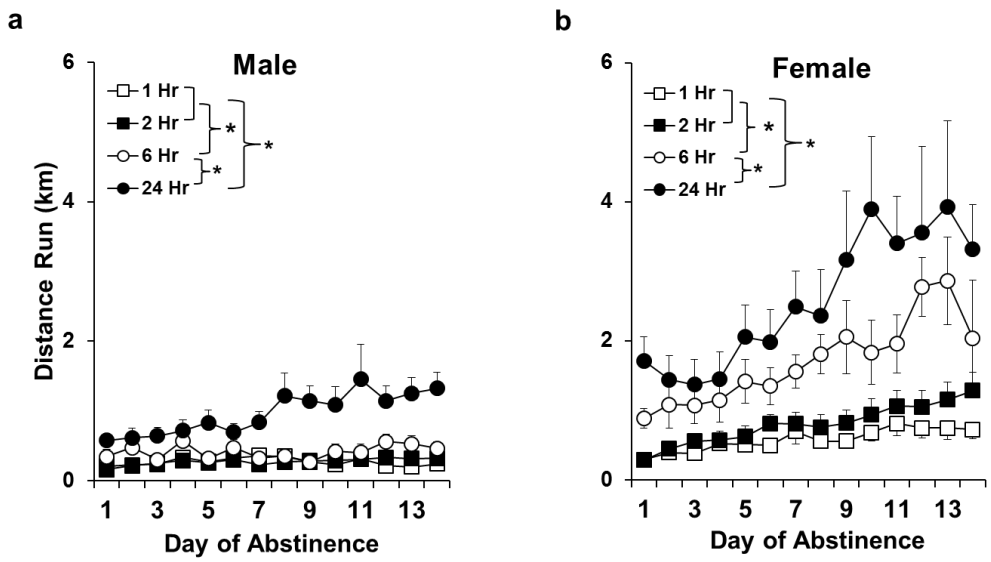

Figure 9- Wheel running dose-dependently affects levels of wheel running in both male (n = 34) and female (n = 37) rats. (left panel) Levels of wheel running are plotted for each of the 14 days during the abstinence period in males. The open squares represent male data points for the 1-hr/day group, filled squares represent male data points for the 2-hr/day group, open circles represent male data points for the 6-hr/day group, and filled circles represent male data points for the 24-hr/day group. (right panel) Levels of wheel running are plotted for each of the 14 days during the abstinence period in females. The open squares represent female data points for the 1-hr/day group, filled squares represent female data points for the 2-hr/day group, open circles represent female data points for the 6-hr/day group, and filled circles represent female data points for the 24-hr/day group. An asterisk indicates a significant group difference ($p < 0.05$). Error bars represent SEM.

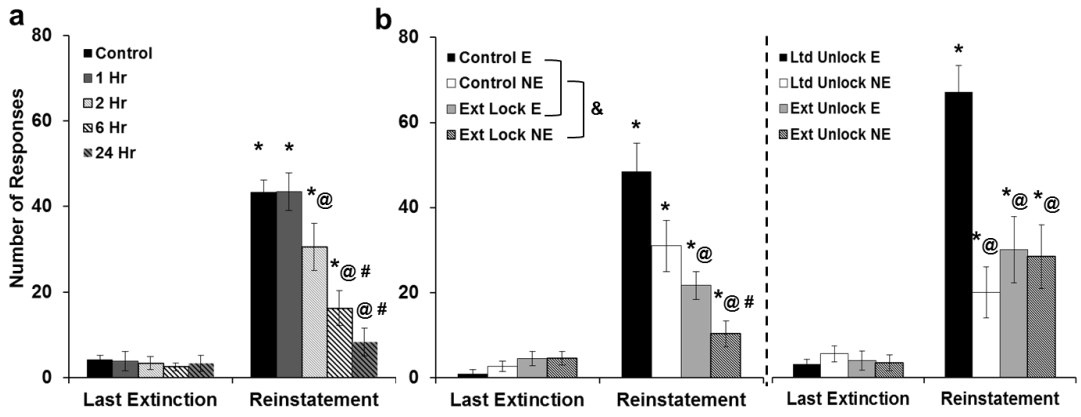

Figure 10- Sex, estrous cycle, and dose-dependent effects of exercise on subsequent cocaine seeking in male (n = 34) and female (n = 37) rats. (a) Number of responses (mean ± SEM) plotted during last extinction and cue-induced reinstatement sessions in males under different control and wheel conditions. Black bar represents the control group, dark grey bar represents male 1-hr/day unlocked wheel group, light grey bar represents male 2-hr/day unlocked wheel group, white and black striped bar represents male 6-hr/day unlocked group, and the grey and black striped bar represents male 24 hr/day unlocked wheel group. An * indicates a significant effect of session, the @ symbol indicates a significant difference from the 1-hr unlocked wheel group, and the # symbol indicates a significant difference from 2-hr unlocked wheel group. (b) Number of responses (mean ± SEM) plotted during last extinction and cue-induced reinstatement sessions in females under different control and wheel conditions by estrous cycle phase. Left panel: Black bar represents the estrus control group, white bar represents non-estrus control group, light grey bar represents estrus extended locked wheel group, and the white and black striped bar represents non-estrus extended locked group. Right panel:

Black bar represents the estrus limited unlocked wheel group, white bar represents non-estrus limited unlocked wheel group, light grey bar represents estrus extended unlocked wheel group, and the white and black striped bar represents non-estrus extended unlocked group. Left panel (10b): An * indicates a significant effect of session, the & symbol indicates a significant difference between estrus and non-estrus locked/ control groups, the @ symbol indicates a significant difference from control estrus group, and the # symbol indicates a significant difference from the extended lock estrus group. Right panel (10b): An * indicates a significant effect of session, the @ symbol indicates a significant difference from the limited estrus group. Error bars represent SEM.

Discussion

Despite the inclusion of female participants in human laboratory studies examining the efficacy of exercise to reduce cocaine relapse vulnerability, few studies have included enough females to examine sex and hormonal influences on its efficacy. Animal studies have recently begun to examine the effects of wheel running, a preclinical model of exercise, on cocaine-seeking in females (Smith et al. 2012); however, there is a lack of direct evidence demonstrating which exercise conditions produce the greatest reduction in relapse vulnerability and if these beneficial effects differ by sex and estrous cycle. Thus, the goal of the present study was to examine sex differences in the dose-dependent effects of wheel running during abstinence on subsequent cocaine-seeking in males and females. Additionally, given findings indicating a critical role of estrous cycle phase on relapse vulnerability and treatment outcome (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007; Feltenstein et al. 2011), we also examined whether the protective effects of wheel running were influenced by estrous cycle phase in freely cycling females. Our study is the first to demonstrate that although females out ran males during 2-hr/day access to an unlocked running wheel, males were more sensitive to the beneficial effects of running at this modest dose condition and subsequently responded less during cue-induced reinstatement (Experiment 1). Notably, 2-hr/day of unlocked wheel running during abstinence was effective in females, but only during non-estrus (Experiment 1) demonstrating that estrous cycle phase influences the beneficial effects of wheel running in females. Consistent with our previous findings (Peterson et al. 2013) we observed a dose-dependent effect of wheel running on subsequent cocaine-seeking in males (Experiment 2) in which 1-hr/day of access to a running wheel did not affect

subsequent cocaine-seeking, 2-hr/day was effective, and 6 and 24-hr/day almost completely suppressed subsequent cocaine-seeking. In contrast, in females, the dose-dependent effects of exercise were influenced by estrous cycle phase in which extended access, but not limited access to a running wheel attenuated estrus-induced increases in cocaine-seeking. Surprisingly, we also observed in females a dose-dependent effect of the locked wheel condition that was also influenced by estrous cycle phase suggesting that in contrast to males, females are sensitive to the presence of the wheel itself (Experiment 2).

Despite lower levels of running than females, males were more sensitive to the beneficial effects of a modest bout of running (2-hr/day). Specifically, males given access to an unlocked wheel for 2-hr/day responded less than their male counterparts that received access to a locked wheel under the same dose conditions (Experiment 1). This finding is consistent with previous reports demonstrating that wheel running has the ability to decrease cocaine's reinforcing effects (Cosgrove et al. 2002; Smith et al. 2008) and cocaine-seeking when given under limited (2-hr/day) access conditions (Lynch et al. 2010). Although 2-hr/day of wheel running during abstinence tended to be effective overall in females, subsequent examination revealed that levels of cocaine-seeking decreased from locked wheel controls but only when females were in a non-estrus phase during reinstatement testing (Experiment 1). This finding suggests that in females the beneficial effects of a modest bout of wheel running is modulated by estrous cycle phase and that during reinstatement testing estrus can negate the protective effects provided by 2-hr/day access to an unlocked wheel.

Consistent with our hypothesis, we also observed sex differences in the dose-dependent effects of wheel running on subsequent cue-induced reinstatement responding.

In males, we observed that longer access (i.e. 2, 6, and 24-hr/day groups; Experiment 2) to an unlocked running wheel progressively decreased responding during reinstatement testing. This effect in males does not appear to be attributable to environmental enrichment, given that male rats in the locked and unlocked wheel groups were exposed to the same environmental conditions and only an effect was observed in the unlocked wheel group. In contrast, estrous cycle phase modulated the protective effects of both locked and unlocked wheel access at higher dose conditions (i.e. extended access; Experiment 2) in females. During reinstatement testing, females in non-estrus phases responded less following limited unlocked wheel access and extended access to both locked and unlocked wheels. Notably, extended access to both locked and unlocked running wheels provided beneficial effects in estrus females and demonstrated that longer access conditions can block estrus-induced increases in cocaine-seeking. Taken together, these findings indicate sex differences in the efficacy of wheel running on cue-induced reinstatement that are dose-dependent and support the hypothesis that its efficacy is mediated by estrous cycle in females.

Females appeared to receive beneficial effects from the presence of the wheel itself with results showing a decrease in cocaine-seeking following extended access to a locked wheel. One possibility to account for this effect is that the locked wheel condition permits limited physical activity within the wheel and thus may serve as a modified exercise condition. To support this notion Koteja and colleagues (1999) reported that within the locked wheel condition animals are still able to be active and readily engage in climbing and hanging activities. Additionally, the greater levels of running observed in females suggest that females are more active than males. Thus, females may have been

more active in the locked wheel condition than their male counterparts and quite possibly more sensitive to this alternative form of exercise. Another possibility is that the locked wheel condition provided environmental enrichment in which females, as compared to males, appear to be more sensitive to the beneficial effects (for review see Girbovan and Plamondon 2013). Further studies are necessary to examine these possibilities and to distinguish the beneficial effects of environmental enrichment versus different types of exercise (wheel running versus climbing/hanging) on cocaine-seeking in females. To circumvent the potential confound of the locked wheel, the present study added a polycarbonate cage condition to control for exercise. Our results suggest that the polycarbonate cage condition may be a more appropriate control for exercise as compared to the locked wheel. This notion is supported by the observation that females were more active during longer access to a locked wheel, which may in fact potentially comprise the main experimental manipulation of the study (i.e. physical activity). Thus, future studies should also include a polycarbonate cage condition when the main experimental variable is physical activity.

Cocaine-seeking has been shown to vary across the estrous cycle in females with reports demonstrating that those in estrus have higher responding in the presence of a priming injection of cocaine (Kippin et al. 2005; Kerstetter et al. 2008; Feltenstein and See 2007), stressor or cocaine associated cues (Feltenstein et al. 2011). In the present study we observed that estrus-induced increases in cue-induced reinstatement responding is decreased following extended access to either a locked or unlocked wheel during abstinence. This finding suggests that higher “doses” of exercise and/or environmental enrichment are required to compensate for hormonal influences during estrus (i.e. high

estrogen levels as compared to progesterone; Feltenstein et al. 2009) that would normally potentiate responding. In contrast, non-estrus females given prior limited access to a running wheel or extended access to a locked wheel demonstrated that less physical activity is required to reduce cocaine-seeking. Further studies are needed to determine the effects of exercise in combination with direct manipulation of hormones in order to establish a causal relationship.

Taken together, our findings suggest differences in the efficacy of exercise that are dose, sex, and estrous cycle-dependent. Given that an alternative form of exercise provided by the locked wheel appeared to be beneficial in females, future studies in humans should examine the use of aerobic versus non-aerobic exercise as an intervention for drug relapse. One caveat to our findings is that it may be difficult in cocaine-dependent individuals to regularly adhere to even modest levels of exercise. However, initial findings in humans are promising among illicit drug-dependent individuals (Brown et al. 2010). Our findings demonstrating a robust impact of estrous cycle phase on reinstatement responding in females suggests that hormones may play a crucial role on treatment outcomes. Thus, future studies should examine the addition of a hormonal supplement paired with exercise as a potential sex-specific intervention for illicit drug-dependent females.

Chapter V

General Discussion and Future Directions

Dissertation Summary

Although there are no approved pharmacotherapies for cocaine addiction, this dissertation proposes exercise as a novel intervention that has the ability to reduce relapse vulnerability and activate the same reward pathways (e.g. DA, Bdnf) as cocaine. To examine the potential efficacy of exercise for relapse prevention I examined the effects of wheel running, an animal model of exercise, on subsequent cocaine-seeking and associated neuroadaptations (i.e. *Bdnf exon IV* expression) in the PFC. This dissertation indicates that multiple factors (e.g. timing of availability during abstinence, dose conditions, and sex) impact the efficacy of wheel running on subsequent cocaine-seeking and that its effects are associated with epigenetic regulation of *Bdnf exon IV* expression in the PFC. Overall, this dissertation establishes exercise as a promising intervention that has the ability to reduce cocaine relapse vulnerability by blocking cocaine-induced neuroadaptations (e.g. Bdnf) that develop over an abstinence period.

Role of *Bdnf* Mediating the Efficacy of Exercise

In chapter II of this dissertation, I sought to determine the most efficacious dose of wheel running on subsequent cocaine-seeking and determine whether its efficacy would be associated with neuroadaptive changes in *Bdnf exon IV* expression in the PFC. My results indicated that in males longer access to a running wheel produced the greatest suppression in cocaine-seeking and that its efficacy was associated with epigenetic regulation of chromatin containing the *Bdnf* gene. The most important finding was that wheel running had the ability to normalize abnormally high levels of Bdnf expression

that is characteristic of prolonged abstinence. These data appeared to be in contrast to previous work demonstrating that wheel running has the ability to upregulate *Bdnf* expression and protein levels in cortical regions including the PFC (Chen et al. 2012; Quire et al 2012). Based on the current results and previous work demonstrating suppression of cocaine-seeking when site-specific infusion of Bdnf into the PFC occurs during early, but not late, abstinence (Berglind et al. 2007) we speculated that the efficacy of wheel running was likely mediated by time-dependent alterations in *Bdnf* expression. Specifically, we proposed that wheel running provided during early abstinence may restore the decrease in *Bdnf exon IV* expression during early abstinence, and in doing so, normalize *Bdnf exon IV* transcription observed following protracted abstinence (see Figure 11 for model). In support of this possibility we also demonstrated that treatment of sodium butyrate, a HDAC inhibitor that, like exercise, modulates gene transcription, including Bdnf exon IV expression, provided beginning during early abstinence mimicked both the behavioral and molecular effects of wheel running. This finding is consistent with other reports showing beneficial effects of HDAC inhibition in reducing cocaine-seeking and extinction of conditioned place preference for cocaine (Malvaez et al. 2010; 2013) and further suggests epigenetic modification of chromatin containing the *Bdnf* gene during early abstinence as a possible mechanism.

Due to the results obtained in chapter II of this dissertation and previous work demonstrating time-dependent behavioral outcomes following site-specific infusion of Bdnf into the PFC (Berglind et al. 2007), we explored the efficacy of wheel running when provided during early versus late abstinence (chapter III). The results indicated that early, but not late, access to a running wheel during abstinence reduced levels of

responding during both extinction and cue-induced reinstatement testing. These data also provided further support for time-dependent alterations in *Bdnf* expression as a potential mechanism mediating the efficacy of wheel running. Based on chapters II and III it appears that wheel running is effective in reducing relapse vulnerability, but that its efficacy is dependent on timing of availability and dose condition. Future studies need to examine the effects of wheel running paired with direct manipulation of *Bdnf* in the PFC to establish a causal relationship. Taken together, these data implicate that exercise as an intervention may be effective in drug-dependent individuals, but that exercise conditions (i.e. type, amount, and intensity) and timing of when exercise is given during abstinence be carefully considered.

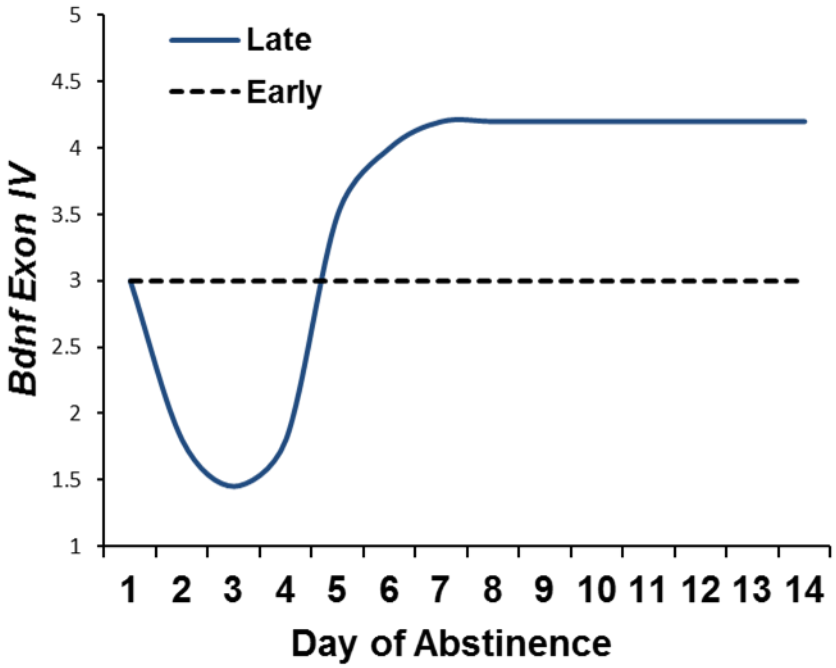

Figure 11- A putative model for the time course of Bdnf exon IV expression mediating the efficacy of exercise during early abstinence. As indicated by our model, exercise provided during late abstinence is not effective because like sedentary conditions it does not block the down-regulation in Bdnf signaling that occurs during early abstinence.

Sex and Estrous Cycle Dependent Influences the Efficacy of Exercise

In chapter IV of this dissertation I sought to examine the impact of sex, dose condition, and estrous cycle phase on the efficacy of cocaine-seeking. Specifically, I demonstrated that although females ran more than males, males were more sensitive to the beneficial effects of running and showed a robust and dose-dependent decrease in cocaine-seeking with longer access resulting in greater suppression. I also demonstrated that limited access to a running wheel during abstinence was effective in females but only when females were in a non-estrus phase during reinstatement testing. This finding suggests that in females the beneficial effects of a modest bout of wheel running is modulated by estrous cycle phase and that during reinstatement testing estrus can negate the protective effects provided by limited access to an unlocked wheel. Notably, we also observed that extended access (i.e. 6 and 24 hr/day) to an unlocked wheel reduced levels of cocaine-seeking in estrus females suggesting that longer access conditions can surmount estrus-induced relapse vulnerability. One interesting finding from this study was that in females there appeared to be a dose-dependent effect of the locked wheel condition that was also influenced by estrous cycle phase, suggesting that in contrast to males, females are sensitive to the presence of the wheel itself.

The finding that females, but not males, were sensitive to the protective effects of the locked wheel may be attributable to sex differences in activity within this condition. This possibility is supported by our current findings in which females displayed greater levels of running and suggest that females were more active than males in the locked wheel. To support this notion Koteja and colleagues (1999) reported that within the locked wheel condition animals are still able to be active and readily engage in climbing

and hanging activities. Thus, females may have been more active in the locked wheel condition than their male counterparts and quite possibly more sensitive to this alternative form of exercise. Another possibility is that the locked wheel condition provided environmental enrichment in which females, as compared to males, appear to be more sensitive to the beneficial effects (for review see Girbovan and Plamondon 2013). Further studies are necessary to examine these possibilities and to distinguish the beneficial effects of environmental enrichment versus different types of exercise (wheel running versus resistance training) on cocaine-seeking in females. Taken together, the data in chapter IV implicate that male drug-dependent individuals may be more sensitive to the beneficial effects of exercise than compared to females. These data also implicate that exercise paired with a hormonal supplement (i.e. progesterone) or alternative forms (i.e. yoga, resistance training) be considered when developing exercise-based interventions in female drug-dependent populations. Overall, the data presented in chapter IV imply that sex and hormonal status greatly impact the efficacy of exercise and suggests that exercise-based interventions be tailored by sex.

Proposed Summary Model for the Differing Efficacy of Exercise on Cocaine Relapse

Based on the data gathered in Chapter II it appears that the effects of exercise are dose-dependent with greatest efficacy observed following longer access (6 and 24 hr/day) conditions. However, in this chapter we reported that while wheel running reduced Bdnf exon IV expression at all access conditions including the 1 hr/day condition, a minimum of 2 hr/day access was required to suppress cocaine-seeking. Although the reason for this

discrepancy is not clear, one possibility is that normalization of both *Bdnf exon IV* expression and of the withdrawal circuitry, through activation of DAergic neurons (Goggi et al. 2003), may be required to attenuate cocaine-seeking. Thus, low access wheel running may normalize *Bdnf exon IV* expression in the PFC, but may not adequately affect DA in the nucleus accumbens, whereas, longer wheel access normalizes both *Bdnf exon IV* expression and DAergic signaling in the mesolimbic system (see Figure 12 for model).

The interpretation for the effects of wheel running on subsequent cocaine-seeking was less straightforward in females and its efficacy appeared to be influenced by estrous cycle phase. Although chapter IV did not report any underlying neurobiology mediating the efficacy of wheel running, it is possible that both DAergic and Bdnf signaling regulate its efficacy in females similarly to those observed in males. Specifically, we propose that the neuro-protective benefits of wheel running in females is in part due to its ability to block the time-dependent decrease in Bdnf exon IV expression observed during early abstinence. This dissertation also proposes that wheel running during abstinences has the ability to block the decrease in DAergic signaling in the mesolimbic reward pathway that during early abstinence and later becomes sensitized during prolonged abstinence (Rossetti et al. 1992) in males and females (see figure 12 for model). However, the neuroadaptative effects of exercise on DAergic and Bdnf signaling appears to be overridden by estrus in rodent females during cue-induced reinstatement. Specifically, we demonstrated that extended, but not limited access to a wheel surmounted the heightened vulnerability observed in females during estrus. Thus, under limited access conditions changes in DAergic and Bdnf signaling during abstinence may

possibly be insufficient to provide sustained neuo-protection when a female is in estrus during reinstatement testing. In contrast, we observed beneficial effects of wheel running during both limited and extended access conditions in non-estrus females. These findings indicate that in females neuroadaptive benefits of wheel running during abstinence, that are mediated through both DAergic and Bdnf signaling, may be surmounted by estrus phase during reinstatement testing in females. Further work is required to examine these possibilities and to determine a causal link between hormonal status, exercise efficacy, and Bdnf signaling.

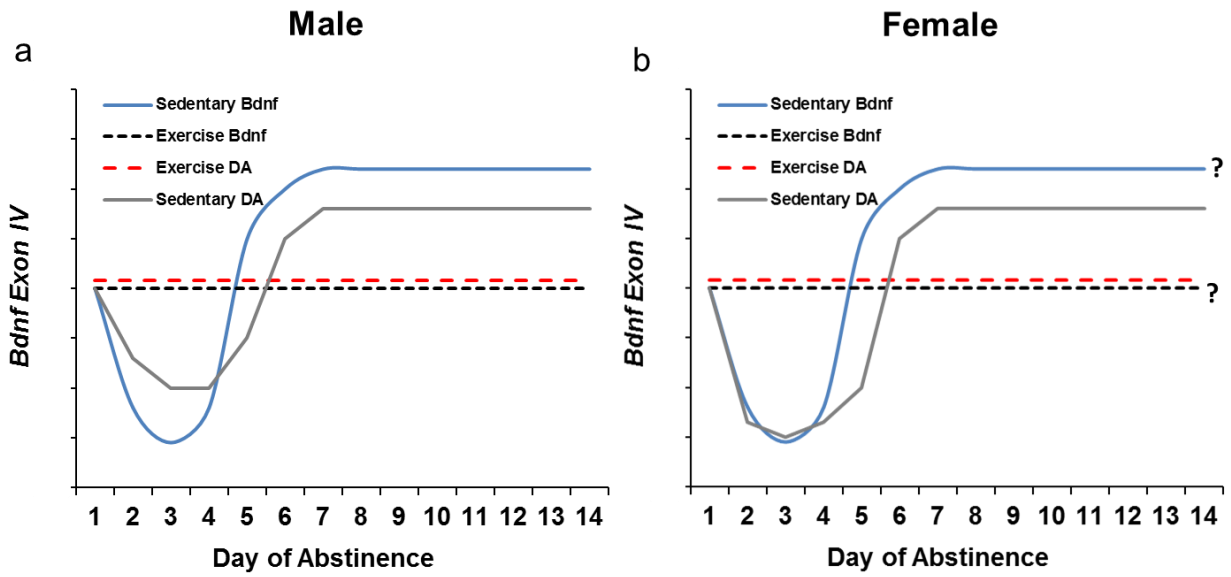

Figure 12- Possible underlying neurobiology mediating the dose, sex, and estrous cycle-dependent effects of exercise on subsequent cocaine-seeking. (a) Male model. Wheel running during early abstinence blocks the downregulation in Bdnf exon IV expression and leads to a normalization of expression following prolonged abstinence, under all dose conditions. However, under the 1 hr/day unlocked wheel conditions we did not observe an effect on levels of cocaine-seeking, thus we proposed the involvement of DAergic signaling within the mesolimbic reward pathway. Specifically, during early abstinence DAergic signaling is reduced during early abstinence (i.e. days 1-7) and later becomes sensitized during prolonged abstinence (Rossetti et al. 1992). Thus, wheel running under the 1 hr/day dose condition, as compared to extended access conditions (2, 6, and 24 hr/day), may not have adequately upregulated DAergic signaling during abstinence and caused this condition to be ineffective. (b) Female model. We propose that possibly the neuro-protective benefits of wheel running in females is in part due to its ability to block

the time-dependent decrease in Bdnf exon IV expression observed during early abstinence (denoted as ?).We propose that in females wheel running also has the ability to upregulate DAergic signaling during abstinence similiarly to that observed in males. However in females, as compared to males, there is a larger decrease in DA during abstinence (Becker and Hu 2008) and this finding may possibly account for the requirement of longer (6 and 24 hr/day in females as compared to 2 hr/day in males) wheel running access being necessary to produce beneficial effects. Additionally, it is possible that the neuroprotective benefits (i.e. changes in Bdnf and DAergic signaling) observed during extended access wheel running is required to surmount estrus-induced vulnerability during reinstatement testing.

Future Directions

Few studies have examined sex differences in the effects of treatment outcomes for cocaine addiction. Although this dissertation establishes sex and hormonal effects on the efficacy of exercise, we did not demonstrate the underlying neurobiology mediating its efficacy in females. Thus, there is a gap in our understanding of potential sex differences in the neurobiological processes mediating the efficacy of exercise. Based on our current results demonstrating sex differences in the efficacy of exercise, it is possible that there are sex differences in the neurobiology that mediate its efficacy. Given that wheel running during abstinence induced normalization of PFC *Bdnf* expression in males, it is possible that the same mechanism may be involved in females. Consistent with this possibility is previous work indicating sex differences in *Bdnf* expression that were regulated by sex and ovarian hormones (i.e. estrogen, for review see Harte-Hargrove LC et al. 2013). Further support for this idea is observed in recent data showing that paternal cocaine self-administration results in epigenetic modification of chromatin containing the Bdnf gene and resistance to cocaine reinforcement in male, but not female, offspring (Vassoler et al. 2013). Thus, future studies should compare the effects of exercise on cocaine-induced alterations in *Bdnf* expression in both males and females to better understand the neurobiological processes mediating its protective effects.

Despite the inclusion of female participants in human laboratory studies examining the efficacy of exercise to reduce cocaine relapse vulnerability, few studies have included enough females to examine sex and hormonal influences on its efficacy. This dissertation revealed that the efficacy of exercise is dependent on sex and estrous cycle phase. One important result observed was that estrus blocked the protective effects

of wheel running when provided during a modest dose condition and suggests that hormonal status influences treatment outcomes. In support of this idea previous work has demonstrated that estrogen increased vulnerability during reinstatement testing conditions and indicates a critical role for hormones in relapse vulnerability (Kerstetter et al. 2008). In contrast, progesterone has been shown to inhibit vulnerability with results showing that it counters estradiol-induced vulnerability under reinstatement testing conditions (for reviews see Anker and Carroll 2010; Carroll and Anker 2009, and Evans 2007). This finding is consistent with our current results demonstrating an effect in non-estrus females that received modest access to an unlocked, but not locked, running wheel during abstinence. Thus, future studies should examine the addition of a hormonal supplement (i.e. progesterone) paired with exercise as a potential sex-specific intervention for illicit drug-dependent females.

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