EFFECTS OF WHEEL RUNNING ON COCAINE SEEKING IN RATS: BEHAVIOR AND NEUROBIOLOGY

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Abstract

Cocaine addiction is a pervasive public health problem, but currently there are no highly effective treatments to reduce its extent or duration. Emerging research in humans and animals suggests that aerobic exercise may decrease drug use and prevent relapse. This set of experiments focused on the use of exercise as a behavioral treatment for cocaine addiction using rodent (rat) models of relapse. Concurrent access to a voluntary running wheel decreased reinstatement of cocaine-seeking behavior in response to a cocaine injection (Experiments 1-3), cocaine-paired cues (Experiment 2-3), and the pharmacological stressor yohimbine (Experiment 2). Wheel running during the withdrawal period also prevented incubation of cocaine seeking or time-dependent increases in reactivity to cocaine-paired cues, a situation that often precipitates relapse (Experiment 5). Further, using pharmacological treatments such as progesterone (Experiment 2) or atomoxetine (Experiment 3) in combination with wheel running led to an additive treatment effect, suggesting a larger role for exercise as a singular or adjunct treatment in the prevention of cocaine relapse. While these behavioral models have revealed exercise to be an efficacious method to attenuate cocaine-motivated behaviors, long-term wheel running also changed cocaine-induced activation of brain reward circuitry (Experiment 4). Using c-Fos immunohistochemistry to evaluate neuronal activation, results demonstrated that exercising and control rats showed differential activation of the nucleus accumbens, caudate putamen, and prefrontal cortex in response to an acute cocaine injection. These results suggest that exercise may alter reactivity to cocaine by inducing plasticity in the mesolimbic dopamine system. Overall, results across these experiments have demonstrated that aerobic exercise has the ability to attenuate activation of the neurobiological substrates of addiction in addition to robustly reducing relapse to cocaine-seeking behavior.

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Introduction

Introduction

Drug addiction is the leading cause of preventable death in the United States (Mokdad et al. 2004), and it is characterized by continued use of a drug despite substance-related problems. These include neglecting important responsibilities, devoting significant resources to using drugs, using in larger amounts or for longer than intended, using despite knowledge of its aggravating effects on persistent physical or psychological problems, the development of tolerance or withdrawal effects, and a persistent desire or repeated unsuccessful efforts to reduce use (American Psychiatric Association 1994). Perhaps the single greatest problem for the treatment of addiction is the high rate of relapse to drug use. Relapse can be precipitated by stress, stimuli and contexts associated with drug use, or by the drug itself; recent data indicate that 80-90% of former users relapse within 1 year of cessation of treatment (Hunt et al. 1971, O'Brien 1997, Brandon et al. 2007, Kirshenbaum et al. 2009). Consequently, relapse rates remain the primary outcome measure of clinical treatment success (Hendershot et al. 2011), and much of preclinical investigation of drug addiction focuses on behavioral and neurobiological mechanisms that trigger relapse as well as treatments to prevent relapse (Epstein et al. 2006). However, for illicit psychostimulants like cocaine, there are currently no FDA-approved pharmacological treatments for relapse. Even for drugs like nicotine, for which there are relatively effective combined pharmacological and behavioral use reduction strategies (Hughes 2009), overall abstinence success rates remain extremely low (Cahill et al. 2012, Stead et al. 2008, Hughes et al. 2007). Thus, there is a critical need for the development of substance abuse treatments that are effective in promoting abstinence and reducing the risk of relapse.

Given the physical and psychological benefits of aerobic exercise, investigations have begun exploring potential treatment applications for exercise as a behavioral intervention for

various psychiatric disorders (USDHHS 1996, Kim et al. 2011, Wigal et al. 2013), and initial findings suggest that exercise may be an effective primary or adjunct treatment for drug addiction and relapse (Ussher et al. 2012, Haasova et al. 2012). In controlled human laboratory studies, cravings for alcohol (Ussher et al. 2004), cigarettes (Daniel et al. 2004), and cannabis (Buchowski et al. 2011) were reduced directly following moderate intensity aerobic exercise, and an increase in daily physical activity reduced alcohol and illicit drug use and craving at 1- to 2-month follow-up (Correia et al. 2005, Roessler 2010). Other work has shown a negative correlation between physical health and fitness and relapse to smoking (Metheny and Weatherman 1998) and a significantly reduced risk of smoking relapse in adults who were physically active (McDermot et al. 2009). During 12 weeks of exercise training, exercisers maintained a longer duration of drug (e.g., alcohol, cocaine, marijuana, opiates, and sedatives) abstinence compared to non-exercisers (Weinstock et al. 2008, Brown et al. 2010), and at 3-month follow-up compared to baseline, exercisers maintained a higher percentage of drug-free days (Brown et al. 2010). Additionally, higher abstinence rates were reported at 6-month follow-up in individuals who underwent a cessation plan incorporating physical activity and traditional behavioral treatment compared to behavioral treatment alone (Horn et al. 2011). Overall, these studies provide evidence that exercise and physical activity may decrease drug intake and help reduce vulnerability to long-term relapse across a range of abused substances.

Nevertheless, while there is evidence of beneficial treatment effects of exercise, there are still other clinical investigations that demonstrate negative or inconclusive effects on drug use and relapse (Ussher et al. 2012). Due to time constraints of clinical research, many of these studies failed to include large enough sample sizes and to verify exercise intensity levels (Ussher et al. 2012, Smith and Lynch 2012). Another limitation of human research is the difficulty of gathering

invasive neurobiological measures that may help uncover the mechanisms of relapse and help inform its treatment. Therefore, preclinical animal work is necessary to examine not only the parameters necessary for the attenuating effects of exercise on drug addiction and relapse but also its mechanism.

Relapse can be examined in a rodent self-administration procedure using the reinstatement model (Stretch et al. 1971, Davis and Smith 1976, de Wit and Stewart 1981). Based on an operant conditioning paradigm, rats are trained to lever press for intravenous infusions of a drug. Following established maintenance of this behavior, drug access is discontinued, and the animal is allowed to extinguish responding. Subsequently, nonreinforced responding on the lever previously used for drug self-administration is reinstated by noncontingent priming injections of the drug itself, by presentation of drug-paired cues (e.g., house light, stimulus lights), or by stressful stimuli (e.g., footshock, yohimbine) (Marchant et al. 2013). It has been shown that stimuli and events that precipitate reinstatement of drug-seeking behavior in rodents also elicit craving and relapse in humans (Shaham et al. 2003). Related to reinstatement, "incubation" of drug-seeking behavior is a phenomenon that also can be modeled in the laboratory and may contribute to persistent vulnerability to relapse (Grimm et al. 2001), and it has been shown to occur in both humans (Bedi et al. 2011) and animals (Neisewander et al. 2000, Grimm et al. 2001) across a range of drug reinforcers. As in the reinstatement paradigm, rats in the incubation model are allowed to self-administer drug for a period of time; then the animals are placed into a forced abstinence or withdrawal period that can last up to 2 months, followed by a drug-seeking test in the presence of drug-paired cues (Lu et al. 2004). Incubation is defined as a time-dependent, progressive increase in magnitude of drug seeking in response to

drug-paired cues over the first few weeks of forced abstinence, and it may help explain why relapse can occur after prolonged drug-free periods (Gawin and Kleber 1986).

Using associated self-administration procedures, several studies have examined the effects of voluntary wheel running as a form of exercise on addiction-related behaviors. For example, wheel running delayed acquisition of cocaine self-administration (Smith and Pitts 2011); decreased intake of amphetamine (Kanarek et al. 1995), methamphetamine (Miller et al. 2011), cocaine (Cosgrove et al. 2002), and ethanol (McMillan et al. 1995, Ehringer et al. 2009); and prevented binge intake of cocaine (Smith et al. 2011, Zlebnik et al. 2012). However, few studies have examined the neurobiological and behavioral treatment effects of wheel running in rodent models of relapse. The following set of experiments outlines the effects of wheel running in both reinstatement and incubation of cocaine seeking models. Specifically, concurrent access to a running wheel – alone or in combination with a pharmacological treatment such as progesterone or atomoxetine – was investigated for its behavioral effects on reinstatement of cocaine-seeking behavior (Experiments 1-3). Additionally, nonconcurrent wheel running was studied for its impact on cocaine-induced activation of brain reward areas (Experiment 4) and the incubation of cue-induced cocaine seeking and associated neurobiological substrates (Experiment 5). These experiments represent a broad approach to the investigation of the influence of aerobic exercise on relapse to drug-seeking behavior, incorporating both translational animal models of addiction treatment and evaluation of the underlying neural mechanisms. Together, they demonstrate promising treatment effects of exercise for the reduction of relapse-related behaviors and provide framework for future inquiry into the neuroadaptations that mediate these effects.

CHAPTER 2

Experiment 1: Effects of concurrent wheel running on extinction and reinstatement of cocaine-seeking behavior in female rats

Zlebnik NE, Anker JJ, Gliddon LA, Carroll ME (2010) Reduction of extinction responding and reinstatement of cocaine seeking by wheel-running in rats. Psychopharmacology 209(1):113-25

Rationale

Early work investigating the effects of exercise on drug-motivated behaviors demonstrated that concurrent wheel running attenuated intake of amphetamine (Kanarek et al. 1995), ethanol (McMillan et al. 1995), and cocaine (Cosgrove et al. 2002). Additionally, another study employing voluntary wheel running in the home cage found that 6 weeks of wheel access decreased breakpoints for cocaine under a progressive ratio schedule (Smith et al. 2008a). However, no study had yet examined the effects of wheel running in an animal model of relapse. Therefore, the goal of Experiment 1 was to examine both concurrent and prior wheel running in a drug-primed reinstatement paradigm for their effects on drug-seeking behavior.

Introduction

Environmental enrichment with dietary (Carroll et al. 2001), social (Bardo et al. 2001; Schenk et al. 1987), and novel (Bardo et al. 2001; Cosgrove et al. 2002; Klebaur et al. 2001) stimuli, as well as opportunities for physical activity (Cosgrove et al. 2002; Smith et al. 2008a) reduce amphetamine and cocaine self-administration in animals, possibly by a reward substitution mechanism at the behavioral and/or neurochemical level (Bossert et al 2005; Kalivas and McFarland 2003; Shalev et al 2002; Solinas et al. 2008). Most of these studies were conducted during a short-access maintenance phase under fixed-ratio (FR) or progressive-ratio (PR) schedules. Except for the use of preferred dietary substances (see Carroll et al. 2001), little is known about how environmental enrichment affects critical transition phases of drug abuse such as acquisition, escalation, extinction, and reinstatement (see review by Carroll et al. 2009a). In one study, Solinas et al. (2008) showed that housing mice in an enriched environment after developing a cocaine-induced conditioned place preference (CPP) prevented cocaine-primed reinstatement of the CPP and reduced activation of corresponding brain circuitry.

In animal studies access to exercise in a running wheel is a positive reinforcer. For example, rats lever-pressed to gain access to a running wheel (Belke et al. 2005, Belke and Wagner 2005), and they escalated running during unlimited wheel access (Lattanzio and Eikelboom 2003). Rewarding effects of the wheel were reflected in a study showing CPP for an environment associated with wheel running (Belke and Wagner 2005; Lett et al. 2000). Initial studies indicated that access to a running wheel reduced the maintenance levels of low dose (0.2 mg/kg) cocaine self-administration in female (but not male) rats under a FR 1 schedule (Cosgrove et al. 2002) and for low (0.3 mg/kg) and high (10 mg/kg) doses of cocaine under a PR schedule in female rats (Smith et al. 2008a). An initial report also indicated that female rats exposed to wheel

running showed lower rates of escalation of cocaine intake during 6-h access than rats with no wheel access (Smith et al. 2011). The present experiment extended these findings of reduced cocaine-seeking behavior due to wheel running to the extinction and reinstatement phases of drug abuse.

Behavioral studies of exercise, drug self-administration, and other rewarding activities have revealed commonalities, interactions, and substitutions among the various behaviors (see Carroll et al. 2009a for a review). That an increasing number of findings from animal research indicated that exercise and other nondrug rewards interfere with drug self-administration suggests common neurobiological mechanisms. For example, brief exposure to exercise increases central dopamine concentrations (Meeusen et al. 2005; Petzinger et al. 2007), and regular exercise increases dopamine concentration and dopamine-binding proteins (Fisher et al. 2004). These changes are similar to the effects of drugs such as cocaine on the mesolimbic and mesocortical pathways (Caine and Koob 1994; Wise et al. 1995; Pich et al. 1997). Recent imaging in human runners also indicates that release of endogenous opioids occurs in frontolimbic brain regions after sustained physical exercise and is correlated with perceived euphoria (Boecker et al. 2008). Since exercise, preferred foods, and other forms of environmental enrichment activate reward mechanisms similar to those activated by drugs of abuse, it is useful to consider physical activity as a substitute for the rewarding effects of cocaine and other drugs as well as a prevention for reinstatement or relapse of drug seeking after a drug-free period.

The purpose of this study was to examine the effect of access to a running wheel during extinction and/or cocaine-primed reinstatement in rats previously trained to self-administer iv cocaine. Cocaine-primed reinstatement is considered to be an animal model of relapse, and understanding and preventing relapse is a major challenge to the successful treatment of drug

abuse. Initial results with dietary substances indicated that environmental enrichment interfered with reinstatement of drug-seeking behavior (Comer et al. 1995; Solinas et al. 2008). In the present study, rats were trained to self-administer iv cocaine (0.4 mg/kg) and then were subsequently divided into 4 groups according to the following 2 x 2 design in which access to wheel running or a locked wheel was given during extinction and/or reinstatement: 1) access to wheel running during extinction from cocaine self-administration and reinstatement of cocaine seeking (WER), 2) access to wheel running during extinction and a locked wheel (did not rotate) during reinstatement (WE), 3) access to the locked wheel during extinction and to wheel running during reinstatement (WR), and 4) access to a locked wheel during extinction and reinstatement (WL; i.e., no opportunity to run). It was hypothesized that the groups with access to running during extinction (WER, WE) and reinstatement (WR, WER) would show reduced responding during these phases compared with group with locked wheel access (WL).

Materials and methods

Subjects

Thirty-seven female 90-day old Wistar rats weighing 250-300 g at the start of the experiment served as subjects (Harlan Sprague-Dawley, Madison, WI, USA). Females were used as they readily acquire wheel running (Jones et al., 1990), they run more than males (Boakes et al. 1999; Cosgrove et al. 2002; Eikelboom and Mills 1988; Lambert and Kinsley, 1993), and they are more sensitive than males to the attenuating effects of wheel running on cocaine self-administration (Cosgrove et al. 2002). Estrous cycle was not monitored to prevent disruption of cocaine- and exercise-maintained behavior by repeated vaginal lavage (Walker et al. 2002). In addition, the animals were housed in their operant chambers during the experiment, and testing

for cycle phase may have disrupted the cycle as well as the cocaine and wheel-reinforced behavior. After arrival, rats were pair-housed in plastic cages and allowed at least 3 days to acclimate before testing. They had free access to pellet food (Rodent Diet 2018, Harlan Laboratories, Madison, WI, USA) and water. Subsequently, rats were removed from the plastic cages and placed in individual operant chambers where they remained for the duration of the study. While in the chambers rats continued to have free access to water, and they were fed 16 g of ground food (LabDiet 5001, Purina Laboratory Chow, Minneapolis, MN, USA) at 3:00 pm daily to maintain them at 85% of their free-feeding body weight. All rodent holding rooms were maintained at 24°C and at 40-50% humidity under a light/dark cycle of 12/12-hr with room lights on at 6:00 am. The experimental protocol (0708A15263) was approved by the University of Minnesota Institutional Animal Care and Use Committee. The experiment was conducted in accordance with the Principles of Laboratory Animal Care (National Research Council 2003), and all laboratory facilities were accredited by the American Association for the Accreditation of Laboratory Animal Care.

Apparatus

Octagonally-shaped operant conditioning chambers consisting of alternating stainless steel and Plexiglas walls were used for housing and testing in the present study. Custom-made, sound-attenuating, wooden boxes equipped with a ventilation fan enclosed each operant conditioning chamber. Operant chambers contained slots that allowed for the insertion of stainless steel panels, a drinking bottle holder, a food receptacle, 2 response levers, 2 sets of stimulus lights, and a house light. The stimulus lights consisted of 3 multi-colored LED lights (red, yellow, and green) located above each response lever, and a single white house light (4.76 W) positioned in the upper corner illuminated each operant chamber. A guillotine-style door,

when opened, allowed access to a free-spinning 35.6-cm diameter running wheel (Med Associates Inc., St. Albans, VT, USA) that was fitted on the left side of each operant chamber. A microswitch recorded quarter-wheel turns, and a lock could be put in place to allow entry but prevent rotation.

During self-administration sessions, a syringe pump (model PHM-100, MedAssociates Inc., St. Albans, VT, USA) was used to deliver response-contingent iv infusions and was located on the outside of the wooden sound-attenuating enclosure. During self-administration sessions, responding on the left lever delivered cocaine infusions through a segment of Tygon tubing (1.52 mm o.d., 0.51 mm i.d., Fisher Scientific, Springfield, NJ, USA) that extended from the 35-ml syringe to a plastic swivel (050-022, Alice King Chatham, Hawthorne, CA, USA) secured to the top center of the operant chamber. An additional segment of tubing, protected by a metal spring-covered tether (C313CS, PlasticsOne, Roanoke, VA), was connected to the opposite end of the swivel and extended into the operant conditioning chamber. Inside the chamber it attached to an infusion harness (Instech Laboratories, Plymouth Meeting, PA, USA) that was fitted to each rat following catheter implantation. The swivel and tether allowed free movement within the operant chamber and easy access to the adjoining running wheel. Data collection and programming were conducted using PC computers with a Med-PC interface (MedAssociates, Inc., St. Albans, VT, USA).

Drugs

Cocaine HCl was supplied by National Institute of Drug Abuse (Research Triangle Institute, Research Triangle Park, NC), dissolved in 0.9 % NaCl at a concentration of 1.6 mg cocaine HCl/1 ml saline, and refrigerated. Heparin (1 ml/200 ml saline) was added to the cocaine solution to prevent catheter occlusion from thrombin accumulation. The flow rate of each

cocaine infusion was 0.025 ml/sec, and the duration of pump activation (1 sec/100 g of body weight) was adjusted to provide a 0.4 mg/kg cocaine dose throughout self-administration testing.

Procedure

Wheel training

Table 2-1 outlines the 6 phases of the procedure. In the first phase, rats were fitted with the infusion harness and tether and fed 16 g of ground food daily. They were allowed access to the wheel during 6-h sessions (9:00 am to 3:00 pm) for approximately 8 days to acclimate subjects to running when connected to the harness/tether system. During training, the house light was illuminated and the door to the wheel was opened. Response levers were present during wheel training, but they remained inactive and responses were not recorded. Acquisition of wheel running was defined as 400 full wheel revolutions in a single session or 100 revolutions per session for 3 consecutive sessions. If an animal failed to reach either criterion within 2 weeks, it was not included in the study.

Table 2-1. Outline of experimental procedure.

Phase (Days)	Wheel Training (~8)	Surgery/ Recovery (~3)	S-A Training (~5)	Maintenance (10)	Extinction (14)	Between-Subjects Reinstatement S S C S C S C (7)	Within-Subjects Reinstatement S C S C+W S C (6)
Group	Dose		0.4 mg/kg, iv		Saline, iv	5, 10, 15 mg/kg, ip	15 mg/kg, ip
WER	Wheel		No Wheel		Wheel		----
WE	Wheel		No Wheel		Wheel	Wheel Locked	Wheel (C+W)
WL	Wheel		No Wheel		Wheel Locked		----
WR	Wheel		No Wheel		Wheel Locked	Wheel	Wheel (C+W)

Surgical procedure

One to 3 days after achieving wheel-running behavior, rats were surgically implanted with an indwelling catheter in the right jugular vein following the procedure outlined by Carroll and Boe (1982). Briefly, rats were anesthetized with a combination of ketamine (60 mg/kg, ip) and xylazine (10 mg/kg, ip) and administered doxapram (5 mg/kg, ip) and atropine (0.4 mg/ml, 0.15 ml, sc) to facilitate respiration. An incision was made lateral to the trachea, the right jugular was exposed, and a small incision was made perpendicular to the vein. The beveled end of a polyurethane catheter (MRE-040, Braintree Scientific, Inc., Braintree, MA, USA) was inserted and then secured to the vein with silk sutures. The free end of the catheter was guided subcutaneously to the midscapular region of the neck where it exited via a small incision and attached to a metal cannula (C3236, PlasticsOne, Roanoke, VA, USA) that was embedded in the infusion harness.

Following the surgical procedure, doors to the wheels remained closed, and rats were allowed a 3-day recovery period during which antibiotic (gentamicin) and analgesic (buprenorphine) medications were administered. Each rat was fitted with an infusion harness and tether that was worn throughout the remainder of the study. After the recovery period, catheters were flushed with a heparinized saline solution at 8:00 am daily to prevent catheter blockage. Every 7 days at 3:00 pm body weights were recorded, and catheter patency was checked by injecting a 0.1-ml solution containing ketamine (60 mg/kg), midazolam (3 mg/kg), and saline (KMS). If a loss of the righting reflex was not manifest upon a KMS catheter patency check, a second catheter was implanted in the left jugular vein following the methods described above, and the experiment resumed in 3 days.

Cocaine self-administration training and maintenance

In the third phase, rats were trained to lever press for iv infusions of cocaine (0.4 mg/kg) under a fixed-ratio 1 (FR1) schedule of reinforcement during daily 6-h sessions (9:00 am to 3:00 pm) that were signaled by illumination of the house light. During sessions, a response on the active/drug-paired lever started the infusion pump and illuminated the stimulus lights located directly above the lever for the duration of the infusion. Responses on the active lever during the length of the infusion (2.5-3.0 sec) were recorded but had no programmed consequences. Responses on the inactive/activity lever illuminated the stimulus lights above that lever but did not activate the pump. Initially, 3 experimenter-delivered priming infusions of 0.4 mg/kg cocaine were administered at the beginning of each training session (9:00 am) followed by the placement of a small amount of peanut butter on the active/drug-paired lever. This training procedure continued until rats earned at least 60 infusions during a single session. Once rats achieved acquisition of cocaine self-administration, they were allowed to self-administer for 10 additional 6-h sessions (maintenance). The door leading to the wheel remained closed throughout self-administration training and maintenance (no wheel access).

Extinction

Following Day 10 of maintenance, cocaine solutions were replaced with saline, and responding produced a saline infusion (FR 1) along with the stimuli described above for maintenance. Based on total cocaine intake during maintenance, rats were separated into 4 groups with the following conditions: 1) access to a running wheel during extinction and reinstatement (WER), 2) access to a wheel during extinction and a locked wheel (unable to rotate) during reinstatement (WE), 3) access to the locked wheel during extinction and to wheel running during reinstatement (WR), and 4) access to a locked wheel during extinction and reinstatement (WL). Groups were compared during the extinction phase for 14 6-h extinction sessions.

Reinstatement

In the reinstatement phase, the stimulus lights, house light, and pump were unplugged to ensure reinstatement responding was due to priming injections and not to drug-associated cues (Larson et al. 2007). Initially, groups were compared in a *between-subjects* design, and subsequently, the WE and WR groups were tested in a *within-subjects* design to evaluate the effect of brief wheel exposure or removal during single reinstatement sessions.

a. Between Subjects Each of the 4 groups were administered saline (S)- or cocaine (C)-priming injections at session onset (9:00 am), and their subsequent cocaine-seeking behavior was tested for the next 6-h (9:00 am to 3:00 pm) for 7 consecutive days. During the first 2 reinstatement sessions, rats were treated with a single S injection, and for the next 5 days they were treated with a single C or S injection on alternating days (i.e., S S C S C S C). Response-contingent drug-paired cues and the house light were deactivated during this time. On the C days rats received priming injections of 1 of 3 randomly-assigned doses of C (5, 10, and 15 mg/kg). Doors to the adjoining wheel remained open during reinstatement testing, but only groups WR and WER could run; the WE and WL groups could enter the wheel, but it did not rotate.

b. Within Subjects For the rats in group WE and WR, three S and three 15 mg/kg C-priming injections (alternating) were administered over 6 days. On the second C priming day, rats in Group WE were allowed access to wheel running (C+W) during the 6-h reinstatement session to determine whether acute exposure to the wheel produced the same effect as chronic exposure in the between-subjects condition for group WER. Similarly, Group WR was given cocaine priming injections on the second and sixth day to determine whether reinstatement would occur without wheel access. Thus, in the within-subjects condition Groups WE and WR received the following priming sequence over 6 consecutive days: S, C, S, C+W, S, C.

Data Analysis

Responses, infusions, and full wheel revolutions during extinction, and responses and wheel revolutions during reinstatement were the primary dependent measures. These measures were analyzed with 2-factor mixed analyses of variance (ANOVA) with group as the between-subjects factor and blocks of days as the repeated measure. Data were grouped in 2-day blocks to reduce daily variability and the number of post-hoc contrasts. Separate 2-factor ANOVAs were used to compare infusion lever and active lever presses during reinstatement with group as the between-subjects factor and the priming injection (e.g., S vs. C) as the repeated measure. Infusion lever and inactive lever presses during reinstatement were also examined using a 3-factor ANOVA with group and lever type (e.g., active vs. inactive) as a between-subjects factor and dose as the repeated measure. A mixed ANOVA was used to compare priming conditions by group for groups WE and WR in the within-subject study. Post hoc tests were performed with Fisher's least significant difference (LSD) protected t-tests, and results were considered significant if $p < 0.05$. Statistical analyses were performed using GB Stat (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Results

All rats consumed the daily 16 g food allotment and did not differ in mean water intake or body weight during the study. Groups also did not differ in number of wheel training days, wheel revolutions during training, or days to acquire cocaine self-administration (Table 2-2).

Table 2-2. Summary of group data.

Group	N	Mean H_2O Consumption (ml ± SEM)	Mean Weight (g ± SEM)	Wheel Training Days (± SEM)	Training Revolutions (± SEM)	Mean Days to Acquire (± SEM)
WR	9	35.59 (± 2.23)	271.82 (± 3.51)	8.11 (± 2.04)	242.54 (± 40.34)	5.22 (± 0.70)
WER	9	35.22 (± 2.35)	269.46 (± 7.58)	3.89 (± 1.14)	298.07 (± 44.17)	4.00 (± 0.17)
WE	9	34.13 (± 2.93)	284.04 (± 4.78)	6.00 (± 1.44)	295.75 (± 95.58)	5.00 (± 1.16)
WL	10	31.76 (± 2.78)	271.06 (± 4.63)	4.44 (± 0.75)	234.01 (± 47.60)	4.44 (± 0.53)

Maintenance

Figure 2-1 shows mean responses (a) and infusions (b), respectively, across the 10-day maintenance period. Separately, responses and infusions were averaged across five 2-day blocks, and there was not a significant main effect of group for either responses or infusions. However, there was a significant main effect of session block for both responses ($F_{4, 184} = 4.81$, $p < 0.01$) and infusions ($F_{4, 184} = 7.61$, $p < 0.0001$). Data were collapsed and analyzed across groups, and a significant effect of session block was found when averaging responses ($F_{4, 184} = 4.86$, $p < 0.01$) and infusions ($F_{4, 184} = 7.53$, $p < 0.0001$) over five 2-day blocks. Post-hoc comparisons showed a significant increase in both responses and infusions during sessions 7-8 and 9-10 ($p < 0.01$) compared with those during sessions 1-2. There were no significant differences in inactive lever presses (data not shown) among groups or across blocks.

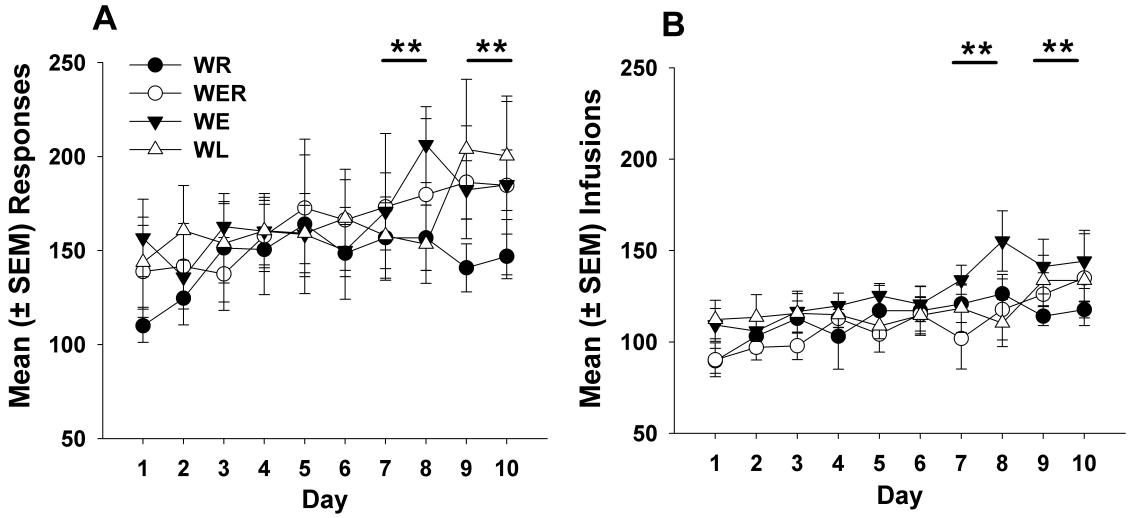

Figure 2-1. Mean (± SEM) responses (A) and i.v. 0.4 mg/kg cocaine infusions (B) during daily 6-h self-administration sessions over the 10-day maintenance period. Session blocks 7-8 and 9-10 (p < 0.01) had significantly more responses (A) and infusions (B) than session block 1-2 when group data were collapsed and analyzed across five 2-session blocks.**

Extinction

Extinction responses (a) and infusions (b) are shown in Figure 2-2. When data were averaged in 2-day blocks a significant reduction in responding occurred on both active and

inactive levers and in saline infusions in rats with wheel access (WER and WE combined) compared with rats without wheel access (WL and WR combined). Since WER and WE did not have significantly different responses or infusions during maintenance (see above) or extinction (data not shown), and both groups underwent the same condition during extinction (i.e., wheel access), their data were collapsed and compared to WL and WR, whose data were similarly combined. There was a significant main effect of group ($F_{1, 258} = 9.64$, $p < 0.01$) and session block ($F_{6, 258} = 42.83$, $p < 0.0001$) and a group x session block interaction ($F_{6, 258} = 9.82$, $p < 0.0001$) for responses. Post-hoc tests revealed significantly higher responding on extinction sessions 1-2 and 3-4 ($p < 0.01$) and sessions 5-6 and 7-8 ($p < 0.05$) for WL and WR combined vs. WER and WE combined (Fig. 2-2A). A similar analysis was conducted for infusions revealing a significant main effect of group ($F_{1, 258} = 9.24$, $p < 0.01$) and session block ($F_{6, 258} = 41.92$, $p < 0.0001$) and a significant interaction ($F_{6, 258} = 7.85$, $p < 0.0001$). Post-hoc comparisons indicated more saline infusions for WL and WR vs. WER and WE during sessions 1-2, 3-4, and 5-6 ($p < 0.01$) (Fig. 2-2B).

Inactive lever presses were compared, and significant main effects of group ($F_{1, 258} = 24.05$, $p < 0.0001$) and session block ($F_{6, 258} = 10.29$, $p < 0.0001$) and a significant group x session block interaction ($F_{6, 258} = 3.68$, $p < 0.01$) (data not shown). Groups with wheel access (WER and WE) made significantly fewer responses on the inactive lever during sessions 1-2, 3-4, 5-6, 7-8, and 11-12 ($p < 0.01$) and sessions 9-10 ($p < 0.05$) than groups with locked wheel access (WL and WR). To assess whether Groups WL and WR combined (locked wheel access) showed preference for the previously drug-paired lever during extinction, active and inactive lever responses were compared ($F_{6, 265} = 15.16$, $p < 0.0001$), with post-hoc tests indicating that significantly more responses were made on the active lever during sessions 1-2, 3-4, 5-6, and 7-8 ($p < 0.01$).

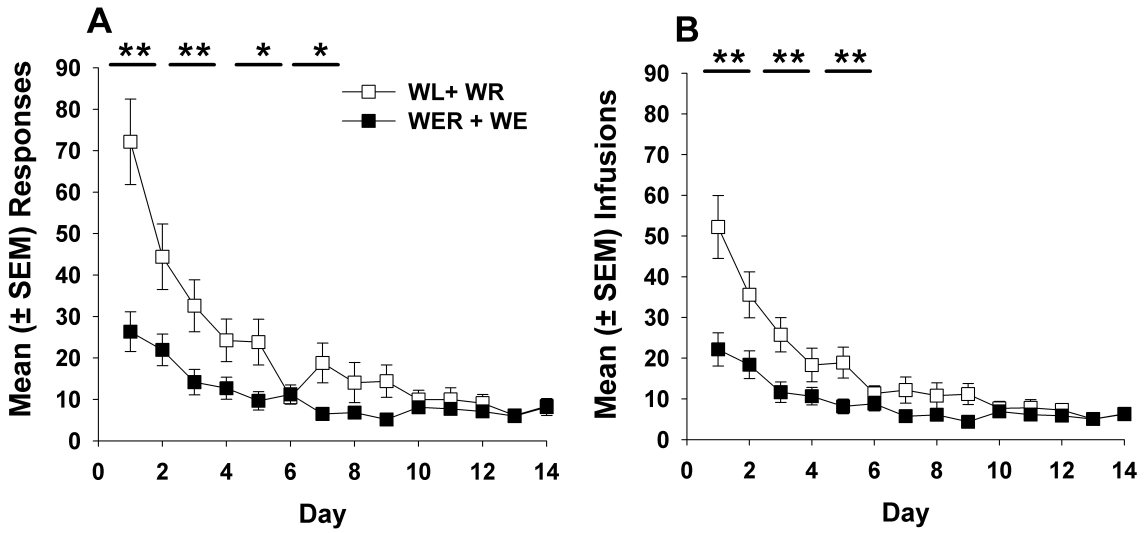

Figure 2-2. Mean (± SEM) responses (A) and iv saline infusions (B) during daily 6-h self-administration sessions over the 14-day extinction period. Groups WL and WR were combined and then, separately, WER and WE were combined, as they showed no significant differences. Following introduction of the wheel on day 1 of extinction, combined Groups WL+WR exceeded combined Groups WER+WE in responses (A) and infusions (B) on session blocks 1 to 2 (p□<□0.01), 3 to 4 (**p□<□0.01), and 7 to 8, (*p□<□0.05). The combined groups also differed on responses on sessions 5 to 6 and 7 to 8 (p□<□0.05) and on infusions on session 5 to 6 (p□<□0.01).**

Wheel revolutions during extinction were averaged in seven 2-day blocks to reduce variability across days, and they were compared between groups WER and WE (Fig. 2-3). There was a significant main effect of session block ($F_{6, 125} = 5.21$, $p < 0.01$), but there was no effect of group or a group x session block interaction. This indicates that a similar increase in revolutions occurred over the 14 days in both groups. To test the hypothesis that removal of cocaine increased extinction responding on the first day, wheel revolutions were compared with a paired t-test on the last day of training and the first day of extinction in groups WER and WE combined, as the groups did not differ, and revolutions were significantly higher on the first day of extinction ($t_{17} = 2.61$, $p < 0.05$). A comparison of revolutions during training (Table 2-2) and on the first day of extinction (Fig. 2-3) for these groups shows that revolutions were nearly twice as high during extinction than training in groups that had wheel access (WE and WER). However, revolutions during extinction only in Group WR were between those of initial training and WE, and they did not differ from either condition.

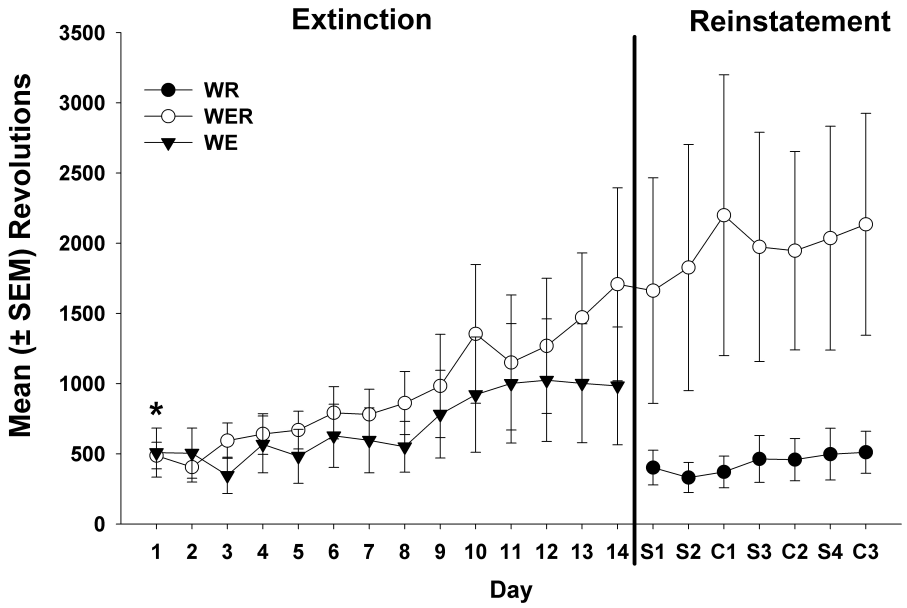

Figure 2-3. Mean (± SEM) wheel revolutions over the 14-day extinction period for groups WR (filled circles), WER (open circles), and WE (filled triangles) and during the reinstatement period for each saline (S) or cocaine (C) priming injection for groups WR and WER. During analysis, groups WE and WER were combined, as they did not significantly differ during cocaine extinction, and when wheel revolutions were compared on the first day of extinction vs. the last day of training (see Table 2-2), they were significantly higher during extinction (*p < 0.05).

Reinstatement

a. *Between Subjects* Figure 2-4A shows responding on the previously-active lever following ip priming injections of S or C during the reinstatement period. Reinstatement began with 2 S-priming injections, and responding following the first priming injection was analyzed to determine the effect of discontinuing unlocked wheel access for group WE after extinction. Group differences were revealed ($F_{3, 39} = 11.85$, $p < 0.0001$), and post-hoc tests showed significantly greater responding for group WE (striped bar) on the first S day vs. WR, WER, and WL (ps<0.01). No significant differences were found in inactive lever responding for the first S priming injection.

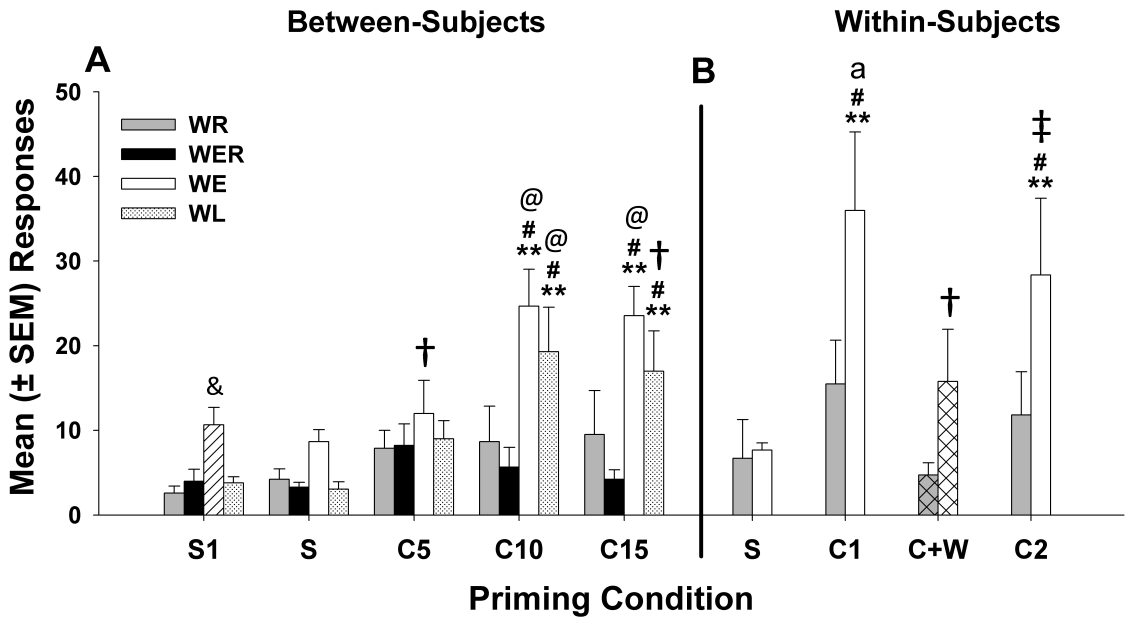

Figure 2-4. Mean (± SEM) responses on the previously active lever (A) following priming injections during reinstatement testing in the between-subjects design with ip saline (S) or 5-, 10-, and 15-mg/kg ip injections of cocaine (C), #p□<□0.01 vs. WER, **p□<□0.01 vs. S, @p□<□0.01 vs. WR, +p□<□0.05 vs. WR. The striped bar indicates the first day after termination of wheel running in WE, and &p□<□0.01 vs. all other groups. Mean (± SEM) responses on the previously active lever (B) following priming injections of saline (S) or two 15-mg/kg cocaine (C1, C2) during a within-subjects reinstatement procedure in groups WE and WR (cocaine and wheel access-cross-hatched bar), **p□<□0.01 vs. S, #p□<□0.01 vs. WR, † p□<□0.05 vs. WR, † = 0.05 vs. WR, ‡p□<□0.05 vs. C + W, a = p□<□0.01 vs. C + W.

Responses averaged over the remaining 3 S-priming days were compared to responses on the 5, 10, and 15 mg/kg dose C-priming days (Fig. 2-4a), and there were significant main effects of priming condition ($F_{3, 147}$= 15.02, p < 0.0001), group ($F_{3, 147}$ = 6.58, p < 0.01), and a significant priming condition x group interaction ($F_{9, 147}$=2.81, p < 0.01). Post-hoc analyses indicated that the 10 and 15 mg/kg C-priming injections resulted in significantly more responding than S priming injections for both groups WL and WE (ps<0.01). In contrast, responding on S- and C-priming days did not differ for WR and WER. Groups WE and WL did not significantly differ in responding after 10 and 15 mg/kg C-priming injections, but both groups responded significantly more than WER and (ps<0.01) at these doses. Compared to WR, WE and WL responded significantly more following the 10 (p < 0.01) and 15 (p < 0.01 vs. WE; p < 0.05 vs. WL) mg/kg priming injections, while WE responded more after the 5 (p < 0.05) mg/kg priming dose as well.

No significant group x priming condition interaction was found for inactive lever responses (not shown), although there were main effects of group ($F_{3, 147} = 4.96$, $p < 0.01$) and priming condition ($F_{3, 147}=3.95$, $p < 0.05$) similar to responding on the previously-active lever.

Inactive and previously-active lever responses during reinstatement were compared in a 3-factor mixed ANOVA. This analysis revealed significant main effects of group ($F_{3, 295} =12.19$, $p < 0.0001$), lever type (e.g., previously-active > inactive) ($F_{1, 295} = 18.73$, $p < 0.0001$), and priming condition ($F_{3, 295} = 15.55$, $p < 0.0001$) as well as significant group x priming condition ($F_{9, 295} = 3.09$, $p < 0.01$) and lever type x priming condition ($F_{3, 295} = 4.04$, $p < 0.01$) interactions (not shown). However, no significant group x lever type x priming condition interaction was found. Finally, when comparing wheel revolutions for WER and WR over the 7-day reinstatement period, there was a near-significant effect of group ($F_{1, 71} = 3.67$, $p=0.0735$). This suggested that running escalated over time in the WER group. Otherwise no significant effects or interactions were found (see Fig. 2-3).

b. Within Subjects Figure 2-4B shows results for 6 rats in WE and all 9 rats in WR for which a subsequent within-subject reinstatement procedure was implemented after the between-groups data were collected. The mean of 3 S-priming sessions was compared to corresponding C-priming sessions (C) and to the C+W (C priming + wheel running access) session in a 2-factor ANOVA. There was a near-significant effect of group ($F_{1, 59} = 3.78$, $p=0.0738$), a significant effect of priming condition ($F_{3, 59}= 12.05$, $p < 0.0001$), and a significant group x priming condition interaction ($F_{3, 59} = 2.92$, $p < 0.05$). WR did not respond differently for S than on any of the C-priming days. In WE, responding did not differ between C+W and S, but there was significantly higher responding for C1 and C2 vs. S ($p < 0.01$) and vs. C+W ($p < 0.01$ vs. C1; $p < 0.05$ vs. C2). Comparing WE and WR, WR responded significantly more following C1 and C2 ($p < 0.01$) and C+W ($p < 0.05$). Analysis of inactive lever responding did not reveal significant differences (data not shown).

Discussion

In the present study there were no differences among the 4 experimental groups in initial exposure to the running wheel (revolutions), in the rate of acquisition of cocaine self-administration, or in the level of cocaine maintenance responding. However, during the subsequent extinction phase, wheel running suppressed drug-seeking behavior. Wheel running significantly reduced extinction responding in WER and WE compared to WL and WR. This finding suggests that wheel exposure reduced elevated drug seeking during this critical phase. Previous studies have shown that access to another type of reinforcer, sweet substances, decreased the extinction of cocaine- (Liu and Grigson 2005) and amphetamine- (Ping and Kruzich 2008) seeking behavior in rats. Similarly, extinction for a sucrose reinforcer was reduced by an enriched environment that included more space, greater social contact, novel objects, and opportunity for exercise (Grimm et al. 2008; Stairs et al. 2006).

In the subsequent reinstatement phase, rats without wheel running access (groups WE and WL) reliably reinstated responding on the previously-active lever. This was in contrast to groups WER and WR that were allowed to run during reinstatement and consequently showed a suppression of cocaine-primed reinstatement. That group WE did not have suppressed reinstatement indicated that recent running access during extinction did not carry-forward into the reinstatement phase. A consistent finding was that the subsequent within-subjects manipulation in group WE revealed that wheel access effectively reduced reinstatement when it was concurrently available during only 1 cocaine-priming session; however, there was not a carry-forward suppressive effect after wheel running access ended and the second C-priming condition occurred. The within-subjects reinstatement study also revealed significantly lower drug seeking in the WR group vs. WE. This could have been due to the more recent exposure to the wheel in the WR group vs. more distant exposure in WE or that exposure during

the between-subjects reinstatement more readily generalized to subsequent within-subjects reinstatement testing in the WR vs. the WE group.

The lack of an enduring suppressant effect of wheel running on reinstatement of cocaine seeking was consistent with the effects of recent but not previous environmental enrichment on sucrose seeking during extinction and reinstatement in rats (Grimm et al. 2008). However, these and the present results were not consistent with findings of earlier studies examining the effects of previous and chronic wheel running on the rewarding effects of drugs of abuse (Chen et al. 2008; Smith et al. 2008a). Several methodological and procedural differences between the present and previous studies may account for the discrepant findings. The most important difference concerns the duration of wheel access between studies. In previous work rats were allowed to run on the wheel for extended periods of time (Chen et al. 2008; Smith et al. 2008a).

In rats, wheel running upregulates the opioid neuropeptide dynorphin (Werme et al. 2000), dopamine neurons (Ahmad et al. 2009), delta FosB (Werme et al. 2002), and brain-derived neurotrophic factor (BDNF) (Khabour et al. 2009; Macias et al. 2007; Neeper et al. 1996; Widenfalk et al. 1999) in areas of the brain associated with reward occurs only after extended periods of free access to exercise in rodents. Additionally, increases in these neurosubstrates, through pharmacological manipulations, led to alterations in cocaine-primed reinstatement in rats (Berglind et al. 2007; Gyertyan et al. 2007; Mantsch et al. 2004; Micheli et al. 2007; Schmidt and Pierce 2006). Although these neural correlates were not analyzed in the present study, it may be assumed that the duration of exposure to wheel running may not have been sufficient to increase these measures to the point where they would produce enduring decreases in subsequent cocaine seeking.

The present investigation extends previous results demonstrating that wheel running decreased concurrent cocaine (Cosgrove et al., 2002), amphetamine (Kanarek et al., 1995), and alcohol (McMillan et al. 1995) self-administration as well as MDMA-induced CPP (Chen et al.

2008) in rats, and it supported findings demonstrating suppression of acquisition of cocaine (Carroll and Lac 1992) and PCP self-administration (Campbell et al., 1998) in rats and monkeys, respectively, using sweetened liquids as alternative reinforcers. While both sweetened liquids (Carroll and Lac 1992; Campbell et al. 1998; Carroll and Campbell 2000; Cosgrove and Carroll 2003; Rodefer and Carroll 1997; Carroll 1985; Kanarek and Marks-Kaufman 1988; Ping and Kruzich 2008) and food (Carroll and Lac 1992; Carroll 1996; Carroll et al. 2001) have been highly effective at interfering with drug self-administration, dietary substances can have adverse consequences if consumed in excess (Avena 2007; Avena et al. 2008; Corwin 2006). The present results and recent work (Cosgrove et al. 2002) indicate that exercise may be an effective and healthier nondrug alternative.

The present results extended the generality of wheel running, as a nondrug alternative reward, to the extinction and reinstatement phases of addiction, and they suggest that a nondrug reward can substitute for a drug reward. Another explanation for the attenuating effects of wheel running on extinction in the WER and WE groups is that cocaine seeking and wheel running were mutually exclusive, and time spent doing one resulted in less time for the other. However, analysis of the time course of wheel- and infusion- maintained behavior indicated that much of the 6-h session was spent on neither activity. For example, the average total during extinction was 50 responses or less, occupying only a small amount of time during the total 6-h session. Thus, the behaviors did not compete for limited time, and this was consistent with previous findings (Cosgrove et al. 2002).

The present findings suggest that rather than the actual behaviors competing, the reinforcing value of the wheel and cocaine reward competed. This interaction was also revealed by the increase in wheel running in group WER and WE from pre-cocaine wheel training (Table 2-2) to extinction (Fig. 2-3) when cocaine access had been removed. Another indication of a possible substitution effect of wheel running with cocaine reward was the resurgence of cocaine seeking (e.g., lever responding) in the WE group on the first day of reinstatement when wheel

running was prohibited. That this resurgence did not occur in the group that was re-introduced to wheel running (WR), the group that continued running in the reinstatement phase (WER), or in the locked wheel (WL) group suggests a reward interaction (wheel x cocaine) effect. When considering nondrug alternatives as a means of reducing drug-seeking behavior, it is important to consider that this may trigger reinstatement of behavior that was previously rewarded by the drug, and extending access to the nondrug alternative (e.g., wheel) is a preventive strategy that could be employed.

Behavioral analysis of resurgence, or the elevation in previously-reinforced behavior following the removal of an alternative reinforcer, has been documented (Leitenberg et al., 1970; Mulick et al. 1976; Carroll, 1985; Carroll et al., 1989). For example, a study by Podlesnik et al. (2006) reported that rats that previously self-administered alcohol showed a resurgence in lever pressing following the removal of a food alternative reinforcer. Others have demonstrated a resurgence of lever pressing for a primary reinforcer following the removal of a secondary reinforcer (Epstein 1983; Leitenberg et al. 1970; Mulick 1976; Wilson and Hayes 1996). The magnitude of resurgence is also dependent on extinction experience, with higher levels of resurgence occurring when there is less of an opportunity to extinguish (Cleland et al. 2000). Thus, the resurgence in responding for the primary reinforcer (drug) in the WE group may have resulted from a lack of extinction experience (Marlatt 1990) due to a greater amount of time being dedicated to wheel running during extinction testing.

Another explanation of the present findings may involve the attenuation of drug-related withdrawal effects by wheel running. Drug withdrawal effects are a primary contributor to elevated extinction responding in animal models (Carroll et al. 2009b; Kelamangalath and Wagner 2009; O'Dell et al. 2007), and reduction of withdrawal effects is integral to the implementation of effective treatment strategies in cocaine addiction (Ahmadi et al. 2006; Kampman et al. 2001a,b). Results from animal and human studies (Alaei et al. 2006; Daniel et al. 2007; Taylor and Katomeri 2007; Taylor et al. 2007; Ussher et al. 2001) indicate that withdrawal

signs and symptoms are reduced following bouts of exercise. This effect may be attributed, in part, to exercise-induced increases of monoamines in brain areas associated with reward. For example, cocaine withdrawal is associated with decreases in dopamine and serotonin in the striatum (Parsons et al. 1995; Rossetti et al. 1992), and continued drug use may be related to avoidance of these negative effects (Koob 2009). Physical activity, on the other hand, increases several monamines, including dopamine, within the striatum of rats and humans (Dishman 1997; Fulk et al. 2004; Hattori et al. 1994; Meeusen 2005; Petzinger et al. 2007), and it decreases stress, anxiety, and depression (Dishman 1997; Fulk et al. 2004; Sarbadhikari and Saha 2006). Higher vs. lower levels of physical activity were also associated with higher self-reported measures of well-being among adolescents (Ussher et al. 2007). Thus, extinction responding in the present study may be described as an attempt to re-establish homeostatic equilibrium following a withdrawal-induced disruption in neural mechanisms that regulate hedonic motivation. Wheel running may have restored this equilibrium thereby decreasing extinction responding.

The present findings offer useful applications for preventing and reducing drug abuse in humans. As mentioned previously, physical activity decreases nicotine craving and withdrawal symptoms (Ussher et al. 2006, 2009; Taylor et al., 2007). A recent investigation using physically-active and -inactive twin cohorts showed that the physically-active cohort was less likely to smoke cigarettes than the inactive cohort (Kujala et al. 2007), and the presence of regular physical activity during adolescence decreased the risk of smoking in early adulthood (Charilaou et al. 2009; Kohornen et al. 2009). Adolescents participating in drug intervention programs that include physical exercise demonstrate lower risk factors for drug abuse and decreased drug use (Collingwood et al. 1991, 2000). Physical activity has also been useful in combating alcohol abuse in inpatient treatment programs (Palmer et al. 1988; Sinyor et al. 1982) and in improving the probability of abstinence up to 3 months after program release (Sinyor et al. 1982).

Exercise has also been used as a supplement to contingency management (CM), a type of substance abuse treatment that rewards periodically-verified abstinence with non-drug alternative

reinforcers (e.g., money or vouchers for retail items). Patients who participated in physical activity in addition to CM intervention had higher drug abstinence rates than those who had CM alone (Weinstock et al., 2008). While CM is highly effective in maintaining abstinence (Prendergast et al., 2006; Lussier et al., 2006), it can be costly (Olmstead and Petry, 2009; Sindelar et al. 2007; Higgins, 1997). In contrast, exercise, by itself or in addition to CM, is an inexpensive and self-maintained behavior that may potentially have greater long-term consequences.

In summary, the present results indicated that access to wheel running decreased the extinction and reinstatement of cocaine-seeking behavior. Reinstatement was reduced by chronic, concurrent wheel running in a between-subjects study and by a single concurrent exposure during a within-subjects procedure. A comparison of 4 groups with differing wheel-running exposure indicated that concurrent wheel running was a key factor, and recent wheel access did not have a carry-forward effect on cocaine-primed reinstatement. The present findings, along with previous reports, suggest that access to wheel running reduces drug-seeking behavior during extinction and reinstatement.

CHAPTER 3

Experiment 2: Effects of the combination of concurrent wheel running and progesterone on reinstatement of cocaine-seeking behavior in female and male rats

Zlebnik NE, Saykao AT, Carroll ME. (2014) Effects of combined exercise and progesterone treatments on cocaine seeking in male and female rats. Psychopharmacology (Berl) 231(18): 3787-98

Rationale

Following the results of Experiment 1 in which concurrent wheel access decreased extinction and cocaine-primed reinstatement of cocaine seeking, interest was generated in determining whether concurrent wheel access also would sufficiently reduce reinstatement precipitated by other relevant stimuli such as stress and cocaine-paired cues. Work by other laboratories had shown attenuation of cue-induced cocaine seeking by both chronic home cage wheel running (Smith et al. 2012) and by home cage wheel running introduced during the extinction period (Lynch et al. 2010), but it was unknown whether concurrent wheel running would have an impact on reinstatement primed by cocaine-paired cues. Given the range of stimuli that have been reported to elicit drug craving in humans (Gawin and Kleber 1987), it was important to examine the treatment efficacy of exercise across many different priming conditions. Therefore, in Experiment 2, a multi-component reinstatement procedure was designed to examine the effects of concurrent wheel running on cocaine seeking in the presence of cocaine, yohimbine (an alpha-2 adrenergic receptor antagonist, considered a pharmacological stressor), cocaine-paired cues, cocaine + cocaine-paired cues, or yohimbine + cocaine-paired cues.

An additional goal of Experiment 2 was to extend earlier work on sex differences in cocaine-motivated behaviors and in treatment receptivity (Lynch and Carroll 2000; Carroll and Anker 2010; Anker and Carroll 2010, 2011). Females have been shown to exceed males in vulnerability to addiction across several phases of the human drug abuse process (Carroll and Anker 2010, Anker and Carroll 2011) and also demonstrate greater attenuation of cocaine intake in the presence of a concurrent running wheel (Cosgrove et al. 2002). Much work links heightened susceptibility to addiction-related behaviors in females to cycling gonadal hormones (Carroll and Anker 2010, Anker and Carroll 2011); for instance, estrogen promotes drug seeking (Larson et al. 2005, Becker 1990, Jackson et al. 2006), while progesterone reduces it (Anker and Carroll 2011, Feltenstein and See 2007, Lynch 2008, Larson et al. 2007, Anker et al. 2012). Moreover, exogenously-administered progesterone has been investigated as a treatment for

cocaine addiction in females (Anker and Carroll 2010, Quinones-Jenab and Jenab 2010, Evans and Foltin 2010, Evans and Foltin 2006, Evans et al. 2002, Sofuoglu et al. 1999, Sofuoglu et al. 2002). Combination treatment strategies incorporating both pharmacological and behavioral therapies have been shown to be more effective than individual treatments alone (Potenza et al. 2011), and given the individual effectiveness of concurrent wheel running and progesterone treatment in females, the aim of Experiment 2 was to investigate the combination of progesterone and concurrent wheel running as a sex-specific dual treatment strategy for cocaine-seeking behavior.

Introduction

Although progress has been made in identifying promising pharmacological or behavioral treatments for drug addiction, no one treatment has been completely effective on its own. Since clinical research suggests that the most successful treatment effects may be achieved using a combination of therapies (Potenza et al. 2011), the goal of the present study was to examine potential sex differences in the combination of pharmacological and behavioral treatment vs. each treatment alone. Several studies indicate that behavioral treatments like cognitive behavioral therapy and contingency management in combination with pharmacological treatments (e.g., methadone, disulfiram, nicotine replacement) helped patients maintain longer abstinence than pharmacological treatment alone (McLellan et al. 1993, Peirce et al. 2006, Ball and Ross et al. 1991, Carroll et al. 1998, Carroll et al. 2004, Bickel et al. 1997). Further, in animal experiments, combining the partial opioid agonist buprenorphine with concurrent access to the nondrug reinforcer saccharin led to an 80-90% reduction in drug use compared to less than 50% reduction for each of the treatments individually (Comer et al. 1996, Rodefer et al. 1997). These initial results suggest that, in both humans and animals, combined drug abuse treatment strategies are more effective than single treatments.

An increasing number of findings indicate that sex differences in drug abuse are influenced by the gonadal hormones estrogen and progesterone (P). While estrogen has apparent potentiating effects on positive subjective effects of drugs in humans (Evans et al. 2002, Sofuoglu et al. 1999, Justice and de Wit 2000) and drug seeking in animals (Larson et al. 2005, Becker 1990, Jackson et al. 2006), P attenuates drug-related responses in both humans and animals (Anker and Carroll 2010, Quinones-Jenab and Jenab 2010, Evans and Foltin 2010, Evans and Foltin 2006, Evans et al. 2002, Sofuoglu et al. 1999, Sofuoglu et al. 2002). In women, high endogenous levels of P were associated with lower cue- and stress-induced cocaine craving (Sinha et al. 2007). Animal models of the human drug abuse process also demonstrate P-mediated attenuation of cocaine-maintained behavior (Anker and Carroll 2011; Feltenstein and

See 2007, Lynch 2008, Larson et al. 2007, Anker et al. 2012). Cocaine-primed reinstatement was reduced in female rats that were treated with P compared to vehicle-treated controls (Anker et al. 2007), and allopregnanolone, a major metabolite of P, attenuated stess-induced reinstatement of cocaine-seeking behavior in female but not male rats (Anker and Carroll 2010). Progesterone and allopregnanolone have been shown to alleviate anxiogenic-like behaviors (Bitran et al. 1995, Brot et al. 1997, Laconi et al. 2001) and blunt the release of CRF following exposure to stress (Drugan et al. 1993, Frye et al. 2006, Owens et al. 1992, Patchev et al. 1994, Purdy et al. 1991). During abstinence, endogenous P has been hypothesized to reduce cue- and stress-induced cravings and relapse by reducing withdrawal-induced HPA activation and mediating components of stress dysregulation (Fox and Sinha 2009, Anker and Carroll 2010).

Another growing body of research has evaluated the physical and psychological benefits of exercise and physical activity and has begun exploring potential treatment applications for exercise as a behavioral intervention (USDHHS 1996, Ussher et al. 2012, Smith and Lynch 2011). Controlled laboratory studies in humans demonstrated that moderate-intensity aerobic exercise decreased cravings for alcohol (Ussher et al. 2004), cigarettes (Daniel et al. 2004), and cannabis (Buchowski et al. 2011), while brief episodes of isometric (Ussher et al. 2006, Ussher et al. 2009) and aerobic (Ussher et al. 2001, Daniel et al. 2004, Williams et al. 2011) exercise also alleviated symptoms of tobacco withdrawal. Studies with animals have also revealed promising treatment effects of exercise on drug-motivated behaviors (Smith and Pitts 2011, Kanarek et al. 1995, Miller et al. 2011, Cosgrove et al. 2002, McMillan et al. 1995, Ehringer et al. 2009, Smith et al. 2011, Zlebnik et al. 2012). Reinstatement of cocaine seeking precipitated by exposure to cocaine (Zlebnik et al. 2010, Smith et al. 2012) or cocaine-paired cues (Lynch et al. 2010, Smith et al. 2012) was attenuated in exercising rats compared to sedentary rats, and exercise over a withdrawal period also decreased subsequent cocaine seeking (Lynch et al. 2010). Several studies have investigated sex differences in the efficacy of exercise as a treatment for drug abuse, and as with pharmacological agents (Campbell et al. 2002, Carroll et al. 2001, Cosgrove and

Carroll 2004), results suggest that exercise attenuates drug seeking more in female than male rats. For example, concurrent access to wheel running (W) decreased intake of cocaine (Cosgrove et al. 2002) and ethanol (Ehringer et al. 2009) more effectively in females than males, and female rats exhibited faster extinction of responding for cocaine self-administration than male rats following chronic W (Smith et al. 2012).

While these results indicate that exercise is an effective sex-specific treatment intervention for drug use and relapse-related behaviors, the mechanism of the effects of exercise on addiction have not been well-studied. However, like P, evidence suggests that exercise reduces stress and anxiety (Asmundson et al. 2013, Sciolino and Holmes 2012). While P has not been used in combination with any other drug abuse treatment, exercise been recommended as an adjunct therapy for standard treatment of anxiety disorders (Sciolino and Holmes 2012) and may also be effective as a supplement to standard behavioral therapies for addiction. Exercise enhanced the effectiveness of behavioral counseling in traditional tobacco cessation programs (Martin et al. 1997) and also had an additive effect with contingency management for the treatment of substance use disorders in an outpatient setting (Weinstock et al. 2008). It is not known whether exercise would improve drug abuse treatment with pharmacological agents such as P and whether these effects would be sex-specific. However, given the promising individual treatment effects of P and exercise in humans and animals, an investigation of their combined therapeutic efficacy is warranted. In the present study, we investigated the treatment effects of W, P, or their combination (W+P) on cocaine-primed, cue-primed, and stress-primed reinstatement of cocaine seeking-behavior in male and female rats. We hypothesized that both W and P would have significant individual treatment effects, but that their combination would be more effective than either individual treatment alone. Further, while both W and P have shown greater treatment efficacy in females compared to males, we hypothesized that the combined treatment would be effective in both sexes, although with a greater effect size in females.

Materials and methods

Animals

Twenty-nine female and 22 male adult Wistar rats were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI, USA) and began behavioral testing around postnatal day 90. Sex differences were investigated in this study because previous work had shown sex differences in avidity for W (Boakes et al. 1999, Cosgrove et al. 2002, Eikelboom and Mills 1988, Lambert and Kinsley 1993) and in the ability of concurrent W to decrease cocaine self-administration (Cosgrove et al. 2002). These studies suggested possible differential exercise treatment effects between males and females. Estrous cycle was not monitored in the females, as animals were housed in their operant conditioning chambers with the attached wheel apparatus during the experiment, and vaginal lavage could have disrupted the cycle as well as cocaine- and wheel-reinforced behavior.

After arrival at the laboratory, rats were pair-housed in plastic cages with free access to laboratory chow (Teklad 2018, Harlan Laboratories, Madison, WI, USA) and water for at least 3-days of acclimation. Upon commencement of behavioral testing, each rat was removed from the plastic cages and placed in individual operant conditioning chambers where it remained for the duration of the study. Once transferred to the operant conditioning chambers, rats continued to have free access to water, and they were fed about 15 min after their daily sessions at 3:15 pm. Females and males were fed 16 g or 20 g, respectively, of rodent meal (Teklad 2018 ground meal, Harlan Laboratories) to maintain them at 85% of their free-feeding body weight. Mean body weights throughout the experiment did not differ among the female groups (FLW = 263 ± 4.62, FW = 254 ± 5.56) or male groups (MLW = 401 ± 6.64, MW = 399 ± 6.01). All rodent holding rooms were maintained at 24°C and at 40-50% humidity under a light/dark cycle (12/12-h) with room lights on at 6:00 am. The experimental protocol (1008A87755) was approved by the University of Minnesota Institutional Animal Care and Use Committee. The experiment was conducted in compliance with the Principles of Laboratory Animal Care (National Academies

Press 2011), and all laboratory facilities were accredited by the American Association for the Accreditation of Laboratory Animal Care.

Apparatus

Animals were housed and tested in custom-built, octagonally-shaped operant conditioning chambers enclosed in wooden sound-attenuating boxes equipped with ventilation fans as previously described (Zlebnik et al. 2010, Zlebnik et al. 2012). During cocaine self-administration sessions, responding on the active/drug-paired lever activated the syringe pump (PHM-100, MedAssociates Inc.) to deliver cocaine infusions through a swivel-tether (375/22PS, Instech, Plymouth Meeting, PA, USA, C313CS-MN, PlasticsOne, Roanoke, VA, USA) infusion system that was attached to an infusion harness (CIH95AB, Instech) worn by the rat. The swivel and tether allowed free movement within the operant conditioning chamber and easy access to the adjoining running wheel to accommodate concurrent drug self-administration and W. Data collection and programming were conducted using PC computers with a Med-PC interface (MedAssociates, Inc.).

Drugs

Cocaine HCl (National Institute of Drug Abuse, Research Triangle Institute, Research Triangle Park, NC, USA) was dissolved in 0.9 % NaCl at a concentration of 1.6 mg cocaine HCl/1 ml saline, and heparin (5 USP/ml) was added to the cocaine solution to prevent catheter occlusion from thrombin accumulation. The flow rate of each cocaine infusion was 0.025 ml/sec, and the duration of pump activation (1 sec/100 g of body weight) was adjusted weekly to provide a 0.4 mg/kg cocaine dose throughout self-administration testing. Progesterone (Sigma Aldrich, St. Louis, MO, USA) was dissolved in peanut oil (0.625 mg/ml) and administered at the 0.5 mg/kg (sc) dose that previously had been shown to decrease reinstatement of cocaine seeking (Anker et al. 2007). Yohimbine (Lloyd Laboratories, Shenandoah, IA, USA) was administered at a dose of 2.5 mg/kg (ip), as prior work demonstrated that this dose reliably reinstated cocaine

seeking alone (Feltenstein and See 2006; Anker and Carroll 2010) and in combination with cocaine-paired cues (Feltenstein and See 2006).

Catheterization surgery

One to three days after achieving wheel-running behavior, rats were implanted with an indwelling catheter in the right jugular vein following previously published methods (Carroll and Boe 1982, Zlebnik et al. 2010, Zlebnik et al. 2012). Following the surgical procedure, doors to the wheels remained closed, and each rat was fitted with an infusion harness and tether that remained in place throughout the remainder of the study.

Procedure

Maintenance of cocaine self-administration

The experimental procedure (Table 3-1) consisted of 5 phases: 1) wheel training, 2) self-administration training, 3) maintenance, 4) extinction, and 5) reinstatement of cocaine seeking. Wheel running acquisition and training of cocaine self-administration followed methods previously published (Zlebnik et al. 2010). During self-administration training and maintenance, sessions began with illumination of the house light, and responses on the active/drug-paired lever started the infusion pump and illuminated the stimulus lights located directly above the lever for the duration of the infusion. Responses on the active lever during the length of the infusion (2-4 seconds for females and 3-5 seconds for males) were recorded but had no programmed consequences. Responses on the inactive lever illuminated the stimulus lights above that lever for the same duration as an infusion but did not activate the infusion pump. Rats were allowed to self-administer iv cocaine (0.4 mg/kg/infusion) for ten 6-h sessions. The door leading to the wheel remained closed throughout the self-administration training and maintenance (no wheel access) phases.

Table 3-1. Experimental timeline.

Phase	Wheel training	Maintenance	Extinction	Reinstatement conditions given in random order				
				Yoh	*Yoh + Cues*	*Cues*	*Coc*	*Coc + Cues*
Days	3	10	14	20 (4 sessions/condition)				
Sessions	6 h/day, 9 am – 3 pm							

Yoh = yohimbine, 2.5 mg/kg, ip; Coc = cocaine, 10 mg/kg, ip; Cues = house light, lever lights, infusion pump

Extinction

Following maintenance, cocaine solutions were removed for the remainder of the study. For 14 sessions, auditory (infusion pump) and visual (house light and stimulus lights) stimuli associated with cocaine self-administration were discontinued to allow rats to extinguish lever pressing. During this period, females (F) and males (M) were each divided into 2 groups, 1 with access to a locked (LW) running wheel (FLW and MLW) and 1 with access to an unlocked (W) running wheel (FW and MW), to assess the effect of concurrent W on extinction and any potential carry-forward effect of W on subsequent reinstatement of cocaine-seeking behavior.

Reinstatement

Next, all groups were tested in a within-subjects procedure for reinstatement of cocaine seeking precipitated by yohimbine (2.5 mg/kg, ip) alone, yohimbine + cocaine-paired stimuli, cocaine (10 mg/kg, ip), cocaine + cocaine-paired stimuli, or cocaine-paired stimuli alone in the presence of W, P (P; 0.5 mg/kg, sc), or W+P. All rats were tested under all 5 priming conditions in nonsystematic order, with the exception that the yohimbine alone priming condition always came first. It was hypothesized that there may be a possible carry-forward effect of chronic W during extinction on yohimbine-primed cocaine seeking during later reinstatement tests, since prior research supported a role for exercise in reducing physiological responses to stress (Sasse et al. 2008, Masini et al. 2011). Nonsystematic treatment sequences within the 5 different priming condition blocks ("X"; e.g., yohimbine alone, yohimbine + cocaine-paired stimuli, cocaine alone, cocaine + cocaine-paired stimuli, and cocaine-paired stimuli alone) were as follows: LW+X, W+X, LW+P+X, W+P+X. On intervening days, saline priming injections were administered as a control condition and to allow responding to extinguish before the next priming injection. Subcutaneous vehicle (peanut oil) injections were administered to control for P injections, and on all days when W was not available, rats had access to a locked running wheel (LW).

Data Analysis

The primary dependent measures were responses during maintenance and responses and wheel revolutions during extinction and reinstatement. For maintenance and extinction, data were grouped into 2-day blocks to reduce daily variability and the number of post-hoc contrasts. These measures were analyzed with 3-factor mixed analyses of variance (ANOVA) with sex and extinction wheel condition (LW vs. W) as the between-subjects factors and blocks of days or treatment during reinstatement (e.g., LW, W, LW+P, W+P) as the repeated measure. Separate 3-factor ANOVA were performed for each priming condition (cocaine, yohimbine, cues, cocaine+cues, yohimbine+cues). Following significant interactions, post hoc tests were performed with Fisher's least significant difference (LSD) protected t-tests, and results were considered significant if $p < 0.05$. Statistical analyses were performed using GB Stat (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Results

Maintenance

Figure 3-1 depicts the mean number of responses for cocaine (0.4 mg/kg/inf) during daily 6-h sessions over the 10-day maintenance period. A significant main effect of sex ($F_{1, 254} = 7.86$, $p = 0.0072$) indicated that females responded more for cocaine than males, and a significant main effect of 2-day blocks ($F_{4, 254} = 2.61$, $p = 0.0367$) indicated that responding for cocaine escalated or increased in males and females over the course of the maintenance period. There was no sex X block interaction. To specifically assess escalation of responding, block 1 (days 1-2) was compared to block 5 (days 9-10) in a separate 2-factor repeated-measures ANOVA. Results demonstrated a significant increase in responding at the end of the maintenance period compared to the beginning of the maintenance period (main effect: $F_{1, 101} = 6.77$, $p = 0.0122$), and this effect occurred for both males and females, as there was no significant main effect of sex or sex X block interaction. Therefore, females responded significantly more for cocaine than males, and both groups escalated their responding over the maintenance period.

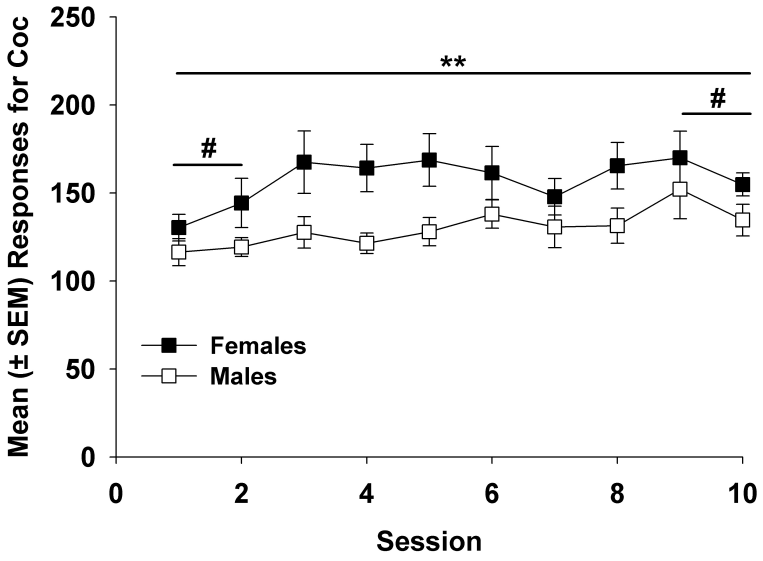

Figure 3-1. Mean (± SEM) daily responses for cocaine (0.4 mg/kg/inf) made by males and females during the 10-day maintenance period. Overall, females made more responses than males ($p < 0.01$), and both females and males significantly increased their responding over the 10-day period (days 1-2 vs. days 9-10, # $p < 0.05$).**

Extinction

Overall, Figure 3-2 shows that females were more resistant to extinction than males, and concurrent access to W reduced extinction responding more in females than males. Mean responses during the 14-day extinction period are shown separately for males and females. A 3-factor (sex X W X block of days) ANOVA revealed significant main effects of W ($F_{1,356} = 9.35$, $p = 0.0037$) and block of days ($F_{6,356} = 30.10$, $p < 0.0001$), and significant W X block of days ($F_{6,356} = 3.40$, $p = 0.003$) and sex X W X block of days ($F_{6,356} = 2.16$, $p = 0.0468$) interactions. Post hoc analyses showed a sex difference in the attenuating effect of W on extinction responding during the initial days of the extinction period. Females with access to W (FW) made fewer unreinforced responses during block 1 (days 1-2; $p < 0.01$), block 2 (days 3-4; $p < 0.01$), and block 6 (days 11-12; $p < 0.05$) compared to females with access to LW (FLW) (Fig. 3-1A); however, there were no differences in extinction responding among the male groups (MW and MLW) (Fig. 3-1B). Additionally during block 1, females with access to LW (FLW) had greater

responding ($p < 0.01$) than males with access to LW (MLW), while females with access to W (FW) had lower responding ($p < 0.05$) than males with access to W (MW). However, while access to W decreased unreinforced responding in females but not males, daily wheel revolutions did not differ between males and females throughout the extinction period (Table 3-2).

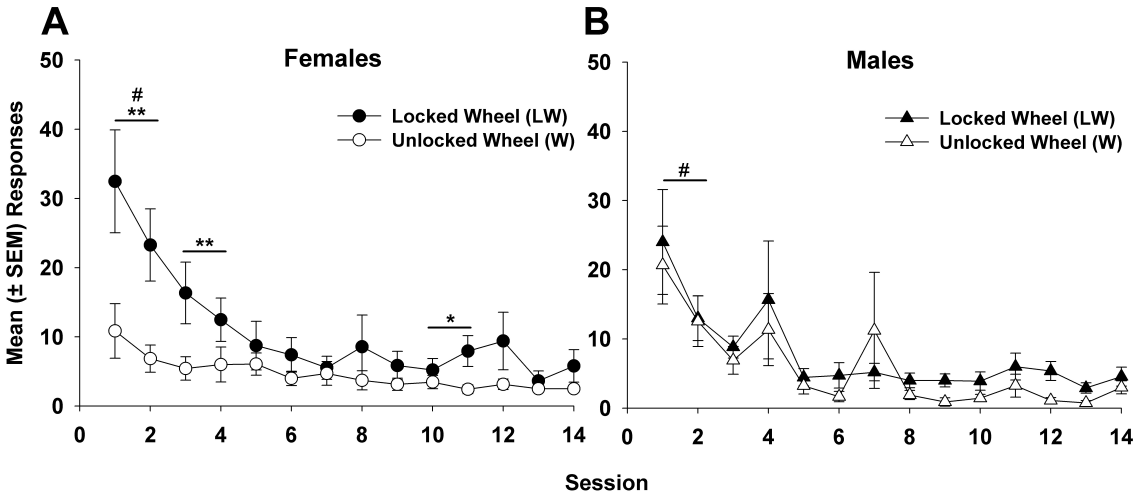

Figure 3-2. Mean (± SEM) daily unreinforced responses during the 14-day extinction period. (A) Females with access to W (FW) made significantly fewer responses than females with concurrent access LW (FLW) during days 1-2, 3-4, and 10-11 ($p < 0.01$, * $p < 0.05$). Further, FW had less responding than MW, while FLW had more responding than MLW during days 1-2 (# $p < 0.05$). (B) There were no differences in responses made by the male groups (MW and MLW).**

Table 3-2. Mean (SEM) wheel revolutinos during extinction and reinstatement periods.

	Extinction	Reinstatement									
		Cues		Yohimbine		Cocaine		Cocaine + Cues		Yohimbine + Cues	
		W	*W+P*	*W*	*W+P*	*W*	*W+P*	*W*	*W+P*	*W*	*W+P*
Females											
FLW	--	271 (177)	233 (72)	435 (100)	654 (155)#	883 (214)&†	934 (252)†	712 (110)†	674 (125)†	683 (188)	665 (218)
FW	421 (72)	609 (289)	889 (286)*#	800 (171)@	842 (224)#@	918 (281)&†	642 (157)†	643 (99)†	715 (140)†	834 (236)	928 (258)†
Males											
MLW	--	273 (86)	324 (85)	348 (50)	394 (70)#	496 (100)&	331 (64)	355 (69)	411 (91)	491 (79)	725 (141)#
MW	245 (84)	199 (72)	513 (154)*#	709 (158)@	804 (220)#@	525 (141)&	314 (121)	322 (115)	376 (130)	637 (181)	515 (149)

* MW+FW > MLW+FLW; # W+P > W; @ MW, FW > MLW, FLW; & W > W+P; † F > M

Cue-primed reinstatement

Reinstatement responding elicited by cocaine-paired stimuli or cues is shown in Figure 3-3A. The effects of treatment with concurrent W, P, or their combination (W+P) on cue-primed reinstatement of cocaine seeking were analyzed by a 3-factor (sex X extinction wheel condition X treatment) ANOVA. Results indicated a significant main effect of treatment ($F_{4,214} = 8.33$, $p < 0.0001$) but otherwise nonsignificant main effects of sex and extinction conditions as well as nonsignificant interactions. Therefore, data were collapsed across sex and extinction wheel condition, and treatment effects were analyzed in a 1-factor repeated-measures ANOVA. Post hoc analyses following a significant F-test ($F_{4,249} = 7.34$, $p < 0.0001$) demonstrated a treatment effect by which animals made significantly fewer responses after treatment with W ($p < 0.05$) and W+P ($p < 0.01$) compared to the LW condition. Therefore, treatment with W alone and W+P significantly reduced cue-primed reinstatement responding in males and females.

Wheel revolutions during cue-primed reinstatement sessions (W vs. W+P only; Table 3-2) also were analyzed by a 3-factor (sex X extinction wheel condition X treatment) ANOVA. As with cue-primed reinstatement responses, there were no main effects of sex or extinction wheel condition. However, there was a significant main effect of treatment (W vs. W+P; $F_{1,77} = 8.71$, $p = 0.0056$) and a significant extinction wheel condition X treatment interaction ($F_{1,77} = 7.96$, $p = 0.0078$). After collapsing across sex, a 2-factor ANOVA revealed a main effect of treatment ($F_{1,77} = 7.99$, $p = 0.0075$) and an extinction condition X treatment interaction ($F_{1,77} = 5.53$, $p = 0.0241$). Post hoc analyses showed greater wheel revolutions ($p < 0.01$) for the males and females that had access to W during extinction (MW + FW) when treated with P than with vehicle. Additionally, when treated with P, rats in MW + FW made significantly more revolutions ($p < 0.01$) than MLW + FLW.

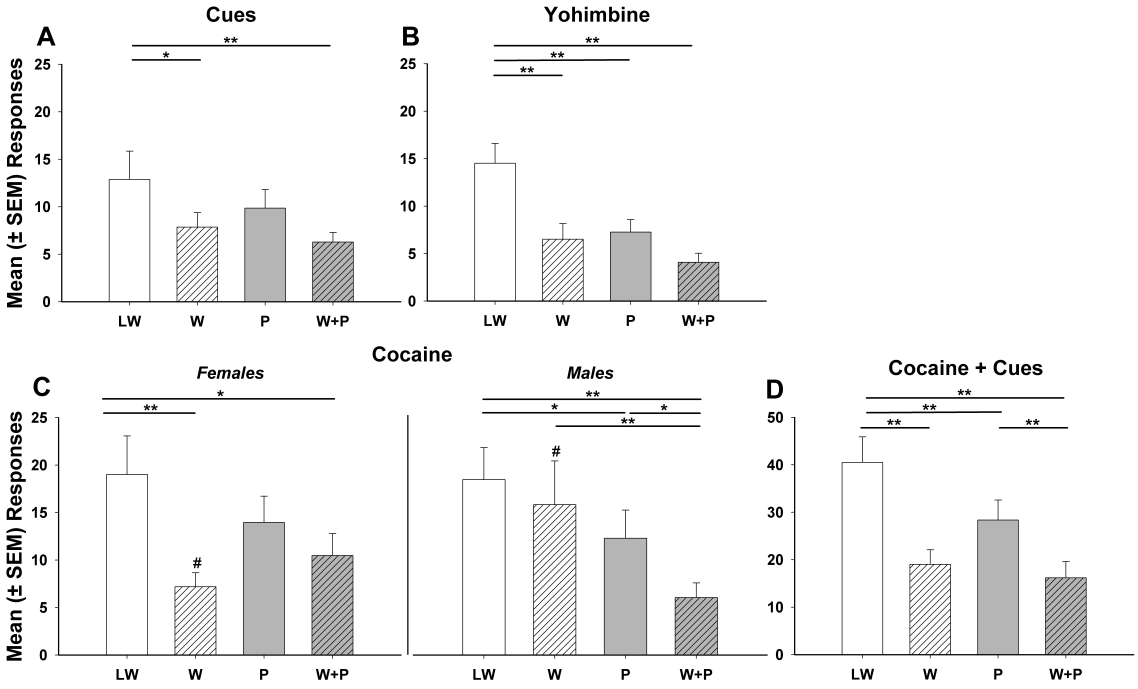

Figure 3-3. Mean (± SEM) responses per treatment session over 4 separate reinstatement priming conditions (e.g., cocaine-paired cues or stimuli, yohimbine, cocaine, cocaine + cocaine-paired cues). As there were no significant main effects of sex or extinction wheel condition (W vs. LW), in most cases data were collapsed across groups to focus on treatment effects. (A) Cocaine-paired cue-primed reinstatement responding was attenuated by W + vehicle treatment (W; * $p < 0.05$) and W+P ($p < 0.01$) compared to control conditions LW + vehicle treatment (LW). (B) Yohimbine-primed reinstatement responding was reduced by W, P, and W+P (** $p < 0.01$) compared to LW. (C) Due to a sex X treatment interaction, males and females were not combined for the cocaine-primed reinstatement condition. Unlocked wheel running access W significantly attenuated responding compared to LW conditions in females but not males (# $p < 0.01$). Among females, W+P also reduced cocaine-primed reinstatement (* $p < 0.05$); however, among males, P (* $p < 0.05$) and W+P (** $p < 0.01$) both decreased responding compared to LW. Further, an additive treatment effect was seen for the W+P treatment combination in males as responding under this condition was significantly lower than under either W (** $p < 0.01$) or P (* $p < 0.05$) conditions alone. (D) Reinstatement responding precipitated by the combination of cocaine priming injection and cocaine-paired cues was reduced by W, P, and W+P (** $p < 0.01$). Further, reinstatement responding under W+P conditions was significantly lower than under P alone conditions, suggesting a greater treatment effect of W+P (** $p < 0.01$).**

Yohimbine-primed reinstatement

Yohimbine-primed reinstatement responding (Fig. 3-3B) also was analyzed with a 3-factor ANOVA, and results revealed only a significant main effect of treatment ($F_{4,249} = 13.31$, p

< 0.0001). Therefore, the sex and extinction condition factors were collapsed, and treatment effects were analyzed with a 1-factor ANOVA. Post hoc tests after a significant F-test ($F_{4,249}$ = 12.87, $p < 0.001$) confirmed a treatment effect of W alone (W; $p < 0.01$), P alone (LW+P; $p < 0.01$), and their combination (W+P; $p < 0.01$) as reinstatement responding under these conditions was significantly less than under LW conditions. These results indicate that all 3 treatments (e.g., W, P, W+P) effectively reduced yohimbine-primed reinstatement.

Analysis of wheel revolutions during the yohimbine-primed reinstatement period (Table 3-2) revealed significant main effects of extinction wheel condition ($F_{1,93} = 6.98$, $p = 0.0115$) and treatment ($F_{1,93} = 4.80$, $p = 0.0341$) but no significant interactions. Data were collapsed across sex, and a 2-factor repeated-measures ANOVA was conducted, and there were significant main effects of extinction wheel condition ($F_{1,93} = 6.13$, $p = 0.0171$) and treatment ($F_{1,93} = 4.27$, $p = 0.0445$) but no significant extinction wheel condition X treatment interaction.

Cocaine-primed reinstatement

Analysis of cocaine-primed reinstatement responding (Fig. 3-3C) revealed a significant main effect of treatment ($F_{4,219} = 19.84$, $p < 0.0001$) and a sex X treatment interaction ($F_{4,219} = 3.19$, $p = 0.015$), but there were no other significant main effects or interactions. Data were collapsed across extinction wheel condition, and results of the 2-factor (sex X treatment) ANOVA showed a significant main effect of treatment ($F_{4,219} = 13.47$, $p < 0.0001$) and a significant sex X treatment interaction ($F_{4,219} = 2.60$, $p = 0.0379$). Post hoc comparisons indicated a differential effect of W in females (FW+FLW) compared to males (MW+MLW): cocaine-primed reinstatement responding under W conditions was significantly lower ($p < 0.01$) than under LW conditions in females but not in males. No other differences were seen between males and females. Among females, in addition to the treatment effect of W, treatment with W+P

significantly decreased reinstatement responding ($p < 0.05$) compared to LW. Among males, P ($p < 0.05$) and W+P ($p < 0.01$) both decreased responding compared to LW. Further, since W+P conditions had significantly lower responding than either W ($p < 0.01$) or P ($p < 0.05$) alone, an additive treatment effect was found for the W+P treatment combination in males. Overall, W attenuated cocaine-primed reinstatement in females but not males, and the combination W+P was significantly more effective than either W or P alone in males.

Wheel revolutions during cocaine-primed reinstatement sessions with access to W (both W and W+P; Table 3-2) were analyzed by 3-factor ANOVA. There were significant main effects of sex ($F_{1,87} = 8.83$, $p = 0.005$) and treatment ($F_{1,87} = 4.43$, $p = 0.0416$) but no interactions; thus data were collapsed across extinction wheel condition for analysis in a 2-factor ANOVA. This analysis resulted in a significant main effect of sex ($F_{1,87} = 6.61$, $p = 0.0138$), indicating that females completed more revolutions than males, and a significant main effect of treatment ($F_{1,87} = 4.57$, $p = 0.0384$), indicating that rats made fewer revolutions when treated with P vs. vehicle. The sex X treatment interaction was not significant.

Cocaine + cocaine-paired cue-primed reinstatement

Reinstatement responding precipitated by the combination of cocaine and cocaine-paired cues or stimuli (Fig. 3-3D) was analyzed by 3-factor ANOVA. As for the reinstatement conditions described above, results indicated a significant main effect of treatment ($F_{4,229} = 22.45$, $p < 0.0001$) but otherwise nonsignificant main effects of sex and extinction conditions. Further there were no significant interactions, so sex and extinction wheel condition factors were collapsed to utilize a 1-factor repeated-measures ANOVA. Following a significant F-test ($F_{4.249} = 20.70$, $p < 0.0001$), post hoc comparisons revealed significant attenuation of responding under W ($p < 0.01$), P ($p < 0.01$), and W+P ($p < 0.01$) conditions compared to LW conditions. Further,

W+P decreased reinstatement responding relative to P alone ($p < 0.01$). While these results indicate significant attenuation of cocaine + cocaine-paired cue-primed reinstatement by W, P, and W+P, they also suggest a greater treatment effect with W+P vs. P alone.

Again, wheel revolutions during the cocaine + cocaine-paired cue-primed reinstatement period (Table 3-2) were also analyzed by 3-factor ANOVA. There was a significant main effect of sex ($F_{1,91} = 13.92$, $p = 0.0006$) but no other significant main effects or interactions. Subsequently, the extinction wheel condition and treatment factors were collapsed, and a 2-tailed Student's t-test was used strictly to compare male vs. female mean revolutions. Females had significantly greater wheel revolutions than males during this reinstatement condition ($t_{88} = 4.12$, $p = 0.0001$).

Yohimbine + cocaine-paired cue-primed reinstatement

Like for other reinstatement priming conditions, yohimbine + cocaine-paired cue-primed reinstatement responding (Fig. 3-4) was analyzed by 3-factor ANOVA, and there was no significant main effect of sex or extinction wheel condition. However, there was a main effect of treatment ($F_{4,244} = 55.78$, $p < 0.0001$) and significant sex X extinction wheel condition ($F_{1.244} = 4.87$, $p = 0.0325$) and sex X extinction wheel condition X treatment ($F_{4,244} = 5.93$, $p = 0.0002$) interactions. Post hoc comparisons revealed significant differences among all 4 groups under LW conditions, likely contributing to factorial interactions. The FW group made fewer responses than both FLW ($p < 0.01$) and MW ($p < 0.01$), and FLW made more responses than MLW ($p < 0.01$). However, interestingly MW made more responses than MLW ($p < 0.01$). There were no other significant differences among the groups. Comparing treatment conditions, all groups showed an attenuation of reinstatement responding under W, P, and W+P conditions relative to

LW conditions. Further, for FLW and MW, responding under the W+P condition was significantly lower ($p < 0.05$) than under the P alone condition.

Results of the 3-factor ANOVA analyzing wheel revolutions during the yohimbine + cocaine-paired cue-primed reinstatement period (Table 3-2) revealed no significant main effects of sex, extinction wheel condition, or treatment, but there was a significant sex X extinction wheel condition X treatment interaction ($F_{1,89} = 4.20$, $p = 0.0469$). Subsequent comparisons demonstrated that FW had higher wheel revolutions than MW when treated with P (W+P; $p < 0.01$), and MLW males had more revolutions when treated with P vs. vehicle (W+P vs. W; $p < 0.05$).

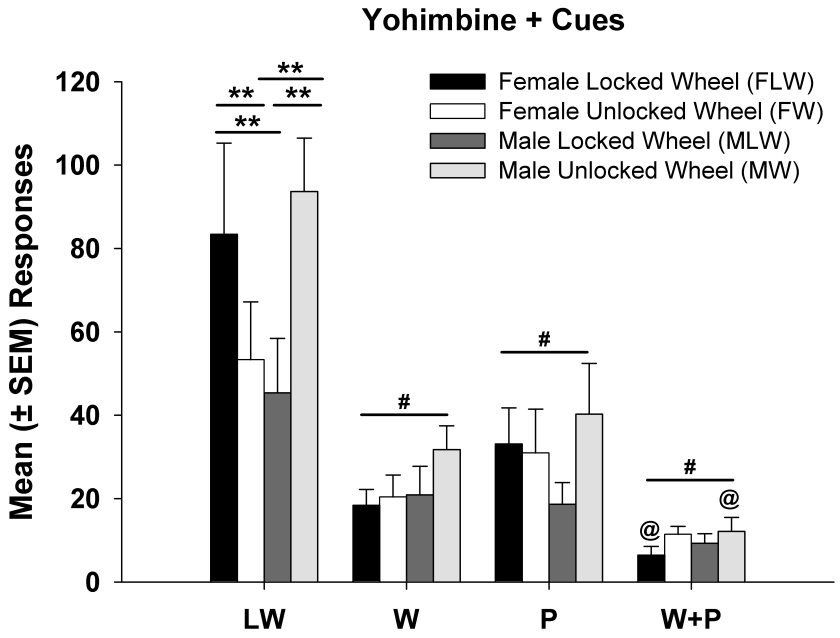

Figure 3-4. Mean (± SEM) reinstatement responses per treatment session for the yohimbine + cocaine-paired cue condition. There were significant differences among all 4 groups under LW conditions: FW made fewer responses than both FLW ($p < 0.01$) and MW, and FLW made more responses than MLW; however, MW made more responses than MLW. There were no other significant differences among the groups. Comparing treatment conditions, all groups showed an attenuation of reinstatement responding under W, P, and W+P vs. LW (# $p < 0.05$). Further, for FLW and MW, there was an enhanced treatment effect of W+P, as responding under that condition was significantly lower (@ $p < 0.05$) than under the P alone condition.**

Discussion

This experiment used a novel treatment approach to attenuate cocaine-seeking behavior in a rodent model of relapse. Wheel running and P, highly effective treatments, were examined separately and in combination for their ability to decrease reinstatement of cocaine seeking by singular priming conditions such as cocaine; a stress-inducing agent, yohimbine; and cocaine-paired cues; and also by compound priming conditions such as cocaine + cocaine-paired cues and yohimbine + cocaine-paired cues. Results confirmed earlier work demonstrating greater treatment effects of W on cocaine seeking in female vs. male rats (Cosgrove et al. 2002). Further, this investigation found that the combination of W and P was more effective in reducing

reinstatement of responding than either treatment alone in rats that were less responsive to each individual treatment (e.g., males) and more effective than P alone following the most challenging priming conditions (e.g., cocaine + cocaine-paired cues, yohimbine + cocaine-paired cues). These results suggest that combining both behavioral (W) and pharmacological (P) treatment strategies may be a highly successful approach to reducing addiction-related behaviors.

The present experiment extended findings from earlier work on sex differences in cocaine self-administration and in the individual treatment effects of W and P on cocaine-seeking behavior. Rats were given long-access (6-h) sessions to cocaine self-administration under a fixed ratio 1 (FR 1) schedule at the 0.4 mg/kg unit dose during the maintenance phase of the reinstatement procedure, and both males and females escalated their responding for cocaine over this period. In accordance with previously published work (Roth and Carroll 2004), females had significantly more responding for cocaine than males over a period as short as 10 days. These differential rates of responding were maintained during the beginning of the extinction phase as females (FLW) had significantly higher rates of extinction responding than males (MLW) during initial sessions following discontinuation of cocaine self-administration. However, all groups reached low levels of responding by the end of the extinction period; and despite having initially greater extinction responding, females were more receptive to treatment with W than males. Although wheel revolutions did not differ among the groups, W significantly attenuated extinction in the FW group but not the MW group. Prior work has demonstrated a reduction in extinction for females with access to W (Zlebnik et al. 2010) and for both males and females with chronic access to W in the home cage (Smith et al. 2012). Although Smith et al. (2012) showed that W decreased extinction responding in both males and females, their results indicated a stronger effect in females than males with a suppression of responding for a greater period of time

(i.e., over more days). Together, the results of the present experiment and those of previous studies demonstrate greater cocaine-motivated responding and greater treatment effects of W in female than male rats.

While there were few differences among male and female rats in the magnitude of reinstatement of cocaine-seeking behavior under control treatment (LW) conditions across all 5 reinstatement priming conditions, there was a difference in their response to treatment with W. Consistent with results during the extinction phase, W significantly reduced cocaine-primed reinstatement of cocaine seeking in female but not male rats. During this reinstatement priming condition and the cocaine + cocaine-paired cue priming condition, females ran more than males (Table 3-2). However, W had equivalent treatment effects on cocaine + cocaine-paired cue-induced responding in males and females, suggesting that the greater treatment effect for cocaine-primed reinstatement in females was not necessarily due to their engagement in greater levels of W than the males. For other priming conditions (e.g., yohimbine, cocaine-paired cues, cocaine + cocaine-paired cues, yohimbine + cocaine-paired cues) in the present experiment, W reduced reinstatement equally well in both sexes. These results suggest that sex differences in the efficacy of exercise to decrease cocaine seeking may be influenced by the conditions provoking reinstatement.

Treatment with P reduced cocaine seeking precipitated by yohimbine, cocaine, cocaine + cocaine-paired cues, and yohimbine + cocaine-paired cues. That this investigation found that exogenously-administered P did not decrease cocaine seeking in response to cocaine-paired cues in rats was in contrast to clinical reports showing reduced cocaine-paired cue-induced craving during phases of high endogenous P (Sinha et al. 2007); however, under control conditions, responding precipitated by cues was relatively low compared to other work on cue-induced

reinstatement collected in the same laboratory (Anker and Carroll 2010, 2011). These results may be explained by the multicomponent reinstatement procedure whereby cues were presented many times over the course of 20 sessions, facilitating extinction to these stimuli.

While most prior work has indicated a significant treatment effect of P on cocaine seeking in female vs. male rats (Carroll and Anker 2010, 2011), P significantly decreased cocaine-primed reinstatement in males, but results demonstrated only a nonsignificant trend for the attenuation of cocaine-primed reinstatement in females. The length and nature of the reinstatement procedure used in the present investigation may have resulted in diminished responding under control treatment (LW) conditions, masking treatment effects in the female groups. In humans, reports regarding the treatment efficacy of P in males have been mixed. While one study found a significant attenuation of positive subjective effects and cardiovascular responses in men (Sofuoglu et al. 2004), another found little to no effect on cocaine's subjective effects (Evans and Foltin 2006) and cocaine use (Sofuoglu et al. 2007) and only dose-dependent decreases in cardiovascular responses to cocaine (Evans and Foltin 2006) in males. These equivocal findings are in accordance with rodent studies demonstrating P treatment as both effective (Romieu et al. 2003) or ineffective (Russo et al. 2010) in decreasing cocaine conditioned place preference in males. Overall, few investigations have examined the role of P to reduce cocaine-motivated behavior in males, and the results of this experiment suggest that treatment with P under the present conditions reduces cocaine seeking in male rats.

In contrast to treatment effects seen with concurrent unlocked wheel access and P separately, treatment with W+P significantly attenuated cocaine seeking compared to control treatment (LW) across all 5 priming conditions, indicating efficacy over a broader range of relapse-provoking stimuli than individual treatments alone. For male rats under cocaine priming

conditions, the combination of W+P was more effective at reducing cocaine seeking than either W or P treatment alone. Additionally, compound priming conditions such as cocaine + cocaine-paired cues and yohimbine + cocaine-paired cues elicited higher rates of responding than singular priming conditions (Feltenstein and See 2006), and the combination of W+P decreased cocaine seeking in response to these stimuli more effectively than P treatment alone. Together, all of these data suggest that the combination of concurrent W+P may be more effective at reducing cocaine seeking than each individual treatment, and this is in accordance with clinical work demonstrating additive or synergistic treatment effects of methadone maintenance (McLellan et al. 1993, Peirce et al. 2006) and tricyclic antidepressants (Kosten at al. 2003, Poling et al. 2006) with behavioral therapies. The present study also extends findings demonstrating greater treatment outcomes for addiction when cognitive (Martin et al. 1997) or motivational (Weinstock et al. 2008) behavioral strategies are combined with exercise. Together, the results of the current experiment and prior work demonstrate greater efficacy from combined behavioral + pharmacological treatments vs. singular treatments.

For individuals in recovery, the combination of behavioral and pharmacological treatments may help overcome frequent challenges to maintaining abstinence. Threats to recovery include drug cravings precipitated by both external (e.g., people, places, things) and internal (e.g., stress) cues, and former addicts must learn to tolerate and resist craving as well as develop healthier patterns of behavior that replace drug reinforcement with alternative nondrug reinforcement (Potenza et al. 2011). The present findings suggest that access to exercise may decrease drug seeking during initial abstinence and that, compared to either treatment alone, the combination of exercise and P may prevent relapse to cocaine-seeking behavior over a broader range of relapse-provoking stimuli, including both external (e.g., cocaine-paired stimuli) and

internal (e.g., cocaine, yohimbine) drug-related cues. Additionally, the incorporation of exercise in a dual treatment plan could accelerate adoption of healthier behaviors and augment attempts to sustain abstinence (Ussher et al. 2012). These treatment effects may be especially effective in individuals with certain vulnerability factors for addiction (i.e., females vs. males), suggesting a customized approach using treatment combinations in order to achieve optimal treatment outcomes. Overall, the present investigation demonstrated that, under certain conditions, combined behavioral (W) and pharmacological (P) interventions were more successful at reducing cocaine-seeking behavior than either intervention alone, and these results may have implications for the treatment of substance abuse in clinical settings.

CHAPTER 4

Experiment 3: Effects of the combination of concurrent wheel running and atomoxetine on reinstatement of cocaine-seeking behavior in female rats selected for high or low impulsivity

Zlebnik NE, Carroll ME (2014) Effects of the combination of wheel running and atomoxetine on cue- and cocaine-primed reinstatement in rats selected for high or low impulsivity. Psychopharmacology (Berl) doi: 10.1007/s00213-014-3744-6

Rationale

Extending the findings of Experiment 3, Experiment 4 examined the combined treatment effects of concurrent wheel running and another pharmacological treatment, atomoxetine, on cocaine-seeking behavior in rats selected for high and low impulsivity, another animal model of differential drug abuse vulnerability. Atomoxetine is a norepinephrine reuptake inhibitor that has been approved by the FDA for the treatment of attention-deficit/hyperactivity disorder (ADHD), but recently, it has been considered as a pharmacotherapy for psychostimulant addiction (Sofuoglu and Sewell 2009, Somaini et al. 2011, Economidou et al. 2009). While several studies examined exercise as an augmentative treatment strategy for the standard treatment of ADHD (i.e., methylphenidate, atomoxetine), there had not been any thorough study of exercise and atomoxetine on drug-seeking behavior. Thus, Experiment 4 assessed the effects of this dual treatment approach on both cocaine- and cue-induced reinstatement.

Introduction

Relapse to drug use is a major challenge to the treatment of addiction, and the development of novel treatment approaches requires greater understanding of the psychological and neurobiological mechanisms underlying vulnerability to substance use and relapse. Accumulating evidence suggests that the most successful treatment strategies involve a combination of behavioral and pharmacotherapies (Potenza et al. 2011, McLellan et al. 1993, Peirce et al. 2006, Ball and Ross et al. 1991, Carroll et al. 1998, Carroll et al. 2004, Bickel et al. 1997), and recent animal work demonstrated that multiple treatments may be combined to generate customized treatment approaches for individuals with certain risk factors for addiction (Zlebnik et al. 2014). Among the major vulnerability factors for addiction and relapse is impulsivity (Perry and Carroll 2008, Carroll et al. 2009). Clinical studies have demonstrated that more impulsive individuals have greater drug use, display more severe withdrawal symptoms (Moeller et al. 2001), experience heightened cravings (Doran et al. 2007), and maintain shorter abstinence periods than lower impulsive individuals (Moeller et al. 2001). Rats screened for high trait impulsivity acquired drug self-administration more quickly (Perry et al. 2005, 2008), self-administer greater amounts of drug (Dalley et al. 2007, Belin et al. 2008), and exhibited higher drug-seeking behavior in an animal model of relapse compared to their low impulsive counterparts (Perry et al. 2008, Economidou et al. 2009, Broos et al. 2012, Diergaarde et al. 2008). Therefore, impulsive behavior not only predisposes an individual to develop substance use problems (de Wit 2009, Jentsch et al. 2014), but it also influences the likelihood of relapse after a period of abstinence (Doran et al. 2007, Moeller et al. 2001, Perry et al. 2008, Economidou et al. 2009, Broos et al. 2012, Diergaarde et al. 2008).

A current candidate pharmacotherapy for psychostimulant addiction is atomoxetine (ATO) (Sofuoglu and Sewell 2009, Somaini et al. 2011), an FDA-approved nonstimulant medication to reduce symptoms of attention-deficit/hyperactivity disorder (ADHD) such as inattention, hyperactivity, and impulsivity. Atomoxetine is a selective norepinephrine (NE) reuptake inhibitor that primarily increases NE in the prefrontal cortex (PFC) (Bymaster et al. 2002). Using ATO, studies have demonstrated reduced impulsivity across a range of behavioral tasks such as the 5-choice serial reaction time (Baarendse and Vanderschuren 2012, Robinson et al. 2008), stop-signal reaction time (Robinson et al. 2008), and delay-discounting (Bizot et al. 2011, Robinson et al. 2008) tasks (but see Baarendse & Vanderschuren, 2012, Broos et al. 2012, Sun et al. 2012). Regarding atomoxetine's effects on stimulant addiction, some clinical investigations have found no therapeutic effect on cocaine use (Levin et al. 2009, Walsh et al. 2013) and the subjective effects of methamphetamine (Rush et al. 2011), while others found reduced physiological and subjective effects of d-amphetamine (Sofuoglu et al. 2009), significant attenuation of alcohol cravings (Wilens et al. 2011), longer abstinence from alcohol use (Benegal et al. 2013), and fewer days of heavy alcohol drinking (Wilens et al. 2008). Similarly equivocal findings were demonstrated in rodents. Treatment with ATO did not affect cocaine self-administration (Economidou et al. 2009), but it did decrease cue-primed cocaine (Economidou et al. 2009, 2011) and heroin (Economidou et al. 2011) seeking in addition to reducing the conditioned stimulus effects of nicotine (Reichel et al. 2007) and nicotine withdrawal symptoms (Davis and Gould 2007). Together, these results argue for a potentially useful role for ATO in decreasing relapse-related behaviors vs. attenuating the acute rewarding effects of drugs of abuse, and further investigations will be needed to better characterize the therapeutic effects of ATO.

However, promising research aimed at quantifying combined treatment effects has shown that using ATO as an adjunct treatment to the standard treatment for ADHD has yielded better outcomes than standard treatment alone (Treuer et al. 2013, Holzer et al. 2013). Additionally, other research found that substance abuse relapse prevention counseling supplemented with ATO maintained longer abstinence than counseling by itself (Benegal et al. 2013). These results contribute to the body of literature supporting enhanced treatment by a combination of behavioral and pharmacological treatments (Potenza et al. 2011) and identify a role for ATO as an adjunct treatment strategy.

In addition to ATO, it has been suggested that substance abuse treatment can be augmented by the addition of aerobic exercise (USDHHS 1996, Ussher et al. 2012, Zlebnik et al. 2014). Like ATO, exercise increases brain catecholamines like NE and DA in the PFC (Ma 2008, Meeusen and de Meirleir 1995, Paluska and Schwenk 2000). Exercise has been shown to reduce cravings for alcohol (Ussher et al. 2004), cigarettes (Daniel et al. 2004), and cannabis (Buchowski et al. 2011), and it also alleviates symptoms of tobacco withdrawal (Ussher et al. 2001, Daniel et al. 2004, Williams et al. 2011). Further, animal models of relapse have demonstrated a reduction in cue- (Lynch et al. 2010, Smith et al. 2012, Zlebnik et al. 2014), stress- (Zlebnik et al. 2014), and cocaine- (Zlebnik et al. 2010, 2014, Smith et al. 2012) induced reinstatement of cocaine seeking by wheel running. As a supplemental treatment, exercise enhanced the effectiveness of behavioral counseling in traditional tobacco cessation programs (Martin et al. 1997) and had an additive effect with contingency management for the treatment of substance use disorders in an outpatient setting (Weinstock et al. 2008). Moreover, it has been suggested that exercise may enhance standard treatment of ADHD symptoms (Kim et al. 2010, Robinson et al. 2012, Wigal et al. 2013). While some have found no additive treatment effects of

exercise and methylphenidate (Medina et al. 2010), recent work in humans found greater attenuation of clinical ADHD symptoms when exercise was administered along with methylphenidate compared to control treatment (Choi et al. 2014). However, to date, the combination of exercise and ATO has not been investigated for its effects on addiction and relapse, but given prior results, its examination is warranted.

In the present experiment, effects of dual treatment with ATO and exercise were examined in high vs. low impulsive rats in an animal model of cocaine relapse. First, rats were screened for high (HiI) or low (LoI) impulsivity based on performance on a delay-discounting task and were subsequently trained to self-administer cocaine. Cocaine access was then discontinued, and animals were allowed to extinguish operant responding. Reinstatement of cocaine-seeking behavior was precipitated by cocaine priming injections or the presentation of cocaine-paired cues during sessions when rats were treated with ATO and/or given concurrent access to a locked or unlocked running wheel. Given previous work, it was hypothesized that the combination of ATO and wheel running would have a greater treatment effect than either treatment alone. Further, based on the effects of ATO and exercise on ADHD symptoms, it was hypothesized that W + ATO would more effectively reduce relapse-related behavior in HiI vs. LoI rats.

Materials and methods

Animals

Twenty-three adult female Wistar rats were used in this experiment (Harlan, Inc., Madison, WI) as prior work has shown that female rats demonstrated greater receptivity to treatment of cocaine seeking by wheel running than male rats (Cosgrove et al. 2002, Smith et al.

2011). Estrous cycle was not monitored to prevent disruption of cocaine- and exercise-maintained behavior by repeated vaginal lavage (Walker et al. 2002).

Before behavioral testing, rats were group-housed in polycarbonate cages with ad libitum access to food and chow (Harlan-Teklad 2018, Harlan, Inc.) in temperature (21-23°C)- and humidity (65%)-controlled colony rooms under a 12-h light-dark cycle (lights on at 6:00 am). All procedures conformed to the eighth edition of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (National Research Council 2011) and were approved by the University of Minnesota Institutional Animal Care and Use Committee. Laboratory facilities were certified by the American Association for the Accreditation of Laboratory Animal Care.

Apparatus

Rats were housed and tested in custom-build operant conditioning chambers as previously described (Zlebnik et al. 2010, 2012). Data collection and programming were conducted using PC computers with a Med-PC interface (MedAssociates, Inc., St. Albans, VT).

Drugs

Cocaine HCl (National Institute on Drug Abuse, Research Triangle Institute, Research Triangle Park, NC, USA) was dissolved in sterile saline at a concentration of 1.6 mg/ml, and heparin (5 USP/ml) was added to the cocaine solution to prevent catheter occlusion from thrombin accumulation. The flow rate of each cocaine infusion was 0.025 ml/s, and the duration of pump activation (1 s/100 g of body weight) was adjusted weekly to provide a 0.4 mg/kg unit dose throughout self-administration testing. Atomoxetine HCl (ATO) was purchased from Tocris Biosciences (Bristol, UK) and dissolved in sterile saline at a concentration of 3 mg/ml.

Catheterization surgery

Rats were implanted with chronic indwelling jugular catheters following methods previously described (Carroll and Boe 1982, Zlebnik et al. 2010). Following the surgical procedure, rats were allowed 3 days to recover while antibiotics (enrofloxacin, 10 mg/kg, sc) and analgesics (buprenorphine, 0.05 mg/kg, sc; ibuprofen, 15 mg/kg, po) were administered. After surgery until the remainder of the experiment, rats wore an infusion harness (CIH95AB, Instech, Plymouth Meeting, PA, USA) and tether (C313CS-MN, PlasticsOne, Roanoke, VA, USA). Catheters were flushed with a solution (0.3 ml, iv) of heparinized saline (20 USP/ml) and cefazolin (10.0 mg/ml) daily to prevent catheter blockage and infection. Rats were weighed, and catheter patency was checked weekly by injecting a 0.1-ml solution containing ketamine (60 mg/kg), midazolam (3 mg/kg), and saline. If loss of the righting reflex did not result from an iv infusion of this solution, a second catheter was implanted in the left jugular vein, and the experiment was resumed following a 3-day recovery period.

Procedure

Table 4-1 outlines the experimental phases: impulsivity screening by delay discounting, running wheel training, and maintenance, extinction, and reinstatement of cocaine-maintained behavior.

Table 4-1. Experimental timeline.

Phase	Delay Discounting	Wheel Training	Maintenance	Extinction	Reinstatement	
					Coc	*Cue*
Days	5	3	10	14	6	6
Sessions	2 h/day	6 h/day				

Delay Discounting

Following acclimation to the laboratory, rats were singly-housed in polycarbonate cages before they began daily behavioral testing on a delay-discounting task in operant conditioning chambers (Perry et al. 2005, 2008; Perry and Carroll 2008). Briefly, sessions were divided into 15 blocks of 4 trials each, and within each block there were 2 forced-choice trials followed by 2 free-choice trials. In all trials, a lever-press response on one lever resulted in the immediate delivery of one 45-mg pellet (Bio-Serv, Frenchtown, NJ); whereas, a response on the other lever resulted in three 45-mg pellets delivered after a delay. Initially, the delay on the delayed reward lever was set at 6 s, but the length of this delay changed based on the animal's performance during the free-choice trials. A response on the small, immediate reward lever yielded a 1-s decrease in the delay to the larger, delayed reward; conversely, a response on the larger, delayed reward lever yielded a 1-s increase in the delay. Each day's session began with the final delay from the previous day's session. These procedures were repeated until the MAD (mean adjusted delay of all free-choice trials) stabilized (differed by < 5 s for 5 consecutive days with no increasing or decreasing trend). The MAD served as a quantitative measure of impulsive choice; rats with MADs < 9 s or > 13 s were considered HiI or LoI, respectively.

Wheel training

Once rats were screened for high or low impulsivity, they were moved to housing in operant conditioning chambers with attached running wheels where they were trained to run following previously-published methods (Zlebnik et al. 2012). Briefly, rats were exposed to an unlocked running wheel for 6-h sessions/day, and acquisition of wheel running was defined as 3 days of > 100 revolutions/session. After meeting this criterion, rats underwent catheter

implantation surgery, and the doors to the running wheels were closed until the reinstatement phase commenced.

Cocaine self-administration acquisition and maintenance

Rats were implanted with jugular catheters following acquisition of wheel running and then trained to lever press for iv infusions of cocaine (0.4 mg/kg) under a fixed-ratio 1 (FR 1) schedule of reinforcement during daily 6-h sessions. Sessions began with illumination of the house light, and responses on the active/drug-paired lever started the infusion pump and illuminated the stimulus lights located directly above the lever for the duration of the infusion. Responses on the active lever during the length of the infusion (2-4 seconds) were recorded but had no programmed consequences. Responses on the inactive lever illuminated the stimulus lights above that lever for the same duration as an infusion, but they did not activate the infusion pump. Initially, 3 experimenter-delivered priming infusions of 0.4 mg/kg cocaine were administered periodically every 2 hours during each training session followed by placement of a small amount of ground food on the active/drug-paired lever. Acquisition was complete when rats earned at least 60 infusions during a single session in the absence of experimenter-delivered priming infusions. Following acquisition, rats were allowed to maintain unlimited self-administration of cocaine for 10 additional 6-h sessions. The door leading to the wheel remained closed throughout the self-administration training and maintenance (no wheel access) phases.

Extinction

Following maintenance, access to cocaine self-administration was discontinued for the remainder of the experiment. Presentation of cocaine-paired stimuli such as the house light, stimulus lights, and infusion pump was also suspended, and rats were given 14 days to extinguish lever pressing.

Reinstatement

In a within-subjects design, reinstatement of cocaine-seeking behavior was induced by experimenter-delivered cocaine priming injections or the presentation of cocaine-paired cues (e.g., house light, stimulus lights, infusion pump). The block of cocaine-primed reinstatement sessions was counterbalanced with the block of cue-primed reinstatement sessions across rats. The treatment sequence within each priming condition block was nonsystematic and included pretreatment with either ATO (1.5 mg/kg, ATO1.5; 3 mg/kg, ATO3, ip) or saline (S) 30-min prior to the start of session and concurrent access to an unlocked (W) or locked running wheel. Rats were exposed to the priming condition (e.g., cocaine-paired cues, cocaine) every other day, and saline or no cue conditions were administered on intervening days to allow for extinction of responding prior to the next cocaine seeking assessment. An example of a priming sequence for an individual rat follows: S+Coc, S, ATO3+Coc, S, W+Coc, S, ATO3+W+Coc, S, ATO3+S, S, ATO1.5+Coc, S, ATO1.5+W+Coc, S, ATO1.5+S, no cue, S+cues, no cue, ATO3+cues, no cues, W+cues, no cues, ATO3+W+cues, no cues, ATO1.5+cues, no cues, ATO1.5+W+cues.

Data Analysis

The primary dependent measures were MADs, infusions during acquisition and maintenance, wheel revolutions during reinstatement, and responses during maintenance, extinction, and reinstatement. Time to acquire cocaine self-administration was analyzed with a Kaplan-Meier survival analysis and log-rank test. For maintenance and extinction, data were grouped into 2-day blocks to reduce daily variability and the number of post-hoc contrasts. Mean adjusted delays were compared with a 2-tailed Student's t-test, and the other measures were analyzed with 2-factor mixed analyses of variance (ANOVA) with phenotype (HiI vs. LoI) as the between-subjects factors and blocks of sessions and reinstatement treatment condition as the

repeated measures. Separate 3-factor ANOVA were performed for each priming condition (e.g., cocaine, cues): phenotype X W access X ATO dose. Comparisons were made using the Newman-Keuls posthoc test, and results were considered significant if $p<0.05$. Statistical analyses were performed using GB Stat (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Results

Delay discounting, acquisition, maintenance, and extinction

In accordance with the impulsivity screening criteria, MADs on the delay discounting task differed significantly between the HiI (4.97 ± 0.44) and LoI (29.13 ± 3.16) rats ($t_{21} = 9.44$, $p < 0.0001$). Time to acquire cocaine self-administration was significantly shorter in HiI vs. LoI rats (Fig. 4-1, $X_1 = 15.57$, $p < 0.001$); all HiI rats met acquisition criteria by session 5, while all LoI rats did not meet acquisition criteria until session 15. However, there were no phenotype differences in responses (Fig. 4-2A) or infusions (data not shown) during maintenance. When data were collapsed across groups, there was a significant main effect of session block ($F_{4,114} = 5.65$, $p < 0.001$), and post-hoc analyses revealed an increase in infusions from sessions 1-2 to sessions 9-10 ($p < 0.01$), indicating an escalation of cocaine intake over the maintenance period. Similarly, there were no phenotype differences in responses during extinction (Fig. 4-2B), but there was a main effect of session block ($F_{6,153} = 4.12$, $p < 0.001$). Once groups were collapsed, there was a significant decrease in responses from sessions 1-2 to 13-14 ($p < 0.01$).

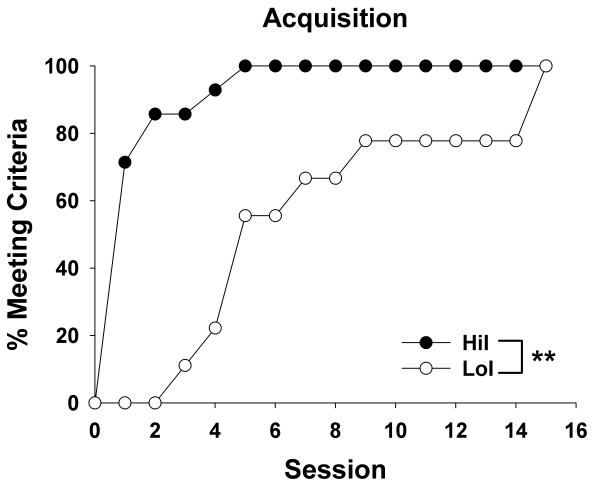

Figure 4-1. Time to meet cocaine self-administration acquisition criteria. HiI rats acquired cocaine self-administration in fewer sessions than LoI rats (p < 0.001).**

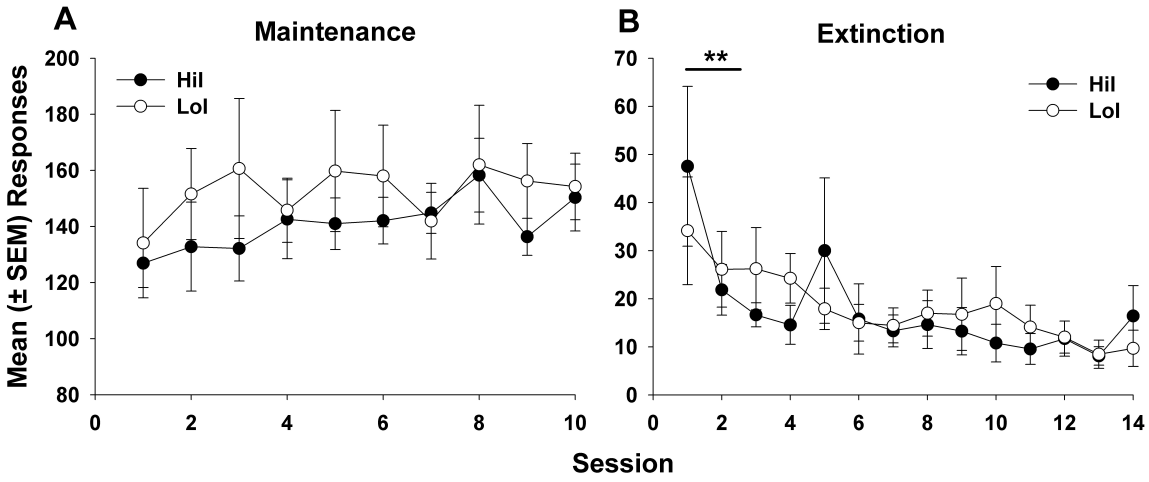

Figure 4-2. Mean (± SEM) responses during the maintenance and extinction periods. (A) There were no group differences in mean (±SEM) daily responses for cocaine (0.4 mg/kg/inf) during the 10-day maintenance period. (B) There were no group differences in mean (±SEM) daily unreinforced responses during the 14-day extinction period. Both HiI and LoI rats significantly reduced their responding from days 1-2 to days 13-14 (p < 0.01).**

Cue-primed reinstatement

Reinstatement responding precipitated by cocaine-paired cues (Fig. 4-3A) was analyzed by 3-factor ANOVA, and results indicated a significant main effect of W ($F_{1,183} = 26.14$, $p < 0.0001$), main effect of ATO ($F_{3,183} = 17.11$, $p < 0.0001$), phenotype X ATO interaction ($F_{3,183} = 3.04$, $p < 0.05$), W X ATO interaction ($F_{3,183} = 15.69$, $p < 0.0001$), and phenotype X W X ATO interaction ($F_{3,183} = 3.76$, $p < 0.05$). Post-hoc analyses showed significantly greater cue-induced reinstatement responding in the LoI rats compared to the HiI rats ($p < 0.01$). However, there were no other phenotype differences. Treatment with ATO1.5 or ATO3 alone significantly attenuated responding compared to vehicle treatment ($p < 0.01$) in the LoI rats, while only ATO1.5 reduced responding compared to vehicle in the HiI rats ($p < 0.05$). Access to W alone ($p < 0.01$) and the combination treatments W+ATO1.5 ($p < 0.05$) and W+ATO3 ($p < 0.01$) decreased cue-primed reinstatement responding in both HiI and LoI rats, and W+ATO3 decreased cue-induced responding more than ATO3 alone ($p < 0.05$) in HiI rats. Therefore, both treatment with A and the combination of W+A were effective in reducing cue-induced cocaine seeking in both HiI and LoI rats.

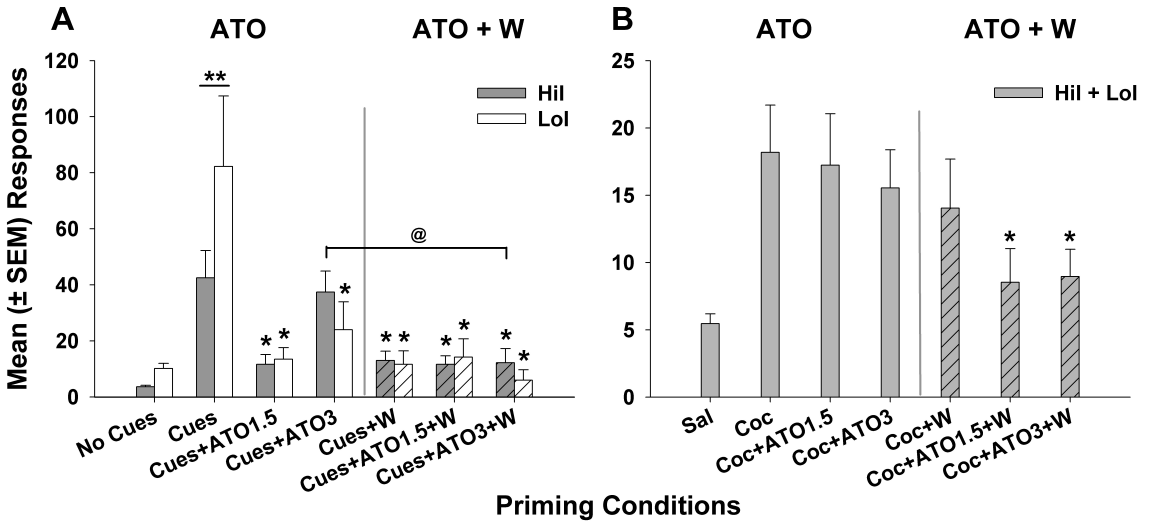

Figure 4-3. Mean (± SEM) responses per treatment session over both reinstatement priming conditions. (A) LoI rats responded significantly more for cocaine-paired cues under control conditions than HiI rats ($p < 0.01$). However, both HiI and LoI rats had attenuated responding following treatment with W, ATO1.5, W+ATO1.5, W+ATO3 (* $p < 0.01$), and LoI rats also had reduced responding following treatment with ATO3 (* $p < 0.01$). The combination treatment W+ATO3 was more effective than ATO3 alone in HiI rats (@ $p < 0.01$). (B) While there were no phenotype differences and none of the individual treatments were effective in reducing cocaine-primed reinstatement, W+ATO1.5 and W+ATO3 both significantly attenuated responding compared to control conditions (* $p < 0.05$), and each was more effective than ATO1.5 alone (# $p < 0.05$) and ATO3 alone (@ $p < 0.05$), respectively.**

Wheel revolutions during W, W+ATO1.5, and W+ATO3 treatment conditions (Table 4-2) were analyzed in a 2-factor ANOVA resulting in a significant main effect of phenotype ($F_{1,68} = 4.63$, $p < 0.05$), but there was no main effect of treatment condition or a phenotype X treatment condition interaction. The treatment conditions were collapsed, and a t-test revealed that LoI rats made significantly more wheel revolutions compared to HiI rats ($t_{62} = 2.71$, $p < 0.01$) during cue-induced reinstatement sessions.

Table 4-2. Mean (SEM) wheel revolutions during reinstatement.

	Reinstatement					
	Cue			*Cocaine*		
	Sal	ATO1.5	ATO3	Sal	ATO1.5	ATO3
HiI	91 (31)	207 (63)	288 (128)	373 (179)	479 (173)	414 (101)
LoI	372 (188)†	645 (290)†	505 (188)†	498 (175)	591 (226)	421 (177)

† Main effect: LoI > HiI

Cocaine-primed reinstatement

Reinstatement of cocaine seeking precipitated by a cocaine priming injection (Fig. 4-3B) was analyzed by 3-factor ANOVA. While there were main effects of W ($F_{1,175} = 8.39$, $p < 0.01$) and ATO ($F_{3,175} = 6.78$, $p < 0.01$), there was no main effect of phenotype or any significant interactions. Data from HiI and LoI rats were collapsed, and subsequent ANOVA [main effects of W ($F_{1,175} = 4.34$, $p < 0.05$) and ATO ($F_{3,175} = 6.38$, $p < 0.01$)] and post hoc analyses did not reveal significant treatment effects from ATO1.5, ATO3, or W alone. However, W+ATO1.5 ($p < 0.05$) and W+ATO3 ($p < 0.05$) were more effective in decreasing cocaine-induced reinstatement than control treatment alone. Wheel revolutions during cocaine-primed reinstatement sessions did not differ between HiI and LoI rats or across treatment conditions. Thus, despite no differences in wheel revolutions during W treatment sessions, the combination treatment W+ATO attenuated cocaine-primed cocaine seeking when each individual treatment (e.g., W, ATO1.5, ATO3) did not.

Discussion

Results of the present study demonstrated phenotype differences in drug abuse vulnerability and significant treatment effects of ATO and aerobic exercise on relapse-related

behavior in HiI and LoI rats. Confirming earlier work (Perry et al. 2005, 2008), HiI rats acquired cocaine self-administration more rapidly than LoI rats. However, across both phenotypes, ATO and W alone selectively attenuated cue- but not cocaine-primed reinstatement of cocaine seeking, and in some instances, these treatment effects were dose-dependent and phenotype-specific. However, regardless of phenotype, the combination of W + ATO markedly reduced both cue- and cocaine-primed reinstatement. These findings suggest that combination treatment approaches may help to overcome a broad range of relapse triggers (e.g., cocaine, cocaine-paired cues) in individuals with differential susceptibility to addiction.

Selective reduction of cue- but not cocaine-primed reinstatement of cocaine-seeking behavior by ATO is consistent with previous research demonstrating attenuation of responding for drug-associated stimuli but not suppression of cocaine self-administration following pretreatment with ATO (Economidou et al. 2009, 2011). While these results suggest little role for ATO in modulating the acute priming effects of cocaine, ATO, when combined with W, was sufficient to reduce cocaine-primed reinstatement responding compared to vehicle treatment. Contrary to earlier work with rats not selected for trait impulsivity (Zlebnik et al. 2010, 2014), W alone did not suppress cocaine-primed reinstatement in HiI and LoI rats in the present experiment. This may have been due to the age of the rats; prior to the self-administration procedure in the current study, the rats were trained on a delay-discounting task and may have been at a slightly older age during reinstatement testing compared to earlier work.

Whereas there were no HiI vs. LoI differences during cocaine-primed reinstatement, phenotype differences were represented in cue-primed reinstatement as the LoI rats had greater cocaine seeking and reached higher levels of wheel running than the HiI rats. Our earlier work suggests that these results are not due to differential locomotor behavior between the HiI and LoI

rats (Perry et al. 2005). However, the discrepancy between the present results and prior work (Diergaarde et al. 2008, Broos et al. 2012) in reinstatement responding among the phenotypes could be due to differences in the delay-discounting procedures used to screen rats for high vs. low impulsivity. Diergaarde et al. (2008) and Broos et al. (2012) both used a fixed increasing delay procedure whereas our work used a self-adjusting delay procedure. During choice trials on the self-adjusting delay procedure, each lever press on the immediate lever reduces the delay on the delayed lever by 1 sec, and each lever press on the delayed lever increases the delay by 1 sec. The LoI rats that were selected based on our screening criteria were accustomed to very long delays (range: 18-42 sec; average: 29) on the delayed lever, and the HiI rats that we selected were accustomed to very short delays (range: 0-9 sec; average: 5 sec) on the delayed lever, resulting in a short delay to reinforcement on both levers for these rats. Therefore, by definition, the LoI rats may be tolerant of waiting for reinforcement and more persistent in their pursuit of reinforcement when it is not readily available, making them more resistant to extinction; conversely, given their history of near-immediate reinforcement on both levers, the HiI rats may not persistently seek reinforcement when it is not readily available, making their extinction more rapid than their LoI counterparts. In support of this, we previously found that female LoI rats had higher extinction responding following termination of cocaine self-administration than HiI rats (Perry et al. 2008). In that study, rats were allowed to extinguish responding to cocaine-paired stimuli during the extinction period, and in the present study, they were not. Consequently, the absence of cocaine-paired stimuli during extinction may account for the lack of extinction responding differences among the phenotypes, and then the presence of cocaine-paired stimuli during cue-induced reinstatement may account for potentiated cocaine seeking in the LoI vs. HiI rats under control treatment conditions.

There were additional phenotype differences in treatment response as LoI rats showed overall better attenuation of responding by treatment with ATO compared to HiI rats. Specifically, cue-primed reinstatement was reduced by both ATO1.5 and ATO3 in the LoI rats, whereas only ATO1.5 reduced cue-primed reinstatement in the HiI rats. However, consequently, in the HiI rats, W+ATO3 was more effective than ATO3 alone in suppressing cue-induced reinstatement responding, indicating a phenotype-specific combination treatment effect as the efficacy of ATO1.5, ATO3, W+ATO1.5, and W+ATO3 was approximately equivalent in LoI rats. In fact, reinstatement remained at very low levels for all conditions incorporating ATO1.5 and W, suggesting floor effects of these individual treatment conditions. However, overall results are consistent with previous reports of treatment effects in other models of individual differences in drug abuse vulnerability. For example, as in the present study, the low vulnerable phenotype showed greater reduction of binge-like cocaine intake by treatment with progesterone (Anker et al. 2012) or baclofen (Holtz and Carroll 2011) compared to the high vulnerable phenotype. More work is needed to fully characterize treatment effects in these models, but results suggest better treatment receptivity in low vs. high susceptible phenotypes.

Differential reinstatement of cocaine seeking and response to ATO treatment between the HiI and LoI rats may be indicative of a possible underlying dissimilarity in neurobiological substrates of addiction and/or impulsivity among these phenotypes. Examining rats selected for HiI and LoI on a delay-discounting task, Regier et al. (2012) found differences in cocaine-induced activation of executive control areas such as the orbitofrontal cortex and cingulate area 1 but not other areas of the PFC such as the prelimbic or infralimbic areas. To date, no one has examined activation of these areas by cocaine-paired cues in HiI and LoI rats, but dose-dependent effects of ATO treatment on cue-induced reinstatement suggest differences in monoaminergic

transmission. Atomoxetine is a selective NET inhibitor which also has a very low affinity for DAT and the serotonin transporter, SERT. At the systemic doses tested in the present experiment (1.5 and 3 mg/kg, ip), ATO has been shown to increase both NE and DA selectively in the PFC but not the striatum (Bymaster et al. 2002). Norepinephrine in the PFC modulates cognitive functioning and has been associated with attention regulation, working memory, and behavioral inhibition (Arnsten 2000, Arnsten and Casey 2011). While results examining the effects of DA receptor agonists and antagonists on delay discounting have been mixed (Hamidovic et al. 2008, Wade et al. 2000, van Gaalen et al. 2006), previous work found decreased impulsive choice during a delay-discounting task with NET inhibition by ATO (Bizot et al. 2011, Robinson et al. 2008, but see also Baarendse and Vanderschuren 2012, Broos et al 2012, Sun et al 2012). Exercise also increases extracellular levels of NE and DA in the PFC (Ma 2008, Meeusen and De Meirleir 1995, Paluska and Schwenk 2000), and it may be by this mechanism that exercise augmented the treatment effects of ATO and resulted in differential levels of wheel running in LoI vs. HiI rats. Additional investigations will be required to outline the mechanism whereby ATO and exercise attenuate cocaine seeking in HiI and LoI rats, but existing data support a role of targeting NE and/or DA transmission in executive control areas.

Together, current and prior work demonstrate promising treatment effects of ATO and aerobic exercise on relapse-related behavior. Importantly, ATO is well-tolerated (Spencer et al. 2001, Quintana et al. 2007, Jasinski et al. 2008), has low abuse potential (Jasinski et al. 2008), and is already approved by the FDA for treatment of ADHD. Likewise, exercise is a low-cost behavioral intervention that conveys psychological and physiological benefits (Garber et al. 2011) that may help protect against relapse such as perceived coping ability (Steptoe et al. 1989), increased self-esteem (Spence et al. 2005), and prevention of cessation-induced weight gain

(Gritz et al. 1989, Klesges et al. 1992). Dual treatment with ATO and aerobic exercise may help former addicts overcome frequent threats to recovery, including both cue- and drug-induced cravings, and may also facilitate the adoption of healthier patterns of behavior. Overall, the present results using combined behavioral (exercise) and pharmacotherapies (ATO) support a customized treatment approach for relapse in individuals with differing vulnerability to addiction (i.e., high vs. low impulsive) and may help to inform substance abuse treatment in a clinical setting.

CHAPTER 5

Experiment 4: Effects of chronic wheel running on cocaine-induced neuronal activation in brain reward areas in female rats

Zlebnik NE, Hedges VL, Carroll ME, Meisel RL. (2014) Chronic wheel running affects cocaine-induced c-Fos expression in brain reward areas in rats. Behav Brain Res. 261:71-8. doi: 10.1016/j.bbr.2013.12.012

Rationale

While Experiments 1-3 focused on the effects of concurrent wheel running on cocaine-seeking behavior, they did not directly address the effects of wheel running on the neurobiological substrates of addiction. Our understanding of relapse-related behavior and its attenuation by wheel running is very limited; thus, the goal of Experiment 4 was to take a step in investigating the effects of chronic wheel running on cocaine-induced cellular activation of brain areas associated with reward processing.

Introduction

A growing body of clinical and preclinical research has shown that physical exercise is effective in preventing and treating drug abuse. Controlled laboratory studies in humans demonstrated that exercise decreased cravings for alcohol (Ussher et al. 2004), cigarettes (Daniel et al. 2004), and cannabis (Buchowski et al. 2011) and alleviated symptoms of tobacco withdrawal (Daniel et al. 2004, Ussher et al. 2006, Ussher et al. 2009, Ussher et al. 2001, Williams et al. 2011). Further, rodent models of the human drug abuse process have shown that exercise in the form of voluntary wheel running delayed acquisition of cocaine self-administration (Smith and Pitts 2011), prevented the escalation of cocaine intake (Smith et al. 2011, Zlebnik et al. 2012), and attenuated reinstatement of cocaine-seeking behavior (relapse) (Zlebnik et al. 2010, Lynch et al. 2010, Smith et al. 2012). Long-term daily wheel running also prevented behavioral sensitization to cocaine (Smith and Witte 2012, Renteria Diaz 2013) and decreased motivation to lever-press for cocaine (Smith et al. 2008a) and heroin (Smith and Pitts 2012) under a progressive ratio schedule.

However, despite promising evidence for exercise in the treatment of drug abuse, not much is known regarding the effects of exercise on the neurobiological substrates of addiction. Drug reward involves activation of the mesolimbic dopamine system, which consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and areas of the limbic forebrain such as the prefrontal cortex (PFC) (Nestler 2001, Everitt and Wolf 2002, Koob and Volkow 2010). Repeated exposure to drugs of abuse such as cocaine or natural rewards such as physical exercise can produce plasticity in this pathway. For example, both chronic cocaine and voluntary wheel running upregulated the transcription factor ΔFosB in the NAc (Hope 1998, Werme et al. 2002, Greenwood et al. 2011), tyrosine hydroxylase in the

VTA (Greenwood et l. 2011, Sorg et al. 1993), and dynorphin in the medial caudate putamen (CPu) (Werme et al. 2000). However, while wheel running can activate and elicit changes in brain areas implicated in the pathophysiology and treatment of addiction, it has not been yet associated with altered neuronal responses to drugs of abuse.

The present study investigated the hypothesis that prior long-term voluntary wheel running would influence cocaine-induced activation of brain reward areas. Rats were given 21 days of voluntary access to a locked or unlocked running wheel before being challenged with a cocaine or saline injection and then sacrificed for immunohistochemical labeling of c-Fos. C-Fos is a transcription factor and a product of *c-fos*, a member of the immediate early gene family (Cochran 1993). C-Fos expression is a measure of neuronal activation in the central nervous system, and stimuli such as drugs of abuse (Cochran 1993), exercise (Vargas-Perez et sl. 2003), and sexual behavior (Bradley and Meisel 2001) elicit its expression in reward processing areas of the brain. We quantified c-Fos immunoreactivity in the NAc, CPu, mPFC, and OFC to investigate whether a history of chronic exercise would alter the activation of this reward circuitry in response to cocaine.

Material and methods

Subjects

Thirty-one female Wistar rats approximately 90 days of age were the subjects in this experiment. Female rats were studied, as they readily acquire voluntary wheel running (Jones et l. 1990) they run more than males (Boakes et al. 1999, Cosgrove et al. 2002, Eikelboom and Mills 1988, Lambert and Kinsley 1993), and they are more sensitive than males to the attenuating effects of wheel running on cocaine-maintained behaviors (Smith et al. 2011, Cosgrove et al. 2002, Thano et al. 2010]. Rats were purchased from Harlan Sprague-Dawley, Inc. (Madison, WI,

USA), and upon arrival in the laboratory, they were pair-housed in standard polycarbonate cages for a minimum of 3 days before the experimental protocol began. Once behavioral testing commenced, animals were singly-housed in operant conditioning chambers for 21 days. Following behavioral testing, rats were singly-housed in polycarbonate cages for 7 days before immunohistochemical processing of brain tissue. Water was available ad libitum at all times; rat chow (Harlan Teklad 2018, Harlan Sprague-Dawley, Inc., Madison, WI, USA) was available ad libitum except during behavioral testing, when it was only freely available outside of daily experimental sessions. Body weights were taken once/week. All laboratory conditions were temperature- and humidity-controlled with a 12-h light/dark cycle with lights on at 6 am.

Drugs

Cocaine HCl was provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC, USA). Cocaine was dissolved in sterile saline at a concentration of 8 mg/ml and refrigerated until use.

Wheel running

During behavioral testing, rats were housed in custom-built, octagonally-shaped operant conditioning chambers with access to an adjoining running wheel (Med Associates, Inc., St. Albans, VT, USA) through a guillotine-style door. Within these chambers, rats had daily access to either a locked or unlocked running wheel for 21 days. The locked running wheel provided daily access to environmental stimulation of the wheel without the ability to run. At session onset (9 am), a house light (4.76 W) illuminated, and the experimenter opened the door to the attached running wheel. Rats had free access to the locked or unlocked running wheel from 9 am until 3 pm (6-h session), when the house light extinguished and the wheel door was closed by the experimenter. Running wheel access was given during the light phase of the light-dark cycle to

correspond to earlier work by our laboratory investigating the effects of wheel running on cocaine-motivated behaviors during this same daily time frame (Zlebnik et al. 2012, Zlebnik et al. 2010, Cosgrove et al. 2002, Larson and Carroll 2005].

Acute cocaine exposure

After 21 days of wheel access, rats underwent a "rest" period of 7 days to allow the acute effects of wheel running to diminish (Rhodes et l. 2003) before being challenged with a cocaine (15 mg/kg, ip) or saline (equivalent volume, ip) injection. The cocaine dose was chosen because it is in the range of doses that elicited differential reinstatement of cocaine seeking in rats screened for high vs. low wheel running (Larson and Carroll 2005), and it was sensitive enough to evoke differential c-Fos activation in brain reward areas in rat models of individual differences in drug abuse vulnerability (Regier et al. 2012). Each cohort consisted of 4-8 rats and included at least 1 rat from each group. The combination of wheel running and cocaine treatment yielded 4 randomly-assigned treatment groups: unlocked/cocaine (N = 9), unlocked/saline (N = 7), locked/cocaine (N = 10), and locked/saline (N = 5).

c-Fos immunohistochemistry

Between 8-10 am, rats were injected with cocaine or saline (i.p.), and 90 min later, they were anesthetized with Sleepaway (26% sodium pentobarbital, 7.8% isopropyl alcohol, 20.7% propylene glycol, and distilled water; 0.5-0.75 ml/animal, i.p., Fort Dodge Laboratories, Fort Dodge, IA) and intracardially perfused with 25 mM phosphate-buffered saline (PBS, pH 7.6) for 2 min followed by 4% paraformaldehyde in 25 mM PBS for 20 min. Brains were post-fixed in 4% paraformaldehyde for 2 h and then transferred to 10% sucrose in PBS overnight. Using a freezing microtome, 40 μm coronal sections were taken at the level of the orbitofrontal cortex (OFC), medial prefrontal cortex (mPFC), caudate-putamen (CPu), and nucleus accumbens (NAc).

Alternate tissue slices were used for immunohistochemical processing. Slices were rinsed in 25 mM PBS with 0.1% bovine serum albumin (BSA) then incubated in rabbit antibody to c-Fos (sc-52, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) at 1:3000 in 25 mM PBS with 0.1% BSA and containing 0.03% Triton X-100 for a total of 48 h (24 h at room temperature followed by 24 h at 4° C). After primary antibody incubation and a series of three 10 min rinses, sections were incubated for 45 min in biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) in 25 mM PBS with 0.1% BSA, rinsed in PBS with BSA, then incubated in avidin-biotin horseradish peroxidase complex (Vector Laboratories) at 1:50 in 25 mM PBS with 0.1% BSA for 45 min with each incubation separated by a series of three 10 min rinses. Sections were then rinsed for 10 min in 0.05 M Tris buffer (pH 7.6) and incubated in 0.08% diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Chemicals, St. Louis, MO, USA) in Tris buffer with 0.003% hydrogen peroxide for 5 min. Nickel chloride (0.03%) was added to the DAB solution to enhance staining. Sections were put through a final series of rinses with Tris and then deionized water. Finally, they were mounted on Adhesion Superfrost Plus slides (Brain Research Labs, Waban, MA, USA) and left to dry overnight. After sections were dry, they were dehydrated, cleared, and cover-slipped using DPX (Sigma-Aldrich Chemicals, St. Louis, MO, USA).

Data analysis

Daily wheel revolutions were recorded for the groups with access to unlocked running wheels, and mean revolutions over the 21 days were analyzed with a 2-tailed Student's t-test. Mean age and body weight were also compared among the 4 groups using separate 2-factor ANOVA with GB Stat (Dynamic Microsystems, Inc., Silver Spring, MD, USA). Differences were considered significant if $p < 0.05$.

Brain regions from all groups were included in the c-Fos analysis. As previously described (Regier et al. 2012), a single tissue section was selected for each brain area to ensure that our cell counts were taken from the same rostral-caudal level for all animals. To validate the rostral-caudal location of the tissue section, we took measurements of the distance between landmarks in which this measurement changes monotonically through the nucleus. For example, at the level of the nucleus accumbens the medial-lateral distance between the medial edge of the anterior commissure and the tip of the lateral ventricle shrinks with the progression through tissue sections in the caudal direction. For the nucleus accumbens and caudate analyses, tissue sections for analysis had to have the same measurement between these structures. The brain regions selected for analysis, with the level relative to Bregma as determined from Paxinos and Watson (Paxinos and Watson 2007), included subregions of the mPFC, such as the cingulate cortex (Cg1) (+2.20 mm), prelimbic area (PrL) (+2.20 mm), infralimbic area (IL) (+2.20 mm); the OFC (+3.70 mm); the dorsomedial and dorsolateral CPu (+1.28 mm); and the NAc shell and core (+1.28 mm). Rectangular counting boxes (300 x 637 μm) were positioned on digital images of each brain area without regard to c-Fos labeling. Representative examples of the coding box placement for the the NAc and the CPu and also the mPFC subregions and the OFC are shown in Figures 5-1 and 5-2, respectively. These digital images then were coded and counted by an individual blind to the coding scheme. Basal c-Fos cell counts among the saline-treated groups were analyzed by a 2-factor (wheel access X brain region) ANOVA to assess potential unstimulated differences in c-Fos labeling. Because significant differences were detected among the saline treated animals, a mean fold change was calculated by dividing all of the individual counts for the rats in the cocaine-treated groups by the mean count for their respective saline-treated group. Subsequently

for each brain area, fold changes for unlocked and locked wheel access groups were compared using 2-tailed Student's t-tests.

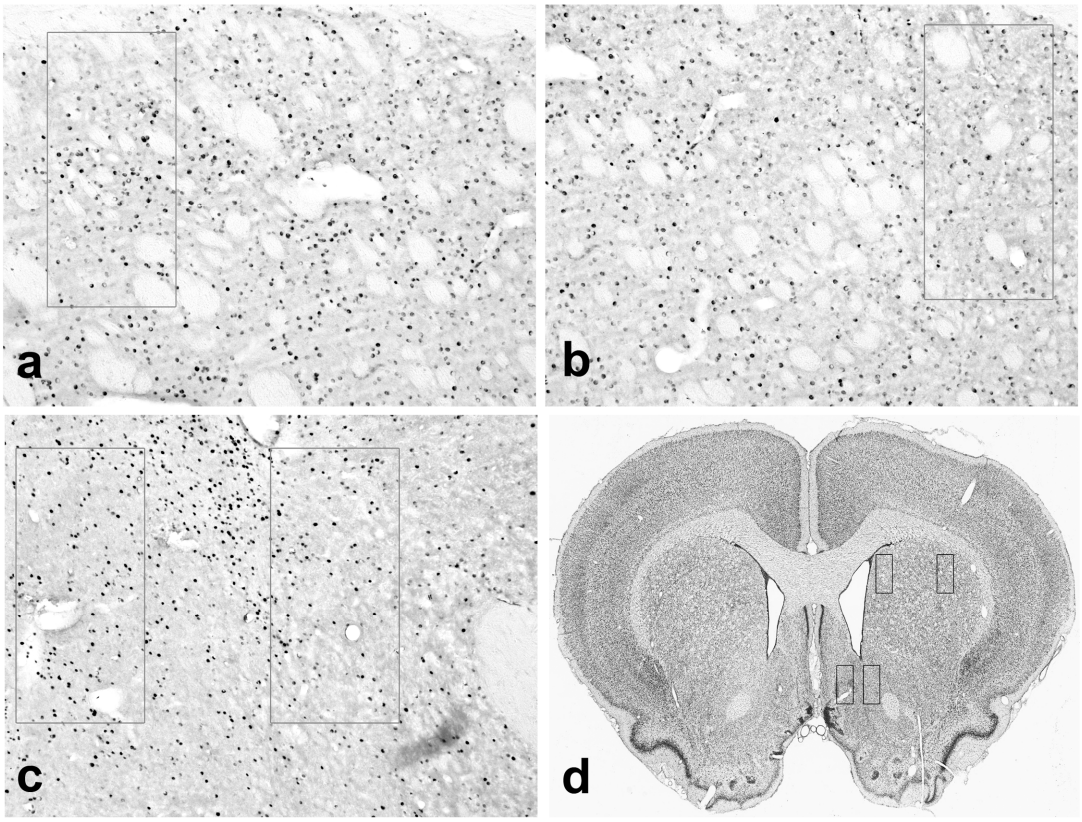

Figure 5-1. Counting box placement for c-Fos-reactive cells of the dorsal and ventral striatum: (a) dorsal medial (DM) CPu, (b) dorsal lateral (DL) CPu, and (c) NAc shell (left box) and core (right box). (d) Image of cresyl violet-stained coronal brain slice at +1.28 mm from bregma (Jones, brainmaps.org database [Jones 2007]).

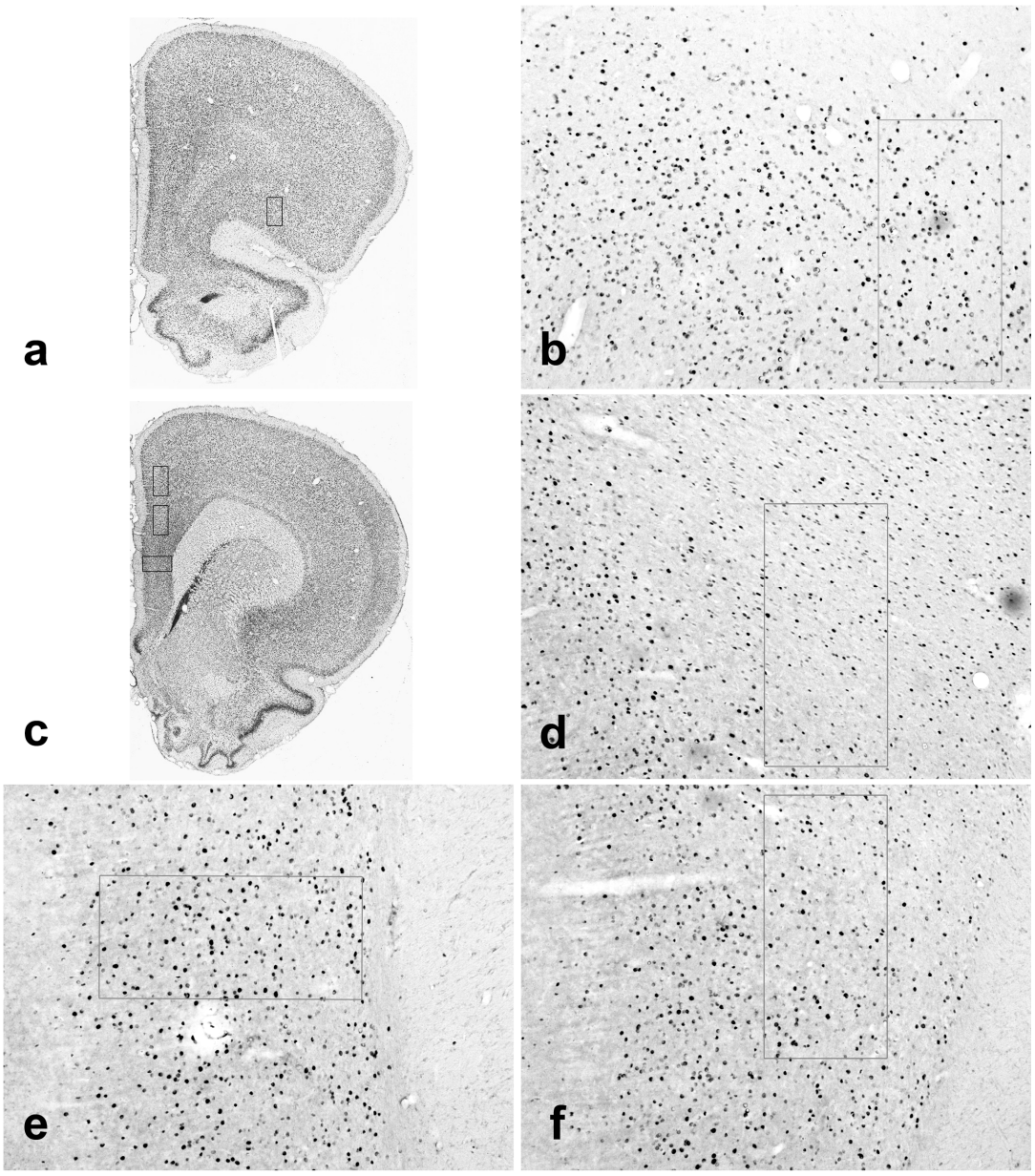

Figure 5-2. Counting box placement for c-Fos-reactive cells of the OFC and mPFC: (a) Image of cresyl violet-stained coronal brain slice at +3.70 mm from bregma (Jones, 2007) where c-Fos-reactive cell counts were taken for (b) OFC. (c) Image of cresyl violet-stained coronal brain slice at +2.20 mm from bregma where c-Fos-reactive cell counts were taken for (d) Cg1, (e) IL, and (f) PrL.

In order to investigate the possible relationship between exercise exposure and c-Fos immunoreactivity in response to cocaine in these brain areas, wheel revolutions for the unlocked wheel access groups (e.g., unlocked/cocaine, unlocked/saline) were correlated with the total c-Fos cell counts for each brain region using separate Pearson's product-moment correlations (R Package 2.13.1, R-project.org).

Results

Wheel running, age, and body weight

Groups with access to unlocked running wheels did not differ in mean (± SEM) wheel revolutions (unlocked/saline: 770 ± 315; unlocked/cocaine: 940 ± 244) over the 21 days of access (Fig. 5-3). Further, none of the groups differed significantly in mean age at the start (unlocked/saline: 92 ± 1; unlocked/cocaine: 92 ± 1; locked/saline: 91 ± 1; locked/cocaine: 91 ± 2) or mean body weight (unlocked/saline: 282 ± 8.22 g; unlocked/cocaine: 285 ± 5.70 g; locked/saline: 289 ± 6.66 g; locked/cocaine: 306 ± 8.00 g) over the course of the experiment.

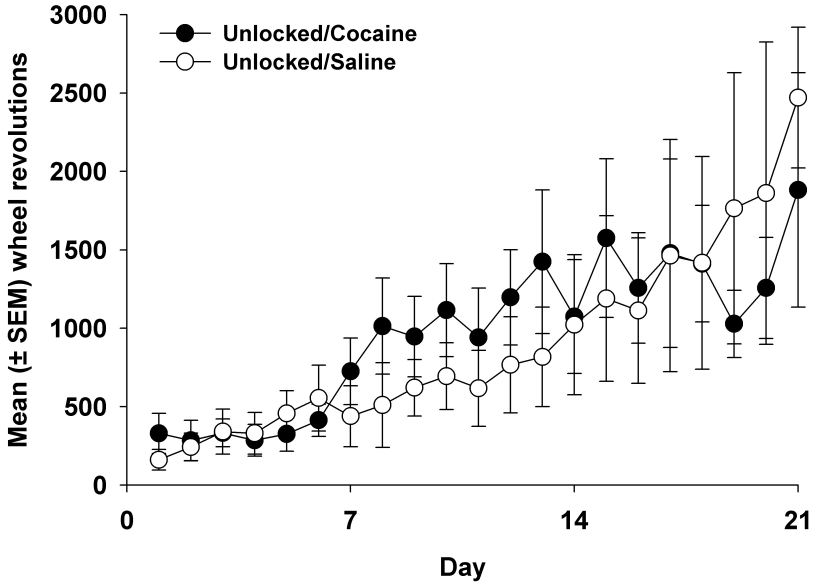

Figure 5-3. Mean (SEM) wheel revolutions during daily 6-h sessions over the 21-day access period did not differ among the unlocked wheel access groups.

Acute cocaine-induced c-Fos expression

Mean total c-Fos cell counts differed among the saline-treated locked and unlocked wheel access groups (baseline conditions; Table 1). There was a main effect for the locked wheel access groups to have greater total c-Fos counts than the unlocked wheel access groups throughout areas of the striatum and the cortex, including NAc shell ($F_{1,30}$ = 53.7, p< 0.0001), NAc core ($F_{1,37}$ = 13.3, p = 0.002), dorsomedial CPu ($F_{1,29}$ = 10.5, p = 0.003), dorsolateral CPu ($F_{1,29}$ =5.75, p = 0.02), PrL ($F_{1,30}$ = 5.17, p = 0.03), IL ($F_{1,30}$ = 5.73, p = 0.02), and also the OFC ($F_{1,29}$ = 4.87, p = 0.04). Further, there was a main effect of greater c-Fos cell counts in cocaine-treated vs. saline-treated groups in the NAc shell ($F_{1,30}$ = 4.85, p = 0.04), NAc core ($F_{1,37}$ = 9.04, p = 0.008), and dorsolateral CPu ($F_{1,29}$ = 20.8, p = 0.0001) of the striatum. However, there was no wheel access X treatment interaction

Table 5-1. Mean (SEM) total c-Fos-reactive cell counts.

Wheel Access	Treatment	NAc		CPu		mPFC			OFC
		Shell	*Core*	*DM*	*DL*	*Cg1*	*PrL*	*IL*	
Unlocked	Saline	40.0 (10.5)	28.3 (3.9)	10.9 (4.9)	3.9 (2.4)	68.9 (12.6)	77.0 (16.3)	69.1 (15.7)	108.7 (36.0)
Unlocked	Cocaine	59.4 (8.9)†	43.8 (5.5)†	30.7 (6.7)	45.8 (10.4)†	91.9 (14.7)	93.9 (11.8)	63.1 (5.2)	149.9 (14.5)
Locked	Saline	66.0 (11.2)*	72.6 (4.7)*	39.8 (7.4)*	23.5 (10.5)*	118.0 (21.3)	135.2 (15.8)*	112.2 (14.6)*	215.4 (16.7)*
Locked	Cocaine	87.0 (5.9)†*	81.9 (4.7)†*	51.3 (7.6)*	75.5 (10.0)†*	98.2 (14.5)	106.3 (15.2)*	79.3 (12.2)*	154.9 (24.3)*

† Main effect: cocaine > saline, $p < 0.05$; * Main effect: locked wheel > unlocked wheel, $p < 0.05$

Given baseline differences in c-Fos-reactive cell counts (Table 5-1), mean fold change in c-Fos cell counts (i.e., increase in cocaine-induced c-Fos cell counts over saline-induced c-Fos cell counts) were analyzed. Mean fold change in response to cocaine differed among animals with access to wheel running compared to controls (Fig. 5-4). The unlocked wheel access group had significantly greater mean fold change in c-Fos expression in the NAc core ($t_{17} = 2.14$, $p = 0.0473$), dorsomedial CPu ($t_{17} = 2.48$, $p = 0.0239$), and dorsolateral CPu ($t_{17} = 3.35$, $p = 0.0038$) subregions of the striatum. Also, while there was only a trend for greater c-Fos expression in the Cg1 ($t_{17} = 2.09$, $p = 0.0516$), there was significantly larger mean fold change in the PrL ($t_{17} = 1.06$, $p = 0.0339$) and OFC ($t_{17} = 4.02$, $p = 0.0009$) in rats with unlocked vs. locked wheel access.

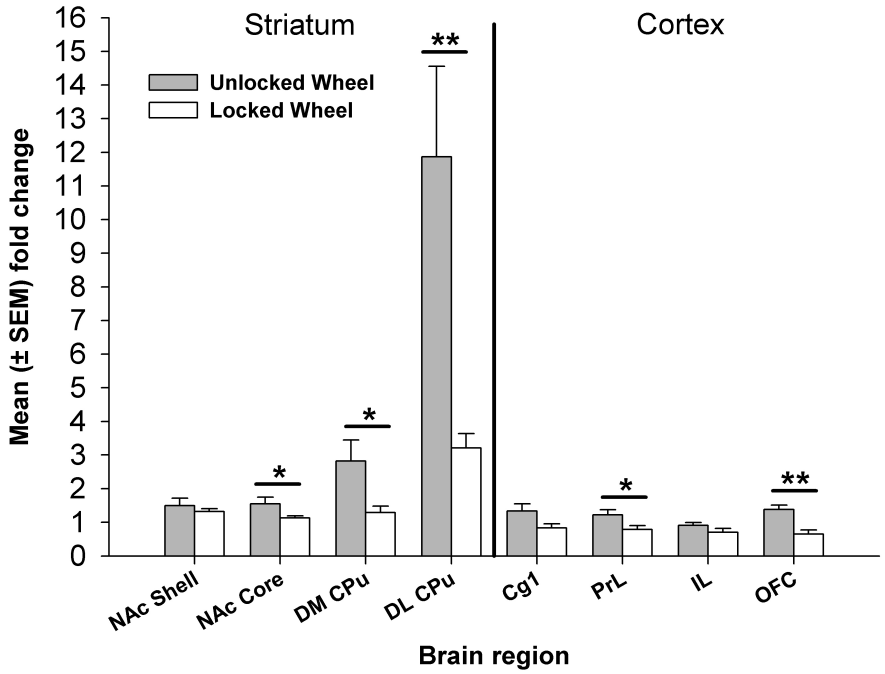

Figure 5-4. Unlocked wheel access rats had greater mean fold change in c-Fos-reactive cell counts in response to cocaine than locked wheel rats in regions of the striatum, such as the NAc core, dorsomedial (DM) CPu, and dorsolateral (DL) CPu (* $p < 0.05$, ** $p < 0.01$). Further, cocaine-induced c-Fos expression was significantly higher in unlocked vs. locked wheel access groups in the PrL subregion of the mPFC and the OFC (* $p < 0.05$, ** $p < 0.01$).

C-Fos cell counts were not significantly correlated with mean wheel revolutions in either the cocaine-treated or the saline-treated groups with access to an unlocked running wheel (data not shown), perhaps due to small sample sizes.

Discussion

The results of the present study indicated that prior chronic exercise (e.g., voluntary wheel running) exposure specifically increased the neuronal response to acute cocaine compared to control conditions (e.g., locked running wheel). Increases in cocaine-induced cellular

activation following 21 days of voluntary wheel running occurred in the NAc core, the dorsomedial and dorsolateral CPu, the PrL subregion of the mPFC, and the OFC. As these brain areas are critical for the development of drug abuse (Nestler 2001, Everitt and Wolf 2002, Koob and Volkow 2010) the results indicate that chronic exercise induces neuroplasticity that may impact the progression and treatment of addiction.

Exercise-mediated changes in the neuronal response to cocaine likely resulted from the considerable overlap among the neurobiological substrates engaged by exercise and cocaine. Initial cocaine reward is hypothesized to depend on dopamine release in the NAc (Koob and Volkow 2010), and the NAc serves a critical role in the acute reinforcing effects of drugs of abuse by integrating input from the PFC, amygdala, and hippocampus and sending output to motor areas (O'Donnell and Grace 1995, Meredith 1999). Wheel running was shown to increase c-Fos (Vargas-Perez et al. 2003) and delta FosB in the NAc core (Werme et al. 2002), or both the core and shell (Rhodes et al. 2003, Greenwood et al. 2011). While the NAc shell appears to be more responsive to the primary reinforcing effects of drugs (Di Chiara et al. 2004, Kelley et al. 2002), the core is thought to mediate responding to cues associated with drugs or natural rewards (Parkinson et al. 1999, Di Ciano and Everitt 2001, Fuchs et al. 2004). Further, the NAc core has also been shown to contribute to cocaine-induced reinstatement. The dorsal mPFC - NAc core - ventral pallidum circuit is critical for the reinstatement of cocaine-seeking behavior elicited by cocaine priming injection (McFarland and Kalivas 2001), and the present results indicate changes in cocaine-induced activation of both the NAc core and the PrL, a dorsal subregion of the mPFC. Given prior work demonstrating an attenuation of cocaine-induced reinstatement with access to a voluntary wheel running (Zlebnik et al. 2010, Smith et al. 2012), the effect of exercise on

reinstatement and other relapse-related behaviors may be partly mediated by its actions in these brain areas.

Related to its role in cocaine-induced reinstatement in animal models, the mPFC is activated during drug- and cue-induced craving in humans (Franklin et al. 2007). Adaptations such as increased phorsphorylation of ERK in the mPFC have been implicated in the incubation of cue-induced cocaine seeking in rats (Koya et al. 2009), and both pERK and cue-induced drug seeking were reduced by daily wheel running (Lynch et al. 2010). Activation of the circuitry from the OFC to the dorsal CPu also appears to be important for drug craving in humans (Franklin et al. 2007, Volkow et al. 1999, Volkow et al. 2005, McClernon et al. 2009), and its disruption has been associated with binge-like drug consumption (Volkow et al. 2004, Volkow et al. 2007) and the development in compulsivity disorders (Volkow et al. 1999). While the results of the current study revealed increases in cocaine-induced activation of the PrL subregion of the mPFC, the OFC, and also the dorsal CPu following exercise vs. control conditions, the nature of this activation is unknown. Many studies investigating chronic drug exposure cite impairments in executive functioning (Koob and Volkow 2010), and binge intake of psychostimulants resulted in poorer performance on PFC-dependent working memory and sustained attention tasks (Briand et al. 2008, George et al. 2008) and OFC-dependent reversal learning tasks (Jentsch et al. 2002, Schoenbaum et al. 2004). A recent study demonstrated decreased excitability of PrL pyramidal neurons following compulsive cocaine intake in rats (Chen et al. 2013), and reversal of this hypoactivity prevented compulsive cocaine self-administration. Interestingly, chronic wheel running reduced damage to serotonergic nerve terminals in the dorsal mPFC subregions Cg1 and PrL and the OFC and to dopaminergic terminals in the dorsolateral and dorsomedial CPu (but not NAc) induced by binge-like administration of methamphetamine (O'Dell et al. 2012). Further,

self-administration studies have demonstrated that access to wheel running prevented compulsive or binge intake of cocaine (Smith et al. 2011, Zlebnik et al. 2012). Thus, exercise may exert its attenuating effects on drug-maintained behaviors by protecting against the neurotoxic effects of drug exposure, and this may help to preserve the integrity of executive control systems including those involving the mPFC and OFC.

The CPu is the brain area perhaps that receives the most overlapping activation from exercise and cocaine. The dorsal striatum, or the CPu, receives convergent input from the motor cortex and thalamus and is the origin of the basal ganglia circuits that are important for motor control (Kreitzer and Malenka 2008). While the CPu has no apparent role in the acute rewarding effects of drugs of abuse, it is involved in habit learning (Volkow et al. 2006, Packard and Knowlton 2002) and is recruited during the development of compulsive drug seeking (Everitt et al. 2008) and drug craving (Volkow et al. 2008, Heinz et al. 2004). The results of the present experiment demonstrating an increase in cocaine-elicited c-Fos immunoreactivity in the dorsomedial CPu in exercising vs. control rats are supported by earlier studies that showed greater Fos-positive (Rhodes et al. 2003) and dynorphin-positive (Werme et al. 2000) cell counts in the dorsomedial CPu following chronic wheel running. Other reports indicated that exercise training elicited adaptations in motor circuits and increased the efficiency of activation of the CPu (Holschneider et al. 2007), including increasing its oxidative capacity (McCloskey et al. 2001). Together, evidence suggests that chronic exercise may induce plasticity in the CPu that alters its reactivity to stimuli such as drugs of abuse.

Overall, the present investigation found evidence that chronic exercise altered baseline neuronal activity and affected cocaine's activation of brain areas critically important for the development of addiction and the precipitation of relapse. Specifically, raw measures of c-Fos

immunoreactivity were lower in exercising compared to control rats following both saline and cocaine priming injections. In the rats with exercise exposure, this implies there was either overall lower basal activity or perhaps lower reactivity to the injection. When counts were normalized to account for different group baselines, acute cocaine elicited a greater increase in c-Fos immunoreactivity in areas of the mesolimbic dopamine pathway in exercising compared to control rats. Taking into account existing work, it appears that chronic cocaine and chronic wheel running affect the c-Fos response to an acute cocaine exposure differently. Interestingly, although an acute cocaine injection will elicit c-Fos activation in a naïve animal, it has been shown that an acute cocaine injection following a period of chronic cocaine exposure does not significantly induce c-Fos in areas of the striatum, indicating that tolerance develops in this response (Hope et al. 1992, Hope et al. 2004, Alibhai et al. 2007). However, in the present experiment, we found significant levels of c-Fos activation following an acute cocaine exposure in the wheel running groups. Therefore, the effects of chronic wheel running on cocaine-induced c-Fos activation may be mediated by different mechanisms than those that mediate the effects of chronic cocaine.

Based on prior behavioral work, it is unlikely that the increase in cellular activation in exercising rats confers general vulnerability to cocaine addiction. While one study found that chronic wheel running increased cocaine conditioned place preference (Smith et al. 2008b), this result is complicated by reports of decreased cocaine conditioned place preference with chronic treadmill running (Thanos et al. 2010) and may be influenced by the timing of exercise, since the immediate after-effects of wheel running itself also can induce place preference (Belke and Wagner 2005). Additionally, an investigation of behaviorally-selected high vs. low wheel runners found greater cocaine self-administration and reinstatement of cocaine-seeking behavior

in the high runners compared to the low runners (Larson and Carroll 2005). These results are consistent with other animal models of individual differences where the high extreme phenotype showed greater susceptibility for addiction (Carroll et al. 2008, Perry and Carroll 2008, Flagel et al. 2009, Belin et al. 2011). However, the results of the present study reflect on the effects exercise experience (locked vs. unlocked running wheel access) rather than avidity for exercise, making direct comparisons difficult.

In contrast, a number of investigations argue for a protective role for exercise in regard to susceptibility to drug-motivated behaviors. Results from self-administration dose-response analyses using a progressive ratio schedule of reinforcement demonstrated that voluntary wheel running decreased the reinforcing effects of cocaine (Smith et al. 2008b) and heroin (Smith and Pitts 2012) by shifting the dose-response curves downward. Additionally, voluntary wheel running reduced intake of cocaine (Cosgrove et al. 2002), amphetamine (Kanarek et al. 1995), methamphetamine (Miller et al. 2012), and alcohol (McMillan et al. 1995, Ehringer et al. 2009, Brager and Hammer 2012) in addition to preventing the acquisition (Smith and Pitts 2011) and escalation of cocaine self-administration (Smith et al. 2011, Zlebnik et al. 2012) and the reinstatement of cocaine seeking (Zlebnik et al. 2010, Lynch et al. 2010, Smith et al. 2012). Therefore, at the behavioral level of analysis, there is much evidence for attenuating effects of exercise on addiction.

Collectively, prior work and current results demonstrate a role for exercise in mediating changes in brain reward circuitry that influence its response to cocaine. Although the present study delineated some of the brain structures affected, further investigations will be needed to determine the mechanism of the effects of exercise. Nevertheless, these results provide support for the neurobiological basis of exercise as a treatment for addiction.

CHAPTER 6

Experiment 5: Effects of chronic wheel running on the incubation of cocaine-seeking behavior in female rats

Rationale

Experiments 1-3 examined the effects of concurrent wheel running alone or combined with a pharmacological treatment on the reinstatement of cocaine-seeking behavior, while Experiment 4 took a new direction and began outlining the effects of chronic wheel running on cocaine-induced brain activation. While earlier Experiments 1-2 focused on concurrent wheel running access and found no carry-forward effects on subsequent reinstatement testing, other work demonstrated that when access to the wheel running was introduced during the extinction period and was available outside of self-administration sessions it was effective in reducing cocaine reinstatement (Lynch et al. 2010, Sanchez et al. 2013). This evidence, along with the results from Experiment 4, indicated that 14-28 days of uninterrupted wheel running following cocaine self-administration may attenuate cocaine-seeking behavior and associated neural activation. Therefore, since concurrent wheel running and cocaine seeking are difficult to distinguish and study at the neural level, the next step in Experiment 5 was to combine examination of both the effects of noncurrent wheel running on cocaine seeking and also on its neurobiological markers. Instead of a reinstatement procedure, an incubation of cocaine seeking procedure was selected, as some effort has been made to identify factors critical for the time-dependent increase in cocaine-seeking behavior (Pickens et al. 2011).

Introduction

Relapse to drug use is a major barrier to the treatment of addiction, with 80-90% of former users relapsing within 1 year of drug use cessation (Brandon et al. 2007, Kirshenbaum et al. 2009). Relapse is often preceded and accompanied by robust craving elicited by drug-paired stimuli and environments, and reports have demonstrated a progressive increase in drug craving or drug seeking over the withdrawal period (Gawin and Kleber 1986, Grimm et al. 2001, Neisewander et al. 2000). In 1986, Gawin and Kleber (1986) sought to characterize drug craving in cocaine-dependent individuals. Their subjects described a period of low craving for cocaine during initial weeks following drug use cessation; however, after a period of abstinence as long as 28 weeks, they experienced the return of intense craving precipitated by cocaine-paired cues. In the laboratory, this phenomenon was reproduced in rats (Grimm et al. 2001, Neisewander et al. 2000) and, subsequently, humans (Bedi et al. 2011, Wang et al. 2013, Li et al. 2014), and it has been referred to as the incubation of drug craving or drug seeking (Grimm et al. 2001). Incubation results from the development of time-dependent, withdrawal-induced neuroadaptations (Grimm et al. 2001, Conrad et al. 2008, Pickens et al. 2011, Wolf and Tseng 2012) and may contribute to relapse after protracted withdrawal (Conrad et al. 2008). Laboratory models of incubation typically compare drug-seeking behavior in response to drug-paired cues during early and late withdrawal from drug self-administration (Grimm et al. 2001). Cue-induced drug seeking has been shown to peak around 2-3 months of withdrawal in both rats (Grimm et al. 2001, Lu et al. 2004) and humans (Wang et al. 2013, Li et al. 2014), and greater drug seeking during late vs. early withdrawal was found using a range of reinforcers including cocaine (Gawin and Kleber 1986, Grimm et al. 2003, Shaham 2002), nicotine (Abdolahi et al. 2010, Bedi et al.

2011), methamphetamine (Shepard et al. 2004, Wang et al. 2013), heroin (Shalev et al. 2001), alcohol (Bienkowski et al. 2004, Li et al. 2014), and sucrose (Grimm et al. 2005).

As it may enhance vulnerability to relapse after an extended withdrawal, incubation of drug seeking is an important factor to consider in designing treatment strategies for drug abuse (Lu et al. 2004, Conrad et al. 2008, Pickens et al. 2011, Chauvet et al. 2012). Thus far, however, very little work has been down to examine treatments to reduce or eliminate the incubation of cue-induced drug seeking. A growing body of research has evaluated the physical and psychological benefits of exercise and physical activity and has begun exploring potential treatment applications for exercise as a behavioral intervention (USDHHS 1996). Results have shown a negative correlation between physical health and fitness and relapse to smoking (Metheny and Weatherman 1998) and a significantly reduced risk of smoking relapse in adults who were physically active (McDermot et al. 2009). Controlled laboratory studies in humans also have demonstrated that moderate intensity aerobic exercise decreased drug cravings (Ussher et al. 2004, Daniel et al. 2004, Buchowski et al. 2011, Prapavessis et al. 2014) and alleviated symptoms of drug withdrawal (Ussher et al. 2001, Daniel et al. 2004, Williams et al. 2011, Prapavessis et al. 2014). Additionally, animal experiments have revealed promising treatment effects of exercise on drug seeking behaviors. Voluntary wheel running decreased reinstatement of cocaine-seeking behavior precipitated by exposure to cocaine (Zlebnik et al. 2010, 2014a; Smith et al. 2012), yohimbine (Zlebnik et al. 2014a), and cocaine-paired cues (Lynch et al. 2010, Smith et al. 2012, Peterson et al. 2014a, Zlebnik et al. 2014a). While the effects of exercise on the incubation of drug seeking are unknown at present, a recent study by Chauvet et al. (2012) explored the effects of housing in an enriched environment that included a running wheel vs. a standard laboratory environment during withdrawal on subsequent cocaine seeking. Animals

housed in the enriched environment had significantly less responding for cocaine-paired cues compared to standard housing controls after both 30 and 60 days vs. 1 day of withdrawal. The treatment outcomes from these studies are promising, suggesting that enriching the environment with opportunities for exercise may have a lasting impact on cue-induced drug seeking and the potential for this drug seeking to increase over time.

Incubation of cocaine seeking has been associated with specific withdrawal-mediated neuroadaptations in the mesocorticolimbic dopamine system, including areas such as the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and central nucleus of the amygdala (CeA) (Pickens et al. 2011). In particular, elevations in phosphorylated extracellular-signal related-kinase (pERK) in the mPFC (Koya et al. 2009) and CeA (Li et al. 2008) were shown to be critical for the development of time-dependent increases in cue-induced cocaine-seeking behavior. Many of these brain areas are affected by wheel running-induced neuroplasticity in their response to cocaine (Zlebnik et al. 2014b), but there have been few investigations into the effects of wheel running on the neurobiological substrates of the incubation of cue-induced cocaine seeking. In a recent study, Peterson et al. (2014a) found that daily wheel running reduced cue-induced cocaine-seeking behavior and BDNF gene expression in the mPFC compared to control treatment conditions. Further, pERK, a downstream mediator of BDNF, has been associated with withdrawal-mediated increases in responding for cocaine-paired cues (Koya et al. 2009), and both pERK in the mPFC and cocaine seeking were reduced by wheel running during the withdrawal period from cocaine self-administration (Lynch et al. 2010). Together, these results suggest a role for aerobic exercise in the attenuation of the incubation of cocaine seeking and its associated neuroadaptations.

In the present study, we examined the effects of aerobic exercise on the incubation of cocaine-seeking behavior and related biomarkers. Rats were given access to a locked or unlocked running wheel during a withdrawal period of 3 or 30 days following cocaine self-administration. Following this period of withdrawal, cocaine-seeking behavior was measured in response to cocaine-paired cues, and the animals were sacrificed for determination of pERK levels in the mPFC and CeA. We hypothesized that unlocked wheel access would attenuate responding for cocaine-paired cues compared to locked wheel conditions and prevent the incubation of cocaine-seeking behavior and attenuate time-dependent increases in pERK.

Materials and methods

Animals

Fifty-three female adult Wistar rats were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI, USA) and began behavioral testing around postnatal day 90. Female rats were studied, as they readily acquire voluntary wheel running (Jones et al. 1990) and run more than males (Boakes et al. 1999, Cosgrove et al. 2002, Eikelboom and Mills 1988, Lambert and Kinsley 1993). Females are also more sensitive than males to the attenuating effects of wheel running on cocaine-maintained behaviors (Cosgrove et al. 2002, Thanos et al. 2010, Smith et al. 2011). After arrival at the laboratory, rats were pair-housed in plastic cages with free access to laboratory chow (Teklad 2018, Harlan Laboratories, Madison, WI, USA) and water for at least 3-days of acclimation. Upon commencement of behavioral testing, rats had free access to water and were fed 16 g of rodent meal outside of behavioral sessions to maintain them at 85% of their free-feeding body weight. Mean body weights throughout the experiment did not differ among the groups throughout the study. All rodent holding rooms were maintained at 24°C and at 40-50%

humidity under a light/dark cycle (12/12-h) with room lights on at 6:00 am. The experimental protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee. The study was conducted in compliance with the Principles of Laboratory Animal Care (National Research Council 2011), and all laboratory facilities were accredited by the American Association for the Accreditation of Laboratory Animal Care.

Apparatus

During wheel training and withdrawal, rats were housed in plastic bins with attached running wheels (ENV-046, MedAssociates, Inc., St. Albans, VT, USA), and during cocaine self-administration, rats were housed and tested in custom-built operant conditioning chambers as previously described (Zlebnik et al. 2010, Zlebnik et al. 2012). Data collection and programming were conducted using PC computers with a Med-PC interface (MedAssociates, Inc.).

Cocaine

Cocaine HCl was provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC, USA) and dissolved in 0.9 % NaCl at a concentration of 1.6 mg cocaine HCl/1 ml saline. Heparin (5 USP/ml) was added to the cocaine solution to prevent catheter occlusion from thrombin accumulation. The flow rate of each cocaine infusion was 0.025 ml/sec, and the duration of pump activation (1 sec/100 g of body weight) was adjusted weekly to provide a 0.4 mg/kg cocaine dose throughout self-administration testing.

Catheterization Surgery

Following wheel training, rats were implanted with a chronic indwelling catheter in the right jugular vein by methods previously described (Carroll and Boe 1982, Zlebnik et al. 2010). Antibiotic (enrofloxacin, 10 mg/kg, sc) and analgesic (buprenorphine, 0.05 mg/kg, sc) medications were administered daily during the 3-day recovery period. Each day, catheters were

flushed with a solution (0.3 ml, iv) of heparinized saline (20 USP/ml) and cefazolin (10.0 mg/ml) to prevent catheter blockage and infection. Weekly, catheter patency was assessed by injecting a 0.1-ml solution containing ketamine (60 mg/kg), midazolam (3 mg/kg), and saline. If loss of the righting reflex did not manifest from an iv infusion of this solution, a second catheter was implanted in the left jugular vein, and the experiment was resumed following a 3-day recovery period.

Procedure

Cocaine self-administration

Following wheel and self-administration training, the experimental procedure (Fig. 6-1) consisted of 3 phases: 1) cocaine self-administration, 2) withdrawal, and 3) test for cue-induced cocaine-seeking behavior. Acquisition of wheel running and training of cocaine self-administration followed methods previously published (Zlebnik et al. 2010, Zlebnik et al. 2012). During self-administration training and maintenance, sessions began with illumination of the house light and extension of the active lever into the operant conditioning chamber. Responses on the active/drug-paired lever started the infusion pump and illuminated the stimulus lights located directly above the lever for the duration of the infusion. Responses on the active lever during the length of the infusion (1 sec/100 g of body wt) and responses on the inactive lever were recorded but had no programmed consequences. Rats were allowed to self-administer unlimited iv cocaine (0.4 mg/kg/infusion) for 6-h sessions (9:00 am – 3:00 pm) over 10 consecutive days.

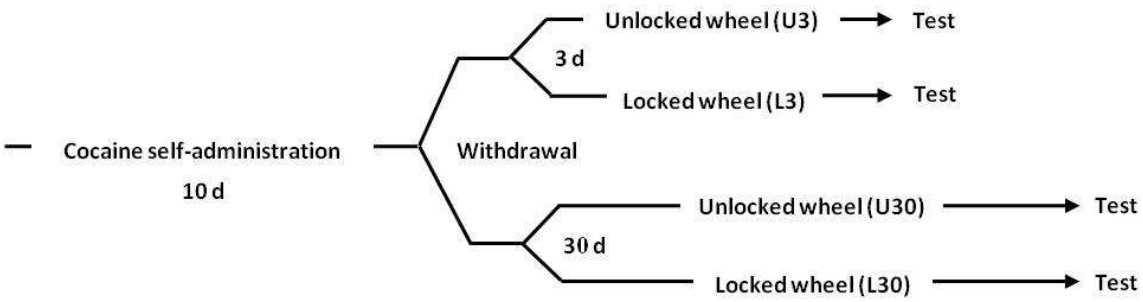

Figure 6-1. Experimental design.

Withdrawal

Following 10 days of cocaine self-administration, catheters were tied off, and rats were randomly assigned to 1 of 4 treatment groups, receiving either 3 or 30 days of withdrawal and either access to a locked (L) or unlocked (U) running wheel: U3, N = 14; U30, N = 12; L3, N = 15; L30, N = 12. Rats then were moved to housing in plastic bins with attached running wheels (MedAssociates, Inc.) and given 6-h daily access to the running wheel (9:00 am – 3:00 pm) (Peterson et al. 2014a,b).

Cue-induced cocaine-seeking test

Rats were returned to the operant conditioning chambers for a 30-min test (Koya et al. 2009) of cocaine-seeking behavior under extinction conditions after 3 or 30 days of withdrawal. The experimental conditions were the same as during cocaine self-administration except that active lever presses no longer resulted in cocaine reinforcement; the house light was illuminated, and responses on the lever previously associated with cocaine infusions activated the sound of the infusion pump and the stimulus lights directly above the lever. Responses on the inactive lever again had no consequences.

Immunoblotting

Immediately following the cocaine-seeking test, rats were rapidly decapitated and their brains were quickly removed. Brains were placed in a brain block on ice and sliced into 2 mm coronal sections at the levels of the mPFC and CeA. Unilateral punches were taken of each of the aforementioned brain areas (1 mm diameter x 2 mm thick) on an ice-cold platform, flash frozen, and stored at -80 °C until tissue processing.

Tissue punches were resuspended in 50 ul of Western blotting processing buffer (1% SDS with 50 mM NaF and 3.3 mM EGTA with added protease and phosphatase inhibitors (Pierce, Rockford IL)) and homogenized using a micro hand homogenizer and stored at -80 °C for later protein assay and Western blotting analysis. Total protein content was measured using a standard DC Protein Assay (BioRad Laboratories) and then analyzed for ERK and pERK protein content. Each experimental group was equally represented on each gel in addition to an appropriate protein ladder. For each brain area being measured, 40 ug of total protein was electrophoretically resolved using SDS-PAGE under reducing conditions on a 12% TGX gel (BioRad Laboratories) for measurement of ERK/pERK/GAPDH at 100 V for 60 min. Resolved proteins were then transferred to a nitrocellulose membrane at 25 V overnight at 4 °C. Nonspecific binding sites were blocked for all membranes with 5% milk in wash buffer (tris-buffered saline + 0.2% Tween-20) for 1 h at room temperature. Membranes being analyzed for ERK/pERK content were also incubated in an appropriate mixture of primary antibodies [ERK 1:2000 Cell Signaling Technology 4696 (mouse), pERK 1:1000 Cell Signaling Technology 9101 (rabbit), GAPDH 1:40,000 Millipore MAB374 (mouse)] in 5% BSA in wash buffer. All primary antibody incubations were performed overnight at 4 °C with shaking. Following primary incubations, all membranes were given appropriate rinses in wash buffer and then incubated in a mixture of infared-labeled secondary antibodies: goat anti-rabbit IRDye 680 (1:20,000 Li-Cor

Biotechnology) and goat anti-mouse IRDye 800 (1:20,000, Li-Cor Biotechnology). The use of species specific IRDyes allows 3 different antigens to be differentially detected simultaneously on the same blot with the use of primary antibodies raised in different species in conjunction with appropriate IRDyes that are excited at different wavelengths. In addition to this, large differences in molecular weight between antigens (i.e., ERK/pERK vs. GAPDH) allows them to be easily identified, removing the need to strip blots for re-probing. Blots were given appropriate washes in wash buffer prior to imaging with a Li-Cor Odyssey Infared Imaging System scanner (Li-Cor, Lincoln, NE).

Behavioral data analysis

The primary dependent measures were responses and infusions during cocaine self-administration, wheel revolutions during withdrawal, and responses during the cocaine-seeking test. For cocaine self-administration, data were grouped into 2-day blocks to reduce daily variability and the number of post-hoc contrasts. Measures were analyzed with 2-factor mixed analyses of variance (ANOVA) with group as the between-subjects factor and blocks of days as the repeated measure. Wheel revolutions during withdrawal were compared between groups by unpaired, 2-tailed Student's t-tests, and responses during the cocaine-seeking test were examined with a 2-factor ANOVA (wheel access X incubation length). Where appropriate, post hoc tests were performed with Dunn's (Bonferroni) procedure, and results were considered significant if $p < 0.05$. To examine the relationship between wheel revolutions during the withdrawal period and subsequent cocaine-seeking behavior, mean wheel revolutions were correlated with mean active lever responses during the cocaine-seeking test using Pearson's product-moment correlations. Statistical analyses were performed using GB Stat (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Protein data analysis

Blots were using a Li-Cor Odyssey Infared Imaging System (Li-Cor Biotechnology, Lincoln, NE) scanner, and images were then analyzed using the Odyssey Application software to determine the integrated density of total protein levels of ERK and pERK. For all Western blots, density measurements for total protein levels were normalized to an internal loading control, GAPDH, and relative levels of pERK/ERK were analyzed by 2-factor (wheel access X incubation length) ANOVA and the Newman-Keuls post-hoc test using GB Stat.

Results

Cocaine self-administration

Fig. 6-2A displays the mean number of daily responses over the 10-day cocaine self-administration period. A 2-factor ANOVA (group X block of days) revealed a significant main effect of block of days ($F_{3,264} = 4.89$, $p < 0.01$) but no significant main effect of group or group X block of days interaction. Data across groups were collapsed and analyzed by 1-factor ANOVA ($F_{4,264} = 5.18$, $p < 0.01$), and results showed significantly greater responding on the last 2-day block compared to the first 2-day block (Days 9-10 vs. Days 1-2, $p < 0.01$), indicating escalation of responding over the self-administration period for all groups. There were no group differences in inactive lever responses (not shown).

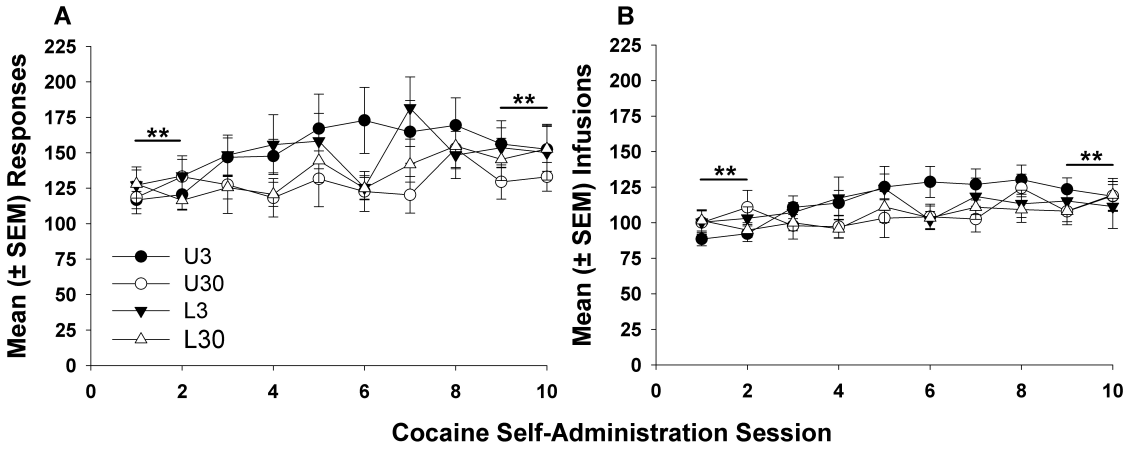

Figure 6-2. Mean (± SEM) daily responses (A) for and infusions (B) of cocaine (0.4 mg/kg/inf) made by all groups during the 10-day cocaine self-administration period. Overall, there were no group differences, and all groups escalated their responding and infusions earned over the 10-day period (Days 1-2 vs. Days 9-10, ** p <0.01).

The mean number of daily infusions (Fig. 6-2B) over the 10-day period was analyzed similarly. Following a significant main effect of block of days ($F_{4,264} = 5.10$, $p < 0.01$) but no other significant main effect or interaction, data were collapsed across group and subsequently analyzed by 1-factor ANOVA. As found with the response data, there was escalation of cocaine intake with a significant increase in infusions from Days 1-2 to Days 9-10 ($p < 0.01$).

Withdrawal

Wheel revolutions during the first 3 days of the withdrawal period (Fig. 6-3A) did not differ between the groups. For the U30 group, wheel revolutions remained stable over the course of the 30-day incubation period with no significant increase in revolutions from Days 1-3 to Days 28-30.

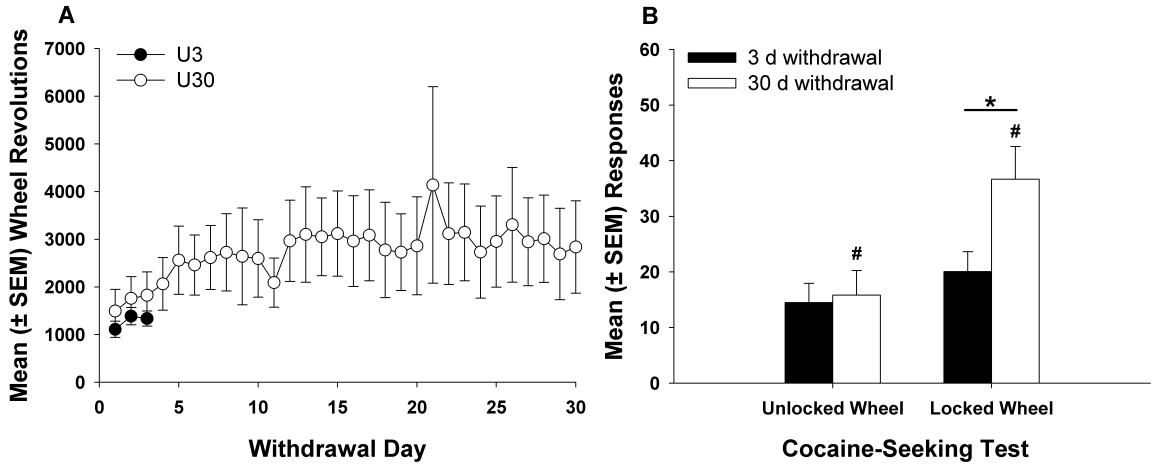

Figure 6-3. (A) Mean (± SEM) wheel revolutions across the first 3 days of the withdrawal period did not differ among the unlocked wheel access groups. (B) Mean (± SEM) responses on the lever previously paired with cocaine were significantly higher for the L30 group compared to the U30 group (# $p < 0.01$). Incubation of cocaine seeking was apparent in the animals with access to a locked running wheel, as the L30 group had considerably more responding than the L3 group ($p < 0.05$). However, this increase in responding was absent in the animals that had access to an unlocked running wheel during the withdrawal period (U3 vs. U30).**

Cue-induced cocaine-seeking test

Fig. 6-3B depicts the number of responses on the previously active lever following reintroduction to the operant conditioning chamber after 3 or 30 days of withdrawal. Data were analyzed by a 2-factor ANOVA, and results revealed significant main effects of wheel access ($F_{1,52} = 10.49$, $p < 0.01$) and incubation length ($F_{1,52} = 4.84$, $p < 0.05$) but no significant wheel access X incubation length interaction. There was a notable increase in cocaine-seeking behavior in the L30 group compared to the U30 group ($p < 0.01$) but no differences between the L3 and U3 groups. The L30 group also exhibited a greater level of responding compared to the L3 group ($p < 0.05$), demonstrating incubation of cocaine-seeking behavior in rats that had access to a locked running wheel. However, for groups that had access to an unlocked running wheel during the withdrawal period, this same increase in cocaine seeking was absent, as there were no significant

differences between the U3 and U30 groups. Inactive lever responding (not shown) was low and did not differ among any of the groups during the cocaine-seeking test.

Wheel revolutions vs. cocaine-seeking responses

Mean wheel revolutions during the entire 3- or 30-day withdrawal period for both unlocked wheel access groups or during the last 3 days of the withdrawal period for the U30 group were not significantly correlated with total responses on the previously active lever during the cocaine-seeking test (data not shown).

pERK/ERK protein levels

For pERK/ERK protein levels in the CeA (Fig. 6-4), there were significant main effects of wheel access ($F_{1,29} = 12.29$, $p < 0.01$) and incubation length ($F_{1,29} = 23.31$, $p < 0.0001$), as well as a significant wheel access X incubation length interaction ($F_{1,29} = 13.70$, $p < 0.01$). Post hoc comparisons showed greater levels of pERK/ERK in the U30 group compared to the U3 and L30 groups ($p < 0.01$). Among all groups, there were no significant differences in pERK/ERK levels in the mPFC (data not shown).

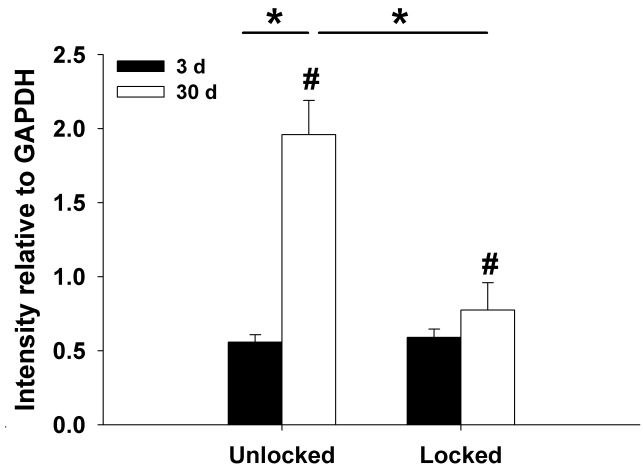

Figure 6-4. Mean (± SEM) relative pERK/ERK levels in the CeA. pERK/ERK levels are elevated during late vs. early withdrawal from cocaine self-administration (# main effect : 30 > 3 days). Additionally, the U30 group demonstrated significantly higher levels of pERK/ERK compare to the U3 and L30 groups (* $p < 0.05$).

Discussion

The primary goal of the present experiment was to investigate the effects of aerobic exercise during a withdrawal period from cocaine self-administration on subsequent relapse-related behavior and protein markers of incubation of cocaine craving. Consistent with prior work, results revealed a robust increase in cue-induced cocaine-seeking behavior and pERK levels in the CeA during late vs. early withdrawal following only 10 days of long access to cocaine self-administration under control conditions, suggesting that even brief exposure to drugs of abuse can produce long-term vulnerability to relapse. Further, access to an unlocked running wheel during 30 days of cocaine withdrawal significantly attenuated cocaine-seeking behavior compared to locked wheel conditions and prevented increases in cocaine seeking over the 30-day withdrawal period. However, while time-dependent increases in cocaine-seeking behavior were reduced following 30 days of wheel running, there was an apparent potentiation of pERK levels under these conditions. Therefore, the present findings suggest that aerobic exercise during

protracted withdrawal may help to reduce relapse-related behaviors, but more work will be needed to delineate its effects on the underlying neurobiological substrates of incubation of cocaine-seeking behavior.

Prior research established reductions in cocaine- (Zlebnik et al. 2010, Smith et al. 2012), yohimbine- (Zlebnik et al. 2014a), and cue- (Lynch et al. 2010, Smith et al. 2012, Zlebnik et al. 2014a) primed reinstatement of cocaine-seeking behavior by wheel running, but the current experiment is noteworthy in its demonstration that wheel running suppressed or prevented the incubation of cocaine seeking over extended withdrawal. In particular, results showed that wheel running reduced cocaine seeking at 30 but not 3 days after discontinuation of cocaine self-administration, indicating that aerobic exercise may not necessarily have a general inhibitory effect on relapse-related behaviors but that its effects may be specific to time-dependent changes in drug seeking that occur over several weeks after cocaine access is terminated. Additional work will be needed to reveal the time course of development of incubation and the critical period of exposure to aerobic exercise with respect to its ability to suppress incubation of cocaine seeking. For instance, only 3 days of wheel running may not have been sufficient to reduce cocaine-seeking behavior compared to locked wheel conditions. However, while some reports suggest that greater access to daily wheel running (6 vs. 1 h/day for 14 days) has a larger attenuating effect on cocaine-motivated responding (Peterson et al. 2014a,b), Smith and Witte (2012) found that only 4 days of housing with a running wheel was enough to suppress motivation to lever-press for cocaine infusions under a progressive ratio schedule. Based on these results, access to wheel running for 6 h/day for 3 days as in the present experiment likely could have been adequate to have an effect on cocaine-seeking behavior. Another explanation for the lack of attenuation in cocaine seeking after 3 days of unlocked vs. locked wheel conditions may be due to floor effects

of cocaine-seeking behavior during early withdrawal. During the initial days following cessation of cocaine self-administration, cocaine seeking has been shown to be low (Grimm et al. 2001) and may have obscured treatment effects of wheel running during early withdrawal. Overall, however, the present results demonstrated that wheel running suppressed cocaine seeking during late compared to early withdrawal, and the results support significant treatment effects of aerobic exercise on relapse-related behavior and its progressive increase over time.

In designing such treatments, it is important to understand how aerobic exercise affects cocaine seeking in terms of the neural processes that underlie time-dependent increases in vulnerability to relapse. Unlike earlier work (Lynch et al. 2010), the present investigation failed to establish decreased levels of pERK in the mPFC following wheel running vs. control conditions during the withdrawal period and also did not replicate the findings of Koya et al. (2009) that demonstrated potentiated levels of pERK during late withdrawal compared to early withdrawal from cocaine self-administration. Following the methods of Lynch et al. (2010), we sampled tissue from the center of the mPFC (i.e., border of the prelimbic and infralimbic regions). However, the greatest time-dependent increases in pERK levels were found mainly in the ventral mPFC (i.e., infralimic region), and our sampling may have diluted any potential attenuating effects of 30 days of wheel running on this measure.

In contrast to the findings in the mPFC, results of the CeA replicated work showing withdrawal-mediated increases in pERK (Lu et al. 2008). However, while we hypothesized that wheel running during the withdrawal period would mitigate increases in pERK, our data revealed significantly higher levels of CeA pERK in the U30 group compared to both the U3 and L30 groups. Therefore, we can conclude that wheel running does not attenuate time-dependent increases in cocaine-seeking behavior by reducing activation of the CeA. Although elevations in

CeA activation have been shown to be critical for the incubation of cocaine (Lu et al. 2009), morphine (Li et al. 2008), and sucrose (Uejima et al. 2007) seeking, it is not the sole mechanism underlying the incubation of reward-seeking behavior. In fact, several brain areas in non-overlapping circuits have been identified for their role in time-dependent increases in cocaine seeking (Pickens et al. 2011). Given the heterogeneity of the brain circuits involved in context-induced and discrete cue-induced drug seeking (Fuchs et al. 2005, Chaudhri et al. 2010, Crombag et al. 2008), engagement of non-overlapping mechanisms may occur during cocaine-seeking tests following reintroduction to the operant chamber (Pickens et al. 2011). Therefore, future work will have to interrogate the involvement of other brain areas to delineate the mechanism whereby chronic wheel running reduces the incubation of cocaine-seeking behavior.

In addition to preclinical work in animals, empirical work with humans has also demonstrated significant reduction of tobacco craving (Ussher et al. 2004, Daniel et al. 2004, Prapavessis et al. 2014) and withdrawal symptoms (Ussher et al. 2001, Daniel et al. 2004, Williams et al. 2011, Prapavessis et al. 2014) following bouts of moderate intensity aerobic exercise. Recent reports indicated that 8 weeks of supervised aerobic exercise in methamphetamine-dependent individuals helped restore D2/3 receptor availability to control levels (Robertson et al. 2013). In addition to notable treatment effects of exercise alone, emerging clinical (Potenza et al. 2011) and preclinical (Zlebnik et al. 2014b) findings suggest that combined behavioral and pharmacological treatments may result in more effective, longer-lasting, and self-maintained treatment outcomes that will reduce relapse to drug use and promote overall better health. Aerobic exercise has been suggested as an adjunct treatment for the standard treatment for anxiety disorders (Sciolino and Holmes 2012), and evidence supports better treatment success when exercise is combined with cognitive (Martin et al. 1997) and motivational

(Weinstock et al. 2008) treatment strategies. While the present results demonstrate a significant treatment effect of exercise during extended withdrawal, there may be an additional role for exercise in augmenting standard addiction treatments and in facilitating the adoption and maintenance of healthier patterns of behavior (Ussher et al. 2012) after prolonged abstinence.

Conclusions

The present results demonstrate a strong role for aerobic exercise in reducing the likelihood of relapse-related behaviors in rats during an extended withdrawal period of 30 days. These findings also indicate that it will be important for future work to not only determine the neural mechanism of the effects of wheel running on time-dependent increases in cocaine seeking but also examine the efficacy of aerobic exercise to decrease incubation of cocaine craving in humans. This persistence of intense craving during protracted withdrawal from drug use presents a major barrier to the treatment of addiction, and implementation of aerobic exercise regimens during the abstinence period may facilitate more long-term successful treatment outcomes.

CHAPTER 7

General Discussion and Conclusions

General Discussion and Conclusions

This set of experiments aimed to broadly examine aerobic exercise as a treatment for cocaine relapse using animal models of cocaine-seeking behavior. Experiments 1-3 employed concurrent access to wheel running to interfere with extinction and reinstatement of cocaine seeking elicited by cocaine, cocaine-paired cues, and/or the pharmacological stressor yohimbine. Experiment 1 examined the effects of concurrent voluntary wheel running alone on extinction and reinstatement of cocaine seeking. Subsequently, Experiments 2 and 3 expanded on the design of Experiment 1 by also examining the combination of wheel running with pharmacological treatments shown to reduce cocaine reinstatement (i.e., progesterone, atomoxetine). Building on these findings and work from other laboratories, Experiments 4 and 5 focused on the neurobiological underpinnings of wheel running's attenuating effects on cocaine-induced cellular activation and cocaine-motivated behavior. Overall, results across these investigations strongly support a role for wheel running in the reduction of relapse-related behavior and also in modulating its associated neural substrates.

These findings are consistent with other preclinical studies examining the effects of aerobic exercise on drug-motivated responding (Table 7-1). In rodent models of the human drug abuse process, access to wheel running reduced acquisition (Smith and Pitts 2011), maintenance (Kanarek et al. 1995, McMillan et al. 1995, Cosgrove et al. 2002, Ehringer et al. 2009, Miller et al. 2011), and escalation (Zlebnik et al. 2012, Smith et al. 2011, Engelmann et al. 2014) of drug self-administration in addition to preventing drug-conditioned place preference (Thanos et al. 2010, Mustroph et al. 2011, Rozeske et al. 2011) and psychostimulant-induced locomotor sensitization (Renteria-Diaz et al. 2013, Thanos et al. 2013, Geuzaine and Tirelli 2014). Further, previous studies in other laboratories have confirmed current results with findings indicating that

chronic voluntary wheel running in the home cage (Smith et al. 2012, Lynch et al. 2010, Peterson et al. 2014a,b) and chronic forced treadmill running (Thanos et al. 2013) attenuated reinstatement of cocaine-seeking behavior. Thus, the present set of experiments contributes to an expanding literature on the beneficial effects of wheel running in preclinical animal models of cocaine abuse and highlights the potential for aerobic exercise as a treatment to reduce or prevent relapse.

Results from human studies have also demonstrated favorable effects of exercise on relapse to drug use. However, the findings are not as straightforward as those for rodent studies. For example, whereas several investigations reported higher abstinence rates at 3 (Marcus et al. 1999, Weinstock et al. 2008, Brown et al. 2010), 6 (Horn et al. 2011), and 12 (Marcus et al. 1999) months following exercise intervention, in the majority of studies no significant effects of exercise on abstinence adherence were reported (for a review, see Ussher et al. 2012, Taylor et al. 2007, Linke et al. 2013). This may be due to a number of factors including small sample sizes, uncontrolled duration and intensity of exercise, inclusion of nontreatment-seeking subjects, and poor methods of abstinence verification. Nonetheless, a consistent feature of experiments with both rodents and humans is that regular exercise needs to be maintained to attenuate relapse-related behavior. Just as wheel running reduced drug seeking as long as it was concurrently available (i.e., absence of carry-forward effects) in Experiments 1-2, rates of abstinence remained higher in individuals who maintained their exercise program on their own beyond the time frame of experimenter-supervised intervention (Marcus et al. 2005).

Table 7-1. Effects of aerobic exercise (wheel running unless otherwise noted) in rodent models of drug abuse.

Model	*Effect*	*Drug*	*Citation*
Locomotor Sensitization	↓	Coc	Renteria-Diaz et al. 2013, Thanos et al. 2013 (treadmill), Geuzaine and Tirelli 2014
Conditioned Place Preference	↓	MDMA	Chen et al. 2008
	↓	Coc	Thanos et al. 2010 (treadmill), Mustroph et al. 2011
	↑	Coc	Smith et al 2008b
	↓	Morphine	Rozeske et al. 2011
Progressive Ratio	↓	Coc	Smith et al. 2008a
	↓	Heroin	Smith and Pitts 2012
Acquisition	↓	Coc	Smith and Pitts 2011
Maintenance (Short Access)	↓	Amph	Kanarek et al. 1995
	↓	Meth	Miller et al. 2011
	↓	Coc	Cosgrove et al. 2002
	↓	EtOH	McMillan et al. 1995, Ehringer et al. 2009
	↑	EtOH	Werme et al. 2002
	--	EtOH	Crews et al. 2004, Ozburn et al. 2008
Escalation (Long Access)	↓	Coc	Smith et al. 2011, Zlebnik et al. 2012
	↓	Meth	Engelmann et al. 2013
Reinstatement (Drug)	↓	Coc	**Experiment 1 (Zlebnik et al. 2010),** **Experiment 2 (Zlebnik et al. 2014a),** **Experiment 3 (Zlebnik and Carroll 2014),** Smith et al. 2012 Thanos et al. 2013 (treadmill)
Reinstatement (Stress)	↓	Coc	**Experiment 2 (Zlebnik et al. 2014a)**
Reinstatement (Cue)	↓	Coc	**Experiment 2 (Zlebnik et al. 2014a),** Lynch et al. 2010, Smith et al. 2012, Thanos et al. 2013 (treadmill), Peterson et al. 2014a,b
	↓	Nicotine	Sanchez et al. 2013, 2014
Reinstatement (Drug + Cue)	↓	Coc	**Experiment 2 (Zlebnik et al. 2014a)**
Reinstatement (Stress + Cue)	↓	Coc	**Experiment 2 (Zlebnik et al. 2014a)**
Incubation of Drug Seeking	↓	Coc	**Experiment 5**

That both exercise and drug intake can be tightly controlled in animal studies may contribute to the consistency of results across rodent models of drug abuse. Indeed, when aerobic exercise was examined for its effects on drug craving in a controlled human laboratory setting, results were much more robust and consistent across studies. Strength of cravings reliably predicted relapse (Doherty et al. 1995, West et al. 1989), and brief episodes of moderate-intensity aerobic exercise (e.g., stationary bike, treadmill) decreased craving for cigarettes (Daniel et al. 2004, Taylor and Katomeri 2007), alcohol (Ussher et al. 2004), and cannabis (Buchowski et al. 2011). In some instances, exercise was as effective or more effective than nicotine replacement therapy in reducing cigarette cravings (Taylor et al. 2007). Craving is often elicited by cues and contexts associated with drug use (Gawin and Kleber 1986), and craving challenged by drug-paired cues such as a lit cigarette (Taylor and Katomeri 2007) or paraphernalia (Buchowski et al. 2011) was attenuated following exercise compared to control conditions. While drug craving is difficult to examine in animals, some have suggested that cue-induced drug seeking may be a model of craving in rodents (Grimm et al. 2001, Lu et al. 2004). Experiments 2, 3, and 5 as well as studies from other laboratories (Smith et al. 2012, Lynch et al. 2010) examined the effects of wheel running on cocaine seeking precipitated by cocaine-paired stimuli and/or contexts and confirmed results from human experiments by demonstrating a decrease in drug-motivated responding. Although there has been limited successful clinical investigation of the effects of exercise on abstinence rates, overall human and rodent laboratory studies support significant reduction of drug craving/seeking by aerobic exercise.

In addition to craving, exercise also decreases the influence of other contributors to relapse, including stress, anxiety, and depression. These contributors are often a consequence of

drug use cessation and comprise the abstinence symptomology of a number of abused drugs (Gawin and Kleber 1986, Piasecki et al. 2000, Shiffman et al. 1996). Stress (Carter and Tiffany 1999, Moran-Santa et al. 2014), anxiety (Moran-Santa et al. 2014), and depression (Abulseoud et al. 2013) are conditions that potentiate craving and have been implicated in relapse during cessation attempts (Chassin et al. 2002, Manning et al. 2005, Abulseoud et al. 2013, Riga et al. 2014, Frankowska et al. 2014). Research indicates that exercise has anxiolytic and antidepressive (Strohle 2009, Saeed et al. 2010, Herring et al. 2010, Babyak et al. 2000) effects. Consistent with the results of Experiment 2 that demonstrated a reduction in stress-induced cocaine seeking by wheel running, studies in abstinent smokers found attenuation of stress during and following brief bouts of aerobic exercise (Ussher et al. 2001, Taylor and Katomeri 2006, Daniel et al. 2006). Although there were no reported significant effects of exercise on anxiety in smokers trying to quit (Taylor et al. 2007, Ussher et al. 2012), exercise reduced negative affect (Bock et al. 1999) and/or increased positive affect (Bock et al. 1999, Taylor et al. 2006, Taylor and Katomeri 2006) in smokers. Additional studies in humans and animals will be needed to fully address the impact of aerobic exercise on relapse-related stress and mood/affect.

Evidence suggests that exercise may enhance the treatment efficacy of pharmacotherapies and behavioral therapies for addiction. In Experiments 2 and 3, wheel running was investigated as an adjunct behavioral intervention for pharmacological treatments that have been shown to reduce reinstatement of cocaine-seeking behavior. While in Experiment 3, assessment of combined treatment efficacy was hindered by floor effects of both individual treatments, Experiment 2 demonstrated that the combination of exercise + progesterone was more effective than either exercise or progesterone alone. These results are supported by clinical work showing better management of substance use disorders when cognitive behavioral therapy (Martin et al.

1997) and contingency management (Weinstock et al. 2008) were combined with exercise programs. Further, the efficacy of exercise itself in promoting abstinence can also be improved by pharmacological intervention. Prapavessis et al. (2007) reported that nicotine replacement therapy augmented the effects of exercise on 12-month abstinence rates over exercise alone. However, they failed to compare the exercise + nicotine replacement therapy group with a group receiving nicotine replacement therapy only; thus, future work should address whether exercise adds additional benefits than those conferred by nicotine replacement alone.

Combination treatment plans allow for customization of addiction treatment and have overall greater success than individual treatments (Potenza et al. 2011). It has been suggested that abstinence from drug use occurs in 3 successive phases: detoxification, tolerance of acute cravings and withdrawal symptoms, and replacement of drug use-related behaviors with healthier and more productive behaviors (Potenza et al. 2011). The heterogeneity of these phases in terms of physiological and psychological processes indicates that each may be best controlled with a different combination of pharmacotherapies and behavioral approaches. Therefore, to achieve optimal outcomes, treatment combinations may be tailored to each phase of abstinence. Given the range of the effects of exercise on drug-motivated behaviors, combinations of drug use cessation strategies that incorporate exercise may exert beneficial effects across all phases of abstinence. In addition to evidence of reduced drug cravings following exercise, data from humans (Bock et al. 1999) and animals (Devaud et al. 2012, McCulley et al. 2012, Miladi-Gorji et al. 2012, Balter and Dykstra 2012, Brocardo et al. 2012) studies indicated an exercise-mediated reduction in withdrawal symptoms and signs across a range of drug classes that may help alleviate discomfort of detoxification during early abstinence. Moreover, aerobic exercise itself is a healthier, rewarding behavior that may help supplant unwanted drug-focused behaviors during

late abstinence. In addition to providing general health benefits (Garber et al. 2008), exercise has been shown to improve self-esteem (Spence et al. 2005) and perceived coping abilities (Steptoe et al. 1989) in individuals trying to abstain from substance use. Therefore, despite the lack of successful clinical studies, the potential for exercise as a singular or supplemental treatment to promote abstinence adherence remains high (Ussher et al. 2012, Linke et al. 2013).

Exercise and combined treatments may not only be tailored to the phase of abstinence but also to the individual. Results from animal studies have revealed individual differences in the efficacy of aerobic exercise to attenuate drug-motivated behaviors. In Experiment 2, wheel running + progesterone was more effective than either alone in male vs. female rats. However, in this study, wheel running reduced cocaine-primed reinstatement in female but not male rats, and this is supported by earlier work demonstrating female-specific reduction of cocaine-reinforced behavior by wheel running. For example concurrent access to a running wheel decreased intake of cocaine (Cosgrove et al. 2002) and ethanol (Ehringer et al. 2009) more effectively in females compared to males. Exercise also had a differential effect in animal models of relapse, as female rats exhibited less extinction and reinstatement of cocaine-seeking behavior than male rats following chronic voluntary wheel running (Smith et al. 2012).

Clinical research may yield significant benefits by pursuing exercise as a treatment for relapse in women. Compared to men, women have lower smoking cessation rates in clinical trials (Perkins 2001), and findings suggest that depression and anxiety (Linke et al. 2013) as well as the fear of weight gain (Perkins et al. 2001) may disproportionately motivate relapse in women. During pregnancy, depression (Gaynes et al. 2005), cravings, and withdrawal symptoms (Dempsey et al. 2002) are further intensified, and evidence suggests that traditional treatments like nicotine replacement therapy are ineffectual in pregnant smokers (Coleman et al. 2012).

However, Prapavessis et al. (2014) recently reported that brief aerobic exercise reduced both tobacco cravings and withdrawal symptoms in abstinent pregnant smokers. Given the low risk of harm and the demonstrated beneficial effects of exercise on mood (Bock et al. 1999, Taylor et al. 2006a,b), withdrawal symptoms (Bock et al. 1999, Devaud et al. 2012, McCulley et al. 2012, Miladi-Gorji et al. 2012, Balter and Dykstra 2012, Brocardo et al. 2012), and weight control (Garber et al. 2008), it may be an attractive female-specific treatment for relapse to drug use. Accordingly, exercise interventions that address these specific concerns in women may enhance abstinence adherence.

Both preclinical (Table 7-1) and clinical (Taylor et al. 2007, Ussher et al. 2012) investigations have outlined a role for aerobic exercise in the attenuation of relapse-related behaviors. However, despite identification of many neuroadaptive changes produced by exercise (Dishman et al. 1997, 2006), the specific neurobiological mechanisms underlying exercise's effects on drug addiction and relapse remain unclear. Aside from Experiment 5, there have been only 2 other preclinical investigations (e.g., Lynch et al. 2010, Peterson et al. 2014a) examining the neural effects of wheel running on cocaine-motivated behavior. However, identification of these mechanisms could further support the use of exercise as a singular or adjunct therapeutic intervention for drug abuse as well as provide insight into novel treatment strategies for other psychiatric illnesses. Research has demonstrated that repeated exposure to both drugs of abuse and voluntary wheel running produced plasticity in the mesolimbic dopamine pathway (Hope 1998, Werme et al. 2002, Greenwood et al. 2011, Sorg et al. 1993, Werme et al. 2000), and Experiment 4 expanded on these findings to investigate the effects of chronic wheel running on activation of this pathway by cocaine. Results demonstrated that increases in cocaine-induced cellular activation after 21 days of voluntary wheel running occurred in the NAc core, the

dorsomedial and dorsolateral CPu, the PrL subregion of the dorsal mPFC, and the OFC. Two of these areas participate in the dorsal mPFC - NAc core - ventral pallidum circuit that is critical for cocaine-primed reinstatement (McFarland and Kalivas 2001). Given prior work demonstrating an attenuation of cocaine-induced reinstatement when there was access to voluntary wheel running (Table 7-1), the effect of exercise on reinstatement and other relapse-related behaviors may be partly mediated by its actions in these brain areas.

While Experiment 4 took a broad approach to examine the effects of exercise on brain reward areas, Experiment 5 took a targeted approach and aimed to investigate the impact of wheel running on specific biomarkers for the incubation of cocaine-seeking behavior. Time-dependent increases in cue-induced cocaine seeking have been associated with phosphorylation of ERK in the mPFC (Koya et al. 2009, Lynch et al 2010) and the CeA (Lu et al. 2005), and while results did not replicate earlier work in the mPFC, they revealed an unexpected potentiation of pERK in the CeA of exercising rats. This finding indicates that wheel running does not reduce incubation of cue-induced cocaine seeing via activation of the CeA. Therefore, the benefit to taking this directed approach to examine addiction-associated neuroadaptations is that it can conclusively exclude potential mechanisms. Current results suggest that future work should focus on other relapse biomarkers to delineate the attenuating effects of exercise and suggest mechanisms for adjunct pharmacotherapy.

In comparison to animal research, human research is limited by the paucity of invasive methods to define and interrogate the neural mechanisms of addiction. While recent rodent work has implicated the glutamatergic system in relapse to drug use (Kalivas 2009, Loweth et al. 2014), there is no appropriate radiotracer to assess glutamateric neurotransmission in PET imaging, and much of the human imaging work has remained focused on the dopaminergic

system (Koob and Volkow 2010). To date, the only human imaging study to examine the effects of aerobic exercise in drug users found that 8 weeks of supervised aerobic exercise in methamphetamine-dependent individuals helped restore D2/3 receptor availability to control levels (Robertson et al. 2013). These findings are especially interesting in light of the rodent work showing that drug-induced deficits in executive functioning are negatively correlated with D2 receptor mRNA in areas such as the OFC and the PFC (Briand et al. 2008). Overall, these results are promising and warrant additional investigation into possible dopaminergic mechanisms of the effects of exercise on drug abuse.

In summary, the results of Experiments 1-5 designate a role for aerobic exercise in the treatment of drug relapse. Specifically, both concurrent wheel running and wheel running outside of experimental sessions prevented reinitiation of cocaine-seeking behavior precipitated by a broad range of stimuli. In some cases, treatment effects were further augmented by concurrent administration of pharmacotherapies shown to reduce cocaine reinstatement. These conclusions are supported by preclinical and clinical work from other laboratories, highlighting the generality and robust nature of these behavioral findings. While there has been little investigation into the neurobiological mechanisms underlying the attenuating effects of exercise on addiction and relapse, present results identified brain areas in which wheel running impacted cocaine-induced neuronal activation and may provide framework for future studies on this topic. Given the strong potential of aerobic exercise to reduce relapse-related behavior, additional investigations are warranted, and new approaches should focus on incorporating well-controlled behavioral measures with examination of relapse biomarkers.

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