CHRONIC FORCED AND FREE EXERCISE ON COCAINE PLACE CONDITIONING AND GLUTAMATERGIC SYNAPTIC PLASTICITY IN THE VTA

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ABSTRACT

Exercise may be useful in preventing substance use disorders and addiction. It is well known that chronic exercise, both forced and voluntary, decreases addiction vulnerability in rodent models and likely in humans. However, the mechanism by which chronic exercise accomplishes this has not been fully evaluated. Here, a conditioned place preference (CPP) paradigm was used to measure the learning of cocaine-associated environmental cues in two groups of rats following a period of both forced and free running. It is shown that rats subjected to chronic forced running displayed no significant deviation in learning of contextual cues paired with cocaine through CPP. On the other hand, rats subjected to chronic free running displayed a notable decrease in acquisition of cocaine-CPP. To investigate the mechanism by which chronic exercise affects cocaine-CPP, we will conduct future electrophysiological studies in the ventral tegmental area (VTA), an area that has been widely implicated in the natural reward circuitry of the brain. The VTA and its role in addiction has been studied extensively by the Morikawa Lab, with one previous study finding that repeated social defeat stress enhances glutamatergic synaptic plasticity in the VTA and cocaine-CPP. Essentially, we expect to observe a decrease in long-term potentiation (LTP) of NMDA-mediated glutamatergic synaptic plasticity in the VTA and consequently, a decrease in cocaine-CPP following both chronic forced and free exercise. If observed, our findings will suggest that decreased plasticity of glutamatergic transmission may be responsible for decreased addiction vulnerability through chronic exercise.

1. OVERVIEW OF ADDICTION

1.1 What is addiction?

You are watching the nightly news and hear yet another case of an opioid overdose. As the news segment progresses, you see the reporters walking around the Austin Recovery center interviewing several recently admitted patients. You notice that every person being interviewed was restless and mentally unsound, exhibiting a variety of odd behaviors ranging from stereotypy to uncontrollable ticks. The news segment then concludes with a message from one of Austin's local physicians describing the gravity of drug addiction in the United States and stating that something must be done to fix this problem. Following this news report, a plethora of questions rush through your mind. What is drug addiction and how can it be treated?

Drug addiction is defined as the behavioral pattern of drug use, characterized by (1) overwhelming involvement with the use of a drug (compulsive use), (2) loss of control in limiting intake, and (3) emergence of a negative emotional state reflecting withdrawal when drug access is prevented (Koob & Volkow, 2010). In the realm of addiction research, most of the primary studies focused on the acute effects of substance use. However, the chronic effects of drug abuse have been of great importance in recent research, in order to develop potential treatments in animal studies and human models. Unfortunately, a definitive cure for addiction has not been established, due to the complexity of the disease and the diverse pharmacological actions of the drugs available. As a result, current research into addiction attempts to elucidate the brain circuitry involved as well as understand the genetic, cellular, and molecular mechanisms that are central in this chronic brain disorder.

A common psychiatric framework that is referenced in regards to drug consumption and both positive and negative reinforcement involves the concept of impulse control disorders and compulsive disorders. Drugs are first taken in an "impulsive" manner, where there is no premeditation or prior thought before committing the action. Pleasure and gratification usually follow drug consumption and thus, impulse control disorders represent the positive reinforcing effects of drugs. Following tolerance to the drug, compulsive disorders begin to arise where the user feels stress and anxiety. This leads one to actively seek out the drug and act out of "compulsion", in order to offer a temporary reprieve from stress despite the negative consequences that may follow (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). As a result, compulsive disorders represent the negative reinforcing effects of drugs. Both impulsive and compulsive disorders are present in varying degrees within the three stages of addiction, known as: "binge/intoxication", "withdrawal/negative affect", and "preoccupation/anticipation".

1.2 Opponent Process Theory and Motivation for Drug-seeking

The motivation for drug-seeking plays an important role in forming addictive behaviors. Richard Solomon, a psychologist in the 20th century, detailed the "opponent process theory" to explain the manner in which emotions are experienced. He believed that emotions acted in pairs and were opposites of one another, where one emotion is experienced and the other is suppressed. For example, if you were studying late at night for a neuroscience exam scheduled for the next morning, your feeling of anxiety would be expressed whereas the feeling of relief would be suppressed. On the other hand, if you showed up to class the next morning and learned

that the exam was postponed for the following week, your feeling of anxiety would be suppressed and relief would be expressed.

This contrast in emotional states is strongly related to the motivation behind drug consumption in addiction. When a drug is first taken, the acute effects are generally associated with pleasure and euphoria, with positive hedonic responses correlating with the intensity, duration, and frequency of the drug stimulus. However, over time, chronic drug use leads to withdrawal and symptoms such as stress and negative affect. This leads to consumption of the drug during withdrawal to eliminate these negative symptoms rather than to experience enjoyment and happiness. Furthermore, with the negative hedonic response growing larger with repeated exposure to the drug, withdrawal completely overpowers the positive effects of the substance taken as well as takes control over the body's other basic needs. With no definitive treatments or cures for addiction, it is necessary to elucidate the brain mechanisms pertaining to acute and chronic drug use.

1.2 Brain circuitry and neurotransmission

As stated above, addiction can be categorized into the three stages of "binge/intoxication", "withdrawal/negative affect", and "preoccupation/anticipation". Research into potential treatments and therapies for addiction have targeted these stages by elucidating the brain circuitry associated with each stage.

In the "binge/intoxication" stage, many studies have noted the importance of dopamine and the mesolimbic dopaminergic system, as well as the central amygdala in regulating the acute effects of abused drugs. Activation of the mesolimbic dopaminergic system has served as a central aspect of the motivation behind drug intake, with connections from the VTA to the

nucleus accumbens (NAc) playing a major role in providing "incentive salience" (Salamone et al., 2007). Incentive salience refers to the desire for a drug, inducing appetitive behavior and driving drug consumption. In regards to dopamine, it has been shown that knockout mice lacking the dopamine D_1 receptor do not self-administer cocaine (Caine et al., 2007). Even though dopaminergic systems have been one of the most cited areas for drug motivation, the central amygdala (CeA) has also been observed to play a role in initial drug reward. One study demonstrated that lesioning the CeA blocked oral self-administration of ethanol in rats (Moller et al., 1997). Therefore, many areas in the brain play a role in drug initiation and acute drug reinforcement.

In the "withdrawal/negative affect" stage, the extended amygdala has been touted as a key structure in integrating brain arousal and mediating negative reinforcement mechanisms. Comprising of the CeA, bed nucleus stria terminalis (BNST), and a subregion of the NAc, the extended amygdala is heavily involved in the limbic system through receiving afferents from the basolateral amygdala and sending efferents to the ventral pallidum and lateral hypothalamus. As a result, this has led many scientists to hypothesize the extended amygdala's role in fear conditioning and stress response (Le Doux, 2000). It is also important to consider the mediation of the hypothalamic-pituitary-adrenal axis by corticotropin-releasing factor (CRF) in regards to withdrawal and negative affect. Several studies have looked into mediation of CRF in dealing with acute withdrawal, with acute ethanol withdrawal in rats being reversed with CRF antagonist administration into the amygdala (Funk et al., 2006). In addition to the extended amygdala and the hypothalamic-pituitary-adrenal axis, the role of dynorphin has been studied in regards to acute drug withdrawal. It has been shown that levels of dynorphin, a class of opioid peptides, are increased in the NAc following increased dopaminergic activation. Exceedingly high dynorphin

levels in the NAc and amygdala are associated with decreased dopaminergic function, present in those displaying cocaine and alcohol withdrawal (Gehrke et al., 2008; Kuzmin et al., 2013).

In the "preoccupation/anticipation" stage, the individual "relapses" and reinstates drug behavior after a period of abstinence. Reinstatement of the drug can be classified in three categories: drug-induced reinstatement, cue-induced reinstatement, and stress-induced reinstatement. With both drug-induced reinstatement and cue-induced reinstatement, several studies have stressed the importance of the glutamatergic pathway from the prefrontal cortex to the NAc, the GABAergic projection from the NAc to the ventral pallidum, and the dopamine projection from the VTA to the medial prefrontal cortex (Macfarland and Kalivas, 2001; Kalivas and O'Brien, 2007). In regards to stress-induced reinstatement, the activation of both CRF and norepinephrine has been cited as important factors in various animal models (Shaham et. al, 2002). With the wide-ranging effects of drugs on various parts of the brain, future treatments for addiction have looked into targeting these particular areas associated with the three stages of addiction.

2. EXERCISE AND EFFECT ON THE BRAIN AND NERVOUS SYSTEM

2.1 Introduction to Exercise

You stop the timer on your watch as you finish the 10-mile trek around Lady Bird Lake. Your heart is racing, yet you feel a burst of adrenaline rushing through your body. You begin to wonder, what was the purpose of all this exercise and running? Is there a particular benefit to raising your heart rate and losing copious amounts of sweat?

In fact, it has been well established that exercise and physical activity reduces the risk of a variety of chronic diseases, including heart disease, obesity, and stroke. With exercise also

protecting the brain from the detrimental effects of aging (Intlekofer and Cotman, 2013), it is important to understand the neurobiological mechanisms in which regular exercise and physical activity confers these advantages. Much of the research in the field of exercise has involved both animal studies and human studies, where researchers can study the physiological and cellular changes from exercise in animal models and the macroscopic changes in cognition and thinking in human models.

2.2 Hippocampus and Spatial Learning

One particular area of interest when studying exercise's effect on the brain is the hippocampus. The hippocampus, essential for spatial navigation as well as formation of memory, can be subdivided into five areas: the dentate gyrus (DG) and four Cornu Ammonis areas (CA1-CA4). The DG is highly specialized in that it continuously generates new neurons, which may have a role in formation of new memories. Areas CA1, CA2 and CA3 have been known to play differential roles in spatial learning through contextual coding as well as the retrieval of extinguished fear responses in rats (Ji and Maren, 2008). Both animal and human studies have documented the changes that arise in the hippocampus through behavioral and imaging studies.

Animal studies have documented the effect of running on the hippocampus. One study showed increased neurogenesis in the dentate gyrus of rats that were subjected to voluntary wheel running (van Praag et al., 1999). Furthermore, spatial discrimination and separation of unique experiences has been studied in mouse models. In another study, both sedentary and running mice were tested on a spatial discrimination task where stimuli of identical shapes and sizes were presented in varying distances from one another on a touch screen (Morton et al., 2006). It was shown that with large distances, both sedentary and running mice performed

equally. However, as the distance between the stimuli decreased, running mice outperformed sedentary mice in recognizing the stimuli. This may have been due to increased neuronal formation in the dentate gyrus that is associated with running. Another study demonstrated that transgenic mice with enhanced adult hippocampal neurogenesis demonstrated improved performance in pattern separation (Sahay et al., 2011). Therefore, exercise's effect on the hippocampus plays a central role in not only neurogenesis, but also spatial discrimination and other important tasks.

In humans, similar tests to those done with animals have studied spatial discrimination. Following several imaging studies, it was confirmed that functional MRI (fMRI) activity in both the DG and area CA3 were associated with pattern separation performance. This was further extrapolated on by a recent study, where young adults subjected to chronic aerobic exercise showed improved performance on visual pattern separation tasks compared to sedentary adolescents (Dery et al., 2013). Another relevant study found that individuals who scored high on the Beck Depression Index performed poorly on this visual pattern separation task, demonstrating that stress and depression may decrease adult neurogenesis (Jacobs et al., 2000). As a result, it is clear that exercise indeed affects spatial discrimination and brain function through possible action on the hippocampus and neurogenesis in the dentate gyrus.

2.3 Growth Factors

In order to study the cellular and physiological changes that occur following prolonged periods of exercise, animal models have been widely studied. In particular, neurotrophins, a family of proteins that regulate the development, survival, and functioning of neurons, have been of interest. The most widely studied neurotrophin in regards to exercise is brain-derived

neurotrophic factor (BDNF) due to its role in supporting the growth and survival of neurons as well as regulating synaptic plasticity. In one of the first studies about exercise and BDNF, it was shown that voluntary wheel-running in rats promoted increased BDNF mRNA levels in a variety of structures in the brain, such as the cerebellum, caudal perirhinal cortex, and hippocampus (Neeper et al., 1995). Further studies have shown that BDNF expression is increased not only in voluntary running, but also forced running models after both short and long periods of exercise (Callaghan et al., 2007; Liu et al., 2009). These findings provide a mechanistic explanation for increased neurogenesis in the hippocampus, as well as offer insight into promotion of neuronal growth and synaptic plasticity.

In addition to BDNF, other trophic factors have been studied for their role in brain regulation. Two trophic factors of interest are vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1). VEGF is elevated in the hippocampus and peripheral areas such as the lungs following exercise, whereas IGF-1 is elevated in a variety of brain areas such as the striatum, hypothalamus, and hippocampus (Tang et al., 2009; Carro et al., 2000). VEGF promotes angiogenesis, the development of new blood vessels, with increasing hippocampal angiogenesis correlating with increased neurogenesis (Van der Borght et al., 2009). On the other hand, neurons with high levels of IGF-1 are known to increase in spontaneous firing and become more sensitive to afferent stimulation, meaning that inputs from surrounding neurons are more easily received (Carro et al., 2000). Hence, exercise has a notable effect on the production of multiple growth factors such as BDNF, VEGF and IGF-1, which all play a central part in neuronal growth and function.

2.4 Synaptic Plasticity and Morphological Changes

Exercise-induced changes in learning and memory have been studied through gene expression, neurogenesis, and most importantly, synaptic plasticity. It is established that synaptic plasticity, mainly in the hippocampus, mediates memory formation. Long-term potentiation (LTP) induction has been studied extensively in hippocampal slices, with several studies finding that there is significant potentiation of the DG synaptic response in young rodents subjected to exercise as well as a reversing of DG LTP diminution in aged rats (Callaghan et al., 2009). On the other hand, area CA1 in the hippocampus displayed no changes in LTP mediated by prolonged exercise (van Praag et al., 1999). This suggests that increased neurogenesis in the DG may play a role in mediating LTP induction. In addition to increased LTP, mRNA levels of NMDAR2B were increased in the DG of runner rats (Farmer et al., 2004). NMDAR2B is a receptor channel that is associated in LTP, with overexpression of NMDAR2B found to enhance LTP induction (Tang et al., 2001).

The study of long-term depression (LTD) has also been relevant in observing the changes in the brain following exercise. LTD effectively serves to weaken synapses and provides a constructive mechanism for LTP to strengthen other synapses, serving as a model for memory formation and encoding of new information. Studies have shown that LTD is generally unaffected by exercise (Vasuta et al., 2007). However, it is interesting to note that mice subjected to running demonstrated LTD that was reliant on activation of NR2A subunit-containing NMDA receptors, which was not the case in sedentary mice. As a result, this suggests that exercise may have an effect on the contribution of NMDA receptor subunits to LTD.

In addition to both LTP and LTD in regards to synaptic plasticity, morphological changes can occur in the brain following exercise. It has been shown that chronic exercise occurring for a period greater than 2 months actually increases the number of pyramidal cells in area CA1 of the

hippocampus as well as spine density of the entorhinal cortex, which has been found to contribute to spatial memory and navigation (Stranahan et al., 2007). Analyses were also conducted on the DG granule cell layers, with findings that the complexity, total length and spine density of dendrites localized in this area increased following running (Eadie et al., 2005). Further studies done on the hippocampus show that shrinkage in dendritic spines are associated with LTD (Zhou et al., 2004) and thus, morphological changes like an increase in dendritic spine length may promote LTP in the hippocampus.

3. INTRODUCTION

3.1 Rational and Purpose

It is well known that exercise has been used as a means for prevention and treatment of various mental disorders including depression (Mikkelsen et al., 2017) and schizophrenia (Curcic et al., 2017). More recently, exercise has also been utilized as treatment in addictive disorders (Lynch et al., 2013; De La Garza et al., 2016) such as the attenuation of nicotine seeking in male-adolescent rats (Sanchez et al., 2013). With physical activity being known to affect the activity of dopaminergic neurons, which play a central role in the formation of addictive behaviors (Hattori et. al, 1994), this has led to the interest of using exercise in the prevention and treatment of addiction and substance use disorders. In order to assess the motivational effects of cocaine on subjects, the conditioned-place preference (CPP) paradigm was generally used in many studies.

Although there are certain studies suggesting exercise increases CPP (Eisenstein et al., 2007; Smith et al., 2008), many reports have demonstrated chronic exercise's negative effect on CPP. In one study, chronic forced exercise during adolescence has been shown to decrease

cocaine-CPP in both male and female Lewis rats (Thanos et al., 2010). Another report demonstrated that chronic wheel running in male C57BL/6J mice accelerates extinction of cocaine-CPP (Mustroph et al., 2011). There are several claims surrounding the neurobiological mechanism underlying cocaine-CPP. One study demonstrated that repeated social defeat stress promotes cocaine-CPP through enhancement of glutamatergic synaptic plasticity (Stelly et al., 2016). However, it is not clear how chronic forced exercise mechanistically acts to decrease cocaine-CPP.

3.2 Mesolimbic Dopaminergic Projection System

The mesolimbic dopaminergic projection system originating in the ventral tegmental area (VTA) plays a central role in the endogenous reward pathway that contributes to addiction. VTA dopaminergic (DA) neurons generally display basal tonic firing, while phasic firing is induced by unexpected rewards (Radulescu, 2010). As a result, these neuronal responses are hypothesized to be the mechanism for Pavlovian cue-reward associations (Tsai et al., 2009). Following a period of repeated cue-reward pairing, conditioned burst responses from dopamine neurons are acquired in response to a predictive reward cue (Schultz, 1998); therefore, this may drive appetitive behavior and serve as the neurobiological mechanism underlying CPP.

Glutamatergic inputs that activate NMDA receptors (NMDARs) have been known to transition DA neurons from tonic firing to bursting (Aggarwal and Wickens, 2011). Therefore, decreased potentiation of cue-driven NMDARs may contribute to decreased acquisition of bursting in DA neurons. Glutamatergic transmission mediated by NMDA receptors undergoes LTP when cue-like glutamatergic stimulation is repeatedly paired with reward-like bursting in dopamine neurons (Harnett et al., 2009). The induction of LTP requires amplification of burst-

evoked Ca^{2+} signals by preceding activation of group I metabotropic glutamate receptors (mGluR1s) coupled to the synthesis of inositol 1,4,5-triphosphate (IP_3). It is important to note that one study demonstrated that the antagonism of mGluR1 impairs cocaine-CPP (Yu et al., 2013). IP_3 receptors (IP_3Rs) detect the coincidence of IP_3 generated by glutamatergic input activating mGluR1s and entry of burst-driven Ca^{2+}. IP_3 enhances Ca^{2+}-induced activation of IP_3Rs by promoting access to stimulatory Ca^{2+} sites, thereby promoting Ca^{2+}-induced Ca^{2+} release from intracellular stores (Taylor and Laude, 2002). It is possible that chronic forced exercise may affect this mechanism, thus affecting CPP.

3.3 Background Information and Hypotheses

In this present study, the effect of chronic forced and free exercise was tested on cocaine-CPP. Glutamatergic synaptic plasticity will be assessed in future studies, using electrophysiological techniques such as patch clamping. The forced running group was subject to daily exercise for 2 weeks, in comparison to previous studies utilizing an exercise regime 5 days a week for a longer period of time (Thanos et al., 2010). Female rats were chosen due to their higher propensity for running (Eikelboom, 1988) and the enhanced efficacy of exercise on cocaine seeking compared to males (Zlebnik et al., 2014). The free running group was subject to daily exercise for 4 weeks and male rats were chosen due to previous literature showing decreased cocaine-CPP in these group of rats (Mustroph et al., 2011). It is hypothesized that both chronic forced and free exercise (1) inhibits the acquisition of cocaine CPP in rats and (2) diminishes NMDAR LTP in the VTA via a decrease in IP_3 sensitivity of IP_3Rs.

4. MATERIALS AND METHODS

4.1 Animals

For the forced running group, female Sprague-Dawley rats (n=12), at 4 weeks of age, were divided into exercise and control groups. Food and water were provided ad libitum, and food intake and body weight were monitored daily. All subjects were housed individually on a 12-hour reverse light dark cycle (lights off: 08:00-20:00 h).

For the free running group, male Sprague-Dawley rats (n=24), at 4 weeks of age, were divided into exercise and control groups. Food and water were provided ad libitum, and food intake and body weight were monitored daily. All subjects were housed individually on a 12-hour reverse light dark cycle (lights off: 08:00-20:00h).

4.2 In vivo drug treatments

All drug and vehicle solutions were administered via intraperitoneal injections. Cocaine was dissolved in 0.9% saline for a dosage of 20 mg/kg for forced runners. Cocaine dosage was then decreased for free runners to 10 mg/kg.

4.3 Procedures

4.3.1 Chronic daily exercise regimen

For the first round of experiments, the experimental rats were subjected to forced exercise via Campden Instrument Forced Exercise Bed apparatus (Figure 1). The apparatus consisted of 6 running wheels (33.99 cm in diameter, 11.18 cm in width) rested on individual tracks that were motorized through an interface that set the exercise speed. All exercise subjects (n=4; 2 rats were

excluded) were conditioned under the same protocol. Exercise began between 08:00h and 13:00h. The subjects were given a 2-day acclimation period, where both exercise and control groups were handled by the experimenter. Following acclimation, forced exercise began at 10 min/day at a steady rate of 3 m/min. The speed and duration of exercise was then raised in small increments to the final speed of 15 m/min for 60 min/day by day 7. The exercise rats were then maintained on this daily exercise regimen for a period of 2 weeks, 7 days a week, prior to conditioned place preference testing. The control group (n=6) were transported to the stationary wheels and remained there for 1 hour following the exercise group.

For the second round of experiments, the experimental rats were subjected to free exercise via metal running wheels (Figure 2). All exercise subjects (n=12) were conditioned under the same protocol, with exercise beginning from 08:00h and finishing at 15:00h. The exercise group were acclimated for several days prior to entering the exercise regimen. Due to the nature of free running, the amount of running by rats in the exercise group was tracked via a magnet and sensor that measured the number of revolutions per hour. The handled controls were then placed in locked running wheels and remained there for the same amount of time as the running undertaken by the free running group.

4.3.2 Conditioned place preference (CPP)

The CPP apparatus (Med Associates, St. Albans, Vermont) consisted of two distinct compartments connected by a small gray middle chamber (Figure 3). One compartment had white walls with grid flooring and lights on, while the other compartment had black walls with rod flooring and lights off. A separate cue (painted ceramic weight) was placed in the rear corner

of each compartment (black weight in white room, white weight in black room) for further differentiation. Data acquisition was recorded using Med PC-IV software.

4.3.3 Pretest phase (Day 1)

Following the 2-week period of forced wheel running and 4-week period of free wheel running, the rats were allowed to explore the entire CPP box for 15 min to test for initial side preference. The percentage of time spent in each compartment was determined following the exclusion of time spent in the middle chamber. Rats that spent more than 35% of the total time in the gray chamber were excluded. Rats with an initial side preference of >60% were also excluded.

4.3.4 Conditioning phase (Days 2-3, Days 5-8)

Following the first day of pretest, the rats underwent a 7-day conditioning phase. On Day 2, 5, and 7, rats were given a saline injection and confined to one compartment. On Day 3, 6, and 8, rats were given an injection of cocaine and confined to the other compartment. Throughout this conditioning phase, rats spent 10 minutes each in the compartments. To ensure no bias in assignment, compartment assignment was counterbalanced such that the rats had, on average, ~50% preference for the cocaine-paired compartment.

4.3.5 Posttest phase (Day 4, Day 9)

Following the first two days of saline and cocaine injection (Day 2 and 3), the rats were subjected to a 15-minute posttest to determine their preference for the cocaine-paired side. After the conditioning phase of Day 5 to 8, rats were subjected to a second 15-minute posttest.

4.4 Statistical analysis

Data is expressed as mean ± SEM. Statistical significance was determined by Student's *t-test* or ANOVA. The difference was considered significant at $p<0.05$.

5. RESULTS

5.1 Primary Results (Chronic Forced Exercise)

5.1.1 Conditioned Place Preference

The effect of forced exercise was tested on the acquisition of cocaine CPP, in which the rats learned to associate a particular context with the rewarding effects of cocaine. Rats underwent forced wheel running for a period of two weeks prior to the 9 days of conditioning as well as throughout the entire conditioning phase. A repeated measures one-way ANOVA demonstrated that the forced running group displayed no particular preference for the cocaine-paired side ($F(2,3) = 0.8998$, $p = 0.4552$) (Figure 4A), whereas there was strong preference for the cocaine-paired side in the handled control group ($F(2,5) = 10.64$, $p = 0.0033$) (Figure 4B).

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on preference for cocaine-paired side. There was a statistically significant effect of day on preference for cocaine-paired side, $F(2, 16) = 5.26$, $p = 0.176$ (Figure 5). There was no significant effect of exercise on preference for cocaine-paired side, $F(1, 16) = 0.06$, $p = 0.8188$. There was no significant interaction between the effects of exercise and day on preference for cocaine-paired side, $F(2, 16) = 0.06$, $p = 0.9376$.

5.1.2 Body Weight

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on body weight. There was a statistically significant effect of day on body weight, $F(29, 232) = 381.28$, $p < 0.0001$ (Figure 6). There was no significant effect of exercise on body weight, $F(1, 232) = 0.29$, $p = 0.6042$. There was a significant interaction between the effects of exercise and day on body weight, $F(29, 232) = 2.96$, $p < 0.0001$.

5.1.3 Food Intake

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on food intake. There was a statistically significant effect of day on food intake, $F(21, 168) = 2.83$, $p = 0.0001$ (Figure 7). There was no significant effect of exercise on food intake, $F(1, 168) = 1.23$, $p = 0.3005$. There was no significant interaction between the effects of exercise and day on food intake, $F(21, 168) = 1.00$, $p = 0.4606$.

5.2 Secondary Results (Chronic Free Exercise)

5.2.1 Conditioned Place Preference

The effect of free exercise was tested on the acquisition of cocaine CPP. Rats underwent voluntary wheel running for a period of four weeks prior to the 9 days of conditioning as well as throughout the entire conditioning phase. A repeated measures one-way ANOVA demonstrated that the free running group displayed a significant preference for the cocaine-paired side ($F(2,22) = 8.141$, $p = 0.0023$) (Figure 8A). There was also a significant preference for the cocaine-paired side in the handled control group ($F(2,22) = 17.75$, $p < 0.0001$) (Figure 8B).

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on preference for cocaine-paired side. There was a statistically significant effect of day on preference for cocaine-paired side, $F(2, 44) = 21.71$, $p < 0.0001$ (Figure 9). There was no significant effect of exercise on preference for cocaine-paired side, $F(1, 22) = 1.457$, $p = 0.2402$. There was no significant interaction between the effects of exercise and day on preference for cocaine-paired side, $F(2, 44) = 2.064$, $p = 0.1391$.

5.2.2 Body Weight

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on body weight. There was a statistically significant effect of day on body weight, $F(34, 770) = 594.7$, $p < 0.0001$ (Figure 10). There was a significant effect of exercise on body weight, $F(1, 770) = 92.5$, $p < 0.0001$. There was no significant interaction between the effects of exercise and day on body weight, $F(34, 770) = 0.761$, $p = 0.8364$.

5.1.3 Food Intake

A mixed two-way ANOVA was used to analyze the effect of exercise and day (pre-test vs. post-test) on food intake. There was a statistically significant effect of day on food intake, $F(2, 66) = 38.02$, $p < 0.0001$ (Figure 11). There was no significant effect of exercise on food intake, $F(2, 66) = 0.972$, $p = 0.3278$. There was no significant interaction between the effects of exercise and day on food intake, $F(2, 66) = 0.1182$, $p = 0.8887$.

6. DISCUSSION AND CONCLUSION

6.1 Primary Results (Chronic Forced Exercise)

6.1.1 Conditioned Place Preference

Here, the handled control group demonstrated increased cocaine-CPP as expected. However, the forced running group garnered a mixture of different results and did not demonstrate reduced cocaine-CPP as was previously predicted. This is in contrast to a previous study reporting chronic forced exercise during adolescence decreased cocaine conditioned place preference in Lewis rats (Thanos et. al, 2010). Both of these studies differed in a variety of factors including duration of exercise, strain of rat, and dosage of cocaine. The factor that may have had the biggest influence on these results is dosage of cocaine, as previous studies have generally used a dosage of 10 mg/kg (Thanos et. al, 2010). The manipulation of cocaine dosage will be considered in future studies.

An important consideration that may have affected the results is indeed sample size limitation and attrition of the rats. When beginning this experiment, two of the six rats in the forced running group were unable to keep up with the preliminary speeds used and had to be excluded from the dataset. This may have occurred due to handling error on the experimenter's part through unintentional fear conditioning of the rats to the forced exercise bed apparatus. In addition, due to the specific protocol stated in section 4.3.3, one more rat was excluded because of its preference for the gray chamber during the pre-test phase of conditioning. With only three rats in the forced running group, the data was undoubtedly affected and may not be an accurate representation of chronic forced exercise's effect on cocaine-CPP. Future studies need to be conducted with a larger sample size and more attention to the handling of rats to prevent attrition.

The handled controls were also a point of discussion. Although the controls were placed in running wheels that were effectively locked in place, this group of rats was still able to run and engage in physical activity. As a result, this may have affected cocaine-CPP results. In order to rectify this in future studies, individual rats in the control group will be placed in cages separate from the home cages for the same period of time as the experimental group are engaged in forced running. However, both groups will still be subjected to the same amount of tactile stimulation and handling during experimentation to account for this confounding variable.

Another factor to be considered is the sex of the rats. With an entirely female sample, this may have also had an effect on exercise's inability to block cocaine-CPP. This is consistent with previous studies stating women have higher vulnerability to psychostimulant drugs than men (Lynch et. al, 2002) and greater preventative effects of exercise on drugs for men (Pate et al., 2000). Clinical studies have also demonstrated that women are subject to greater cravings of addictive substances than men (Flores et. al, 2017). As a result, future studies including both male and female rats are needed to assess sex differences in eliciting cocaine-CPP.

6.1.2 Body Weight

Throughout the course of the study, both groups of rats gained a significant amount of weight as expected. However, exercise did not have a significant effect on weight. This finding is inconsistent with other reports demonstrating that chronic forced exercise causes a significant decrease in body weight (Levin and Dunn-Meynell, 2004). However, it is important to note that exercise was voluntary and the duration of wheel running was longer (6 weeks) compared to daily 2-week forced running in this study. Although there was a significant interaction between the effects of exercise and day on body weight, this is likely due to experimental error. As

confirmed by the Bonferroni posttests, there was a significant difference in body weight on day 26, which was when several of the handled control rats had no access to water. Therefore, this may have affected the results of the mixed two-way ANOVA.

6.1.3 Food Intake

Food intake was erratic throughout the course of the study, which is consistent with findings from other reports for female rats (Thanos et. al, 2010). Exercise was found to not have any significant effect on weight, which is inconsistent with what was expected.

6.2 Secondary Results (Chronic Free Exercise)

6.2.1 Conditioned Place Preference

Similar to chronic forced exercise, the free running group of rats demonstrated no significant difference in acquisition of cocaine-CPP compared to the handled controls. However, it is important to note that based on the Figure 9 graph, exercise did seem to have an effect in conditioning of the rats. There is a notable delay in conditioning of free runners as seen by the Post 1 data points in Figure 9. Although the interaction between exercise and day was statistically insignificant ($p > 0.05$) as measured by the mixed two-way ANOVA, the p value increased in significance compared to the data from primary results with chronic forced exercise. If the same trend in cocaine-CPP is observed in future studies, increasing the sample size of rats may also increase the statistical significance of the data. All in all, it is clear that by manipulating the variables of interest from the chronic forced exercise study, this positively affected our results with the desired findings.

First, with a greater sample size (12 rats per group), the CPP results are more accurate and representative of the rats' behavior. Additionally, the handler for the free running protocol was different and may have been more successful in avoiding fear conditioning of rats to the running wheels, leading to the exclusion of no rats from either the exercise or CPP protocol. It is important to note that lowering the cocaine dosage from 20 mg/kg to 10 mg/kg may have also played an important role in decreased cocaine-CPP in free runners.

Second, the handled controls were placed in locked running wheels. The metal running wheels shown in Figure 2 differed from those in Figure 1 in the sense that the rats were physically unable to move or engage in physical activity due to the design of the wheels. As a result, this accounted for the issue noted in the primary study and may have played a part in affecting the data.

Third, the sex of the rats was switched completely to male Sprague-Dawley rats. Although previous literature has stated that females are generally better runners than males (Eikelboom, 1988), males have been known to be less vulnerable in regards to substance abuse (Pate et al., 2000). In future studies, both genders should be accounted for to determine if gender plays a role in cocaine-place conditioning.

Lastly, the duration and method of running was changed from two weeks to four weeks and from forced running to voluntary wheel running. With certain studies using a running protocol of 6 weeks for chronic forced running (Thanos et al., 2010), it is clear that four weeks of running is sufficient for suppressing cocaine-CPP which is consistent with one recent study (Mustroph et al., 2016). However, it is important to consider that changing the method of running may have had an effect in producing this result.

6.2.2 Body Weight

Throughout this study, both groups demonstrated an increase in weight, as observed by the statistically significant effect of day on body weight. Furthermore, exercise was found to have a significant effect on body weight as well, which is consistent with other studies stating that chronic exercise decreases body weight (Levin and Dunn-Meynell, 2004). By having a longer period of time for running (4 weeks as opposed to 2 weeks), this may have played a role in this significant decrease in body weight shown for free runners compared to the handled controls.

6.2.3 Food Intake

Similar to the primary study, food intake was also very erratic for both handled controls and free runners. Again, exercise was found to not have a significant effect on food intake, which is inconsistent with what was expected.

6.3 Expected Decrease of NMDAR LTP and Learning of Cocaine-Associated Cues

In relation to addiction and substance abuse, both human and animal studies have been used to observe exercise's effect on this complex disease. With human studies, one longitudinal study has shown that increasing levels of exercise from adolescence to adulthood actually predicts a decrease in rates of illicit drug use during adulthood (Terry-McElrath and O'Malley, 2012). Twin studies have found that with certain cases of discrepancies in physical activity for both twins, the more active twin has a decreased risk for illegal drug use in adulthood compared to the less active twin (Kujala, 2007). With animal studies, researchers have utilized conditioning paradigms such as CPP to observe the animals' behavior following prolonged periods of

exercise. It has been shown that treadmill exercise in rats attenuate self-administration of morphine (Hosseini et al., 2009). One other study has also found that concurrent access to a running wheel attenuates self-administration of methamphetamine as well as alcohol (Miller et al., 2012; Ehringer et al., 2009). Therefore, these findings highlight the potential of exercise and physical activity to reduce the reinforcing effects of drugs of abuse.

Although there has been a plethora of studies conducted on exercise and drug initiation in individuals, a clear cellular mechanism for the inverse relationship between drug use and exercise in humans has not been studied extensively. One recent study attempted to elucidate changes in the mesolimbic dopaminergic pathway that occur following exercise in rats (Robison et al., 2018). Utilizing autoradiography and ligands that are D1R-like and D2R-like, they observed that compared to sedentary rats, rats subjected to forced exercise demonstrated a decrease in both D1R and D2R binding in the nucleus accumbens shell and the olfactory tubercle, both of which are central in the brain reward pathway. Other studies have demonstrated the importance of exercise in modulating various targets and enzymes in the mesolimbic dopaminergic pathway. In one study with obese mice, it was found that moderate treadmill exercise increased the levels of dopamine, tyrosine hydroxylase (TH) and D2 receptor expression in the VTA-nucleus accumbens system (Chen et al., 2017). Another study with adolescent rats showed that 6 weeks of voluntary wheel running also increased TH levels in the VTA, as well as increased delta opioid receptor levels in the NAc (Greenwood et al., 2011). As a result, these findings bolsters the hypothesis that aerobic exercise may contribute to the attenuation of drug-seeking behavior and that further research is necessary to elucidate the exhaustive effects of exercise on the brain and body.

Exercise has been shown to have beneficial effects on cognition and neuroplasticity in various areas of the brain (Hötting et al., 2013). In this study, it is expected that chronic forced exercise mediates a decrease in the induction of LTP of NMDAR mediated transmission in VTA DA neurons. Rats subjected to chronic forced and free wheel running are expected to display diminished acquisition of cocaine-CPP, a paradigm and form of Pavlovian conditioning that necessitates NMDAR dependent bursting in VTA (Zweifel et al., 2009; Wang et al., 2011; Whitaker et al., 2013).

It has been reported that prolonged periods of exercise increased hippocampal brain-derived neurotrophic factor (BDNF) levels essential in regulation of synaptic plasticity and memory (Vaynman et al., 2004). Another study reported that exercise increased both responsiveness and induction of LTP in the dentate gyrus of hippocampus (Radahmadi et al., 2016). If chronic forced exercise was shown to diminish NMDAR LTP in VTA DA neurons, this would suggest that exercise has varying effects on learning and memory across different areas of the brain.

In contrast to the traditionally accepted mechanism of CPP suppression due to the alteration of the brain reward pathway and dopaminergic transmission, many studies have demonstrated the importance of VTA regulation in suppressing CPP, such as through the blocking of Ca^{2+}/calmodulin-dependent protein kinase II (Liu et al., 2013) or increased phosphorylation of translation initiation factor eIF2α (Huang et al., 2016). In regards to the mechanistic effect of exercise on suppressing CPP, studies have suggested the upregulation of D2 receptors in the striatum as a possible mechanism for attenuation of CPP (Thanos et al., 2010). Other studies have also demonstrated that increased adult hippocampal neurogenesis is not required for exercise to abolish cocaine-CPP in mice (Mustroph et al., 2015). However, the

establishment of a specific mechanism by which exercise suppresses CPP is still unclear. It has been shown that repeated stress enhances VTA glutamatergic synaptic plasticity in increasing cocaine-CPP (Stelly et al., 2016). If the rats subjected to forced running in this study demonstrated a decrease in cocaine-CPP as predicted, this would imply that decreased NMDAR LTP in VTA DA neurons may act as a potential mechanism behind this observation. As a result, this will further bolster the theory that decreased plasticity of glutamatergic transmission in the VTA serves as the underlying neurobiological mechanism behind exercise suppressing CPP.

With exercise contributing to NMDAR-dependent hippocampal LTP (Radahmadi et al., 2016; Lüscher and Malenka, 2012), how does chronic forced exercise lead to decreased NMDAR-dependent LTP in the VTA? Prolonged periods of exercise may induce decreased PKA activity or levels of PKA, leading to the decreased sensitization of IP_3Rs and thus, a decrease of NMDAR LTP in VTA. With reports demonstrating that antagonism of mGluR1 impairs acquisition of cocaine-CPP (Yu et al., 2013), chronic forced exercise may play a role in affecting this component. It is important to note that voluntary exercise has been shown to increase levels of mGluR5, a subtype of metabotropic glutamatergic receptor, in the dentate gyrus (Farmer et al., 2003). Future studies are needed to elucidate exercise's specific role on the expression and/or synthesis of mGluRs.

6.4 Conclusion

In summary, this study demonstrated the following: (1) Chronic forced exercise had no significant effect on the acquisition of cocaine-CPP and (2) chronic free exercise demonstrated a notable effect in acquisition of cocaine-CPP. Although both studies produced statistically insignificant results, it is clear from the graph in Figure 9 that there is a notable delay in

conditioning and learning for free runners compared to handled controls from pre-test to post-test 1. Due to the highly variable nature of animal studies and conditioning, these findings provide more insight into the manner in which one should manipulate the experimental protocols for future reference.

6.5 Future Directions

In the future, electrophysiological analysis will be used to determine the effects of exercise on the brain, specifically on the VTA. It is expected that both chronic forced and free exercise diminishes NMDAR LTP in the VTA. This finding, if observed, would suggest that prolonged exercise may contribute to decreased drug reward-learning rather than alteration of the brain reward pathway and dopaminergic transmission. This may serve as the underlying neurobiological mechanism by which chronic forced and free exercise decreases vulnerability to addiction and substance use disorders.

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9. FIGURES AND TABLES

Figure 1. Campden Instrument Forced Exercise Bed apparatus.

Figure 2. Free running wheels (rats in side compartment) used for voluntary exercise protocol.

Figure 3. Photograph of CPP apparatus. Colored ceramic weights can be seen in rear of each compartment.

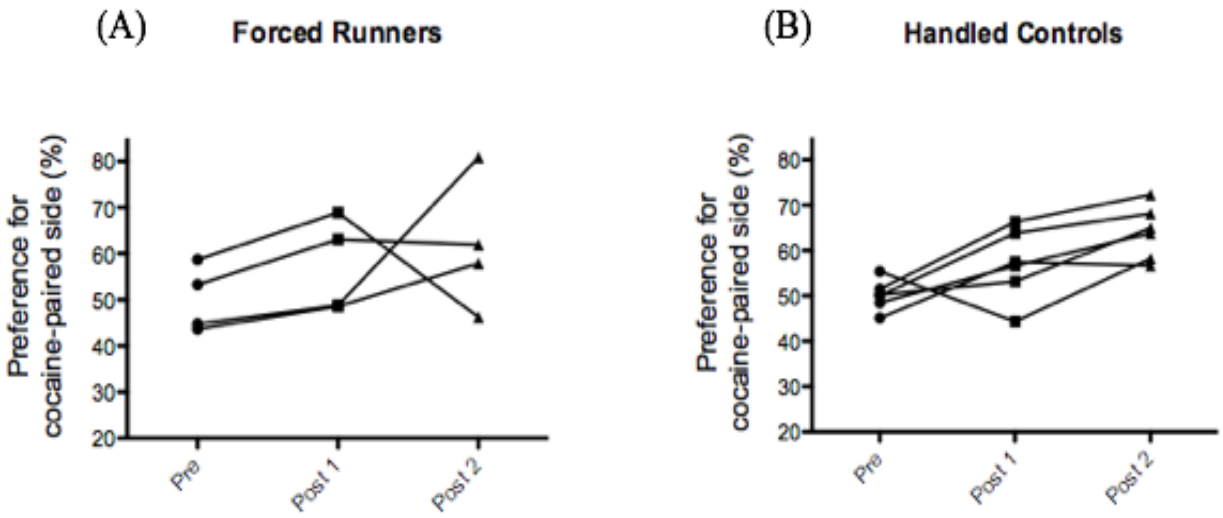

Figure 4. There was a variety of mixed preferences for group of forced runners. Handled controls demonstrated strong preference for cocaine-paired side over course of study.

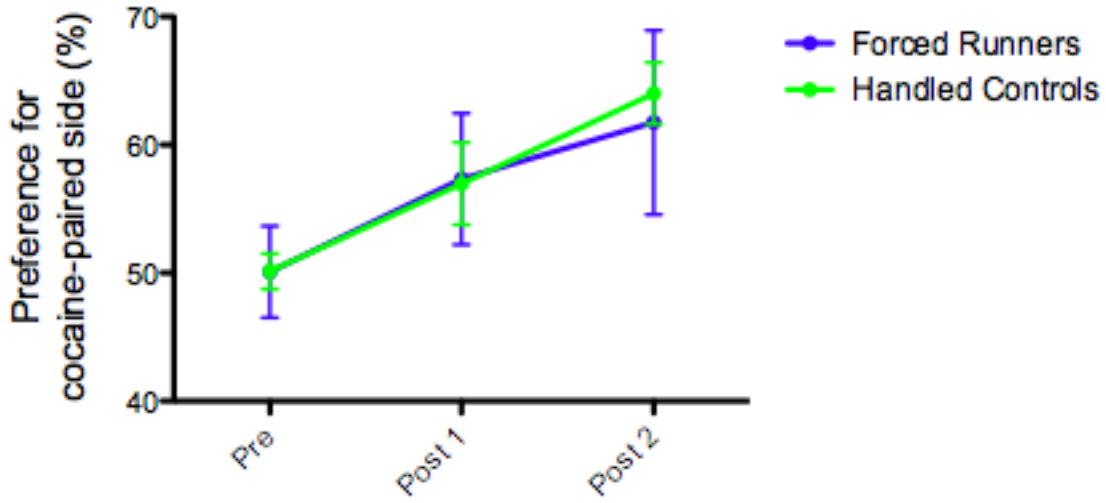

Figure 5. There was a statistically significant effect between day and preference for cocaine-paired side for both forced runners and handled controls.

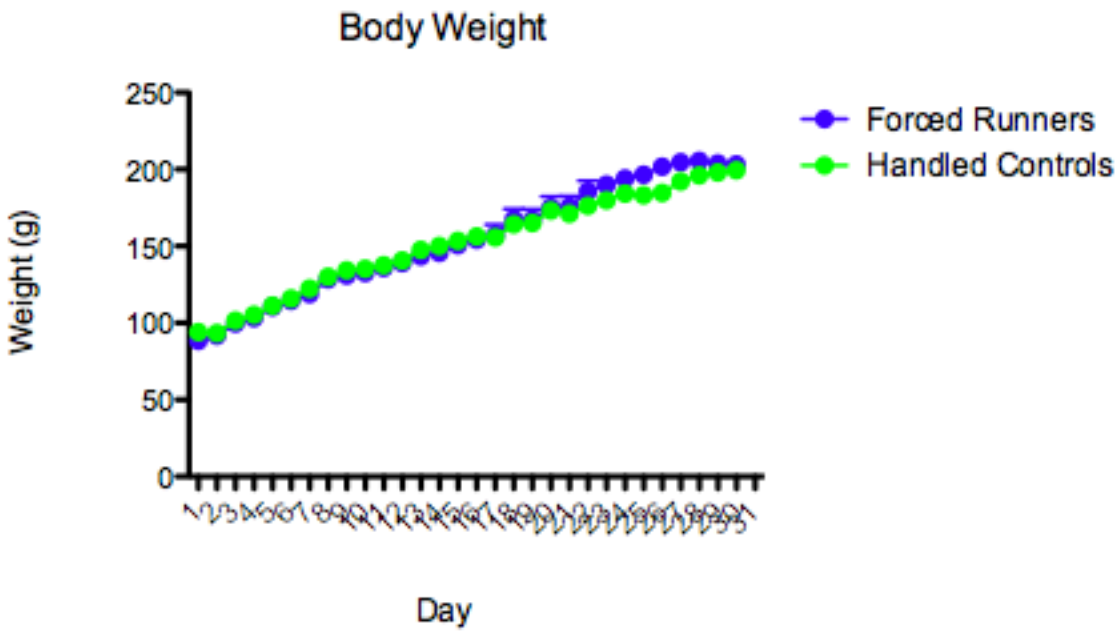

Figure 6. Body weight of forced runners and handled controls over course of study. Day had a significant effect on body weight ($p < 0.0001$).

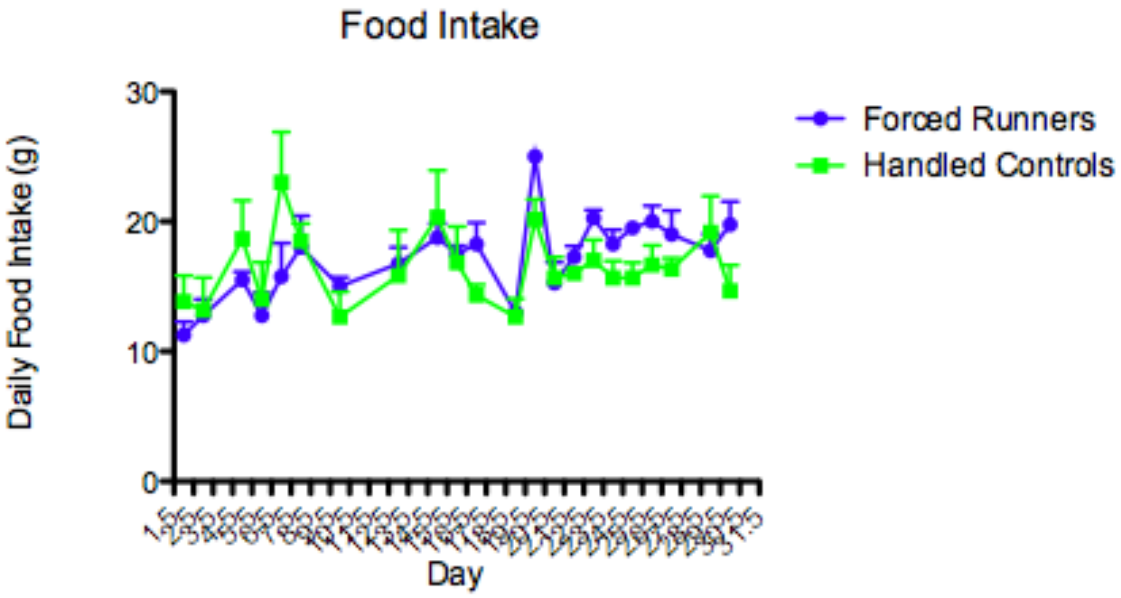

Figure 7. Mean food intake of forced runners and handled controls throughout course of study. There was no significant effect of exercise on food intake ($p = 0.3005$).

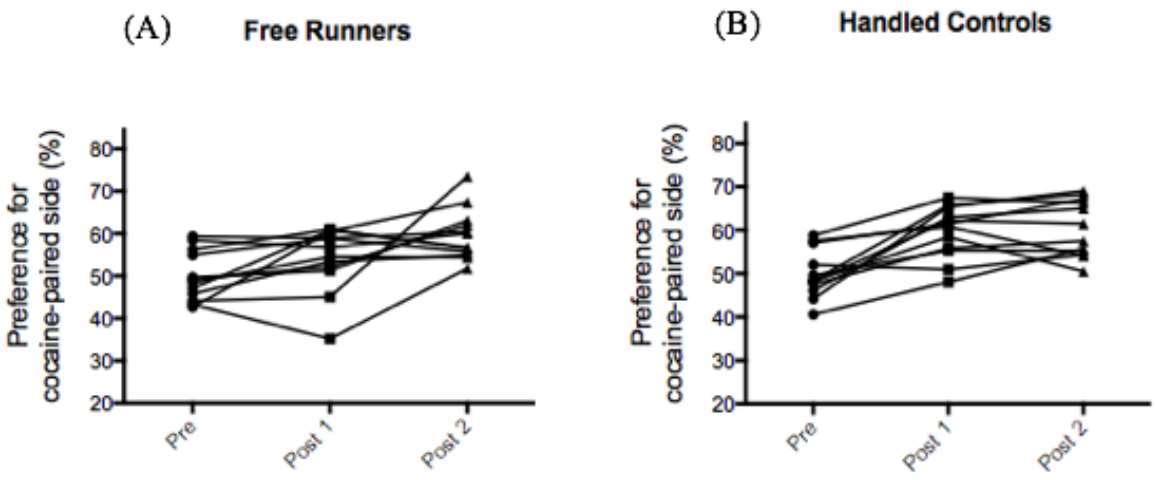

Figure 8. Free runners demonstrated a significant increase in cocaine-CPP from pre-test to post-test 2. Handled controls demonstrated strong preference for cocaine-paired side over course of study.

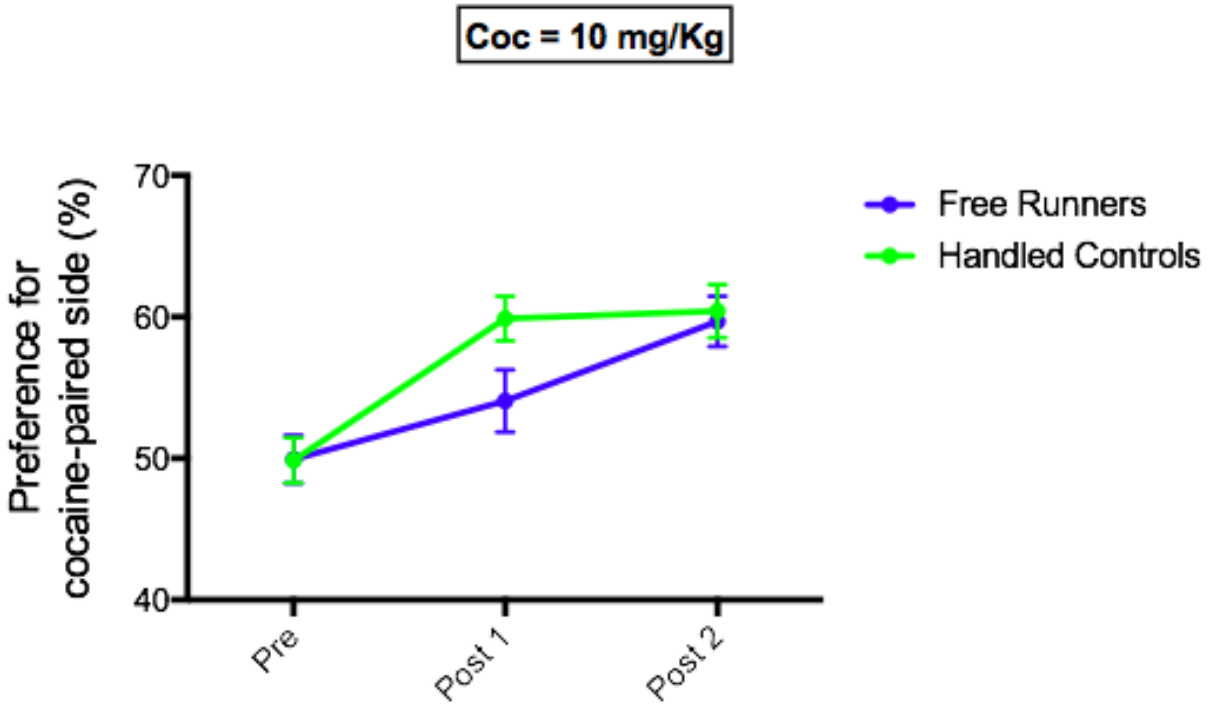

Figure 9. There was an increase in cocaine-CPP for free runners. Handled controls demonstrated strong preference for cocaine-paired side over course of study. It is important to note that following post-test 1, there is a clear delay (although statistically insignificant) in acquisition of cocaine-CPP in free runners compared to handled controls.

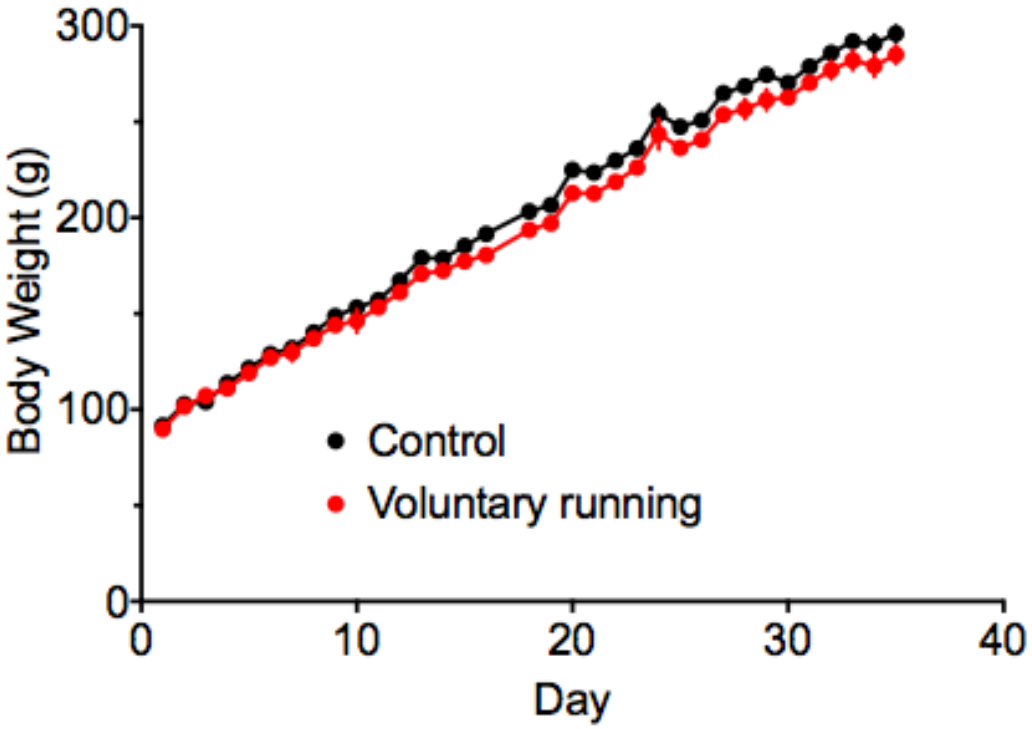

Figure 10. Body weight of free runners and handled controls over course of study. Day had a significant effect on body weight ($p < 0.0001$).

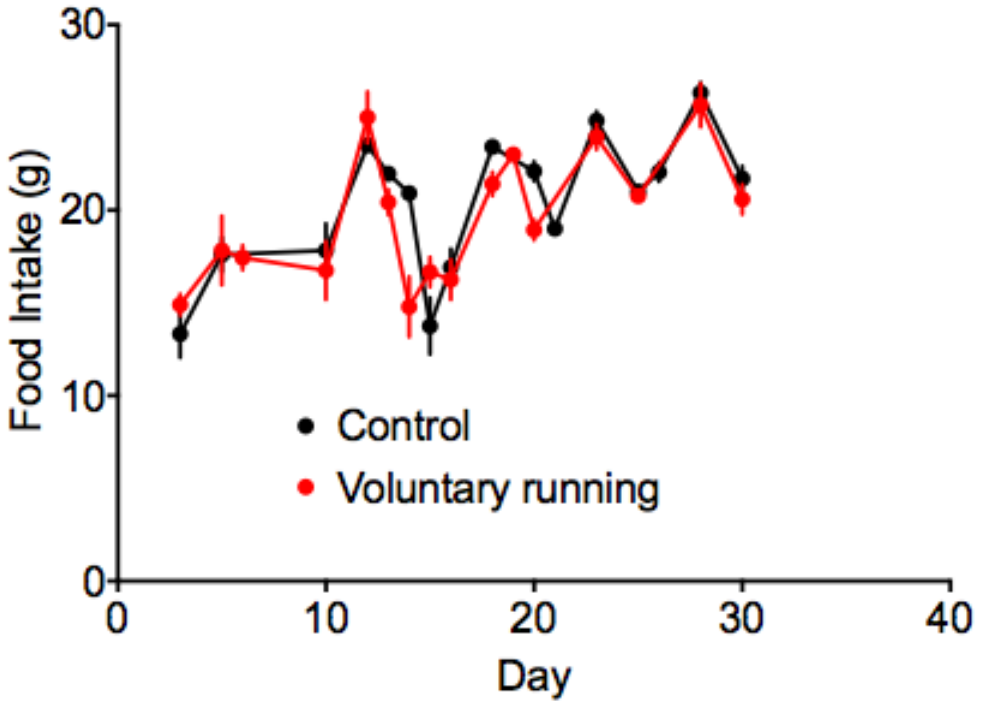

Figure 11. Mean food intake of free runners and handled controls throughout course of study. There was no significant effect of exercise on food intake ($p = 0.3278$).