INVESTIGATION OF THE BEHAVIORAL PROCESSES AND NEUROBIOLOGICAL SUBSTRATES INVOLVED IN THE MOTIVATION FOR VOLUNTARY WHEEL RUNNING IN THE RAT

by

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A Dissertation submitted to the

Graduate School-Newark

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Behavioral and Neural Sciences

written under the direction of

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Newark, New Jersey

January, 2013

ABSTRACT OF THE DISSERTATION

Investigation of the behavioral processes and neurobiological substrates involved in the motivation for voluntary wheel running in the rat

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Rats engage with voluntary running wheels spontaneously and come to run tremendous, stable distances over the first 3 weeks of wheel exposure, with males and females showing significant differences in this behavior. Though voluntary running has been utilized extensively to study its effects on the body and brain, less has been done to examine the behavior itself, and specifically lacking are studies that focus on the motivation for the behavior and any gender differences within. Here, I investigate, in a comprehensive, quantitatively comparable manner, details of voluntary wheel running and variables that affect it in both male and female Sprague Dawley rats. Using both unconditioned and conditioned techniques, I explore my primary hypothesis that voluntary wheel running is a motivated behavior with positive incentive salience, with a focus on the motivation for this behavior during both the acquisition and habitual phases of running. I then utilize these behavioral techniques to explore the involvement of discrete brain regions in the motivation for voluntary wheel running. Results from this work support the hypothesis that voluntary wheel running is a motivated behavior with positive incentive salience. The data reveal that females acquire the behavior more quickly and during habitual phases of running, run significantly farther distances at faster rates. Additionally, I show using high-performance liquid chromatography that participating in voluntary wheel running throughout life alters neurotransmitter content in brain areas including the caudate putamen, ventral tegmental area, medial prefrontal cortex (mPFC) and medial preoptic area, and that engaging in this motivated behavior throughout life alters both the neurochemical and behavioral responsiveness to an acute dose of cocaine. Though males and females show an equally robust conditioned place preference for the total experience of running during the acquisition phase, the reinstatement of running after a period of forced wheel abstinence is greater for females than males, with males showing a stronger preference for the aftereffects of wheel running. Finally, I reveal for the first time that the prelimbic mPFC and nucleus accumbens core may be necessary for the motivation for voluntary wheel running.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Joan I. Morrell, my thesis adviser and doctoral mentor. I deeply appreciate her academic leadership and her willingness to explore a new arena of research with me, namely the motivation for voluntary wheel running. She has been tremendously giving in her time, effort and energy in leading me through my doctoral work, and for that, I am truly grateful. She helped me achieve a life's goal of mine, attaining a doctorate, and I am forever in her debt for this level of giving. Additionally, I would like to thank my thesis committee chair, Dr. Mauricio Delgado, and committee members, Drs. Elizabeth Abercrombie, Denis Paré, and Laszlo Zaborsky, for dedicating their time to enhance my intellectual development and improve my thesis work. I would specially like to thank my external thesis committee member, Dr. Rudy Eikelboom, a true expert in the field of voluntary wheel running. I would also like to thank Dr. Bart Krekelberg for recommending me to receive a Rutgers University Presidential Fellowship, which has supported me financially through my PhD years. Additionally, I would like to thank Drs. Ian Creese and Paula Tallal for their leadership of the Behavioral and Neural Science Program and all of my professors and mentors in the program who guided me through my first several years of Neuroscience classwork. I would also like to thank my family for fully supporting me in pursuing all of my dreams and allowing me to get to this stage of my academic career. Finally, I would like to thank my husband, Dr. Daniel F. English, whom I met my first year of graduate school. We spent many hours together forming the basis of our neuroscience knowledge by studying for our class, Foundations of Neuroscience. We then formed a home and family, which supported me through my PhD years, and I am forever grateful to him for that.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

LIST OF ILLUSTRATIONS

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

CHAPTER 1: INTRODUCTION

Voluntary wheel running confers a variety of beneficial effects on the brain and behavior of rodents and serves as a preclinical model for human exercise

Julia C. Basso

Reduced or insufficient physical activity is a hallmark of the modern sedentary lifestyle. The negative effects of physical inactivity include obesity, ischemic heart disease, stroke, diabetes, hypertension, high cholesterol, cancer, osteoporosis, musculoskeletal disorders, neurological disorders, and mental health disorders such as anxiety and depression (see Kruk, 2009 for review). This results in the annual spending of an estimated $75 billion in medical costs due to physical inactivity. Remarkably, the New England Journal of Medicine reports that after decades of increased life expectancy as society and medicine developed, it is likely that there will be a trend-breaking decrease in life expectancy in the United States in the 21st century due to obesity, and a healthier lifestyle including consumption of better foods and an increase in physical activity is needed to overcome this problem (Olshansky et al., 2005).

The Center for Disease Control (CDC) reports that physical activity helps promote both physical and mental health. For example, physical activity helps maintain healthy body weight and promote healthy bones, muscles, and joints, reduces injuries from falls in older adults, improves sleep, reduces the risk of developing obesity, diabetes, high blood pressure, cardiovascular disease, osteoporosis, and cancer, reduces feelings of depression and anxiety, and reduces the risk of dying prematurely or from heart disease (www.cdc.gov). The American Heart Association recommends a daily regimen of at least 30 minutes of moderate to vigorous exercise, determined by an increase in heart rate to 50-85 percent of maximum. However, 74% of adults do not attain this level of physical activity and 25% do not participate in any level of physical activity at all

(CDC; US Department of Health and Human Services). Additionally, physical inactivity is more common among women than men and increases with age. Considering these statistics, it is evident that, as a population, we have a problem with our motivation to exercise. Therefore, understanding the behavioral and neural mechanisms underlying the motivation to engage in voluntary exercise is essential.

Voluntary wheel running in rats is a pre-clinical model for exercise in humans Voluntary wheel running in the rat has been proposed as a viable pre-clinical model for voluntary exercise in humans (Eikelboom, 1999). Additionally, as mentioned above, the modern human lifestyle is marked by low levels of activity with ample access to high caloric foods, a combination that leads to poor physical and mental health. The current common lifestyle of the laboratory rat is quite similar, showing low levels of activity in a confined space with constant access to rat chow. Therefore, these studies focused on voluntary wheel running in the adult rat that up to that point only experienced a sedentary lifestyle. Some consideration was also given to the behavioral and brain alterations that occur when voluntary exercise begins early in life.

General hypothesis for my thesis work After reading the literature dating back to 1898, I adopted the hypothesis that voluntary wheel running is a motivated behavior with positive incentive salience for rats. Though much work has been done utilizing this behavior to alter body, brain and behavioral mechanisms, less has been done to investigate the behavior in its own right. What data exists is limited by its predominant use of one gender (males), as well

as inconsistent control over essential variables including age, light-dark cycle, period of wheel availability, housing conditions and the running wheel apparatus itself. Generally, the voluntary wheel running data was not comprehensive and because of the inconsistent experimental conditions, was difficult to compare across studies. Studies suggested that gender differences existed; however, few studies directly compared males and females. Therefore, I sought to investigate the behavior of voluntary wheel running in a comprehensive, quantitatively comparable fashion, as well as quantify the motivation for this behavior through well-established protocols. Additionally, I hypothesized that because the behavior is different between genders, a gender difference must exist in the motivation to engage in voluntary wheel running. I further hypothesized that because voluntary wheel running is a motivated motor behavior, certain brain regions traditionally thought to be involved in motor and motivational processes would be involved in the regulation for the motivation of this behavior as well. I hypothesized that participating in an activity that stimulates these motor and motivational substrates throughout life may alter these circuits, thus altering its responsiveness to other stimuli with incentive salience at adulthood.

A brief overview of and orientation to my thesis work For my thesis work, I utilized voluntary wheel running in Sprague Dawley male and female rats to investigate this motivated behavior and the brain substrates underlying it. First (Chapter 2), I explored the details of an extensive battery of variables that constitute the features of the behavior of voluntary wheel running in both males and females. I found that males and females interact with the wheel differently in

terms of their daily bouting patterns, distances, times and rates run, and that variables such as hormonal status (i.e., gonadal hormones), wheel availability, short- and long-term forced wheel abstinence, and wheel apparatus significantly affect this behavior. Second (Chapter 3), I examined how participating in voluntary wheel running throughout life affects certain brain regions with motor and motivational functions as well as their preference for an acute initial dose of a pharmacological stimulant (cocaine) with known incentive salience. To do this, I studied the baseline monoamine content in these areas via high-performance liquid chromatography as well as the monoamine content when the animals were challenged with a low dose of cocaine. I also examined, using a conditioned place preference (CPP) model, their preference for a similar low dose of cocaine. These experiments revealed that the caudate putamen is altered by a lifetime of activity and that certain other brain regions such as the nucleus accumbens shell, ventral tegmental area, and medial preoptic area react differently in active versus sedentary animals when challenged with cocaine. Additionally, lifelong activity affects the preference for drugs of abuse, shifting the dose response curve to the right, with active animals preferring higher doses of cocaine. Third (Chapter 4), I sought to prove that voluntary wheel running is a behavior with positive incentive salience. I utilized a conditioned place preference (CPP) model to examine the motivation for running during the acquisition period and the return of the wheel after a period of forced wheel abstinence to examine the motivation for running during its habitual state. Here, I prove for the first time via CPP that the experience of the acquisition of wheel running has positive incentive salience for

both genders. Additionally, I show that rats display a robust rebound running response after return of the wheel from a period of forced wheel abstinence, a demonstration that the habitual phase of wheel running has high incentive salience for both genders. Surprisingly, though males and females display statistically different wheel running behavior, their measures for the motivation for wheel running proved quite similar. In contrast to this, using a CPP model, only males demonstrated that the aftereffects of wheel running had positive incentive salience. For females, I explored a variety of variables altering the nature of the CPP for the aftereffects of running, but no evidence was found that this aspect of the running experience had incentive salience for them. In Chapter 5, I examined through transient inactivation, brain regions underlying the motivation for this behavior. Specifically, I studied the prelimbic and infralimbic medial prefrontal cortex (mPFC) and the nucleus accumbens (NA) core and shell. I found that the prelimbic mPFC and NA core may be necessary for the reinstatement of or increase in wheel running behavior seen after a period of forced wheel abstinence.

Previous research shows that physical activity in the form of voluntary wheel running and forced treadmill running affects the brain and behavior in a variety of ways Physical activity is known to enhance behavior and cognition. For example, physical activity decreases depression (Solberg et al., 1999; Greenwood et al., 2003; 2005; Duman et al., 2008) and anxiety (Dishman et al., 1996; Binder et al., 2004; Duman et al., 2008), and improves attention (Hoffmann et al., 1987; Hopkins et al., 2009), learning and memory (van Praag, 2008).

Additionally, physical activity has been shown to induce a variety of molecular and physiological changes in the brain, providing possible mechanisms to explain the behavioral effects described above. For example, physical activity enhances neurogenesis in the dentate gyrus of the hippocampus, a brain area involved in learning and memory (Kempermann et al., 1997; 1998; Kempermann, 2003; see van Praag et al., 2008 for review; van Praag et al., 2009; Lafenetre et al., 2010), with neurogenesis showing a positive correlation to distances run (Allen et al., 2001). Physical activity also enhances neuronal maturation and increases dendritic density, arborization, complexity and length (Redila & Christie, 2006; Zhao et al., 2006; Stranahan et al., 2007; Lafenetre et al., 2010). Long-term potentiation, a physiological mechanism considered essential to learning and memory, is also enhanced by physical activity (van Praag et al., 1999; Farmer et al., 2004; O'Callaghan et al., 2007). A prevalent view in the literature is that consequent to all of these neuromolecular changes, physical activity improves behavioral measures of learning and memory (Lafenetre et al., 2010).

Physical activity also increases the availability of certain neuronal growth factors, known as neurotrophic factors, such as brain derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and nerve growth factor (NGF), in specific areas of the brain such as the hippocampus and cortex (Neeper et al., 1995; 1996; see Cotman & Engesser-Cesar, 2002 for review; Smith & Zigmond, 2003; Tajiri et al., 2009), with mRNA levels showing a positive correlation to distances run (Cotman & Engesser-Cesar, 2002). Physical activity

also increases gene expression of a variety of genes involved in plasticity, synaptic trafficking, signal transduction, and transcriptional regulation (Tong et al., 2001; Molteni et al., 2002). Many of the beneficial effects of physical activity are thought in part to be due to increases in these growth factors.

Physical activity also improves both physiological and cognitive functioning in a variety of rodent disease models such as Alzheimer's disease (Adlard et al., 2005; Parachikova et al., 2008; Nichol et al., 2008; 2009; Yuede et al., 2009; see Zhong & Weisgraber, 2009 for review), Parkinson's disease (Dobrossy & Dunnett, 2003; Fisher et al., 2004; Smith & Zigmond, 2003; O'Dell et al., 2007; Petzinger et al., 2007; Mabandla et al., 2009; Tajiri et al., 2009), Huntington's disease (Pang et al., 2006; van Dellen et al., 2008), and traumatic brain injury (Griesbach et al., 2004; 2007; 2009).

Physical activity also causes a variety of changes in the neurotransmitter and neuromodulator systems in the brain including dopamine, serotonin, norepinephrine, gamma-aminobutyric acid (GABA), glutamate, oxytocin, endogenous opioids, and endogenous cannabinoids. These changes are discussed more extensively in Chapters 3 and 5.

These data suggests that the utilization of the body in an intense, aerobic manner causes significant changes in the brain. Considering that the physical body can produce such robust brain changes, it is important to understand why rats voluntarily engage in running in the first place and if some of these brain changes, specifically the alterations seen with neurotransmitters and/or neuromodulators, might underlie the motivation for this behavior.

Basic concepts regarding the hedonic and motivational properties of rewarding stimuli give a context in which to understand the motivation for voluntary wheel running

Reinforcement and reward A reinforcing stimulus is one that increases or decreases the frequency of a particular behavior. The reinforcing stimuli may be positive or negative, as in the presentation of a reward or the removal of a punishment. Aversive stimuli, such as foot-shock, can also be reinforcing as aversive stimuli will increase the frequency of avoidance or fearful behaviors. A rewarding stimulus is one that is perceived as positive, has positive affective value or hedonic properties, is pleasurable, or increases the probability of stimulus-contingent responses (White, 1989; Everitt & Robbins, 2005; Salamone et al., 2007). Reinforcing stimuli, as would be the case in positive reinforcement, can be rewarding as well. Reward contains three components, an associative/predictive learning component, a hedonic component, and a motivational component (see Kringelback and Berridge, 2010 for review).

Hedonic and motivational components of reward - liking versus wanting Berridge and Robinson (1998) developed a theory that a rewarding stimulus has two components, namely the hedonic component and the motivational component. The responsivity to a particular rewarding stimulus with hedonic properties is termed *liking*, whereas motivation for this rewarding stimulus is termed *wanting*. Though liking is a subjective concept, it is objectively measured through affective tone, such as taste reactivity (gaping, tongue protrusions) in response to a sweet taste. Wanting is examined through a variety of quantifiable

measures such as consumption of the stimulus or operant responding or conditioned place preference for the stimulus. That is, these measures are able to quantify the amount of incentive salience attributed to a particular stimulus. Berridge (2007) considers incentive salience a conditioned, motivated response to a rewarding stimulus, and as such, is affected by previously learned associations and the current physiological state of the animal.

Liking generally precedes wanting, as a rewarding stimulus may gain incentive salience over time. During acute exposures to a stimulus, liking and wanting may occur concurrently; however, after prolonged exposure to the stimulus, these two components may become dissociated. For example, initial use of a drug may be accompanied by both liking and wanting of the substance; however, after chronic use, tolerance to the liking develops while wanting increases. That is, the incentive salience or the motivational drive to obtain and use the drug increases over time without any increase in perceived hedonic quality of the drug.

Appetitive and consummatory behaviors Sherrington (1906) developed a theory that all behaviors are in one of two categories, appetitive or consummatory. Appetitive behaviors precede interactions with the stimulus and represent anticipatory or seeking states. Sherrington's appetitive component of behavior resembles Berridge's motivational component of reward. Upon approach of the stimulus, a variety of consummatory behaviors may occur, including consumption of, withdrawal from, or aggression towards the stimulus. The term consummatory, however, does not refer to consuming a stimulus, but

the use of energy to interact with the stimulus. Once this consummatory behavior is complete, the appetitive value of the stimulus changes, and in the absence of the stimulus, the appetitive phase can recommence presumably at a different liking and wanting set point.

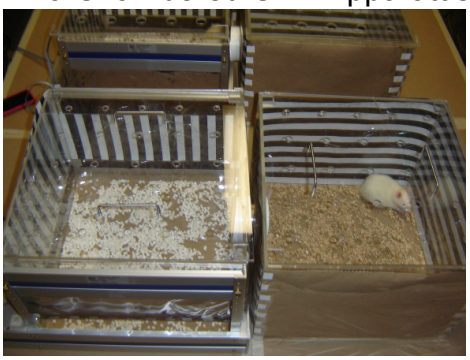
Consummatory behavior is measured via observable behaviors or physiological responses involving interaction with the stimulus. For example, in my thesis work, I examined the consummatory behavior of voluntary wheel running after a period of forced wheel abstinence. The appetitive behavior is measured via two quantifiable measures, operant responding or conditioned place preference, often used to measure responses antecedent to attaining the stimulus. In an operant paradigm, animals can be trained to lever-press for a rewarding stimulus. The amount and rate of lever pressing as well as the number of lever presses that the animal is willing to make to obtain the stimulus (the breakpoint) represents the animal's motivation for the stimulus. Measuring motivation through operant responding can be complicated by the fact that once the animal achieves access to the stimulus, this affects the appetitive value of the stimulus. Conditioned place preference (CPP) is another paradigm to measure the wanting, appetitive or motivational value of a stimulus. The post-conditioning test is conducted in the absence of the stimulus, thus the motivational value for the stimulus is not affected by consumption of it. The CPP paradigm, which is used extensively in this thesis, is discussed below.

Conditioned place preference paradigm The motivational properties of a natural or pharmacological stimulus can be measured through the conditioned

place preference paradigm. In this paradigm, a stimulus, the unconditioned stimulus (US), is paired with a neutral environment featured with unique contextual cues. Over several sessions of the US-chamber pairings, the once neutral environment acquires the motivational value of the US, thus becoming a conditioned stimulus (CS). Therefore, in the absence of the US, the appetitive or motivational value of the US can be measured by analyzing the amount of time spent in the stimulus-associated chamber. The conditioned place preference paradigm can be utilized with either two or three chambers. Our lab has traditionally utilized a three-chamber CPP apparatus; however, for this thesis, I designed a two-chamber CPP apparatus to test the incentive salience of the wheel running experience. The two CPP apparatuses are shown below.

Three-Chambered CPP Apparatus

Two-Chambered CPP Apparatus



The CPP paradigm has been utilized to establish the motivational value for naturally occurring stimuli such as voluntary wheel running, pups, food, sex, and hormones (Mehrara & Baum, 1990; Oldenburger et al., 1992; Maes & Vossen, 1993; Fleming et al., 1994; Mattson et al., 2001, 2003; Wood, 2004; Seip et al., 2008a; Pereira & Morrell, 2010; Greenwood et al., 2011), as well as for pharmacological stimuli, such as cocaine, amphetamine, opiates, morphine,

ethanol, and nicotine (Bardo et al., 1995; Tzschentke, 2007; Seip et al., 2007; 2008b).

Mesocorticolimbic dopamine pathway The mesocorticolimbic dopamine pathway is involved in the hedonic and motivational properties of both natural and pharmacological stimuli. The mesocorticolimbic dopamine pathway originates in the ventral tegmental area (VTA) and has dopaminergic connections to both limbic and forebrain structures. Specifically, the ascending VTA dopaminergic projections synapse in the nucleus accumbens, ventral regions of the caudate putamen, frontal cortex, amygdala and olfactory tubercle (Nestler et al., 2001). A proportion of the mesocortical dopamine projections are a component of the medial forebrain bundle, a band of ascending and descending dopaminergic, serotonergic and noradrenergic fibers that synapse on the lateral hypothalamus and brainstem tegmentum and when stimulated is rewarding and causes increases in voluntary wheel running (Schwarzberg & Roth, 1989; see Wise and Rompre, 1989 for review). Adjacent to the VTA is the substantia nigra (SN), which also contains dopaminergic neurons in its compacta segment (SNc). These neurons primarily make dopaminergic projections to the caudate putamen, and are thus generally considered by to be involved more in the regulation of voluntary movement than in reward learning and motivation. However, this strict division between the function of dopamine originating in the VTA versus SNc is not absolute, as demonstrated by reward related firing of SNc DA neurons (Schultz et al. 1993) and dorsal striatal involvement in reward processing (Balleine et al. 2007).

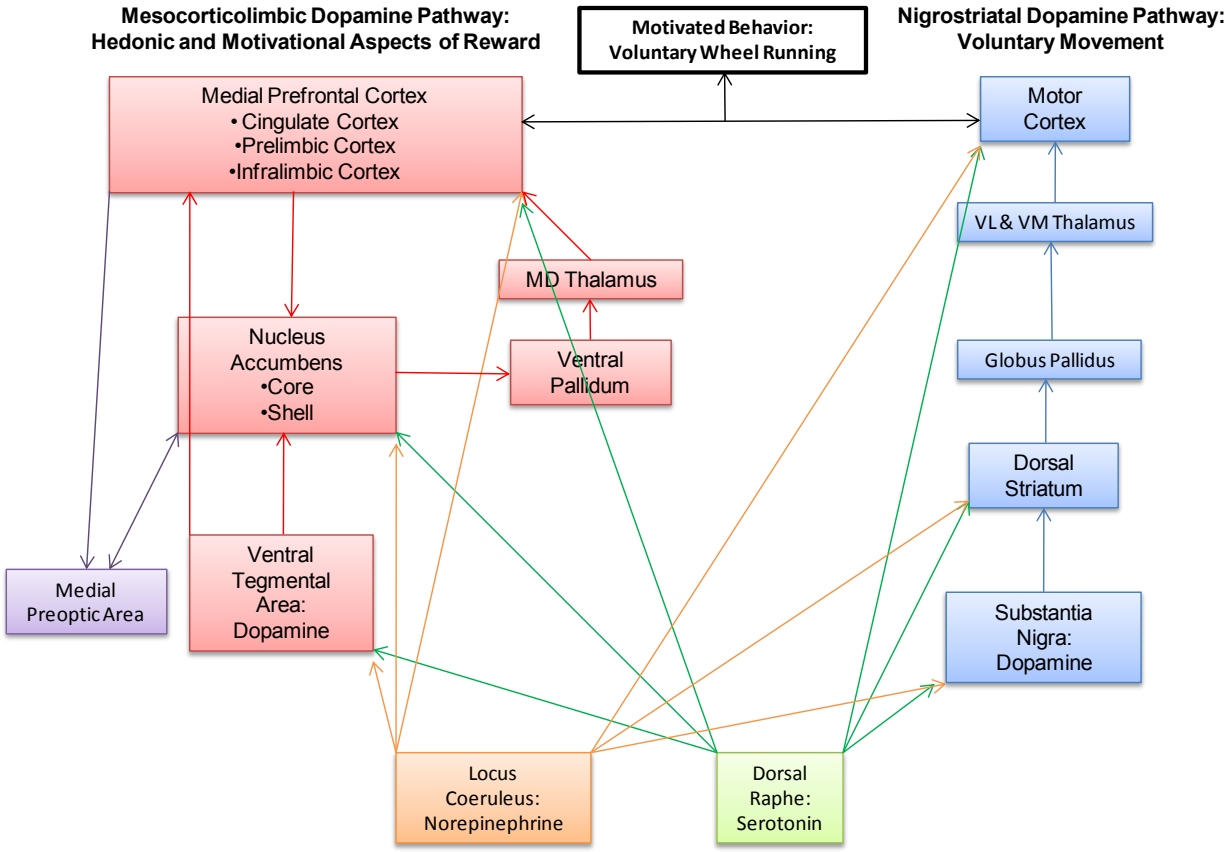
This dopaminergic system was traditionally thought to be involved in appetitive or motivational responses to both natural and pharmacological reward-related stimuli, as dopamine neurons first fire in response to a reward and subsequently to the presentation of predictors of the reward, and blockade of DA signaling by neuroleptic drugs can be used to eliminate the reinforcing properties of previously rewarding stimuli (see Kringelback & Berridge, 2010 for review).

More recent evidence suggests that dopamine is neither necessary for generating normal pleasure sensations nor sufficient to enhance pleasure (see Kringelback & Berridge, 2010 for review). For example, pharmacological lesioning of dopamine neurons or dopamine signaling blockade by neuroleptic drugs does not impair the hedonic liking reaction of rats to a sweet taste (Pecina et al., 1997; Berridge & Robinson, 1998). Additionally, raising dopamine levels either genetically or pharmacologically does not increase hedonic liking reactions of rats to a sweet taste (see Kringelback & Berridge, 2010 for review).

Based on these data, Berridge and colleagues propose that dopamine is involved in the motivation for or incentive salience of reward-related stimuli (Berridge, 2007). Considering that CPP is thought to measure the incentive salience of reward-related stimuli, mesocorticolimbic dopamine is most likely involved in the development of CPP for both natural and pharmacological stimuli, including the CPP for the wheel running experience. The involvement of dopamine, as well as other neuromodulators, in the motivation for voluntary wheel running is discussed in Chapters 5 and 6.

The following circuit diagram (Figure 1) depicts the mesocorticolimbic and nigrostriatal dopaminergic pathways that are involved in hedonic and motivational aspects of rewarding stimuli and voluntary movement respectively, thereby in conjunction producing motivated behaviors. The same circuit is thought to underlie the motivation for voluntary wheel running in the rat. Several of these areas are investigated throughout this thesis, specifically in Chapters 3 and 5, and this circuit serves as a basis for choosing these areas.

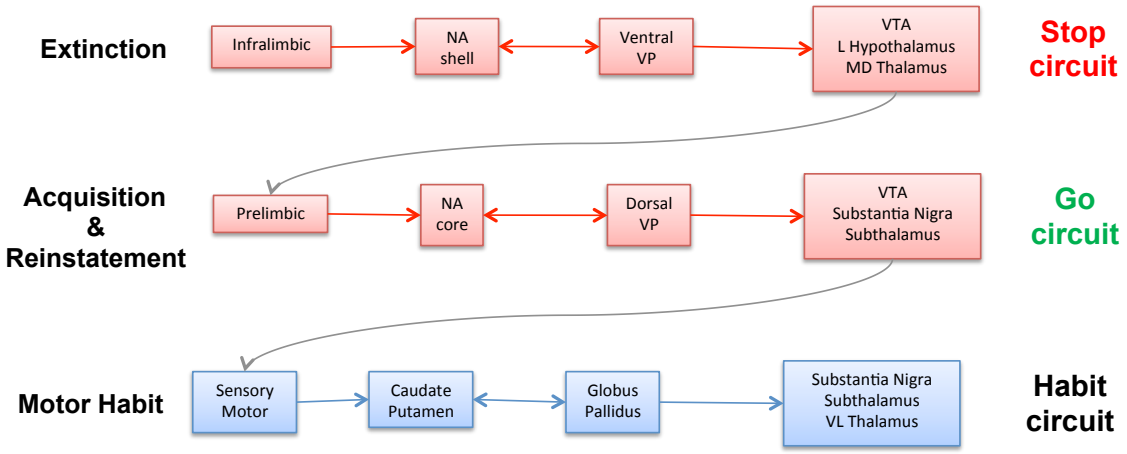
Figure 1



More specifically, I adopted the following model from Kalivas (2008) to explore specific brain regions directly involved in the motivation for voluntary wheel running. The "go circuit", as I term it, is involved in the acquisition and

reinstatement of motivated behaviors, where as the "stop circuit" is involved in the extinction of these behaviors. As shown in Figure 2, these circuits are interconnected and when the behavior is acquired and becomes habitual, a different circuit termed the habit circuit takes over. I hypothesized that just as the prelimbic medial prefrontal cortex and nucleus accumbens core are involved in the reinstatement of drug taking after a period of forced drug abstinence, these areas would be involved in the reinstatement of wheel running after a period of forced wheel abstinence. I explored this hypothesis through experiments involving transient inactivation of these specific areas.

Figure 2



Cocaine as an example of a pharmacological stimulus that engages the mesocorticolimbic dopamine pathway I was specifically interested in how engaging in the motivated behavior of voluntary wheel running throughout life affects the neurochemical milieu of some of these regions as well as the behavioral and neurochemical responsiveness to a pharmacological stimulus with known incentive salience at adulthood. I chose cocaine for these purposes.

Cocaine or benzoylmethylecgonine ($C_{17}H_{21}NO_4$) is an alkaloid substance derived from the coca plant. This pharmacological stimulus, which induces euphoric states, is a psychomotor stimulant, appetite suppressant, and local anesthetic. Cocaine exerts its effects on a variety of areas including the ventral tegmental area, nucleus accumbens, striatum, and frontal cortex (Carelli et al., 2000; Nestler et al., 2001; Borgland et al., 2004; Kumar et al., 2005; Schilstrom et al., 2006), and during the expression of a cocaine-associated chamber preference, in the absence of cocaine itself, neuronal activation is evident in these regions as well as in the basolateral amygdala and medial preoptic area (Mattson & Morrell, 2005).

Cocaine is a dopamine, serotonin, and norepinephrine reuptake inhibitor, binding to and blocking the membrane transporters of these monoamines (Uhl et al., 2002). When cocaine is bound to these monoaminergic transporters (DAT, SERT, and NET), dopamine, serotonin, and norepinephrine are unable to be transported back into the presynaptic terminal and thus remain longer in the synapse, whereby they bind to and stimulate their respective receptors on the postsynaptic cell for longer than is the physiological norm. Each monoamine transporter contributes to specific aspects of cocaine's mechanisms of action. For example, DAT knockout mice (as well as SERT knockout mice) do not show cocaine-induced hyperlocomotion; however, they still show a CPP for cocaine, suggesting that dopamine is involved in the locomotor-activating properties of cocaine rather than the incentive salience of cocaine (Sora et al., 1998; Medvedev et al., 2005). Interestingly, the CPP for cocaine is extinguished in

DAT/SERT double knockout mice, suggesting that the integration of the dopaminergic and serotonergic circuitry is needed for the expression of the motivational aspects of this psychostimulant. Further, NET function may contribute to cocaine's aversive properties (Uhl et al., 2002).

I explored how engaging in a motivated behavior throughout life affects the neurochemical content of motivationally related brain regions (Chapter 3). I utilized cocaine as a pharmacological stimulus with positive incentive salience to challenge the mesocorticolimbic system, which may have undergone particular alterations in animals reared with access to a wheel. I examined these alterations both behaviorally, through a CPP model, and neurochemically using HPLC.

In conclusion, the present proposal seeks to investigate the behavior of, the motivation for, and the neural mechanisms underlying voluntary wheel running. To do this, Chapter 2 describes the pattern of voluntary wheel running from its acquisition to its stabilization in males and females and explores the effects of a variety of variables on the behavior. Chapter 3 examines the effects of engaging in a lifetime of voluntary wheel running on the neurochemical milieu of certain brain regions involved in motor and motivational functions, and the neurochemical and behavioral responsiveness to a pharmacologically salient stimulus at adulthood. Chapter 4 explores measures to quantitate the incentive salience of voluntary wheel running, specifically examining differences between males and females, and Chapter 5 utilizes these techniques to investigate the brain substrates underlying the motivation for this behavior. Collectively, my

thesis work gives a comprehensive picture of the behavior of voluntary wheel running, placing it in the framework of a motivated behavior with positive incentive salience, and suggesting brain regions that may be necessary to support the motivation for this behavior.

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CHAPTER 2

Investigation of voluntary wheel running in the male and female Sprague Dawley rat: influence of gonadal hormones, interrupted wheel access, and wheel apparatus

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Abstract

Voluntary wheel running in rodents has been used extensively to induce both overt changes in behavior and covert changes in the body and brain, but few studies have examined in a systematic and quantitatively comparable manner how variables such as gender, hormonal state, wheel availability, and wheel apparatus affect wheel running behavior. We studied wheel running in male and female Sprague Dawley rats (each group, *n*=4-20) by determining the distance run, time spent running, rate of running, and bouting patterns from first exposure to the wheel to acquisition of stabilized or habitual running. Gender differences emerged during the first 24 hours of wheel interaction and remained after running patterns stabilized (2-3 weeks). Females acquired the behavior more quickly and traveled significantly farther daily distances by running longer and at faster rates. The stage of the ovarian cycle markedly influenced running, with proestrus females running farther, longer, and faster in more frequent bouts than metestrus females or males. Gonadectomy significantly decreased all aspects of running; however, gender differences not attributable to circulating hormones remained: female running still surpassed that in males. In experienced runners, after weeks of deprivation of wheel availability, they quickly regained maximal running patterns, whereas months of deprivation returned them to a wheel-naïve state. Limited daily wheel availability (30 minutes or 2 hours) led to running a greater percentage of wheel access time, even eliminating bout running, but the rate of running was always slower than in the *ad libitum* runner. Subjects with alternate-day wheel access ran the same distance as *ad libitum* runners on wheel access days but adopted a different pattern of rate of running during wheel availability. Overall, rats with limited wheel access showed remarkable alterations in time and rate of running, which appeared to be an anticipatory response to removal of the wheel. Wheel running also was impacted by the type of apparatus utilized, which must be taken into consideration when comparing data across studies. Together, this information provides new evidence that voluntary wheel running habits are flexible and can be shaped by specific protocol choices. These data will help to inform future investigations of the CNS mechanisms underlying the motivation to engage in voluntary wheel running.

1. Introduction

Wheel running is a robust spontaneous voluntary activity for many animals, including rodents, avians, marsupials, Erinaceinae, carnivores, and non-human primates (see [1] for review). Rats are commonly reported to run 1-15 kilometers (0.6-9.3 miles) per day [2,3], with an early report of a marathon-scale 43 kilometers (26.7 miles) in a 24-hour period [4]. The prevailing interpretation is that voluntary [1] but not forced [5-7] wheel running has positive incentive salience for rodents, as rats lever-press for access to a wheel [8-12] and show a conditioned place preference for the aftereffects of the wheel [3,13-15]. We are interested in how CNS motivational processes support wheel-running behavior.

We hypothesized that gender, hormonal status, wheel availability, running experience, wheel deprivation, and wheel apparatus would impact wheel running and be important to its motivational processes, possibly by influencing differences in the finer-grained aspects of running responses or patterns. However, while devising experiments to investigate the neural substrates of the motivation for wheel running, we found that although voluntary wheel running in rodents has been studied since the late 1800s [16] and studies are extensive (710 citations in PubMed; [17,18]), the existing literature does not provide an adequate level of quantitative comparisons and fine-grained analysis. Therefore, we undertook a comprehensive set of experiments to test the impact of variables such as those mentioned above on wheel running in rats using modern data acquisition and analyses, resulting in directly comparable parametric and quantitative data across variables.

Wheel running is widely used as a tool to induce changes in particular dependent variables in the brain or body, with voluntary wheel running in rodents altering behavioral measures of anxiety and depression, attention, learning and memory, neurogenesis and neuronal maturation, neuronal branching, synaptic plasticity, and levels of growth factors [19-42]. Furthermore, voluntary wheel running confers functional and neuroprotective effects in animal models of neurodegenerative disorders such as Alzheimer's, Huntington's, Parkinson's diseases and ischemic stroke [41,43-49]. However, in this type of work, it is often used without consideration of variables that regulate the wheel running itself and thus the amount necessary to initiate or maintain such running-dependent changes. Thus, the outcomes of the current study will be useful to those studying wheel running-induced variables in such diverse and clinically significant areas of research.

Several studies have examined running behavior itself, including variables that influence the amount of voluntary wheel running; however, only a few [2,18,50-54] provide quantitative parametric comparisons. Variables that are considered to influence wheel running include age, endocrine status, presence of a sexual partner, pregnancy, food availability, limited availability of the wheel, changes in the light-dark cycle, voluntary versus forced use of the wheel, and wheel shape and size [3,4,7,14,17,18,51,53-64].

The parametric utility and level of detail in this running literature in rats are limited by the predominant use of males, the primary emphasis on distance run, the use of different wheel apparatuses with variable amounts of wheel availability

(hence the often unacknowledged impact of deprivation from wheel running), various amounts of running experience, differences in light-dark cycle, and in order to increase distances run, the added impact of food and/or water deprivation. Furthermore, studies conducted before 1950 often used housing or running wheel conditions that are no longer acceptable (i.e., animals may have been stressed due to aspects of the apparatus or small housing units). Thus, it is difficult to gather comparable data across these wide-ranging studies.

We determined how gender, hormonal status, wheel access, and wheel apparatus affect the emergence and achievement of stabilized voluntary wheel running in Sprague Dawley rats by using a systematic and quantitatively comparable assessment. These experiments provide analysis beyond simple measures of total distance run to time spent running, running rate, fine details of the daily cycle of running, and bouting patterns, beginning from the very first hour of interaction with the wheel. The fine-grained examination of the variables that impact running show how running patterns, which are altered by gender and wheel availability, can be tailored to optimize their use in models that investigate different ways in which running affects behavioral outcomes as well as the neurobiology of the brain.

2. Method

2.1 Subjects

Data were collected from treatment groups, typically 8-20 animals per treatment group per gender (in a few subgroups *n*=4, as described below). Male and female Sprague Dawley rats (original stock from Charles River Laboratories,

Kingston, NY, USA) were bred in our colony at the Rutgers University Laboratory Animal Facility (Newark, NJ, USA) (accredited by the American Association for Accreditation of Laboratory Animal Care). All animals were kept on a 12-hour light-dark cycle (lights on at 7:00 am; unless otherwise noted) in a room at 22(±1)°C and given *ad libitum* access to water and rat chow (Lab Diet 5008, PMI Nutrition International, LLC, Brentwood, MO, USA). Daily checks were conducted for health and availability of food and water, and weight was measured and animal husbandry performed twice per week. All animals were healthy and had normal body weight throughout all experiments. Animal care and experimental procedures performed in this protocol were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were reviewed and approved by the Rutgers University Animal Care and Facilities Committee. Care was taken to minimize the suffering and curtail the number of animals utilized.

2.2. *Running wheel apparatuses*

Animals were housed in either an AccuScan Instruments (Columbus, OH, USA) VersaMax Animal Activity Monitor (wheel: 25-cm diameter, stainless steel mesh floor; home cage: 40 cm long x 40 cm wide x 30 cm wide) or a Med Associates Inc. (St. Albans, VT, USA) ENV-046 Activity Wheel with Plastic Home Cage for Rat (wheel: 35.6-cm diameter, 4.8-mm stainless steel grid rods with 1.6-cm spacing, 12-gram freewheeling drag; home cage: 48.26 cm long x 26.67 wide cm x 20.32 cm high with a 7.2 cm wide x 10.2 cm high opening to wheel). The resistance of both running wheels was low and equivalent, and no extra weight or resistance was placed on either wheel. Both housing apparatuses were lined

with woodchip bedding (Beta chip, Northeaster Products Corp., Warrensburg, NY, USA) with *ad libitum* food and water at all times. For both systems, data were captured electronically. In the AccuScan system, 16 infrared beams lined each axis of the box. Each box was connected through wires to the computer, and data were captured through Windows-based software, VersaMax and VersaDat. In the Med Associates system, an LCD digital counter captured wheel turns. The wheel was connected through wires to the computer, and data were captured through the Windows-based software, MedPCIV. Most of the data are from the AccuScan system, and all comparisons are made within apparatus, except for the analysis across the two apparatuses. The accuracy of the computer recorded wheel turns was confirmed at the start and finish of each experiment.

2.3 Procedures

2.3.1 Fundamentals of wheel running

Emergence and stabilization of wheel running: At age 65 days, females (*n*=6) and males (*n*=6) previously housed in shoebox cages and naïve to running wheels were placed in the AccuScan Instruments system boxes with running wheels at ~12:00 pm (lights on at 7:00 am, off 7:00 pm). Animals remained in these home cages for 21 days, except for husbandry (animals removed at most 1 hour). After 21 days or longer of *ad libitum* access to the wheels, animals were returned to shoebox cages.

Running patterns from 3 weeks up to 15 weeks: Four females from the above experiment then experienced a period of long-term wheel deprivation (see below) followed by an additional 3 weeks of wheel running. They were then moved to

the Med Associates running wheel system and remained there for up to 12 additional weeks of wheel exposure (15 continuous weeks of running). Data were recorded daily. In a second set of experiments, at the day of weaning (postnatal day [PND] 21), two groups of four females (*n*=8) were placed in the AccuScan Instruments system with a running wheel (enrichment objects included cardboard or Plexiglas tubes or balls or manzanite). Daily running distance was recorded periodically (approximately once per week once animals reached ≥PND 65) by removing an individual animal and placing it in the Med Associates wheel for 24 hours. These animals were exposed to the running wheel for up to 15 weeks, i.e., ~9 weeks after reaching adulthood (PND 65).

2.3.2. Impact of gonadectomy on running behavior

Rats experienced in running (21-38 days of wheel access, continuously or for 30 minutes or 2 hours per day, see below) underwent gonadectomy using aseptic conditions according to standard procedures (*n*=4 males, *n*=4 females) [65]. Animals were anesthetized with 1.0 mL/kg of a mixture of ketamine HCl (75.0 mg/mL), xylazine (7.5 mg/mL), and acepromazine maleate (1.5 mg/mL) and housed singly until wounds healed. Sham surgeries involved opening the body wall or scrotal sac and handling the ovaries (*n*=1) or testes (*n*=1) with surgical instruments, then replacing them and closing the area using wound clips. After wounds healed and animals returned to presurgical weight, they were housed in pairs to avoid social isolation. To allow endogenous levels of gonadal hormones to fall to undetectable levels, 1 month elapsed and then animals were allowed *ad libitum* access to running wheels for 3 weeks.

2.3.3. Effect of estrus cycle stage on running

Females (*n*=8, 65 days old) raised with wheels since PND 21 were housed individually in the Med Associates system for a 24-hour period. To determine the stage of their cycle, vaginal lavage was performed by standard procedure [65] uniformly each day during the middle of the light cycle (12:00-1:00 pm), i.e., the normal rest period. The slides were examined by one observer using a Zeiss bright-field microscope and confirmed by a second observer. Cell cycle stages were determined by comparison with photomicrographs in *The Laboratory Rat* [66] and *The Laboratory Rat: Volume I Biology and Diseases* [67]. Samples were analyzed randomly, and both observers were unaware of experimental conditions. Because of the dynamics of the continuous estrus cycle [68], each sample is considered in the context of the sequential days of sampling, and therefore represents not a single absolute stage of the estrus cycle but a process of transition from metestrus-diestrus (labeled metestrus on Figure 1C), diestrus-proestrus (diestrus on graph), proestrus-estrus (proestrus on graph), and estrus-metestrus (estrus on graph).

2.3.4 Effect of limited or interrupted running wheel access

Running with limited daily wheel availability: Females (65 days old) were randomly assigned to two groups, 30-minute (*n*=8) or 2-hour (*n*=8) runners, and housed in pairs in shoebox cages. Females were chosen for this experiment because they were observed to be more robust runners than males (see results section 3.2.1). Additionally, as mentioned earlier, most voluntary wheel running work has been conducted using males; specifically, Eikelboom [53,54] examined

limited daily wheel access in males. Thus, using females is a novel approach. Each day for 38 days, 2 hours after lights off, i.e., the normal active period, animals were placed in either running wheel apparatus and allowed to run for 30 minutes or 2 hours.

Alternate-day wheel availability: Females (*n*=20; 65 days old) were given access to the AccuScan system with running wheels for 24 hours every other day for 21 days of total running. They were specifically given access during the light cycle (~12:00 pm) so that a change in running could be seen if a rebound running response occurred (rats with *ad libitum* access do not run or run minimally [<0.1 km] during this phase of the light cycle). On alternate days, they were housed in shoebox cages. This protocol resembled that of the rats with *ad libitum* access rather than the protocol with limited wheel availability, as their wheel cage served as a home cage environment. Here we were able to analyze the effects of 24 hours of wheel deprivation on both the overall pattern of running and the rebound response of running.

Impact of long-term wheel deprivation: The four females that underwent the 3-week running procedure described above (*emergence and stabilization of wheel running*) were then deprived of the wheel for 4-6 months. During that time, they were housed two per cage in shoebox cages. Subsequently, they were allowed *ad libitum* access to the running wheel for an additional 3 weeks.

Impact of short-term wheel deprivation: After 20 days of alternate-day wheel availability (10 days of running), females (*n*=4) were deprived of wheel access for 1 week by being placed in shoebox cages. They were then returned to the wheel

for an additional 20 days of alternate-day running. Subsequently, they were deprived of wheel access for 2 weeks by being placed in shoebox cages.

Effects of different wheel apparatuses: Males (*n*=12) were exposed to either running wheel apparatus for 3 weeks. One group in the AccuScan system (*n*=4) received running wheel access on consecutive days, whereas the other group in the Med Associates system received running wheel access on alternate days (*n*=8). After the males completed their 3 weeks of running, they were transferred to the alternate system and given *ad libitum* access to the wheel for 1 more week. Additionally, females (*n*=4) with a 3-week running history followed by 6 months of wheel deprivation, followed by another 3 weeks of running in the AccuScan system were then transferred to the Med Associates system for an additional 3 weeks of running.

2.4 Analytic Approaches and Statistical Analysis

All wheel running data were analyzed by examining wheel turns each minute of the day that the animal had access to the wheel. The computer software captured running wheel data in time bins of 1 minute. Through observation, we found that a few wheel turns were made by the rat interacting with the wheel from the inside or outside rather than running in it; these distances were not significantly different from zero. Daily distance run was calculated by summing total wheel turns in each 24-hour session. Wheel turns were then converted into distance (kilometers) by multiplying this number by the circumference of the wheel. Daily time in the wheel was calculated by counting the number of minutes that the rat ran at least 1 wheel turn in each 24-hour period. Daily rate of running

was calculated by dividing daily distance run by daily time spent running in the wheel. For our analyses, we defined a running bout as a sequence of time bins (1 minute each) that had one or more wheel turns recorded. The duration of these bouts and the distance run during each bout were calculated as above. The rate of the bouts, termed *bout rate*, was calculated by dividing the bout distance by the bout time. Although bout rates are similar to the hourly or daily rates, due to the way these values were calculated, they are not intended to be exactly the same. Unless the term *bout rate* is used, we are referring to an hourly or daily rate.

All statistical analyses were conducted using the computer software IBM® SPSS® 16.0 graduate pack, 17.0, or 19.0 (Chicago, IL, USA), or GraphPad Prism® 5.0 (LaJolla, CA, USA) (linear regression analyses only). A significance value of $p \leq 0.05$ was used for all statistical analyses; data met the tests of normalcy and homogeneity of variance and so were analyzed with parametric tests. *Numerical data*: An independent samples *t*-test was used to determine all statistical significance between one measure in two separate groups. A repeated-measures analysis of variance (ANOVA) was used to determine statistical significance between measures sampled in the same set of animals on two or more occasions. If data did not meet normality (t-test) or sphericity (repeated measures ANOVA), corrections such as Greenhouse-Geisser (repeated measures ANOVA) were used. Linear regression was used to determine statistical significance between independent groups of animals where data were repeatedly sampled over an extended period, for example, 21 or more

days, and it was appropriate to fit a trend line to the data. A Kolmogorov-Smirnov two-sample test (K-S tst) was conducted when statistically significant differences needed to be determined between two frequency distributions.

3. Results

3.1 First response to the wheel in both genders Whether first exposure to the wheel occurred in the light or dark period, the initial interaction by both genders occurred within 2.5 minutes. Subsequently, both genders produced their first wheel turn by walking, followed by running within the first hour, covering, on average, 138 meters at 4 meters per minute in 30 minutes of running. During the first 24 hours of wheel access, females spent a longer time running (280 versus 174 minutes, $t(6)=3.279$, $p=0.017$) and showed trends of running 2.3 times farther (1.6 versus 0.7 km, trend $t(6)=2.311$, $p=0.060$) at a rate 1.4 times faster (5.5 versus 4.0 m/min) than males (*n.s.*).

3.2 Gender comparisons in fundamentals of the emergence and stabilization of wheel running

In both genders, all features of running (i.e., distance, time, and rate) increased in the manner described below for the first 3 weeks of wheel availability, then stabilized for the remaining 12 weeks of these experiments. At all stages of the emergence of running up to and including stabilized running, 90% or more of the running was done in the dark phase of the light-dark cycle. During the emergence of stable running, no correlation between body weight and the fundamental parameters of running were seen in either gender. However,

thereafter, particularly in males with increasing age and/or body weight (~≥500 g), a significant negative correlation was seen with running distance (data not shown).

3.2.1 Daily distance run As seen in Figure 1A and B, a gradual and steady increase in average daily distance run, an independent measure derived from wheel turns, occurred from the first day of wheel exposure in wheel-naïve rats for males ($F(1.250,33.757)=27.939$, $p<0.001$) and females ($F(1.338,36.116)=10.605$, $p=0.001$). Running distance stabilized at the end of the first 3 weeks of *ad libitum* exposure to the wheel and remained the same for up to 15 weeks of wheel exposure. At the end of 3 weeks (Figure 1B, day 21 of running), females ran on average 1.5 times farther daily than males (males 4.3 kilometers [2.7 miles]; females 6.6 kilometers [4.1 miles] per day). At each weekly time point, females ran farther than males (Figure 1A).

3.2.2 Time and time of day spent running This variable was derived from the number of 1-minute bins with 1 or more wheel turns. At 3 weeks of wheel exposure, males and females spent equal time running on the wheel per day (males 4.3 hours, females 3.7 hours, calculated as a weekly average). Statistically significant gender differences occurred only at week 2 (females > males), as females reached their peak time spent running by week 2 and males by week 3. Most running (90%) was done during the dark phase; both genders conducted up to 60% of their total running in the first 6 hours of the dark period and the remainder, but significantly less (30-35%), in the last 6 hours (Figure 2 A, B, C).

3.2.3 Rate of running Both males ($F(2,50)=33.149$, $p<0.001$) and females ($F(1.336, 36.075)=23.424$, $p<0.001$) increased their average daily running rate during the first 3 weeks of wheel exposure, with females peaking at week 2 and males at week 3. At all time points, females ran at significantly faster average rates than males (week 1 $t(35.608)=-5.001$, $p<0.001$; week 2 $t(37.450)=-7.513$, $p<0.001$; week 3 $t(54)=-4.066$, $p<0.001$). At 3 weeks of wheel exposure, mostly during the dark cycle, females ran on average 1.5 times faster (19.2 m/min [0.7mi/hr]) than males (13.5 m/min [0.5mi/hr]) (Figure 2B & C), with males running even more slowly during the early and late portions of the dark cycle. Additionally, the average fastest rate run by females (50.0 m/min), that is, the average fastest rate achieved by each individual during days 14-21 of wheel exposure, was 1.5 times greater than the average fastest rate run by males (32.7 m/min) ($t(43.931)=10.720$, $p<0.001$).

A frequency distribution histogram of the number of minutes spent at various rates of running (meters per minute) demonstrated that overall, females spent more minutes running at higher rates and males spent more minutes running at lower rates (Figure 3A) (K-S tst, $p<0.05$). The similar initial modes (0.8 m/min) of both genders was due to the number of minutes spent running 0.1-4 m/min and were observed to be mostly due to walking or slow running in the wheel. Although these values could include some wheel turns that were caused by the rats' interaction with the wheel other than walking/running in the wheel, our observation indicated that this is not common. At rates above 4 m/min, it is

unlikely that wheel turns were caused by interactions other than walking or running.

3.2.4 Rats run in bouts Intact males and females ran in short periods or bouts (Table I) separated by periods in which they rested, ate, drank, groomed, or otherwise locomoted. The pattern of running in bouts interspersed with other activity occurred at a similar frequency of 4 bouts per hour averaged overall for the dark period; this rate was similar between genders when analyzed by averaging female activity across all 4 days of the ovarian cycle (see section 3.3). Although the average number of bouts did not vary significantly across the dark period, the average length of a bout, the time in between bouts, and the distance traveled during bouts varied markedly across the dark period (Table 1).

3.3 Influence of gonadal hormones on stabilized running Our intention was to apply the same quantitative analysis utilized to examine running in the intact animal to determine the effect of hormones on habitual running. For this analysis we used either intact females with proven cycle state and at least 3 weeks of running experience or separate groups of intact males and females that were gonadectomized after an initial running experience of at least 30 days.

The stage of the ovarian cycle markedly influenced several key features of the daily running pattern. Because significant differences in average daily distance run were found only between the metestrus and proestrus periods (Figure 1C), we focused on those parts of the cycle. Proestrus females spent almost double the time (6.05 hr) running 3 times longer distances (9.4 km) (Figure 1C) at 54% faster rates (25 m/min) than their metestrus counterparts

(time t(10)=7.196, p<0.001; distance t(6.014)=4.293, p=0.005; rate t(10)=2.645, p=0.025). Males and metestrus, diestrus, or estrus females ran similar daily distances (Figure 1C, males indicated by dashed line) for similar time intervals (3.7 hr daily) and at similar rates of running (13.5 m/min). During all stages of the ovarian cycle, the light-dark pattern of running was the same (i.e., highest distances, times and rates during the 1st hour of the dark period with subsequent declines thereafter).

Gonadectomy-induced changes included the distance run, time spent running, rate of running, and frequency of running bouts. Gonadectomy decreased wheel running dramatically and permanently in both genders with virtually no trace of their re-achieving their prior running capacity. After gonadectomy, the maximum daily distance run was never over 1 km for ovariectomized females and 0.5 km for orchiectomized males (Figure 1A), both significantly different from their intact counterparts. A gender difference in distance run (females > males) persisted after gonadectomy and was statistically significant (week 3, t(28.047)=4.915, p<0.001). The impact of gonadectomy on distance run was dramatic: ovariectomized females ran 12 times less distance than intact females or sham-operated controls (week 3, t(28.113)=7.019, p<0.001), whereas orchiectomized males ran 30 times less distance than intact males or sham-operated controls (week 3, t(27.033)=7.909, p<0.001). Similar to intact rats, gonadectomized rats did most (60%) of their running in the first 6 hours of the dark period (Figure 2). Ovariectomized females ran significantly

more than orchiectomized males in the first and second 6 hours of the dark cycle (Figure 2A).

Time spent running decreased from weeks 1 to 2 for orchiectomized males ($F(1,23)=12.756$, $p=0.002$) and ovariectomized females ($F(1,23)=39.490$, $p<0.001$) and subsequently remained stable at this reduced level. Overall, orchiectomized males spent 7% of their time running (on average 0.81 hr per 12-hour dark period) compared to 36% (4.3 hr) for intact males, and ovariectomized females spent 10% of their time running (on average 1.2 hr running per 12-hour dark period) compared to 31% (3.7 hr) for intact females (intact versus orchiectomized males, week 3, $t(28.734)=12.210$, $p<0.001$; intact versus ovariectomized females, week 3, $t(38.419)=8.775$, $p<0.001$). Ovariectomized females spent significantly more time running than orchiectomized males at all time points (week 1 $t(4.834)=42.355$, $p<0.001$; week 2 $t(27.440)=3.877$, $p=0.001$; week 3 $t(35.133)=2.853$, $p=0.007$), a gender difference that only emerged after removal of the gonadal hormones. Gonadectomy also significantly reduced the daily rate of running, from the intact average of 13.5 m/min (males) and 19.2 m/min (females) to 3.0 m/min for orchiectomized males (week 3, $t(42)=8.0141$, $p<0.001$) and 6.8 m/min for ovariectomized females (week 3, $t(54)=11.3652$, $p<0.001$). Gonadectomized animals continued to run in bouts, which were significantly different from their intact counterparts in terms of number, time, distance and rate (Table 1).

3.4 Influence of interrupted wheel access In these experiments, we tested how various forms of wheel access interruption altered running patterns in

experienced runners. In order to examine these interruptions with a robust running model, these experiments were carried out in females because of their greater distance and speed and because they were subject to less chance of body weight gain-induced confounds of these measures.

3.4.1 Influence of wheel deprivation In experienced runners, both short (1-2 weeks) and long periods (up to 6 months) of wheel deprivation impacted all measures of running immediately after the wheel was returned, with the length of the deprivation determining the extent of impact. In all cases of deprivation, running distance in the first 24 hours after wheel return plummeted to 58% (1 week), 63% (2 weeks) and 70% (4 months) of the daily maximum prior to deprivation. The distance achieved in this 24-hours after wheel return was equivalent to distances run during the first week of wheel exposure in wheel naïve animals. If the wheel deprivation period was short (≤ 2 weeks), then daily distance run quickly recovered (including time spent running and rate of running), such that within 48 hours of wheel re-exposure, a return to maximum running was seen. In contrast, a long wheel deprivation period (4 to 6 months) returned the rat to naïve running levels, as seen on all measures, which again required a 3-week period to re-establish a stable maximum.

3.4.2 Influence of limiting the amount of daily access Daily limits on wheel availability profoundly affected all running variables (distance, time, rate, and bout pattern).

3.4.2.1 Distance Similar to females with *ad libitum* access to the wheels, runners given 30 minutes of daily wheel access during the dark period reached their peak

distance run at week 2, whereas animals given 2 hours of wheel access continued to increase running distances through week 4 (Table 2). Unsurprisingly, two-hour runners ran significantly more than 30-minute runners during the 1st, 3rd and 4th week; however, during the second week, these groups ran similar distance, which the 30-minute group ran in 25% of the time. Once running habits were established (by week 3), in a comparison that was proportional to the time the wheel was available (i.e., the first 30 minutes of wheel availability), the 30-minute runners ran significantly farther than the 2-hour ($t(54)=1.975$, $p=0.053$) or *ad libitum* runners ($t(54)=3.056$, $p=0.003$).

3.4.2.2 Time The percentage of time spent running during wheel availability was also shaped by wheel availability (Table 2). Thirty-minute runners consistently spent a larger percentage of their allotted time running than 2-hour runners (week 1 $t(54)=5.103$, $p<0.001$, week 2 $t(29.907)=8.638$, $p<0.001$, week 3 $t(54)=3.854$, $p<0.001$). That is, by day 5, and consistently thereafter, 30-minute runners spent 90-100% of their wheel access time running, whereas 2-hour runners reached a maximum of 63% of their time spent running much later (week 4). *3.4.2.3 Rate* Limited wheel availability also markedly altered the overall rate of running (Table 2). Thirty-minute runners ran at a consistently faster rate than 2-hour runners throughout the first 3 weeks (week 1 $t(43.883)=2.659$, $p=0.011$; week 2 $t(54)=5.442$, $p<0.001$; week 3 $t(43.594)=2.586$, $p=0.013$), reaching their peak rate of 14 m/min by week 2. Meanwhile the 2-hour runners reached a rate of 11 m/min by week 3, and only in their fourth week of wheel availability ran at a pace equal to that of 30-minute runners (i.e., 13.7 m/min). However, even when

the overall pace was similar, detailed differences remained in that 2-hour runners decreased their running rate over time compared to 30-minute runners ($F(10.829,400.662)=25.513$, $p<0.001$), even during the first 30 minutes of wheel availability ($F(3.887,143.812)=12.313$, $p<0.001$).

A frequency distribution histogram of the minutes spent at various rates further demonstrates that the distribution of running rates was markedly slower in both classes of short-term runners (Figure 3A versus C). Analysis of a comparable 30-minute time period in *ad libitum* runners showed a mean running rate of 26.9 m/min, with a mode of 1.6 m/min, whereas the mean rate of the 30-minute runners was 15.8 m/min, with a mode of 12.3 m/min, and the mean rate of the 2-hour runners was 14.8 m/min, with a mode of 0.8 m/min (K-S tst, n.s.). Compared to the *ad libitum* runners, which had the fastest individual rate of 61 m/min, the 2-hour short-term runners were also slower with the fastest rates of the fastest individual being 51.7 m/min; however, one 30-minute runner ran an extraordinary 171.4 m/min, producing the fastest rate seen in all experiments.

3.4.2.4 Bouts The 30-minute wheel availability also dramatically altered the bout running patterns compared to *ad libitum* or 2-hour availability (Table 1). Specifically, 30-minute runners took no break in running, resulting in only 1 bout per 30-minute period (*ad libitum* versus 30-minute, $t(4.0)=3.354$, $p=0.028$). Thus, the bouts of 30-minute runners were 6.4 times longer (30.0 min), with animals running 5.8 times farther (587 m) than *ad libitum* runners (Table 1).

3.4.3 Influence of alternate-day access Regardless of whether rats had access to wheels every day for 3 weeks or alternate days for 3 weeks, they came to run

the same distance and time such that at their peak, both groups spent 4 hours running about 4.4 km/day (averaged across the days of the cycle). Alternate-day access, however, affected the pattern of emergence of running (Figure 4A), ultimate running rate, and the light-dark cycle pattern of running (Figure 4B & C).

In females with *ad libitum* access, peak distance was achieved in 2 weeks, but alternate-day females required 3 weeks to reach this same peak (Figure 4A). Alternate-day runners reached their peak time running (4 hrs) by week 1, whereas *ad libitum* runners reached this same peak after 2 weeks of wheel exposure. *Ad libitum* and alternate-day runners each reached their peak average running rate by week 2 (week 1 to 2 $F(1,15)=33.931$, $p<0.001$); however, at week 2 and thereafter, *ad libitum* runners ran 19.2 m/min or about 22% faster on average than alternate-day runners (15.7 m/min) (week 2, $t(41.788)=2.933$, $p=0.005$; week 3, $t(50)=2.487$, $p=0.016$). Similarly, the average and the overall fastest rate of the *ad libitum* runners were 30% (50 versus 38.5 m/min) and 28.2% (61 versus 47.6 m/min) faster than the alternate-day runners.

Our protocol choice to return the wheels during the light (normally resting) phase revealed a prominent feature of the impact of alterernate-day wheel availability (Figure 4B & C). Immediately upon wheel return, the females had a robust running response (170 m), which was significantly greater than normally resting *ad libitum* runners left undisturbed in that hour (0 m). As seen in Figure 4B, the alternate-day runners then ran at declining rates and returned to their normal resting behavior in about 1 hour. However, at the end of the light period, they recommenced running sooner than *ad libitum* runners in anticipation of

lights off and the usual peak running period. Thus, alternate-day runners ran significantly more in the light period than *ad libitum* runners.

Unlike *ad libitum* runners, running in alternate-day runners did not decrease during the dark period but rather remained stable, generally at a lower hourly rate, with alternate-day runners covering significantly less distance than *ad libitum* runners during the first 6 hours of the dark cycle (Figure 4B & C). For example, in the first 6 hours, *ad libitum* runners ran approximately 34% faster than alternate-day runners (22.7 m/min versus 16.9 m/min, $t(50)=3.654$, $p=0.001$), covering 2.9 versus 1.8 km. For most of the second half of the dark cycle, *ad libitum* runners ran at faster rates (18.4 m/min versus 13.8 m/min, $t(50)=3.226$, $p=0.002$), traveling a similar distance (~1.6 km). Then remarkably, during the last hour of the dark period, the alternate-day runners burst into activity and ran 8 times more than *ad libitum* runners (323.5 m versus 40.6 meters) at a 3.5-times faster rate (12.9 versus 3.7 m/min, $t(50)=-7.661$, $p<0.001$).

The number of bouts run was the same for alternate-day and *ad libitum* runners (Table 1). For most of the dark period, the duration of the bouts and the distance run were also the same except in that remarkable last hour of the dark period during which alternate-day runners had bouts 2.5 times longer (5 minutes), achieving 10.5 times the distance (99 meters), compared to their *ad libitum* counterparts. Thus on average in alternate-day runners, the length of bouts did not change across the dark period, whereas in *ad libitum* runners, it decreased ($F(2,40)=4.215$, $p=0.022$).

A final point can be made about the difference in visibility of the distinct 4-day cycling pattern of data from *ad libitum* runners (Figure 1C) versus alternate-day runners, which had one of two patterns. If the alternate-day access occurred during proestrus and metestrus, significant changes could be seen in distance run from day to day (i.e., due to comparison of proestrus versus metestrus), whereas if the alternate-day access occurred during estrus and diestrus, only moderate, seemingly stable levels were seen.

3.5 Influence of different wheel apparatuses Female rats of comparable age, which were wheel naïve at the start of running, achieved stable running in a similar time frame (2 weeks) in either the AccuScan or Med Associates apparatus (Figure 5A) (AccuScan week 1-2: distance $F(1,27)=21.472$, $p<0.001$, time $F(1,27)=15.084$, $p=0.001$, rate $F(1,27)=32.786$, $p<0.001$; Med Associates week 1-2: distance $F(1,55)=53.338$, $p<0.001$, time $F(1,55)=8.177$, $p=0.006$, rate $F(1,55)=53.680$, $p<0.001$). However, the females ran farther for significantly longer times at faster rates in the Med Associates apparatus than the AccuScan apparatus. During week 1, rats exposed to the Med Associates apparatus ran on average 4.4 hr/day, approximately 4.8 km (3.0 mi), at a rate of 18 m/min, which was 3 times the distance ($t(77.866)=-6.743$, $p<0.001$) and 50% more time spent running ($t(82)=-4.614$, $p<0.001$) at almost double (1.8 times) the rate ($t(81.920)=-5.789$, $p<0.001$) as those in the AccuScan apparatus. At their peak (2 weeks and thereafter), compared to AccuScan runners, Med Associates runners traveled double the distance (9.5 km/day) ($t(64.268)=-6.436$, $p<0.001$), spent 43% more

time running (5.35 hrs/day) (t(82)=-4.484, p<0.001), and ran at 1.5 times the rate (29 m/min) (t(82)=-7.340, p<0.001).

Similar to rats in the AccuScan wheels (as in our findings in the prior sections of this paper), rats in the Med Associates wheels ran in similar numbers of bouts during all periods of the dark cycle (Table 1). Compared to AccuScan runners, Med Associates runners covered longer distances due to more time spent running, with significance by the first hour of the dark period (bout time t(42.563)=-2.474, p=0.017; bout distance (t(41.088)=-2.937, p=0.005). Similar to AccuScan runners, both the distance achieved and the length of the bouts decreased over the dark period (bout time (trend) F(1.388,23.604)=3.621, p=0.057; bout distance F(1.292, 21.968)=5.646, p=0.020).

We also explored running responses in a crossover repeated measures design. The results showed (Fig 5B) that sequence of apparatus exposure matters. After 3 weeks of wheel exposure, males transferred from the Med Associates to the AccuScan system for a fourth week of running ran significantly less in this fourth week in the AccuScan Apparatus (F(1,27)=32.456, p<0.001). This was contrary to the expectation that distance run would either increase or remain stable (if a maximum distance was already reached) from week 3 to week 4. If rats transferred from one wheel apparatus to another needed to re-learn or re-acquire habit levels of running, it would be expected that daily distance run over the first week would appear similar to rats that were naïve to the wheel; however, this was not the case. Naïve runners placed in either the Med Associates wheel (1.0-3.0 km/day: F(1,7)=12.840, p=0.009) or the AccuScan

wheel (0.6-1.2 km/day: $F(1,3)=13.990$, $p=0.033$) showed a significant increase in distance run over the first 7 days of running; however, when experienced (3 weeks) Med Associates runners were placed in the AccuScan system, they did not show an increase in running distance over the first week in the new apparatus; rather, these rats remained at a constant distance of 0.5 km/day, running 45% less distance in this first week than naïve AccuScan runners ($t(44.406)=4.042$, $p<0.001$).

When the opposite sequence was used, i.e., from the AccuScan to the Med Associates system, daily running distance increased 1.6 times, such that animals ran 7.4 km in a 24-hour period. The distance run, which had stabilized by week 3 in the AccuScan system, continued to increase for another 2 weeks (week 1 versus week 2 in Med Associates $F(1,6)=8.832$, $p=0.025$) and then re-stabilized at a higher daily distance of approximately 12 km/day, which was significantly greater than the maximum reached in the AccuScan apparatus ($F(1,6)=20.601$, $p=0.004$).

4. Discussion

Overview

We conducted a systematic analysis of the impact of gender and gonadal hormone status on voluntary wheel running from its emergence to its stabilization in wheel-naïve animals. We examined the impact of early running experience as well as short- and long-term wheel deprivation. Because our analysis included the time spent running and bouting patterns, we could calculate the rate of

running to reveal how changes in these parameters underlie the differences in distance run that we observed across groups. With few exceptions, our data agree with the literature, most of which predominantly used males and measured distance run. However, we provide novel information for females and reveal how changes in specific aspects of running behavior lead to gross changes in distance run.

Rate of running

We used multiple measures of the rate of running to examine as fully as possible this important variable that is at the root of the differences in wheel running distances across groups. For example, averaged across the light-dark cycle, females run faster than males. We also know that rats run in bouts and that this averaged rate includes both running and non-running bouts, so it is also useful to examine the average rate of running within a running bout. To provide additional understanding of gender differences in running rate, we calculated group average of the fastest rate run from analysis of running rate per minute and identified the fastest rate run by the fastest individual. We also generated frequency distribution histograms of rates of running to get a complete picture of how much the various possible rates of running were utilized and how these were impacted by variables such as gender and wheel availability. In the literature there is limited analysis of the time spent running and the rate of running, but what there is, for example Eikelboom and Mills [18], generally accords with our findings on gender differences in *ad libitum* running. Given the technical limits of data collection that are manifested in all wheel running apparatuses used to date,

the issue of whether rate of running is in fact a measure of the instantaneous running rate has been raised by Eikelboom [69]. He makes the point that within each data collection bin, whether it is 1 minute or 5 seconds, the actual maximal rate could be different if the subject runs constantly at a given rate or runs faster some of the time and slower the rest of the time, features that might vary over experimental groups, masking to a certain extent the instantaneous rate of running by the averaging process. We acknowledge this technical limit on the measure of the instantaneous rate of running, but we postulate that even with this limit, such rate analysis is still useful for determining the impact of gender and wheel access on running behavior and suggest that the detailed analysis of frequency distribution of the rates of running offers additional insight into such rate differences. Thus, in agreement with Koteja et al. [70], we argue that the rate differences, even given the technical limits, offer insight into running processes.

First response

We provide a systematic analysis of initial wheel interaction, which is often overlooked [1]. Our protocol which used both a novel cage (future home cage) and novel wheel demonstrated that both genders have considerable spontaneous avidity for interaction with the wheel. Our current data generally accord with those of others using the same protocol (i.e., a novel wheel in a novel home cage). Male mice first approached a wheel within a few minutes and ran on the wheel within the first 30 minutes [71,72]; in our studies, this was also the case with male and female rats. In the context of our earlier work, our data also demonstrate that such outcomes are altered if the protocol involves

introducing a novel wheel into an established home cage. In that situation, the rats take 2.5 times longer to interact with the wheel [73] and run ten times less in the first 24 hours [64], in accord with expectations from classic studies of neophobia by Barnett [74].

Fundamentals of gender differences in the emergence of stabilized running

Our findings accord with those of others who report that 3 weeks of *ad libitum* wheel access is sufficient to produce a stable running distance in the male and female rat [2-4,18,50,51,53,54,75]. Our fine-grained analysis of the emergence of running shows that females reached their peak of running in terms of distance, time spent running, and rate of running a week earlier than males (2 versus 3 weeks), a distinction not previously made with such systematic comparison to clearly demonstrate this gender difference. Thereafter, the overall pattern of running does not change with further experience in either gender. In one study of male mice, the daily time spent running indicated that the distance run remained stable throughout the wheel exposure period of 14 days [76], which seemingly conflicts with our findings and those of others indicating that stable running requires longer to emerge in male rats. Our data also extend those prior findings by demonstrating that running remains stable for up to 15 weeks in animals up to 12 months old, and that up to that point, there is no correlation between body weight and distance run. Thereafter, however, as body weight increases, running decreases. We also found that a stable running pattern emerges regardless of the apparatus used, and most curiously, that emergence

of running and adult running patterns are not impacted if the running is started as early as PND 21.

The fact that stabilized or habitual running required several weeks to emerge in wheel-naïve animals suggests that CNS components known to mediate learning and action in response to new stimuli, such as the medial prefrontal cortex and ventral striatum, are involved initially, with habitual running possibly involving dorsal striatal regions. Ultimately the training of the motor cortex and its efferent systems is the outcome of such a progression. We speculate that progressive involvement of these CNS areas emerges as running develops into a habit in a process analogous to the process by which the CNS mediates drug taking [77]. We do not rule out the role of corollary peripheral changes that likely also occur, such as joint, muscle, and skeletal toning and strengthening, but suggest that both CNS and peripheral changes may be necessary as the basis for forming habitual running. Collectively, our data also show that the rate of running can be readily altered, even for stabilized running, which suggests that both dorsal striatal and cerebellar circuits might be involved in the timing and precise motor patterns needed to so flexibly alter the rate of running and its patterns.

As rats are a nocturnal species, 90% of voluntary wheel running occurs during the dark period [18,78], with distances run generally decreasing over the dark cycle [4]. In addition our work shows a gender difference in the temporal pattern of running; namely, females surpass males in distance run by running significantly more during the second half of the dark cycle. In accord with others

[18], we found that the distance run per bout, the number of bouts, and the rate of running within a bout decrease over the dark period; however, our analysis of the cycle (see below) newly demonstrates that examining only averages across the estrus cycle masks a gender difference in bouting across the dark period.

Although some rats and mice have been bred to exhibit more uniformly high running behavior [79,80], in commonly used populations of outbred rats, we agree that voluntary wheel running can vary between individuals but is stable within individuals [2,18,68,81-85]. For example, a classic study of voluntary wheel running in the experienced female rat reports common daily ranges of 5-10 miles, and up to one extraordinary 27 miles in a 24-hour period [4], with male rats having a range of 2-5 miles run per day [4]. The minimum daily average distances run can be similar for males and females, but females usually considerably exceed males in the maximum distance run and in the maximum rate of running and its frequency across and within individuals. Our work also revealed a significant influence of apparatus on the range of individual responses, particularly the maximal distance and its root variable speed.

Estrus cycle is one source of gender differences in stabilized running Our data show that on average, intact females run farther than intact males given the same wheel availability. This finding accords with the limited systematic comparisons available in the literature, which include a classic work [4], another based on only 15 days of running from a naïve state [18], and lastly, certain comparisons that can be surmised across independent studies that included mostly only males [2,53-54,75]. Our data demonstrate that the greater distance

run by females is due to a generally faster rate, even when averaged across all days of the typical 4-day female estrus cycle, in accord with Eikelboom & Mills [18], the only prior measure of gender difference in rate. We further demonstrated that the female rate is faster not only on average, but also in measures of average fastest rate, distribution of running speeds, and the maximal speed of the fastest runner.

Previous studies show that ovarian hormones significantly affect the distance traveled in voluntary wheel running, such that females run drastically different daily distances across the estrus cycle, with the greatest distance occurring during the proestrus period immediately before ovulation [4,55,56,58,86,87]. Our data confirm those findings showing that from the metestrus to the proestrus period, the daily distance run doubles. We further newly prove in a quantitatively comparable manner, that the lowest distance run by females (metestrus period) is similar to the maximal amount run by males.

When we examined the time spent running across genders, we found that males and females run for similar amounts of time if the days of the female cycle are averaged, but that there is a gender difference in the time spent running on the different days of the cycle that is masked by this averaging. We have generated novel data showing that the greater distance covered by females is due to limited periods in the female's cycle, most notably proestrus, in which females run substantially longer than males (or females in metestrus) and at faster average rates.

Generally in accord with our data, Eikelboom & Mills [18] found that the number of bouts, when averaged across the running period, is similar across genders; however, the duration of the bouts, the distance run per bout, and the rate of running is higher in females, even when averaged across the days of the cycle. In our bout analysis, we found that the proestrus female runs in longer bouts and at a faster rate per bout, revealing the fundamental gender difference that results in a greater distance covered per bout than the male (or metestrus female). We also newly show that a rat increases her distance run in proestrus by both a small increase in average total time run, and importantly, by an increased rate and more and longer bouts of running.

Curiously, although the prevailing view is that the most running during the cycle occurs during proestrus, one study in rats reported the highest daily running distances during estrus [88]. We suggest that the 10:14 hour light-dark cycle used in that study, which is significantly different from the more standard 12:12 hour light-dark cycle, possibly affects running and certainly influences estrus cycle readings, given the continuous cyclic nature of the hormonal changes across the days of the estrus cycle [68]. Additionally, those authors did not indicate the crucial consideration, the time of day that their vaginal smears were taken. These speculations might explain the discrepancy between Gerall et al. [88] and most of the literature, including our work.

Since metestrus running patterns mimic male running patterns, collectively with the proestrus data, this suggests that, in the gender differences in running, male versus female differences in body weight or muscle mass or tone before 15

weeks of age are not as important as daily cyclic differences in running. It also is difficult to argue that the effects of gonadal steroid hormones across males and females are solely due to effects on peripheral muscle or other tissue. Rather, we speculate that contemporaneous circulating gonadal steroid hormones across genders and during the estrus cycle alter particular CNS processes that are the core source of such differences in running. Such effects might be due to the action of steroid hormones on neuronal activity via their known receptors in the striatum, possibly including both the nuclear receptors and membrane receptors, which are likely to mediate very rapid steroid [89-91].

We found that knowing the features of running during the entire hormonal cycle readily allows one to predict the day of the estrus cycle, in any given data set, to the extent that this allows for predictive or retrospective choices in choosing data points at desired cycle points. In general, the features of this pattern can be seen after 1 week of wheel exposure, although it is more fully developed after 2 weeks, when intact females have fully stabilized running.

Adult gonadal hormones are not the sole basis of gender differences in running

Previous reports showed that gonadectomy of adult females and, in rare studies that included males, reduced daily running distance by 60-95%. Notably ovariectomized females show a flat pattern of daily wheel running, with complete disappearance of the 4-day rhythmic running cycle [4,88]. We revisited these findings because the studies on males were done 85 years ago using home cage conditions unlikely to meet modern standards and because systematic gender comparisons were not done in prior literature.

We confirmed prior findings for distance and demonstrated that removal of the gonadal hormones significantly decreased distance run by decreasing time spent running and running rate at all time points by approximately 90%, with dramatic alterations in all features of bouting. Certain earlier studies allowed rats to begin running almost immediately after gonadectomy; however, it is now understood that behavioral changes from gonadectomy or steroid hormone replacement therapy in gonadectomized subjects requires several weeks to manifest [92]. Thus, prior studies likely included data points before complete washout of the effects of the hormones. Assuming that gonadectomies were complete, this likely explains why Richter [4] reported that in some individuals more running occurred after gonadectomy. In contrast, we allowed hormone levels and hormone-dependent peripheral and brain effects sufficient time to decrease before running measures recommenced.

Removal of the gonadal hormones did not affect the pattern of running over the light-dark cycle, with gonadectomized males and females still conducting 90% of their running during the dark period in a similar but blunted bout pattern compared to intact males and females, a finding not previously reported. Also not previously reported is the fact that gender differences in voluntary wheel running survive gonadectomy, such that ovariectomized females still run farther by running faster and for longer periods of time than orchiectomized males. This indicates, as others have theorized before [1], that voluntary wheel running is not solely regulated by contemporaneously circulating gonadal hormones. Our data support the hypothesis that there may be either

early developmental effects of gonadal steroid hormones, as suggested by classical behavioral neuroendocrine perspectives for sexual behavior, or that chromosomally regulated processes independent of gonadal steroid hormones are at work, as suggested by new understanding of the action of Y-chromosomal factors independent of gonadal steroids [93-95].

Additional new information from our study is that gonadectomy in males and females eliminated the natural progression of increased running in experienced runners after a period of wheel deprivation.

Our analysis of the natural pattern of *ad libitum* running across the genders in the intact and gonadectomized states revealed that the gender differences in distance run were due to the fact that females run at a faster rate, with the additional feature that the time spent running and the bouting patterns vary across the estrus cycle. The fact that such gender differences in running do not depend entirely on contemporaneous levels of gonadal steroid hormones was uncovered by analyzing gonadectomized animals. The fact that gender and hormonal status so readily alter features of habitual running suggests that gonadal steroid hormones regulate particular CNS regions. As suggested by others, one possible site of interaction is the striatum, particularly its dopaminergic modulation, which is strongly regulated by gonadal steroid hormones [89,90], although other studies suggest that the medial preoptic area and anterior hypothalamus might be the site of such interactions [96]. We will conduct future studies to determine hormonal and brain region interactions on running behavior.

Influence of interrupted wheel access

Overall, among the most significant new information we provide with our data on running responses to interrupted wheel access are marked alterations in rate of running (generally slower), changes in bouting features, and changes in details of the overall pattern of running.

While investigators studying the impact of voluntary wheel running commonly utilize a daily routine of limited availability of the wheel [1], the only prior systematic work on this topic was done in experienced male runners [50-52] but only distance run was explored. We add data on female responses and fine-grained dimensions of analysis on time, rate, and bouting features, which are novel points in the literature.

Our findings suggest that daily workout routines of limited time never allow the subjects to achieve certain measures of maximal training fitness, such as maximal speed. This might be important for studies that use running behavior to alter some dependent biological event in subjects, and might provide insight into rodent motivation to use the wheel. It is also intriguing to consider what both of these end points might mean for humans.

Similar to other studies using limited wheel availability [14,54], we found that the period in which maximal running distance emerges depends on the daily wheel availability schedule. For example, we found that very short wheel access (30 min/day) induced the emergence of maximal or stable distances run in a very short time (1 week), whereas it took animals with 2-hour access significantly longer (3 weeks) than either the very short access or *ad libitum* runners to reach

their maximal running distance, time, and rate. These findings suggest that the limitations of short-term running can be met by adjustment of the brain and body systems more quickly, whereas the demands of longer-distance running on a daily basis seen in *ad libitum* runners take those systems longer to adjust.

Particularly intriguing new information is the drastic extent to which rats given short wheel access times altered the time, the rate, and bouting pattern of running. Rats given 30-minute or 2-hour access had similar running rates that are about 25% slower than those of rats with *ad libitum* access, but they used a greater percentage (90-100% in 30-minute runners and 63% in 2-hour runners versus 38% *ad libitum* dark period) of their time running, even to the point of running constantly without bouting. Because this difference in running rate occurs by the third day of wheel availability, we suggest that rats quickly learn and keep track of the amount of time per day that they have access to the wheel.

Additionally, 30-minute runners had high, stable running rates during those 30 minutes, whereas 2-hour runners decreased their running rate during the wheel availability time, even during the first 30 minutes. This suggests that rats learn that wheel availability is limited, and they adjust their running rates to obtain a particular amount of running within the allotted time frame. This might be anticipatory running that is based on a planning process in which the rats modify their running pattern to maximize their running because they anticipate the removal of the wheel.

The final area of intermittent wheel access examined was the effect of providing wheels on alternate rather than consecutive days. We found that

distance run increased immediately during the first hour after wheel return; however, this immediate increase in running did not affect the total daily distance run, in accord with the findings of others [3,51]. Thus alternate-day runners keep track of how much they run in a given 24-hour period, including running in the light period after return of the wheel and running in the dark period, which is hours after the initial running spurt, thus keeping track of their daily distance regimen. We find that in comparison to *ad libitum* runners, alternate-day runners alter their running pattern by running significantly less during the first 6 hours of the dark period and significantly more during the 12-hour light period.

Our findings generally agree with those of Mueller et al. [52] for ultra-short wheel deprivations (1-10 hours); they found a positive correlation between deprivation and distance run in the first 24 hours after return of the wheel; namely, the longer the wheel deprivation, the more drastic the rebound running effect and the greater the percentage of the daily distance run immediately after return of the wheel. However, our data newly show that the rate and pattern of running that occurs over the dark cycle differs significantly from *ad libitum* access. With *ad libitum* access, wheel running decreases over the dark cycle, but with alternate-day access, wheel running remains stable over the entire dark cycle and peaks in the final hour of wheel availability. The idea that rats remember and thus can regulate in this way the amount of running they conduct during the day despite the availability of the wheel is a new concept. We suggest this is a complex form of anticipatory response.

The fact that in several different data sets, these limited-interval runners readily altered their patterns of running by modifying both the entire 24-hour pattern of running and their bout patterns suggests that these responses can be considered anticipatory, consistent with the hypothesis that learning and memory circuits are at work together with features of the medial prefrontal executive planning and execution system. Such anticipatory behaviors provide researchers with a tool by which to alter the voluntary running parameters of subjects, enabling more precise control of how running affects various dependent measures under study.

Most intriguing to our initial purpose of establishing a set of data from which we could test the motivational processes at work in wheel running are these anticipatory responses, including the considerable alacrity of repeated robust rest-period running, the substantial alteration in bouting (hence rest periods within running), and the substantial alteration in the entire 24-hour pattern of running in the alternate-day runners. All of these findings suggest motivational forces of material strength, i.e., a positive incentive salience for wheel running or its after-effects sufficient to organize daily behavioral patterns that are distinct from those of the *ad libitum* runner. This is consistent with our emerging data using a conditioned place preference model, which also indicates a considerable positive incentive salience of running and its after-effects [97,98]. The prevalent understanding of motivational circuits in the mammalian brain make it reasonable to suggest that medial prefrontal, ventral striatal, and ventral tegmental components of the neural circuit that are known to mediate motivated

responses may be involved here. Indeed, our emerging data on the involvement of the prelimbic and infralimbic subregions of the medial prefrontal cortex and the core and shell components of the nucleus accumbens specifically support these speculations about the neural basis of the incentive salience of wheel running [98].

Another important general finding of the interrupted running data is determining how long running effects last. We found that experienced runners exhibited a material residual effect of running for up to 2 weeks of wheel deprivation, since they regained their maximal running patterns only 48 hours after the return of the wheel, much faster than maximal running emerges from the naïve state. These findings in females accord well with those of Mueller and colleagues [52] in males. We also found that the time course for acquisition and stabilization of voluntary wheel running is exactly the same in naïve runners and experienced runners subjected to a long period (4-6 months) of wheel deprivation. The nature of the mechanism of the residual effect of prior running experience is not known, but it is possible that changes in both brain and body are at work. Our data provides the information of how long these residual effects last, and when they are no longer present, offering at least a temporal framework for the cellular or molecular mechanisms at work.

The data from alternate-day runners compared to *ad libitum* runners further suggest that the emergence of running and its stable form are sturdy behavioral patterns not significantly impacted by whether the running occurs in the single home cage or if the animals are routinely moved from shoebox home

cages to running chambers every other day for a month or more, or if the running occurs in different apparatuses. The lack of home cage effect can be seen by comparing *ad libitum* runners, which were housed in single home cages with wheels, with alternate-day runners, which were moved from home cage to the AccuScan apparatus. We also saw all the same patterns of running with the Med Associates apparatus, which has very different features. The design of the Med Associates apparatus allows the animals to live in a shoebox cage with a wheel attached, but has a feature that allows wheel access with a minor change in environment without a change in cage.

Different apparatuses

Our finding that rats run significantly different daily distances, achieved by longer faster running in similar bout patterns in different wheel apparatuses, is to our knowledge the first side-by-side quantitative comparison of this nature in rats. Other researchers have shown that other rodent species (e.g., mice and hamsters) had varied preferences for different types of wheels [61,63].

To our knowledge, any differences in the work required (effort) for the rats to run in these two different apparatuses does not appear to explain the difference in distance run. Both are designed to have minimal friction, and although the Med Associates wheel is a bit heavier than the AccuScan wheel, the rats ran greater distances in the Med Associates wheel, suggesting that this is not a factor. If preference for the Med Associates wheel is concluded by the fact that rats run a greater distance in this apparatus compared to the other, and if the workload is ruled out, other wheel features must be considered. Possibly, the

rats prefer the slightly larger diameter wheel or wheels with metal rods rather than metal mesh floors, or the architecture of the wheel is preferred because it has a slightly enclosed wheel chamber separate from the living chamber, as in the Med Associates apparatus. This wheel also provides airflow from air outside of the home cage, which might enrich the environment. Additionally, the Med Associates wheels have remarkable stability during running due to construction details. In contrast, the AccuScan wheel is slightly smaller, within the larger living chamber, is not enclosed, and might be less stable at its center axis attachment point. These data suggest that it is important to acknowledge that different wheel apparatuses impact the behavior of voluntary wheel running, at least in terms of the daily distance run. This is important for investigators who have more than one type of running wheel apparatus in their laboratory or are comparing their data to those of others. The impact of apparatus can be considered in the context of our finding that rats alter the distance they run by altering the time, rate, and bout pattern of their running. Thus, different apparatuses may induce responses across these variables that are important to the different running-dependent end points of a variety of paradigms. These data also suggest that the differences in absolute distance run and speed run found in the literature are likely due to use of different apparatuses. Although there is considerable cohesion across the literature on the principles of voluntary wheel running in the rat, the exact running distance per day (as well as exact details of the daily running time and rate, though limited in examination), must be expected to vary widely, and they certainly do.

Final points

In addition to the utility of these data to the preclinical study of running-induced variables in the brain and body, we agree with Eikelboom [99] that voluntary wheel running with *ad libitum* access to food and water as implemented in these studies has potential as a preclinical model for voluntary exercise in humans. Considering that 80% of adults in the United States do not achieve the level of physical activity (aerobic and muscle-strengthening) recommended by the American Heart Association and 25% do not attain any level of physical activity indicates that despite the knowledge of its beneficial effects, there is a need for such a preclinical model to understand the behavior and its motivational basis. Even taking into consideration the arguments of Sherwin [1] that wheel running in laboratory rats is a "contrived" response to the wheel in laboratory conditions, we argue that both the robust unconditioned responses to the wheel that we examined in this work as well as our emerging work using conditioned measures of motivation [97,98] indicate that wheel running has very high incentive salience in rats. Tapping into understanding the CNS components that underlie this incentive salience in our emerging studies [98] may be relevant both for the study of motivated responses to other stimuli as well as potential for informing the human problem of too little voluntary physical activity in certain segments of our population.

Acknowledgements

We thank Dr. Mariana Pereira for her scholarly contributions to this work and Leah Dziopa and Sabina Advani for their technical assistance.

TABLE 1

Table 1: Bout running variables over the course of the dark cycle in males and females in a variety of running protocols

	Intact Males and Females Metestrus and Proestrus (1st hour only)			Gonadectomized Males and Females		
Variable	**1st hour dark**	**6th hour dark**	**12th hour dark**	**1st hour dark**	**6th hour dark**	**12th hour dark**
Bout number	6.3 (±0.8) / 4.3 (±0.8) 6.0 (±0.9) / 5.2 (±1.0)	4.0 (±0.8) / 3.3 (±1.3)	3.5 (±0.7) / 2.8 (±1.1)	3.0 (±0.9) / 5.0 (±1.8)	1.8 (±1.1) / 2.3 (±1.1)	0.5 (±0.3) / 3.0 (±1.5)
Bout time (minutes)	5.7 (±0.8) / 5.2 (±0.5) 4.4 (±0.5) / 6.7 (±1.1)	4.9 (±1.0) / 5.2 (±0.9)	2.0 (±0.6) / 2.0 (±0.3)	1.6 (0.3) / 2.9 (±0.6)	1.3 (±0.3) / 1.6 (±0.5)	**0.5 (±0.3) / 2.5 (±0.5)**
Bout distance (meters)	89.3 (±22.7) / 116.0 (±18.5) **71.2 (±15.7) / 183.5 (±44.2)**	88.4 (±27.7) / 131.1 (±33.1)	8.5 (±4.6) / 9.4 (±4.2)	**3.7 (±1.6) / 30.5 (±8.2)**	4.1 (±2.3) / 13.2 (±6.7)	**0.4 (±0.2) / 21.3 (±5.6)**
Bout rate (meters/minute)	**11.6 (±1.7) / 16.1 (±1.3)** **12.9 (±1.6) / 23.0 (±2.4)**	13.2 (±2.4) / 17.3 (±2.8)	2.8 (±0.9) / 2.8 (±0.7)	**1.7 (±0.3) / 8.4 (±1.2)**	2.4 (±1.2) / 5.1 (±1.7)	0.8 (±0.0) / 7.1 (±1.4)
Inter-bout number	6.3 (±0.9) / 4.3 (±0.8) 6.7 (±0.9) / 4.8 (±0.9)	3.3 (±1.0) / 4.3 (±0.5)	3.0 (±0.8) / 4.2 (±0.7)	4.0 (±0.9) / 5.5 (±1.6)	2.8 (±1.1) / 3.0 (±1.1)	1.5 (±0.3) / 4.0 (±1.5)
Inter-bout interval (minutes)	4.1 (±0.8) / 8.3 (±2.3) 5.1 (±0.9) / 4.9 (±1.5)	13.5 (±4.6) / 9.1 (±2.0)	18.0 (±4.6) / 12.7 (±2.8)	13.8 (±2.9) / 8.3 (±3.1)	20.9 (±6.3) / 18.7 (±4.7)	**39.7 (±8.7) / 13.0 (±3.8)**

	Alternate-Day Access Females			Limited Access Females 30 Minutes / 2 Hours	MedAssociates Females		
Variable	**1st hour dark**	**6th hour dark**	**12th hour dark**	**1st hour dark**	**1st hour dark**	**6th hour dark**	**12th hour dark**
Bout number	4.0 (±0.4)	2.3 (±1.0)	5.3 (±1.7)	**1.0 (±0.0) / 3.8 (±0.8)**	3.8 (±0.5)	2.5 (±0.3)	1.9 (±1.2)
Bout time (minutes)	5.5 (±1.3)	6.3 (±1.2)	5.0 (±0.6)	**30.0 (±0.0) / 9.9 (±2.5)**	10.0 (±1.9)	8.8 (±1.9)	3.2 (±0.6)
Bout distance (meters)	90.3 (±31.2)	142.5 (±32.8)	99.1 (±16.3)	**586.5 (±46.4) / 110.2 (±33.3)**	308.6 (±63.8)	259.2 (±69.6)	17.7 (±7.3)
Bout rate (meters/minute)	12.5 (±2.1)	21.6 (±3.6)	16.8 (±1.7)	**19.6 (±1.5) / 8.0 (±1.2)**	24.0 (±2.3)	22.0 (±3.0)	4.4 (±0.9)
Inter-bout number	3.5 (±0.3)	2.8 (±0.9)	5.3 (±1.4)	**0.0 (±0.0) / 3.5 (±1.0)**	3.6 (±0.6)	2.0 (±0.3)	2.5 (±0.9)
Inter-bout interval (minutes)	10.9 (±3.3)	16.1 (±6.4)	6.4 (±2.4)	NA / 6.5 (±2.0)	6.2 (±1.1)	19.1 (±4.4)	21.4 (±5.8)

*** Note that bold comparisons are significantly different (p<0.05)**

Intact males and females: The time spent running and distance run decreased as the dark period progressed for both males (bout time $F(2,22)=3.676$, $p=0.042$; bout distance (trend) $F(2,22)=3.058$, $p=0.067$) and females (bout time $F(2,40)=4.215$, $p=0.022$; bout distance $F(2,40)=5.530$, $p=0.008$), but the pattern within each hour was similar across genders, with the exception that the bout rate was significantly faster for females during the 1st hour of the dark period.

Metestrus versus proestrus: The number of bouts per hour was the same during the metestrus and proestrus periods; however, the time spent running, the average distance run, and the rate achieved during these bouts was significantly greater during the proestrus period (bout time (trend) $t(65)=1.943$, $p=0.056$; bout distance $t(37.540)=2.394$, $p=0.022$); bout rate $t(53.725)=3.559$, $p=0.001$).

Gonadectomized males and females: The number of bouts in the ovariectomized females was not significantly less than in the intact females, but orchiectomized males ran significantly fewer bouts than intact males in the first hour of the dark period ($t(6)=2.751$, $p=0.033$) and thereafter. The time spent running and the distance run during bouts was markedly and significantly less for ovariectomized than for intact females (bout time 6th hour $t(30.869)=3.485$, $p=0.001$; bout distance 1st hour $t(28.797)=2.189$, $p=0.037$, 6th hour $t(24.812)=3.487$, $p=0.002$) and orchiectomized compared to intact males (bout time 1st hour $t(29.550)=4.624$, $p<0.001$, 6th hour $t(14.396)=3.624$, $p=0.003$; bout distance 1st hour $t(23.226)=3.771$, $p=0.001$, 6th hour $t(12.172)=3.037$, $p=0.010$). The time spent running and the distance achieved during bouts was also significantly less for orchiectomized males compared to ovariectomized females (bout time 12th hour $t(15)=2.271$, $p=0.038$; bout distance 1st hour $t(20.411)=3.208$, $p=0.004$, 12th hour $t(12.043)=3.731$, $p=0.003$).

Limited Access Females: The bouting pattern of 2-hour runners was similar to that of *ad libitum* runners and thus significantly differed from 30-minute runners (bout number $t(3.0)=3.667$, $p=0.035$; bout time $t(14.0)=-7.918$, $p<0.001$; bout distance $t(17)=-6.866$, $p<0.001$; bout rate $t(17)=4.737$, $p<0.001$).

TABLE 2

Table 2: Influence of limiting the daily amount of wheel access on distance, time, and rate

	Distance				Percentage of Time	Rate	
Amount of Daily Running	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 3**	**Week 3**	**Average Fastest Rate**
30 Minutes	231 m	**417 m**	399 m	410 m	95%	15 m/min	30.5 m/min
2 Hours (30-minute comparison)	304 m	523 m	778 m (302 m)	**1149 m**	63%	10.9 m/min	27.6 m/min
Ad Libitum: 2-hour comparison (30-minute comparison)	344 m	**1021 m**	808 m (226 m)		38% 31% / 33%	19.2 m/min	50 m/min

*** Note: 2-hour and 30-minute comparisons refer to distance only. Bold numbers signify peak distance.**

Distance: Similar to *ad libitum* runners, 30-minute runners reached their peak distance run by week 2 (week 1 versus 2 $F(1,27)=35.002$, $p<0.001$), whereas 2-hour runners reached their peak distance run by week 4 (week 1 versus 2 $F(1,27)=9.405$, $p=0.005$; week 2 versus 3 $F(1,27)=8.530$, $p=0.007$; week 3 versus 4 $F(1,27)=4.236$, $p=0.049$). Two-hour runners ran significantly more than 30-minute runners during the 1st (t(48)=-2.086, $p=0.042$), 3rd (t(41.361)=-4.359, $p<0.001$) and 4th week (t(33.839)=-4.503, $p<0.001$). In the same 2-hour time period, *ad libitum* runners ran significantly more only during the second week (t(38.380)=-2.354, $p=0.024$).

Time: Once running patterns were established, 30-minute and 2-hour runners spent a larger percentage of their access time running compared to their *ad libitum* counterparts, which spent only 38% of their time running (active 12-hour dark period) (30-minute t(54)=4.610, $p<0.001$; two-hour t(54)=7.130, $p<0.001$). When the same time periods of the dark cycle were analyzed, *ad libitum* runners spent only 33% (30 minutes) and 31% (2 hours) of their time running, which is again less than both groups of limited-access runners.

Rate: Once running was established (during the 3rd week), 30-minute runners ran 22% slower than *ad libitum* runners (n.s.), whereas 2-hour runners ran 50% slower than *ad libitum* runners (t(46.280)=-7.506, $p<0.001$). The average fastest rate of running in 30-minute and 2-hour runners was also significantly slower than *ad libitum* runners (30 minute t(54)=-3.440, $p=0.001$; 2 hour t(54)=-9.742, $p<0.001$).

FIGURE 1

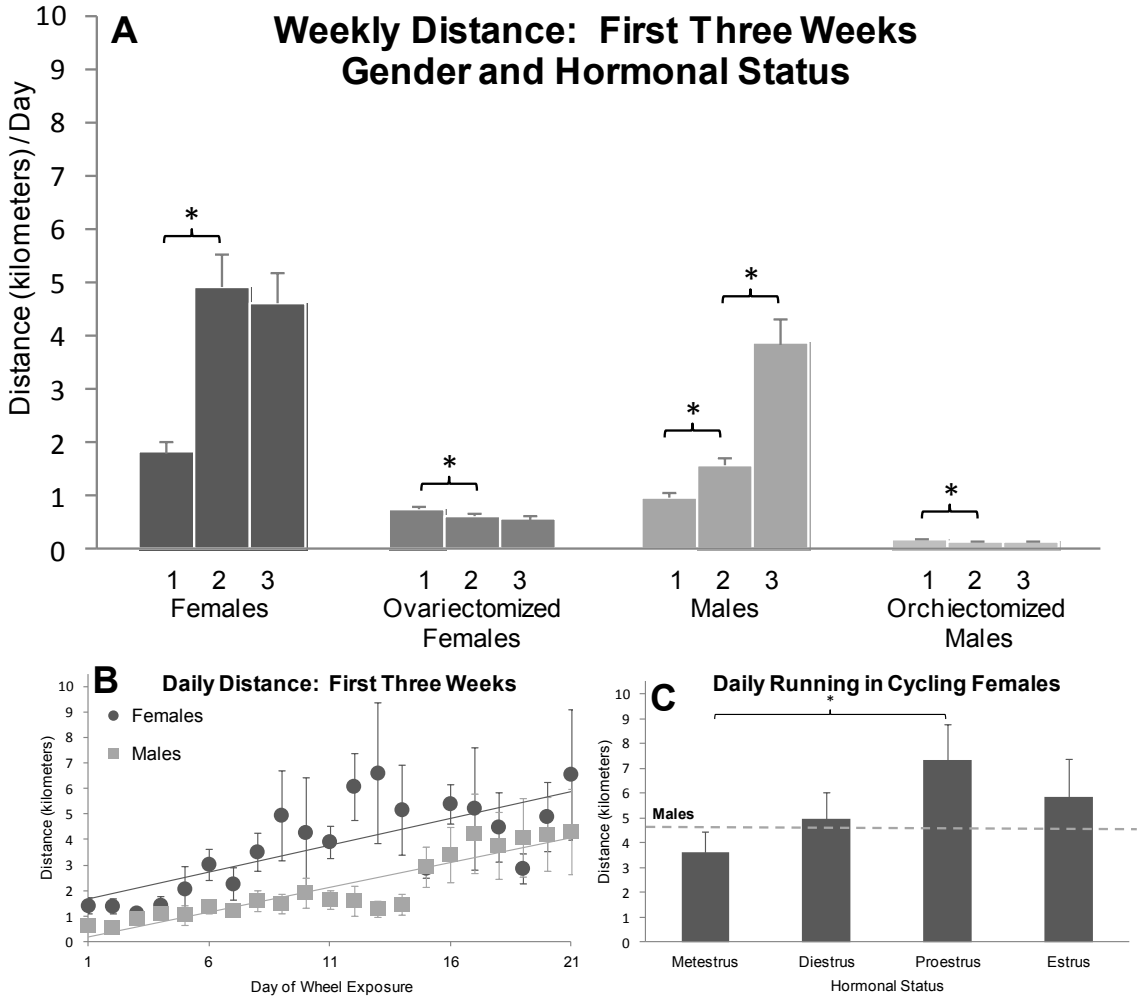

Figure 1 (A). Average (± SEM) distance (kilometers) run in a 24-hour period during the 1st, 2nd and 3rd week of wheel exposure in intact females, ovariectomized females, intact males, and orchiectomized males. Males increased daily distance run throughout the first 3 weeks (week 1 versus 2 $F(1,27)=6.352$, $p=0.018$; week 2 versus 3 $F(1,27)=26.401$, $p<0.001$); females reached peak daily distance run by week 2 (week 1 versus 2: $F(1,27)=21.472$, $p<0.001$). Females ran significantly more during the 1st ($t(37.082)=3.665$, $p=0.001$) and 2nd ($t(30.914)=5.437$, $p<0.001$) week of running. After gonadectomy, decreases in daily distance run were seen from week 1 (intact versus ovariectomized females $t(33.181)=4.756$, $p<0.001$; intact versus orchiectomized males $t(29.172)=8.393$, $p<0.001$). (B). Average (± SEM) daily distance run (kilometers) over the first 21 days by intact females and males. (C). Average (± SEM) daily distance run by habitual females during the four-day cycling period: metestrus, diestrus, proestrus and estrus; habitual males indicated by dotted line.

FIGURE 2

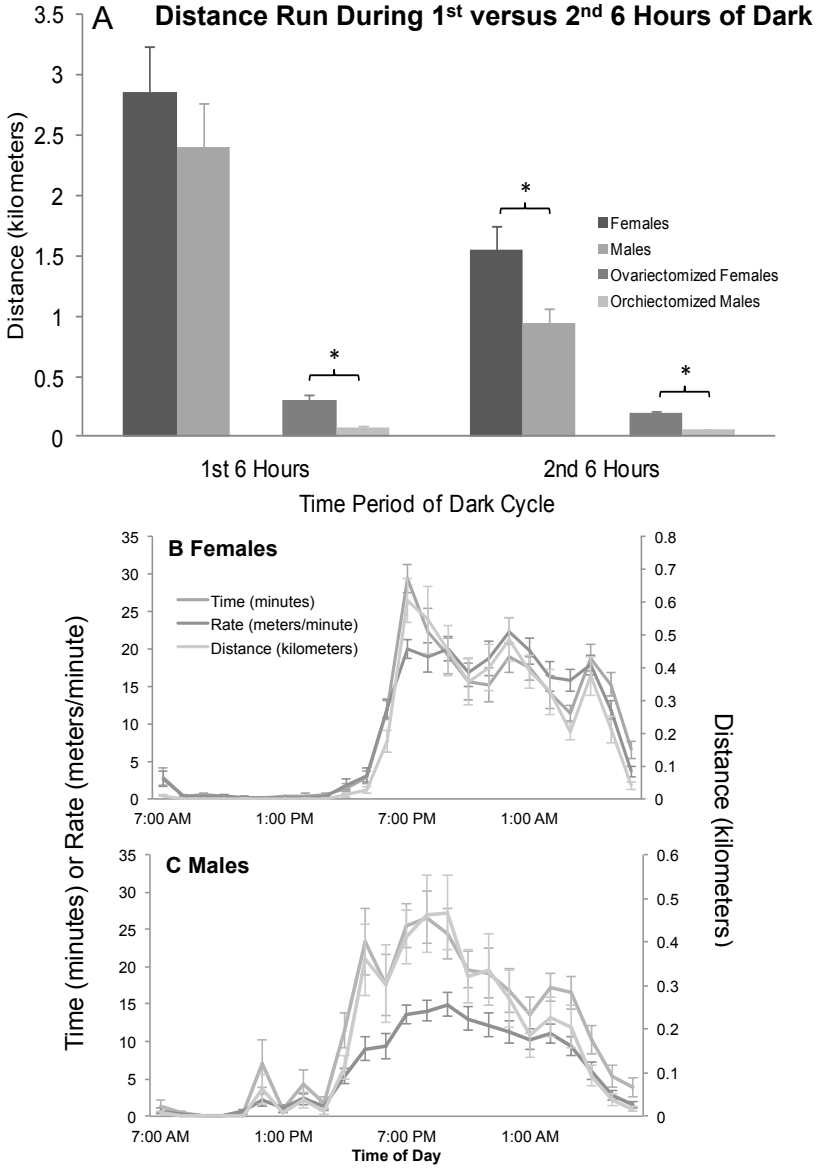

Figure 2 (A). Average (± SEM) distance (kilometers) run during the 1st and 2nd 6 hour period of the dark cycle for intact females, intact males, ovariectomized females, and orchiectomized males during the 3rd week of running. Animals ran significantly farther in the 1st versus 2nd 6 hours of the dark cycle (females $F(1,27)=37.176$, $p<0.001$; males $F(1,26)=53.539$, $p<0.001$; ovariectomized females $F(1,27)=7.220$, $p=0.012$; orchiectomized males $F(1,15)=8.669$; $p=0.010$). Males ran significantly less distance than females during the 2nd 6 hours of the dark period ($t(53)=2.656$, $p=0.010$); orchiectomized males ran significantly less than ovariectomized females during the 1st ($t(28.630)=4.370$, $p<0.001$) and 2nd ($t(30.303)=4.854$, $p<0.001$) 6 hours of the dark cycle. (B). Average (± SEM) hourly time (minutes), rate (meters/minute), and distance (kilometers) run during the 24-hour light-dark cycle for females and males during the 3rd week of running.

FIGURE 3

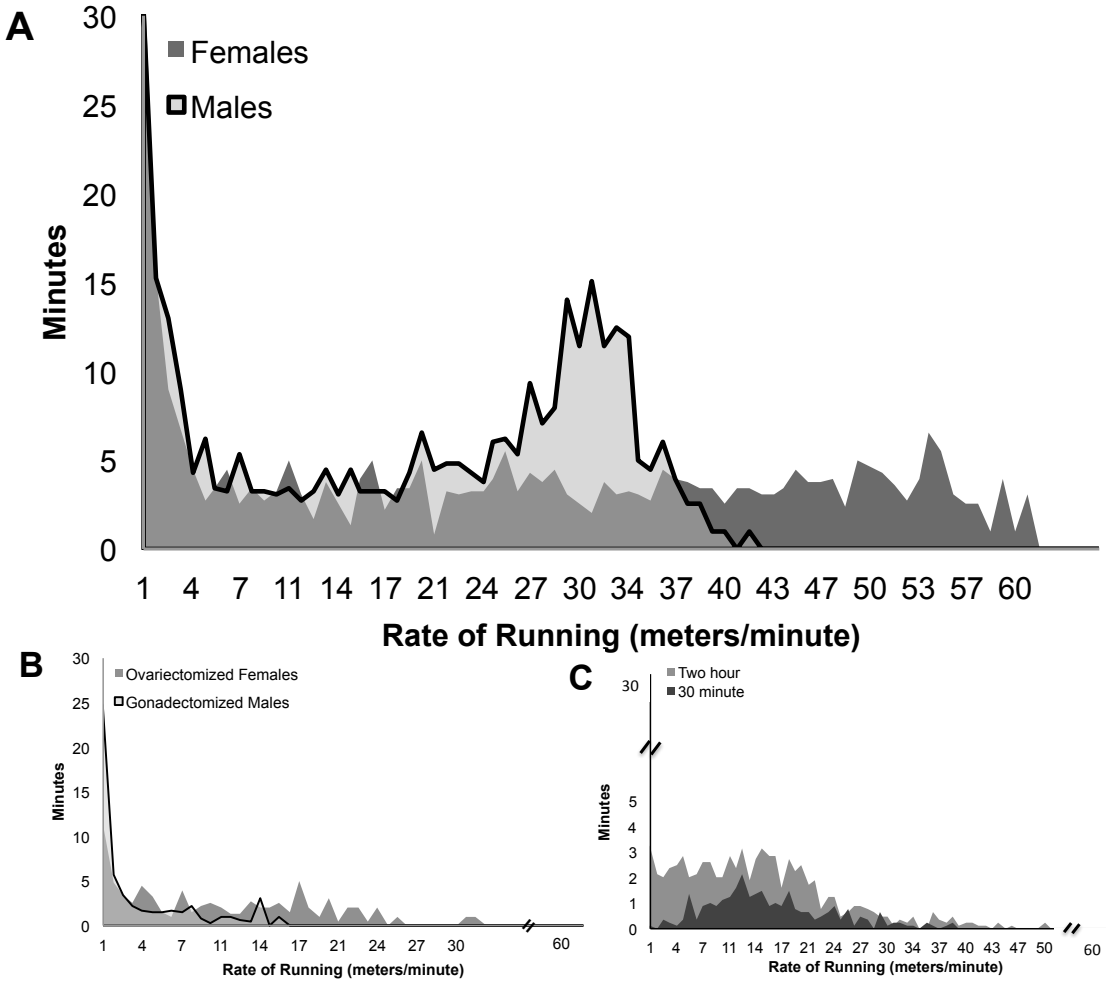

Figure 3 (A). Histogram of the number of minutes spent running a particular rate (meters/minute) for females and males. After the initial mode (0.8 m/min), frequency distribution of minutes females spent running at particular rates was distributed fairly evenly, with an absolute maxima of the fastest individual running up to, but not beyond, 61 m/min (mean 25.9 m/min). In contrast, in addition to a similar initial mode (0.8 m/min), males had an additional significant mode, not present in females, at 32 m/min and an absolute maximum of the fastest individuals running up to, but not beyond, 42 m/min (mean 19.4 m/min). (B). The effect of gonadectomy on rate distribution. The frequency distributions of the rates of running for ovariectomized females and orchiectomized males were significantly different (K-S tst, $p<0.05$), with ovariectomized females (mean 11 m/min, mode 0.8 m/min) more frequently running faster than orchiectomized males (mean 2 m/min, mode 0.8 m/min). (C). The effect of limited daily access (two-hour versus 30 minute) in intact females on rate distribution.

FIGURE 4

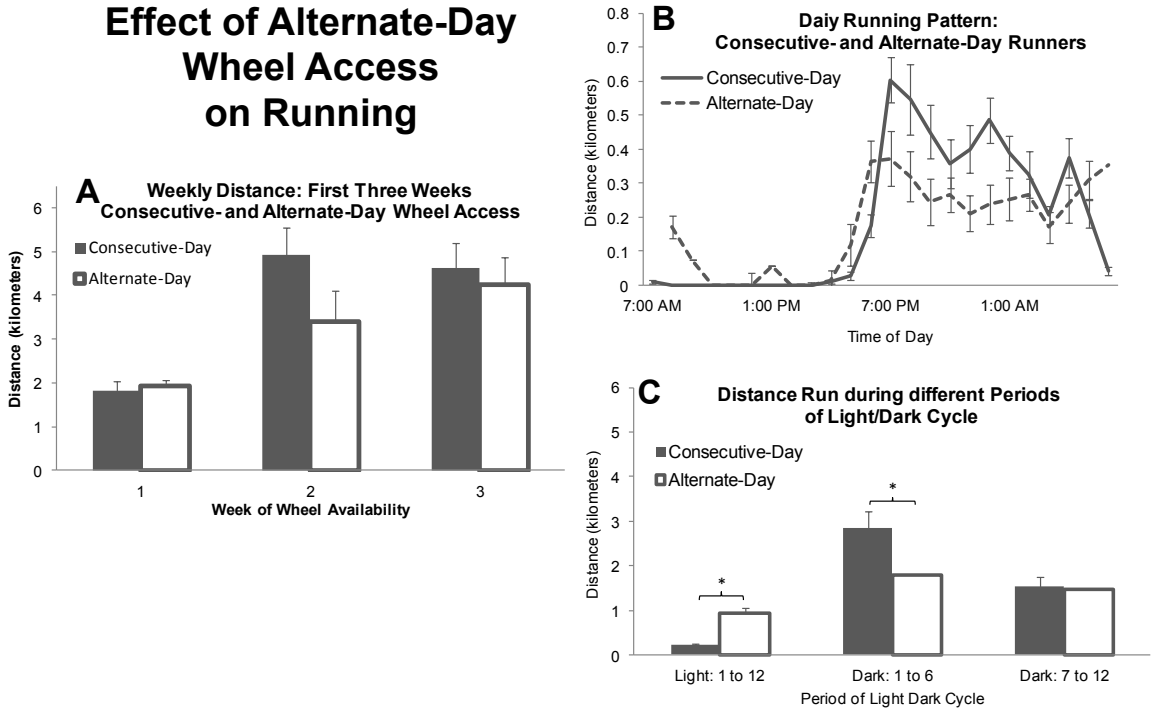

Figure 4 (A). Average (± SEM) distance (kilometers) run in a 24-hour period during the 1st, 2nd and 3rd week of wheel exposure for females given consecutive- or alternate-day wheel exposure. *Ad libitum* runners reached peak distance run by week 2 whereas alternate-day runners reached peak distance run by week 3 (week 1 to 2: $F(1,15)=11.358$, $p=0.004$; week 2 to 3: $F(1,15)=4.712$, $p=0.046$) (B). Average (± SEM) hourly distance (kilometers) run during the 24-hour light-dark cycle for females given consecutive- or alternate-day wheel exposure. Alternate-day runners ran significantly more during the first (7:00 am, $t(59.096)=11.700$, $p<0.001$) and last hour of the day (6:00 am, $t(25.418)=-5.332$, $p<0.001$) . (C). Average (± SEM) distance (kilometers) run during the light period and the 1st and 2nd 6-hour period of the dark cycle in females given consecutive- or alternate-day wheel exposure. Compared to *ad libitum* runners, alternate-day runners ran significantly more in the light period ($t(43.427)=-5.039$, $p<0.001$) and significantly less in the first six hours of the dark period ($t(50)=2.193$, $p=0.033$) .

FIGURE 5

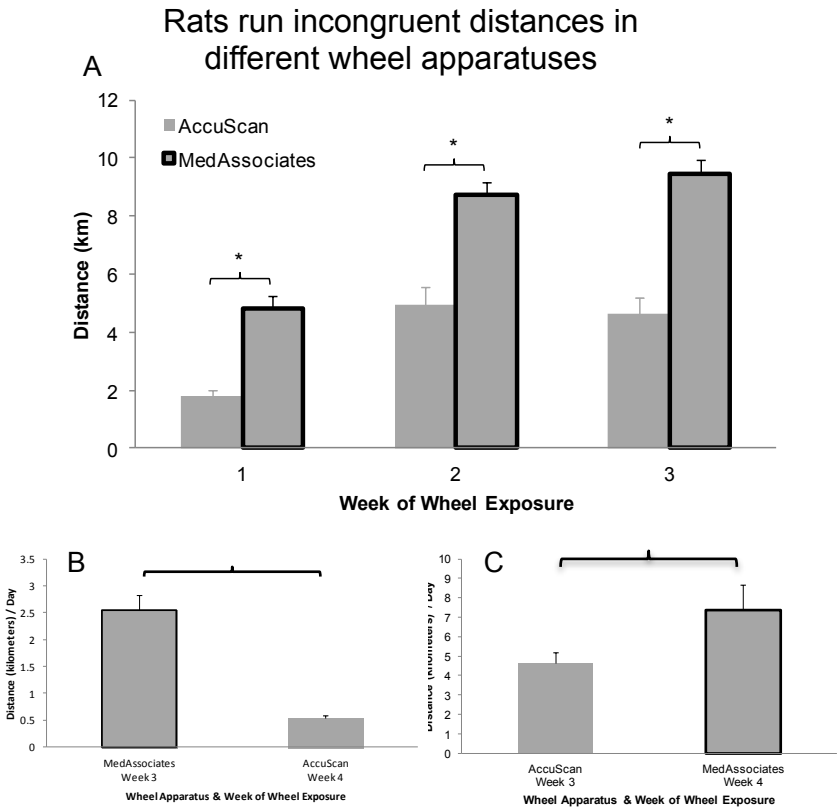

Figure 5 (A). Average (± SEM) distance (kilometers) run in a 24-hour period during the 1st, 2nd and 3rd week for females in the AccuScan or Med Associates system. (B). Average (± SEM) distance (kilometers) run by males when transferred from the Med Associates system (week 3) to the AccuScan system (week 4). (C). Average (± SEM) distance (kilometers) run by females in a 24-hour period when transferred from the AccuScan system (week 3) to the Med Associates system (week 4).

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CHAPTER 3

Voluntary lifelong activity in the rat alters monoamine content in brain regions with motor and motivational functions, monoamine responses to acute cocaine challenge, and cocaine-seeking behavior

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Elizabeth D. Abercrombie & Joan I. Morrell

Abstract

Participating in physical activity is important for physical and mental wellbeing, and engaging in exercise throughout childhood and adolescence is key to maintaining these behaviors in adulthood. Physical activity is also used as a method to help decrease cravings and enhance recovery in drug and alcohol rehabilitation facilities, but little is known about how participating in physical activity throughout lifetime affects initial drug interactions. Activity, both in the form of forced treadmill running and voluntary wheel running, has been shown to increase neurotransmitters and metabolites in discrete brain regions and to alter the incentive salience of drugs of abuse. Here we examine the neurochemical effects of lifelong voluntary activity on brain regions that function in motor and motivational circuits, and how initial exposure to a pharmacological stimulant affects active versus sedentary subjects both neurochemically and behaviorally. We utilized two groups of female Sprague Dawley rats raised from postnatal day 21 in either an environment that promoted activity or sedentary behavior. At adulthood, we tested differences in the content of dopamine, serotonin, norepinephrine and their metabolites in the caudate putamen (CP), ventral tegmental area (VTA), nucleus accumbens core (NAc) and shell (NAs), medial prefrontal cortex (mPFC), and medial preoptic area (mPOA). We also tested the neurochemical response to a low initial dose of cocaine (5.0 mg/kg), as well as the incentive salience of the low end of the dose response curve of cocaine (0.5, 1.0 and 5.0 mg/kg) using a conditioned place preference model. Compared to their sedentary counterparts, active animals displayed an increase in neurotransmitter and metabolite content in the CP, VTA, mPFC and mPOA. Although acute cocaine alone had a minimal neurochemical effect on sedentary subjects, in active subjects acute cocaine decreased neurotransmitter and metabolite content in the VTA and mPOA, while increasing certain levels in NAs. Additionally, active subjects found higher doses of cocaine to be more salient than lower doses, which was not the case for sedentary subjects, revealing an activity-induced shift in the lower end of the cocaine dose response curve. Based on cocaine plasma levels, we also found that these results were due to CNS rather than peripheral mechanisms. The present results suggest that participating in activity throughout development may alter the motivational circuitry and its responsiveness to the incentive salience of pharmacological stimuli in adulthood.

1. Introduction

Participating in physical activity throughout life is important for acquiring and sustaining physical and mental health in humans. Beginning to exercise during childhood is key to continuation of this behavior into adolescent and adult life (Telama et al., 1997; Kjonniksen et al., 2009), and the Center for Disease Control and Prevention recommends that youth, defined as ages 6 to 17 years, obtain at least 60 minutes of physical activity per day. Many drug and alcohol rehabilitation facilities incorporate exercise as a part of the daily regimen of activities to help decrease cravings and enhance recovery, and it is relevant to our focus that exercise is suggested as a preventative measure against drug and alcohol dependency (Brown et al., 2009; Buchowski et al., 2011; Haasova et al., 2012; Neale et al., 2012). Although there has been emphasis on fully developed drug dependency or addiction, it is important to recognize a potentially more tractable treatment point that is relevant to our work, the initial phase of drug use, where lower, sampling doses are used sporadically leading to potential drug dependency (Gawin, 1991). Furthermore, the number of people who engage in occasional or recreational use of substances with abuse potential, including cocaine, is much larger than the number of people who are diagnosed clinically with substance dependency or addiction (Warner et al., 1995; SAMSHA, 2001-2003; O'Brien and Anthony, 2005). The impact of physical activity in the context of initial or occasional use of drugs of abuse is therefore an important area for preclinical study, as it might be a consideration in approaches to dependency prevention in humans.

In this study, we asked whether lifelong voluntary activity rather than sedentary behavior alters baseline adult neurotransmitter content in brain regions related to motor and motivational processes. Additionally, we examined whether this activity history alters the neurotransmitter content and cocaine-seeking response to an initial cocaine challenge. To date, few preclinical studies have addressed the biological basis underlying the interaction between physical activity, particularly throughout development, and subsequent challenge with drugs of abuse. Studies using conditioned place preference (CPP) to assess the incentive salience of drugs of abuse require only a few exposures to drug, and therefore can be used to assess more acute or initial phases of drug-induced changes. The impact of activity on CPP for drugs has been assessed in rodents that participate in forced treadmill running both during adolescence and adulthood. For example, rodents that experience forced running during adolescence show a decreased CPP for cocaine (Thanos et al., 2010), and those that experience forced running at adulthood show a decreased CPP for methamphetamine, with longer periods of forced running corresponding to a lower CPP (Chen et al., 2008).

A marked disadvantage of forced exercise is that it is a known stressor in rodents (Moraska et al., 2000; Brown et al., 2007), and so others have used voluntary wheel running to avoid this complication. Voluntary wheel running in rodents has been suggested as an animal model for voluntary exercise in humans (Eikelboom, 1999) and is known to have a myriad of positive effects on the body, brain and behavior of rodents, including decreased anxiety and

depression, improved attention, and enhanced learning and memory (Hoffmann et al., 1987; Dishman et al., 1996; Sherwin et al., 1998; Solberg et al., 1999; Greenwood et al., 2003; 2005; Binder et al., 2004; Bjornebekk et al., 2005; van Praag et al., 2005; 2008; Kronenberg et al., 2006; Duman et al., 2008; Hopkins et al., 2009; Robinson et al., 2011). Additional beneficial effects of activity can be seen in rodents raised in enriched environments with a wheel since they show a decreased CPP for a variety of drugs of abuse, including cocaine and opioids (Xu et al., 2007; El Rawas et al., 2009; Solinas et al., 2009). While the majority of the preclinical studies examining the initial phases of drug responses suggest that increased activity in rodents is associated with lowered preference for drugs of abuse, one study reports an increased CPP for some doses of cocaine in rats exposed to a running wheel throughout rearing (Smith et al., 2008).

The effect of activity on the chronic phases of drug challenge has also been examined using models of advanced drug dependency. Both voluntary wheel running and forced treadmill running, either throughout adolescence or during adulthood, have been shown to decrease self-administration of certain drugs of abuse such as cocaine and morphine (Cosgrove et al., 2002; Hosseini et al., 2009), with higher runners showing lower breakpoints (Smith et al., 2008). Physical activity has also been shown to affect drug consumption such that when rats are concomitantly given the opportunity to engage in voluntary wheel running and consume substances, oral alcohol and amphetamine consumption decrease (Kanarek et al., 1995; Ehringer et al., 2009).

Along with impacts on behavioral responses to drugs of abuse, researchers have examined the impact of activity on monoamine content levels in the brain. For example, forced treadmill running during adulthood increases monoamine levels in specific brain regions (Hattori et al., 1994; Meeusen et al., 1997; Hasegawa et al., 2000), with a positive correlation seen between neurotransmitter/metabolite levels and speed of the treadmill (Freed & Yamamoto, 1985; Hattori et al., 1994). Additionally, both voluntary wheel and treadmill running during adulthood induces significant changes in monoamine systems (De Castro and Duncan, 1985; Samorajski et al., 1987; Wilson & Marsden, 1995; Dunn et al., 1996). A few studies have demonstrated altered monoamine content of the brain (i.e., primarily decreases) in response to drugs of abuse, even with acute challenges (Einhorn, 1988; Festa et al., 2004). In a rare examination of neurotransmitters levels in active subjects that are drug challenged, exercised rats show lower immediate (30 minutes after drug challenge) extracellular levels of dopamine to amphetamine challenge but higher subsequent (6 hours after drug challenge) levels of extracellular dopamine compared to their sedentary counterparts, suggesting that long-term forced exercise leads to decreased release and reuptake of dopamine (Marques et al., 2008).

The present work compared two groups of rats, active subjects, which were offered an environment that induced voluntary activity, and sedentary animals, which were housed in standard cages with no activity offered. Both groups were subsequently tested to determine how they responded to an acute

dose of cocaine, a pharmacological stimulus with known incentive salience that blocks reuptakes of monoamines leaving excess neurotransmitter within the synapse. We hypothesized that these active rats would have altered content of neurotransmitters and their metabolites in brain regions involved in motor and motivational processes. Further, we hypothesized that these activity-induced differences would provide a basis for an altered response to an acute cocaine challenge both at the neurotransmitter level and with regard to the incentive salience of the cocaine stimulus. We also hypothesized that the activity-induced cocaine response alterations would be due to changes in the CNS rather than peripheral changes in cocaine metabolism.

We chose the postmortem analysis of tissue punches to provide regional content of the monoamine family using high-performance liquid chromatography (HPLC), a method with which we have prior experience (Olazábal et al., 2004). This enabled us to get a picture of the simultaneous changes that occur with these experimental conditions in multiple regions of key importance in motor as well as motivational pathways. Because we wanted to maximize the lifetime experience differences between the activity and sedentary conditions, we formed the groups as early as possible, the day of weaning (postnatal day (PND) 21). Our prior work (Smith and Morrell, 2007) demonstrates that rats can wheel run competently and do so robustly in the type of wheels used in this experiment (PND 18-19) (Smith and Morrell, 2007), and furthermore, that young animals respond with increased activity to cagemate conspecifics and all forms of objects in the home cage (Smith and Morrell, 2011). We used a low dose acute cocaine

exposure, proposed to model that of sampling or initiation doses from the human condition (Morrell et al., 2011), and we used a motivational test, conditioned place preference, which is commonly interpreted as a measure of a stimulus seeking response and with which our laboratory has considerable experience (Mattson et al., 2003; Tzschentke, 2007; Seip et al., 2008).

2. Method

2.1 Subjects

Data were collected from treatment groups described below. Female Sprague Dawley rats (original stock from Charles River Laboratories, Kingston, NY, USA) were bred in our colony at the Rutgers University Laboratory Animal Facility (RAF) (Newark, NJ, USA) (accredited by the American Association for Accreditation of Laboratory Animal Care). All animals were kept on a 12-hour light-dark cycle (lights on at 7:00 am) in a room at 22(±1)°C and given *ad libitum* access to water and rat chow (Lab Diet 5008, PMI Nutrition International, LLC, Brentwood, MO, USA). Daily checks were conducted for health and availability of food and water; weights were taken and animal husbandry performed twice per week. All remained healthy and of normal body weight throughout the experiments. Animal care and experimental procedures performed in this protocol were in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were reviewed and approved by the Rutgers University Animal Care and Facilities Committee. Care was taken to minimize the suffering and curtail the number of animals used.

2.2 Housing environments

At the day of weaning (PND 21), females were placed in either an activity-inducing environment (translucent boxes [40 cm long x 40 cm wide x 30 cm high], providing wheel and objects, and 4 cagemates) (n=12) or a sedentary environment (opaque cages [47 cm long x 25.5 cm wide x 23 cm high], no wheel, no objects, and two cagemates to avoid the stress of single housing) (n=16). Objects provided were either a cardboard tube or a crawl ball and manzanite sticks (Animal Specialties and Provisions, LLC, Quakertown, PA).

We previously demonstrated (Basso and Morrell, 2010) that regardless of whether females were raised in the active housing condition, as used in this work, or were only given access to wheels (no cagemates, no objects) at adulthood (PND 65), they displayed similar distances, rates, and patterns of running. Both groups achieved stabilized daily distances run of 4 to 8 kilometers per day in adulthood, as determined using the AccuScan running apparatus (AccuScan Instruments [Columbus, OH, USA] VersaMax Animal Activity Monitor [wheel: 25-cm diameter, stainless steel mesh floor; home cage: 40 cm long x 40 cm wide x 30 cm wide]) (Basso and Morrell, 2010). Running capacity of the youngest subjects (PND 21) was likely dependent upon the mesh floor of these wheels versus the more common bar flooring of other wheel products. Robust interaction with the objects was evidenced through observation and their destruction by chewing; robust cagemate interactions were also observed in both group-housed animals.

All animals remained in their housing environment until ~PND65. At this time, all animals were returned to standard, home cage environments (2 per cage). To avoid confounds of acute exercise, they remained there for 72 hours prior to the following procedure.

2.3 Procedure

2.3.1 Analysis of brain tissue via HPLC

Removing the brain: At ~PND 68, rats were given an intraperitoneal (IP) dosage of 5.0 mg/kg cocaine HCl or an equivalent amount of 0.9% saline and individually placed in shoebox cages. Thirty minutes later, rats were sacrificed with a guillotine (Harvard Apparatus), the brain quickly removed, placed in a chilled stainless steel brain blocker, and cross-sectioned into blocks (7-10mm) with chilled razor blades. These blocks were rapidly frozen in powdered dry ice and subsequently stored in a -80° C freezer.

Punching and homogenizing: Brains were removed from the -80° C freezer and were equilibrated at -20° C overnight. Brain samples were then mounted onto the cryostat's specimen holders with water and dry ice and placed in the -13° C microtome. For sampling of particular subregions, 300 micron cryostat sections were prepared according to the locations specified and procedures described in Palkovits and Brownstein (1988). As soon as the sections were made, all punches (using the Tel Pella, Inc. [Redding, CA] Harris Uni-Core™ punch tools - 0.5, 1.0 and 2.0 mm diameter) were taken bilaterally directly on the microtome knife. After the tissue was punched, it was placed in a chilled Eppendorf tube, which had been previously weighed. All tools that were used in the process of

punching (punches, paint brushes, slides, and Eppendorfs) were kept frozen in the microtome so as to allow the tissue to transfer easily into the Eppendorf. Samples for the medial prefrontal cortex were taken from sections at ~A3000 μm from bregma; three 0.5 mm punches were taken bilaterally along the medial cortical boarders. Samples for the nucleus accumbens were taken from sections at ~A2100 μm from bregma. One bilateral 0.5 mm punch was taken of the nucleus accumbens core, and one bilateral 1.0 mm punch was taken of the nucleus accumbens shell. The caudate putamen was sampled bilaterally with a 2.0 mm punch at ~A1800 μm from bregma. The medial preoptic area was sampled at ~0 μm from bregma with a 1.0 mm punch that was positioned over the midline. The ventral tegmental area was sampled at ~P5400 μm from bregma. It was punched one time with the 1.0 mm tool positioned over the midline. After each area of interest was punched for neurotransmitter sampling, each brain slice was mounted on a subbed Fisher Scientific (Pittsburgh, PA) premium microscope slide and saved for imaging at a later point. Immediately after punching, Eppendorf tubes were placed onto dry ice and then weighed again. Brain tissue was then homogenized using a 0.1 N $HCLO_4$ and 100 μM EDTA homogenizing solution in a concentration of 20 μL solution per mg of brain punch. All tubes were then placed back on dry ice and samples were stored in the -80° C freezer. To validate location, punched slices were later stained and examined with a microprojector and microscope by two separate observers naïve to results.

High-Performance Liquid Chromatography: We utilized HPLC to analyze tissue levels of the catecholamine neurotransmitters, norepinephrine (NE) and dopamine (DA), and their metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the monoamine neurotransmitter, serotonin (5-HT) and its metabolite, 5-Hydroxyindoleacetic acid (5-HIAA). Before analysis of our samples, 20 µl of a standard solution, containing a known concentration of each neurotransmitter and metabolite, was injected into the ESA, Inc. (Chelmsford, MA) Coulochem® II HPLC machine with electrochemical detection (Antec-Leyden, VT-03 flow cell). The potential of the working electrode was +600 mV. A reverse-phase column (Varian, Brownlee, RP-18, Velosep, 3 µm, C18, 100 Å) was used, and a filtered mobile phase containing 3.3 ml of a 0.4 M sodium octyl sulfate, 1 ml of 0.1 M EDTA, 75 ml of MeOH, and NaAc 13.61 g (pH=4.2) was pumped (LC-10AD VP Shimadzu) at a flow rate of 0.7 ml per minute. After processing of the standard solution, experimental samples were removed from the -80° C freezer, placed in dry ice, and quickly melted by hand. They were then centrifuged at 15,000 rpm for 20 minutes. The supernatant (20 µl) was then pipetted and injected as above. All neurotransmitters and metabolites were identified by the retention time and quantified by calculating the area under the particular curve associated with the substance eluting off the column. These calculations were based in relation to the standard sample. All data was captured and analyzed using eDAQ Pty Ltd. PowerChrom® 280 hardware and its associated software.

All further details of the methods and analysis of brain tissue via HPLC are as in Olazábal et al. (2004), including the statistical analyses used.

2.3.2 Conditioned place preference (CPP)

This procedure was adapted from our prior work using CPP methodology designed to examine the incentive salience of pharmacological and natural stimuli (Mattson et al. 2001; 2003; Seip and Morrell, 2007). These publications include the rationale for and further methodological details for our use of CPP, including extensive coverage of the statistical analyses particular to these methods. Subjects raised in either an active (n=47) or sedentary (n=56) environment were used in adulthood (~PND 65).

Apparatus: The Med Associates three-chambered CPP apparatus consisted of three Plexiglas chambers of the same size, each uniquely cue-decorated. The center chamber acted as a passageway as well as a neutral space that prevented post-conditioning preferences based on forced choice (Seip and Morrell, 2007). Infrared beams lined each of the chambers, and beam breaks from the rats' bodies recorded the animals' time in each chamber. This data was collected via a computer interface with the MED-PC® Version IV Research Control & Data Acquisition System.

Pre-conditioning baseline: Each animal was placed in the center chamber and was allowed to roam freely between the three chambers for 60 minutes. Time spent in each chamber was recorded automatically. Pre-conditioning sessions were prior to drug exposure.

Conditioning sessions: For all conditioning sessions, animals were injected with either saline or cocaine and isolated in one of the side chambers for 30 minutes by lowering the guillotine doors. Saline was always administered in the morning conditioning session, whereas cocaine was always administered in the afternoon conditioning session.

Conditioning began the day following the pre-conditioning session. At ~10:00 am, subjects were given an IP injection of saline and placed in their most-preferred side chamber and allowed to remain there for 30 minutes after which they were returned to home cages. At ~1:00 pm, subjects were given an IP injection of 0.5 mg/kg (wheel n=27; no wheel n=32), 1.0 mg/kg (wheel n=12; no wheel n=16), or 5.0 mg/kg cocaine (wheel n=8; no wheel n=24) and placed in their least-preferred side chamber, allowed to remain there for 30 minutes, and subsequently returned to home cages. This process was repeated for a total of four conditioning days, with four saline conditioning sessions and four cocaine conditioning sessions.

Post-conditioning test of CPP: The day after the fourth conditioning day, animals were exposed to a 60 minute post-conditioning (CPP test) session, which was conducted free of any stimulus. Subjects were placed in the center chamber and allowed to roam freely between the three chambers for 60 minutes. Time spent in each chamber was recorded automatically.

Cocaine: Cocaine hydrochloride in a highly purified powdered form was obtained from the National Institute of Drug Abuse (Research Triangle Park, NC, USA). Before IP injection, cocaine HCl was placed into 0.9% saline to obtain a 1.0 or

4.5 mg/mL solution. A 1.0 mg/mL solution was made if the subsequent injection included the 0.5 mg/kg dosage. Subsequently, 0.5, 1.0 or 5.0 mg/kg cocaine HCl was injected into the rat intraperitoneally, with each of these doses representing independent groups.

Analyses and chamber assignments: Data was analyzed using two techniques, individual chamber preference and group chamber time. Individual chamber preference was calculated by examining the time spent in each chamber during the pre- and post-conditioning sessions (Mattson et al., 2001; 2003). Group chamber times were calculated by averaging across animals, the time spent in each chamber. Together, these two measures were used to understand the preference of each individual animal as well as the group preference as a whole.

Briefly, a stringent previously developed quantitative criterion was used to categorize individuals as having a particular preference (Mattson et al., 2001; 2003). The criterion to be categorized as having a chamber preference was that the individual had to spend ≥30 minutes in one chamber, and this time also had to be ≥25% larger than that of the second greatest chamber time. If these two criteria were not met, the animal was categorized as showing no preference. Four preference categories were possible (left, center, right, or no preference). After naïve individual chamber preference from the pre-conditioning session was established, animals were assigned to receive cocaine in their least-preferred side chamber. If an animal showed a preference for the center or showed no preference, then cocaine was assigned to one of the side chambers at random.

2.3.3 Analysis of plasma levels of cocaine At adulthood (~PND 65) sedentary (n=8) and active (n=12) females were injected with 5.0 mg/kg cocaine intraperitoneally. Cardiac blood was collected 15, 30 or 60 minutes after cocaine injection (sedentary 15 (n=2), 30 (n=3), 60 (n=30); active 15 (n=4), 30 (n=4), 60 (n=4)). All technical aspects of blood sampling, extraction of plasma, and shipping of samples were carried out as in our prior published work (Wansaw et al., 2005). When all samples were collected, they were packed in dry ice and sent to the Center for Human Toxicology at the University of Utah for processing by Dr. David Moody. Plasma samples were analyzed via liquid chromatography – tandem mass spectrometry for cocaine and its main metabolites, benzoylecgonine, ecgonine methyl ester, and norcocaine as previously described by Lin et al. (2001), including the methods of statistical analysis. All drug and metabolite concentrations are reported in ng/mL.

2.4 General methods of statistical analysis Most statistical analyses were conducted using the computer software IBM® SPSS® 21.0 (Chicago, IL, USA). A significance value of $p \leq 0.05$ was used for all statistical analyses. For data that met the requirements of parametric statistical tests, in general, analysis of variance was used followed by appropriate post-hoc testing including the independent samples t-test. If data did not meet normality (t-test) or sphericity (repeated measures ANOVA), corrections such as Greenhouse-Geisser (repeated measures ANOVA) were used. For categorical data, non-parametric tests were used including a Fisher's exact test for within groups or a one-tailed test for significance of proportions between groups. Further details of the

statistical methods used for data yielded by each type of data can be found in the references to our prior work cited in each methods section above.

3. Results

3.1 Alterations in total neurotransmitter content between females raised in active or sedentary conditions in brain regions involved in motivation and voluntary movement and the effects of a low dose of cocaine

Table I provides concentrations of all neurotransmitters and their metabolites by region. All values that reach statistical significance are noted in bold and provide demonstration of the effects of the lifelong opportunity to engage in activity, an acute dose of cocaine at adulthood or their synergy. The sections below discuss the statistically significant changes. Selected points are illustrated with graphs.

Caudate Putamen (CP): In the CP, active subjects had increased total levels of DA (sedentary:active; t(11)-4.486, p=0.001) and its two metabolites, DOPAC (trend, t(11)=-2.154, p=0.054) and HVA (t(11)=-3.092, p=0.010), as well as NE (t(5.849)=-2.698, p=0.0370) (Table 1; Figure 1). Regardless of whether animals were raised with or without a wheel, cocaine had no effect on total content levels in the CP (sedentary:sedentary+cocaine; active:active+cocaine). One difference to the acute cocaine response was found between the sedentary and active groups, in that one metabolite, HVA, was greater for active compared to sedentary subjects (sedentary+cocaine:active+cocaine; t(12)=-2.391, p=0.034). Further, in the case of a comparison between animals that received only one of the two stimuli, active subjects showed greater total content levels of DA and its metabolites compared to sedentary animals administered an acute dose of

cocaine at adulthood (active:sedentary+cocaine; DA trend t(12)=2.106, p=0.057; DOPAC t(12)=2.232, p=0.045; HVA t(12)=4.068, p=0.002).

Ventral Tegmental Area (VTA): In the VTA, lifelong activity had a minimal impact on total neurotransmitter or metabolite content, in that only total DOPAC content was significantly higher in active subjects versus their sedentary counterparts (Table 1; sedentary:active; t(12)=-2.453, p=0.030).

In sedentary animals, acute administration of cocaine at adulthood had no effect on total neurotransmitter or metabolite content (Figure 2B; sedentary:sedentary+cocaine;). However, in active subjects, cocaine lowered all transmitter and metabolite levels, reaching statistical significance in the case of DA metabolites and NE (Figure 2A; active:active+cocaine; DOPAC t(10)=2.466, p=0.033, HVA t(5.000)=3.026, p=0.029, and NE (t(10)=2.527, p=0.030). Further, when the acute cocaine response was compared across sedentary and active groups, neurotransmitter and metabolite content in the VTA was lower in active subjects compared to their sedentary controls (sedentary+cocaine:active+cocaine; HVA t(7.000)=2.360, p=0.050; 5-HIAA t(8.275)=2.920, p=0.019; NE t(12)=4.369, p=0.001). Additionally, animals that received both stimuli (i.e., were active and were given an acute dose of cocaine) showed lower total neurotransmitter and metabolite content compared to stimulus naïve animals (sedentary:active+cocaine; HVA t(7.000)=4.176, p=0.004; 5-HT (t(12)=3.831, p=0.002; NE t(12)=4.084, p=0.002).

Nucleus accumbens core (NAc): In the NAc, neither activity nor an acute dose of cocaine at adulthood had an effect on total neurotransmitter or metabolite

content (Table 1; sedentary:active; sedentary:sedentary+cocaine; active:active+cocaine). When the acute cocaine response was compared between active and sedentary groups, only one difference was seen in that 5-HIAA content in the NAc was lower in the active subjects (sedentary+cocaine:active+cocaine $t(11)=2.599$, $p=0.025$).

Nucleus Accumbens shell (NAs): In the NAs, active subjects were not different from sedentary subjects in their neurotransmitter or metabolite content (Table 1; sedentary:active). An acute dose of cocaine at adulthood in sedentary animals had no effect (Figure 3B; sedentary:sedentary+cocaine). However in active subjects, total 5-HT content was greater after cocaine administration (Figure 3A; active:active+cocaine; $t(10)=-2.203$, $p=0.052$). Cocaine challenge revealed that DA content in the NAs was higher in active compared to sedentary subjects (Figure 3A; sedentary+cocaine:active+cocaine $t(12)=-2.171$, $p=0.051$).

Medial prefrontal cortex (mPFC): In the mPFC, active subjects had a minimal impact on total neurotransmitter or metabolite content. The only difference was in one metabolite, 5-HIAA content, which was significantly higher in active compared to sedentary subjects (Table I; sedentary:active; $t(12)=-2.305$, $p=0.040$). Acute administration of cocaine at adulthood had no effect on total neurotransmitter or metabolite content (sedentary:sedentary+cocaine; active:active+cocaine).

Medial preoptic area (mPOA): In the mPOA, active subjects had decreased levels of DA (sedentary:active; $t(10)=2.934$, $p=0.015$), but increased levels of DOPAC ($t(10)=-3.158$, $p=0.010$) (Table 1). In sedentary animals, acute cocaine

administration at adulthood decreased DA (sedentary:sedentary+cocaine; t(12)=3.153, p=0.008); however, in active subjects, acute cocaine administration at adulthood decreased DOPAC (active:active+cocaine; t(10)=2.438, p=0.035), 5-HT (t(10)=2.570, p=0.028) and NE (t(10)=2.967, p=0.014) (Figure 4). Animals that received both stimuli (i.e., were active and were given an acute dose of cocaine) had lower total DA content as compared to stimulus naïve animals (sedentary:active+cocaine; t(10)=2.880, p=0.016). In the case of a comparison between animals that received only one of the two stimuli, sedentary animals administered an acute dose of cocaine at adulthood showed lower total NE content as compared to active subjects (active:sedentary+cocaine t(12)=2.456, p=0.030).

3.2 Peripheral metabolism of cocaine in lifelong active versus sedentary animals

No differences were found in the plasma concentration of cocaine or any of its metabolites at the three time points sampled, except for benzoylecgonine at one time point (Figure 5). The plasma concentration of benzoylecgonine 15 minutes after cocaine injection was significantly higher in females with a history of lifelong activity as compared to their sedentary counterparts (t(4)=-3.173, p=0.034). Of particular interest, no trends or statistically significant differences were found in the active versus sedentary groups at the 30-minute time point, as this is the conditioning time for the place preference tests (Figure 5).

3.3 Preference for a cocaine-associated chamber in lifelong active versus sedentary animals

Preference for a cocaine-associated chamber (0.5, 1.0 and 5.0 mg/kg in independent groups) was examined in females raised in active or sedentary conditions. To determine if animals were conditioned during CPP training and if they developed a conditioned place preference for the chamber associated with cocaine, chamber preference and group time for the animals in each independent group were compared before and after conditioning.

0.5 mg/kg intraperitoneal cocaine: Females raised in sedentary conditions showed successful conditioning in terms of both individual preference ($p=0.004$) and group chamber time ($F(2,62)=3.670$, $p=0.031$), and spent significantly more time in the chamber associated with 0.5 mg/kg cocaine at the post-conditioning session as compared to the pre-conditioning session ($F(1,31)=5.106$, $p=0.031$). This was not the case for females with a lifelong history of activity as they did not show a conditioning effect for this dose of cocaine.

1.0 mg/kg intraperitoneal cocaine: Females raised in sedentary conditions showed a conditioning effect in terms of both individual preference ($p=0.012$) and group chamber time ($F(2,30)=6.174$, $p=0.006$), with females spending significantly more time in the chamber associated with 1.0 mg/kg cocaine at the post-conditioning session as compared to the pre-conditioning session ($F(1,15)=7.685$, $p=0.014$). Females with a lifelong history of activity showed a conditioning effect only in terms of group chamber time ($F(2,22)=4.925$, $p=0.017$); however, as a group, they did not spend significantly more time in the cocaine-associated chamber at the post-conditioning session compared to the pre-conditioning session.

5.0 mg/kg intraperitoneal cocaine: Females raised in sedentary conditions did not show a conditioning effect for a dosage of 5.0 mg/kg cocaine; however, females raised with a wheel showed a strong conditioning effect (individual preference $p=0.001$; group chamber time $F(2,14)=34.943$, $p<0.001$) and spent significantly more time in the chamber associated with 5.0 mg/kg cocaine at the post-conditioning session as compared to the pre-conditioning session ($F(1,7)=39.748$, $p<0.001$).

4. Discussion

Overall our findings demonstrate that subjects with a history of activity from early life have altered neurotransmitter and metabolite content primarily in the CP, with some effects in the VTA, mPFC, and mPOA, as adults. On this background of activity-induced effects, acute challenge with cocaine reveals further differences in neurotransmitter and metabolite content in the NA, the VTA and the mPOA in active compared to sedentary subjects (see summary Table 3). Furthermore the CPP data demonstrate that active subjects have altered cocaine-seeking responses compared to sedentary subjects. While it is true that there is one significant increase in the level of benzoylecgonine, a bioactive metabolite of cocaine (Schuelke et al., 1996), at one time point, the bulk of the data show that plasma levels of cocaine and metabolites are virtually identical in active compared to sedentary subjects. Thus, these data rule out the possibility that these CNS and cocaine-seeking behavioral differences are due to the effects of the activity experience on peripheral cocaine metabolism, for example, by the liver. Rather, this information suggests that equal amounts of cocaine are

available to the CNS in both sedentary and active subjects, which then respond differently dependent upon the underlying effects of activity or its absence within the CNS.

The data on regional differences in the content of neurotransmitters and metabolites across conditions are generally congruent with what is known about the anatomy of these brain regions, as dopaminergic projections from the VTA and substantia nigra pars compacta (SNc), noradrenergic projections from the locus coeruleus, and serotonergic projections from the dorsal raphe are all found to these regions in different densities. As to functions of these areas, the caudate putamen is well understood as a region important in regulation of movement (Rothwell, 2011), and data suggest a role in reward seeking for the dorsal striatum (Balleine, et al 2007). Additionally, the participation of the mPFC, NA, and VTA in motivated responses, particularly effortful aspects of motivated processes, is well established (Berridge, 2007; Salamone et al., 2007).

Activity effects on neurotransmitter content Overall our findings show notable increases in dopamine, norepinephrine, and their metabolites in active over sedentary subjects in the CP, VTA, mPOA, and a single increase of a serotonin metabolite in the mPFC. To our knowledge, no studies have examined the effect of voluntary activity from pre-pubertal age to adulthood, but others have examined the effects of long-term activity in adults. In general, our data accord with the findings of others that compared active to sedentary groups and showed increases in neurotransmitter content, particularly increases in dopamine and norepinephrine. For example long-term voluntary wheel running at adulthood increases total whole

brain content of dopamine (De Castro and Duncan, 1985) and total norepinephrine content in the whole brain, cortex, midbrain, pons medulla and spinal cord (Brown and Van Huss, 1973; Dunn et al., 1996). Furthermore, rats that voluntarily run in a wheel for extended periods of time during adulthood show significant decreases in total brain dopamine receptor densities (De Castro and Duncan, 1985), which may be viewed as an expected corollary of increased DA content.

Other prior studies show that even forced treadmill running increases total as well as synaptic amounts of dopamine, norepinephrine, serotonin, and their metabolites (see Chaouloff, 1989 and Meeusen et al., 2001 for review). These increases are found in the frontal cortex, striatum, nucleus accumbens, hippocampus, hypothalamus, midbrain, brain stem, and spinal cord; however, some variations are reported. Acute forced treadmill running (20-60 minutes) during adulthood increases both total and extracellular levels of dopamine, serotonin, norepinephrine and their metabolites in the dorsal and ventral striatum and hippocampus (Hattori et al., 1994; Wilson and Marsden, 1995; 1996; Meeusen et al., 1997; Hasegawa et al., 2000; Goekint et al., 2011), with a positive correlation in the ventral striatum seen between neurotransmitter/metabolite content and speed of the treadmill (Freed & Yamamoto, 1985; Hattori et al., 1994). Additionally, long-term forced treadmill running has been shown to increase total norepinephrine concentration in the pons and spinal cord and increase total metabolite content in the frontal cortex, hippocampus, and pons (Dunn et al., 1996).

Additional evidence to suggest that the monoamine system is involved, altered and/or regulated by voluntary wheel running comes from different genetic lines of mice that display different patterns of running in terms of daily distance, duration, and speed (Lightfoot et al., 2004; Knab et al., 2009). These various inbred strains of mice are known to differ in dopaminergic anatomy of the midbrain as well as expression of tyrosine hydroxylase (TH) and D1 receptors (high running mice show lower TH and D1 gene expression), and Knab and Lightfoot (2010) suggest that the voluntary running differences may be due in part to the differences in the dopamine system between these inbred lines. Additionally, a high-running line of mice (hyperactive) that have been bred for over 31 generations show increased levels of total baseline dopamine, norepinephrine, and metabolites in the NA and, dopamine, serotonin, and metabolites in the CP (Mathes et al., 2010). Upon administration of dopaminergic agonists and antagonists, these various genetic lines of mice show differential responses in terms of their voluntary wheel running behavior (Rhodes et al., 2001; 2003). Further, mice categorized as low- versus high-runners within a particular strain show different dopaminergic drug-induced running responses, with low active mice actually running more in response to dopaminergic agonists (Schumacher et al., 1994), most likely because increased activity levels correlate with decreased dopaminergic functioning (Knab et al., 2009).

Together with our work, these prior studies conclude that physical activity, whether in the form of forced treadmill running or voluntary wheel running increases both synthesis and turnover of the monoamine neurotransmitters,

dopamine, norepinephrine and serotonin, in a variety of discrete brain regions. The present study adds support to this view, additionally suggesting that an environment that stimulates activity throughout rearing increases monoamine neurotransmitter synthesis and turnover in brain regions associated with mediating movement and motivation.

Activity induced changes of neurotransmitter content revealed by response to cocaine challenge Animals raised with the opportunity to engage in physical activity also have an altered monoaminergic response to an acute cocaine challenge. Specifically, in the VTA, nucleus accumbens core, and mPOA, there is a decrease in monoamine content, whereas in the nucleus accumbens shell, both serotonin and dopamine increase. This is the case whether the comparison is to active subjects without cocaine or sedentary subjects that have had an acute dose of cocaine. These data suggest a pattern of system-wide alterations in monoamine content in motivationally related areas, which may be the basis for the behavioral differences seen with the CPP tests for the incentive salience of cocaine. The data demonstrate that no simple one-to-one pattern of changes are found across regions that have diverse roles in motivational processes. Additionally, the data provide an overview of changes within a network of regions that might be individually examined in future microdialysis studies to give a more dynamic picture.

Few other studies on the impact of drugs on neurotransmitter levels in the brains of active versus sedentary rats have been conducted. The work that has been done also shows a general pattern of neurotransmitter decrease in response

to drug. After a long-term regime of forced treadmill exercise, subjects show lower immediate extracellular levels of dopamine but higher continuous levels of extracellular dopamine compared to their sedentary counterparts, suggesting that activity leads to decreased release and reuptake of dopamine (Marques et al., 2008).

To provide perspective on how acute cocaine alters the brains of active subjects, it is helpful to consider how acute cocaine alters neurotransmitters in the brain of sedentary animals. Much of the literature focuses on neurotransmitter and receptor changes induced by chronic cocaine exposure, and very little on the changes that can be seen with acute cocaine. While our work showed only one change, a decrease in the neurotransmitter content of the mPOA, others who used a significantly higher dose of cocaine found increases in dopamine in the CP and decreases in levels of dopamine, serotonin and their metabolites in the NA of female rats (Festa et al., 2004). Previous research has also shown that cocaine reduces dopamine release in the NA (Einhorn, 1988). Taken together with our findings, these data suggest that long-term activity alters the cocaine-response set point in these brain regions. We suggest that the higher doses of cocaine reveal that motivationally-related regions are very responsive, even in the simple case of a single acute drug challege. This implies that initial responses to a drug challenge may be capable of initiating events of significance in motivational pathways.

mPOA responses are novel but not entirely unexpected Our data demonstrate that active subjects had decreased DA and increased DOPAC in the mPOA. In an effect generally similar to the response of cocaine challenge in the NAc and VTA,

cocaine challenge in active subjects decreased norepinephrine, serotonin and DOPAC in the mPOA. This region, rich with androgen and estrogen receptors, is very well known to be involved in the motivational aspects of reproductive behaviors, like the expression of sexual behavior in males and maternal behavior in females (Sachs and Meisel,1988; Lonstein and Morrell, 2007; Paredes, 2009; Pereira & Morrell, 2011). Less well known is a role for the mPOA in estrogen-induced voluntary wheel running and it's likely significance in running alterations in distance run across the normal female estrus cycle (Fahrbach et al., 1985; Basso and Morrell, 2010; Spiteri et al., 2012).

Prior studies have shown that brief periods of forced treadmill running increase extracellular levels of DA, DOPAC and HVA in the mPOA and anterior hypothalamus, a very closely related region (Hull et al., 1995; Hasegawa et al., 2000). Additionally, long-term voluntary wheel running in adulthood has been shown to increase total norepinephrine concentration in the nearby ventral hypothalamus (Samorajski et al., 1987).

Both the mPOA and the VTA have reciprocal connections with the NAs, which also shows altered responses to cocaine challege in rats with a history of activity. Evidence suggests that the NAs is involved in the incentive salience of both pharmacological and natural stimuli, and that responses to these stimuli increase extracellular dopamine in this region (Di Chiara and Bassareo, 2007). Possibly these three structures have a role in the altered incentive salience for cocaine seen in active rats.

Activity-induced changes in behavioral responses to sampling or challenge exposure to cocaine Our preclinical cocaine-seeking model focuses on the processes of motivated choices during the initial or acute stages of cocaine exposure, an aspect of cocaine exposure that is generally less studied in behavioral examinations of the impact of cocaine in the rat. Our findings here suggest that the lowest levels of cocaine are not salient in active females, but are in sedentary ones, and further, remarkably, that a history of activity boosts the salience of the somewhat higher dose of cocaine. Using very low and very few doses of cocaine, we have shown that compared to their sedentary counterparts, subjects with an active history find the lowest doses of cocaine (0.5 and 1.0 mg/kg, plasma levels 25-40 ng/ml) to have less incentive salience and the higher but still remarkably low dose of 5.0 mg/kg (plasma level 120 ng/ml) to have more incentive salience. Thus, it appears that the dose response curve of the incentive salience of low, sampling doses of cocaine is shifted toward the right or higher dose in active subjects. These findings generally accord with our prior extensive work with postpartum female rats showing that the dose response curve of the salience of cocaine in a CPP test varies in a parabolic dose-response curve across a plasma level of 25-400 ng/ml of cocaine, and that CPP can be established with only 2 to 4 cocaine exposures (Seip et al, 2008; Morrell et al., 2011). This shift in the dose response curve of cocaine salience in active subjects may be related to the altered cocaine-induced monoamine response in motivationally related brain regions in these active animals compared to their sedentary counterparts. This is the first study to report that voluntary wheel running throughout rearing alters the

conditioned place preference for the lower, sampling doses of the psychostimulant, cocaine, and that a shift in the dose response curve across these doses appears to be at work.

Studies testing the effect of only one or two drug doses in active rodents report report a decreased conditioned place preference for a variety of drugs of abuse including cocaine, morphine, and heroin (Xu et al., 2007; El Rawas et al., 2009; Solinas et al., 2009). Smith et al. (2008) were the only other group to investigate the effect of voluntary wheel running throughout rearing on a cocaine CPP. Their voluntary running wheel protocol was almost exact to the one utilized in the present work, except that rats were housed individually. Similar to our results, Smith et al. (2008) found that rats raised in active conditions showed a stronger preference for a place associated with higher doses of cocaine than their sedentary counterparts, with a significant effect seen at the 10.0 mg/kg cocaine dosage. Smith et al. (2008) failed to see a significant effect with the 5.0 mg/kg dosage; however, this may be due to the fact that they raised their animals in isolated conditions, which have been shown to have distinct effects on conditioned place preference for a variety of drugs (Schenk et al., 1983; Wongwitdecha & Marsden, 1996; Courdereau et al., 1997).

Thanos et al. (2010) reported that forced treadmill running during rearing (up to 1 hour per day) decreases a conditioned place preference for 25.0 mg/kg IP cocaine, and Solinas et al. (2009) reported that rats raised in enriched environments with a wheel show a decreased conditioned place preference for both 10.0 and 20.0 mg/kg IP cocaine. A differential effect may be seen for the

conditioned place preference of the 10.0 mg/kg dosage between Smith et al. (2008) and Solinas et al. (2009) because of the difference in rearing environment. Additionally, Solinas et al. (2009) included along with the running wheel, a small house and 4 to 5 enrichment objects that were exchanged weekly with novel objects.

Even in the case of chronic drug self-administration, both voluntary wheel running and forced treadmill running administered either throughout adolescence or during adulthood have also been shown to decrease self-administration of certain drugs of abuse such as cocaine and morphine (Cosgrove et al., 2002; Hosseini et al., 2009), with higher runners showing lower breakpoints (Smith et al., 2008). Physical activity has also been shown to affect drug consumption in that when rats are concomitantly given the opportunity to engage in voluntary wheel running and consume substances, oral alcohol and amphetamine consumption decrease (Kanarek et al., 1995; Ehringer et al., 2009).

While the bulk of the preclinical literature suggests that increased activity in rodents is associated with lowered preference for drugs of abuse, we suggest that this view may be too simple. Both our work, demonstrating that the dose response curve for establishing a CPP to cocaine is shifted with lifelong activity, and that of Smith et al. (2008) suggest that a history of activity cannot be relied upon to universally reduce the incentive salience of cocaine. These data emphasize the importance of including a sufficient range of doses to probe for the possibility of a shift in the response curve.

Interpretation of postmortem neurotransmitter analysis The neuropharmacological methods used here capture one time point in the course of neurotransmitter metabolism and provide, by the analysis of both the neurotransmitters and their metabolites, a view of the pharmacokinetics at that time point. This approach allows the simultaneous analysis of multiple brain regions of interest, in effect providing a blueprint of brain regions likely to underlie behavioral differences in active and sedentary subjects, including differences in responses to a cocaine challenge. Collectively these data suggest that both in vivo microdialysis and/or electrophysiological studies on regions within this blueprint are likely to yield information on neurotransmitter release and neuronal activity patterns.

The present work revealed that a history of lifelong activity alters the neurochemistry of the brain in motor and motivationally related areas as well as the cocaine response both in terms of neurochemical and behavioral measures. Considering our neurochemical results, which showed differences between sedentary and active animals with just one dose of cocaine, we suggest that behavioral differences between active and sedentary rats emerge even as early as this first dose. In our test for the incentive salience of cocaine, active rats found 5.0 mg/kg cocaine to be more rewarding than sedentary rats, and with this same dose, for example, neurotransmitter and metabolite content in the VTA decreased in active but not sedentary animals. Some speculations can be made as to what the nature of the the cell biology at work is in the impact of activity on the brain as well as cocaine-induced response differences between active versus sedentary animals.

In terms of the cell biology of the neurons, at the time the tissue punches were taken, the neurotransmitters and their metabolites were captured from both within the terminals and within the synapse. Regarding the greater baseline neurotransmitter content in the CP in active versus sedentary animals, it is straightforward to suggest that with a long-term history of activity, the number of nerve terminals and synapses increases due to activity-induced synaptogenesis and even more speculatively neurogenesis. This is a likely hypothesis as others have shown that voluntary wheel running induces neurogenesis and synaptogenesis in the dentate gyrus of the hippocampus (Redila and Christie, 2006; Zhao et al., 2006; Stranahan et al., 2007; van Praag et al., 2008), which is a brain region integrally related to and directly connected with the motor and motivational structures examined here (Sesack and Grace, 2010). Additionally, alterations in synaptic metabolism, either involving the neurons of origin or local interneurons, might be part of the regulation of changes in neurotransmitter and metabolite content.

In the case of acute cocaine challenge, the cell biology underlying the neurotransmitter and metabolite changes we and others see is unknown. Certainly, activity-induced changes in neuronal activity might be at work. In one possible example, the VTA is a dopaminergic output region involved in the ascending reward pathway to the NA and prefrontal cortex, and when rats are given an acute dose of cocaine, the basal firing rate of VTA dopamine neurons decreases (Einhorn, 1988). Inevitably, neurotransmitter transmission and metabolism within the VTA is regulated by VTA autoreceptors as well as reciprocal

projections from the NA, which are also affected by cocaine (Einhorn, 1988). The fact that the neurochemical cocaine response in the VTA is different in active versus sedentary animals suggests that lifelong activity may alter the responsiveness of VTA dopamine neurons to a pharmacological stimulus with known incentive salience. Therefore, it is possible that the ongoing exposure to a rewarding experience (i.e., wheel running (Lett et al., 2000; 2002; Greenwood et al., 2011; Basso and Morrell, 2010; 2012)) throughout development alters the reward circuitry and its subsequent responsiveness to rewarding stimuli at adulthood.

Another possibility is that there might be a particular role for the glia cells in active subjects. Animals reared with a voluntary running wheel or in an enriched environment with a wheel show increased levels of gliogenesis in the hippocampus as well as the cortex (van Praag et al., 2000; Ehninger & Kemperman, 2003; Steiner et al., 2004; Villeda and Wyss-Coray, 2008). If this is the case, other brain regions, including those we sampled, may have more abundant glia, leading to the altered cocaine responses we see. That is, because glia are known to help with the clearance and breakdown of neurotransmitters from the synaptic cleft (Haydon et al., 2009), if active animals have more glia, then when cocaine is in the CNS, neurotransmitters and their metabolites could potentially be cleared from the environment faster than in sedentary animals with fewer glia, leading to the lower neurotransmitter/metabolite levels we see in our work, for example, in the VTA in active animals given cocaine.

A final spectulation arises for the human condition when we take the perspective that physical activity is a stimulus with positive incentive salience, itself a rewarding experience (Basso and Morrell, 2010), and that it may be regulated by or stimulate areas involved in the motivational brain circuitry (Basso and Morrell, 2012). We speculate that if we engaged in a practice of physical activity throughout our childhood and adolescent lives, these systems would be different in their responsiveness to all incentives at adulthood. That is, they would respond differently to other stimuli, such as palatable foods or pharmacological substances with known incentive salience such as drugs and alcohol. The current work sheds some preclinical light on this idea, suggesting that if we engage in a lifetime of physical activity, just as our bodies would be toned, our motivational brain circuitry would also be shaped by this process.

Acknowledgements

We thank Dr. Mariana Pereira for her scholarly contributions to this work and Anna Chavez, Leah Dziopa, and Sabina Advani for their technical assistance.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

TABLE 1

The Effect of an Active Environment throughout Development and/or an Acute Dose of Cocaine at Adulthood on Total Content of DA, 5HT, NE, and their Metabolites in Motivational Brain Regions

Region	Treatment	Dopamine	DOPAC	HVA	Serotonin	5-HIAA	Norepinephrine
Caudate Putamen	Sedentary (n=7)	9684.8 (±767.3)	1464.7 (±176.5)	3548.8 (±373.5)	1104.1 (±315.1)	265.8 (±26.4)	129.8 (±26.5)
	Active (n=6)	**14904.8 (±882.8)*[a]**	**2696.6 (±586.4)+[a]**	**5522.2 (±536.0)*[a,d]**	645.3 (±62.6)	322.3 (±14.9)	**385.7 (±91.1)*[a]**
	Sedentary + Cocaine (n=8)	**10385.9 (±1721.6)+[b]**	**1306.7 (±317.2)*[b]**	**2848.6 (±404.3)*[b,e]**	1043.3 (±248.1)	265.4 (±33.7)	238.5 (±68.6)
	Active + Cocaine (n=6)	11860.2 (±1698.8)	2321.8 (±578.1)	**4550.0 (±623.9)*[c]**	505.5 (±52.9)	282.2 (±13.1)	**390.1 (±128.2)+[a]**
Ventral Tegmental Area	Sedentary (n=8)	137.0 (±18.2)	49.2 (±3.5)	114.8 (±17.3)	116.9 (±7.5)	69.8 (±9.1)	93.0 (±10.9)
	Active (n=6)	143.3 (±32.1)	**68.2 (±7.7)*[a]**	84.8 (±14.0)	126.7 (±45.2)	85.3 (±17.7)	77.9 (±15.3)
	Sedentary + Cocaine (n=8)	132.2 (±35.2)	64.8 (±14.0)	92.4 (±21.1)	109.5 (±21.2)	78.5 (±9.0)	109.2 (±13.5)
	Active + Cocaine (n=6)	90.7 (±15.6)	**41.4 (±7.7)*[b]**	**42.5 (±0.00)*[a,b,c,d]**	**55.8 (±15.6)*[a]**	**51.2 (±2.7)*[c]**	**34.3 (±7.9)*[a,b,c,d,f]**
Nucleus Accumbens Core	Sedentary (n=7)	1703.7 (±703.8)	442.6 (±125.3)	680.4 (±169.3)	219.7 (±64.0)	130.0 (±20.8)	79.5 (±21.1)
	Active (n=7)	589.5 (±83.2)	361.8 (±65.5)	555.7 (±72.9)	124.6 (±13.5)	105.8 (±14.7)	94.4 (±13.2)
	Sedentary + Cocaine (n=8)	959.8 (±276.1)	323.2 (±87.2)	412.2 (±62.4)	175.8 (±33.6)	126.4 (±10.9)	128.8 (±35.8)
	Active + Cocaine (n=5)	729.3 (±289.6)	261.0 (±109.9)	462.1 (±99.1)	144.5 (±39.8)	**82.6 (±12.1)*[c]**	83.7 (±17.9)
Nucleus Accumbens Shell	Sedentary (n=7)	181.7 (±107.1)	115.0 (±54.5)	161.0 (±48.1)	58.4 (±20.8)	60.2 (±6.6)	58.2 (±22.1)
	Active (n=6)	55.8 (±48.7)	85.6 (±23.5)	106.9 (±26.0)	30.4 (±8.5)	60.3 (±10.0)	35.4 (±5.9)
	Sedentary + Cocaine (n=8)	45.5 (±20.3)	65.1 (±19.7)	85.9 (±8.5)	35.6 (±6.5)	62.0 (±9.8)	39.2 (±7.4)
	Active + Cocaine (n=6)	**143.8 (±45.2)+[c]**	88.0 (±19.2)	114.4 (±18.7)	**55.0 (±7.2)+[b]**	59.2 (±5.6)	42.8 (±5.3)
Medial Prefrontal Cortex	Sedentary (n=7)	12.9 (±2.9)	25.1 (±11.8)	117.5 (±29.4)	73.2 (±11.6)	76.4 (±8.3)	105.7 (±12.8)
	Active (n=7)	10.5 (±2.5)	37.0 (±7.4)	59.5 (±8.4)	65.9 (±6.4)	**115.9 (±15.0)*[a]**	102.3 (±6.4)
	Sedentary + Cocaine (n=8)	13.3 (±2.3)	26.9 (±4.3)	69.5 (±15.9)	61.9 (±12.5)	77.9 (±15.4)	97.1 (±11.4)
	Active + Cocaine (n=5)	11.0 (±2.2)	36.8 (±14.6)	50.8 (±8.5)	80.5 (±19.2)	98.0 (±7.1)	93.6 (±14.4)
Medial Preoptic Area	Sedentary (n=6)	50.9 (±5.1)	15.6 (±2.0)	68.6 (±16.5)	122.0 (±42.1)	56.0 (±8.0)	624.0 (±129.4)
	Active (n=6)	**33.0 (±3.3)*[a]**	**28.2 (±3.5)*[a]**	42.9 (±0.0)	114.7 (±16.9)	71.5 (±14.8)	753.7 (±80.4)
	Sedentary + Cocaine (n=8)	**28.6 (±4.8)*[a,d]**	21.8 (±4.5)	75.4 (±14.6)	117.9 (±27.2)	48.3 (±7.4)	**537.6 (±47.1)*[b]**
	Active + Cocaine (n=6)	**25.9 (±7.0)*[a,d]**	**17.6 (±2.6)[b]**	57.2 (±14.3)	**57.7 (±14.3)*[b]**	40.3 (±5.5)	**416.7 (±80.2)*[b]**

*** p<0.05, + p<0.059**
a = statistically significant comparison to sedentary group in t-test
b = statistically signficant comparison to active group in t-test
c = statistically significant comparison to sedentary+cocaine group in t-test
d = statistically significant comparison in one-way anova post-hoc comparison to sedentary
e = statistically significant comparison in one-way anova post-hoc comparison to active
f = statistically significant comparison in one-way anova post-hoc comparison to sedentary + cocaine

Table 1 Average amount (±SEM) of neurotransmitter or metabolite content (picograms/milligram of tissue) in the caudate putamen, ventral tegmental area, nucleus accumbens core and shell, medial prefrontal cortex and medial preoptic area in animals reared without a wheel (sedentary), with a wheel (active), without a wheel and given an acute dose of 5.0 mg/kg cocaine at adulthood (sedentary+cocaine), or with a wheel and given an acute dose of 5.0 mg/kg cocaine at adulthood (active+cocaine). All statistically significant differences are noted in bold.

TABLE 2

Table 2: Effect of Wheel Availability on Conditioned Place Preference for Cocaine		
Dosage of Cocaine	**Active Environment**	**Sedentary Environment**
0.5 mg/kg	**No CPP**	**CPP**
1.0 mg/kg	**No CPP**	**CPP**
5.0 mg/kg	**CPP**	**No CPP**

Table 2 Animals raised with a wheel displayed a conditioned place preference for the highest dose of cocaine tested (5.0 mg/kg), whereas animals raised without a wheel displayed a conditioned place preference for the two lower doses of cocaine tested (0.5 and 1.0 mg/kg).

TABLE 3

Treatment Effect	Statistical Comparison	Brain Region	Neurotransmitters and/or Metabolites	Direction of Effect
Active Environment	*Sedentary versus Active*	CP	DA, DOPAC, HVA	↑
			NE	↑
		VTA	DOPAC	↑
		mPFC	5-HIAA	↑
		mPOA	DA	↓
			DOPAC	↑
Acute Cocaine	*Sedentary versus Sedentary+Cocaine*	mPOA	DA	↓
Cocaine on Active Baseline	*Active versus Active+Cocaine*	VTA	NE	↓
			DOPAC, HVA	↓
		NAs	5-HT	↑
		mPOA	5-HT	↓
			NE	↓
			DOPAC	↓
Cocaine-Activity Synergy	*Sedentary+Cocaine versus Active+Cocaine*	CP	HVA	↑
		VTA	NE	↓
			HVA	↓
			5-HIAA	↓
		NAc	5-HIAA	↓
		NAs	DA	↑
Synergy of Both Versus Single Stimuli	*Sedentary versus Active+Cocaine*	CP	NE	↑
		VTA	5-HT	↓
			NE	↓
			HVA	↓
		mPOA	DA	↓
	Active versus Sedentary+Cocaine	CP	DA, DOPAC, HVA	↓
		mPOA	NE	↓

Table 3 (Discussion Table) The effect of being raised in an active environment, an acute dose of cocaine at adulthood, or the combination of both treatments on neurotransmitter and/or metabolite increase or decrease in motivational brain regions. All changes noted are statistically significant ($p<0.05$).

FIGURE 1

An active environment increases neurotransmitter and metabolite content in the caudate putamen

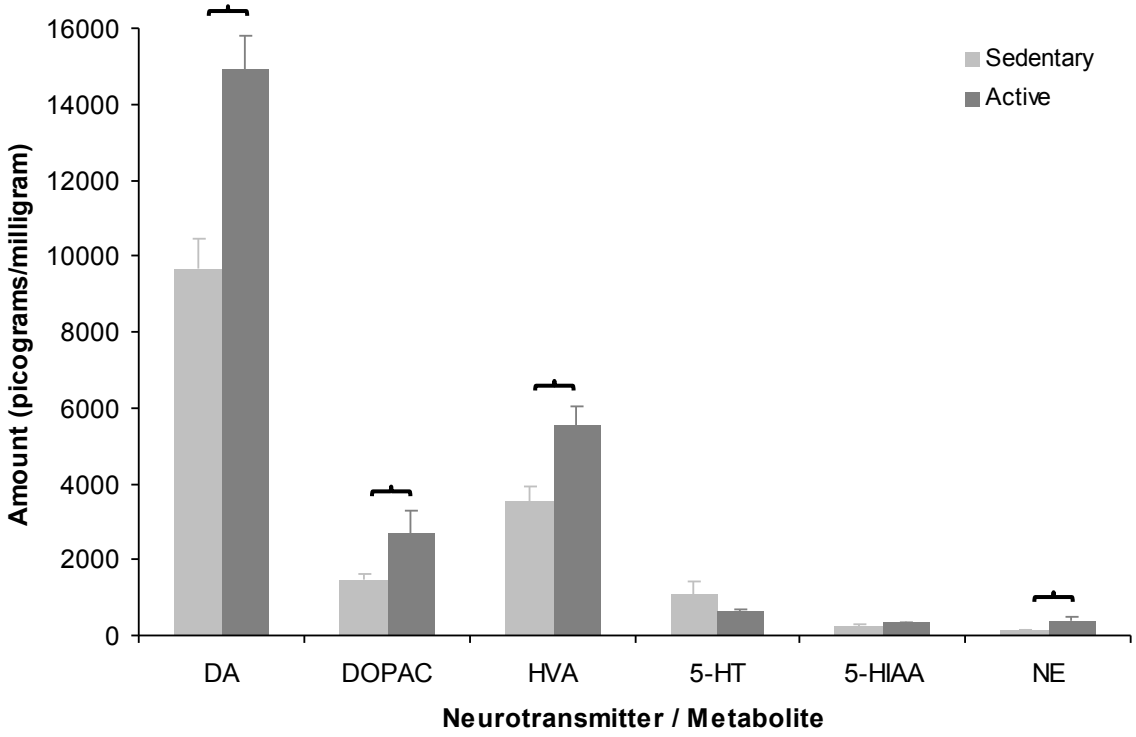

Figure 1 Average amount (±SEM) of neurotransmitter or metabolite content (picograms/milligram of tissue) in the caudate putamen in animals reared without or with a wheel.

FIGURE 2

Acute cocaine administration in active animals decreases neurotransmitter/metabolite content in the ventral tegmental area

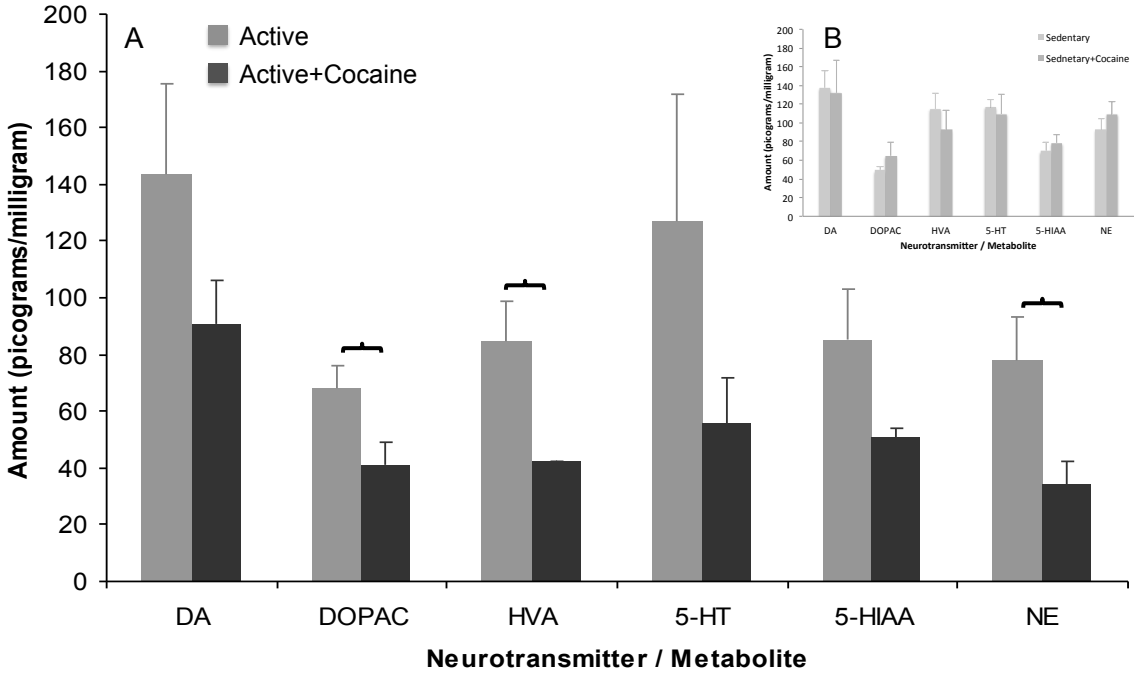

Figure 2 Average amount (±SEM) of neurotransmitter or metabolite content (picograms/milligram of tissue) in the ventral tegmental area in animals reared (A.) with or (B.) without a wheel and given either saline or an acute dose of 5.0 mg/kg cocaine at adulthood.

FIGURE 3

Acute cocaine administration in active animals increases rather than decreases neurotransmitter/metabolite content in the nucleus accumbens shell

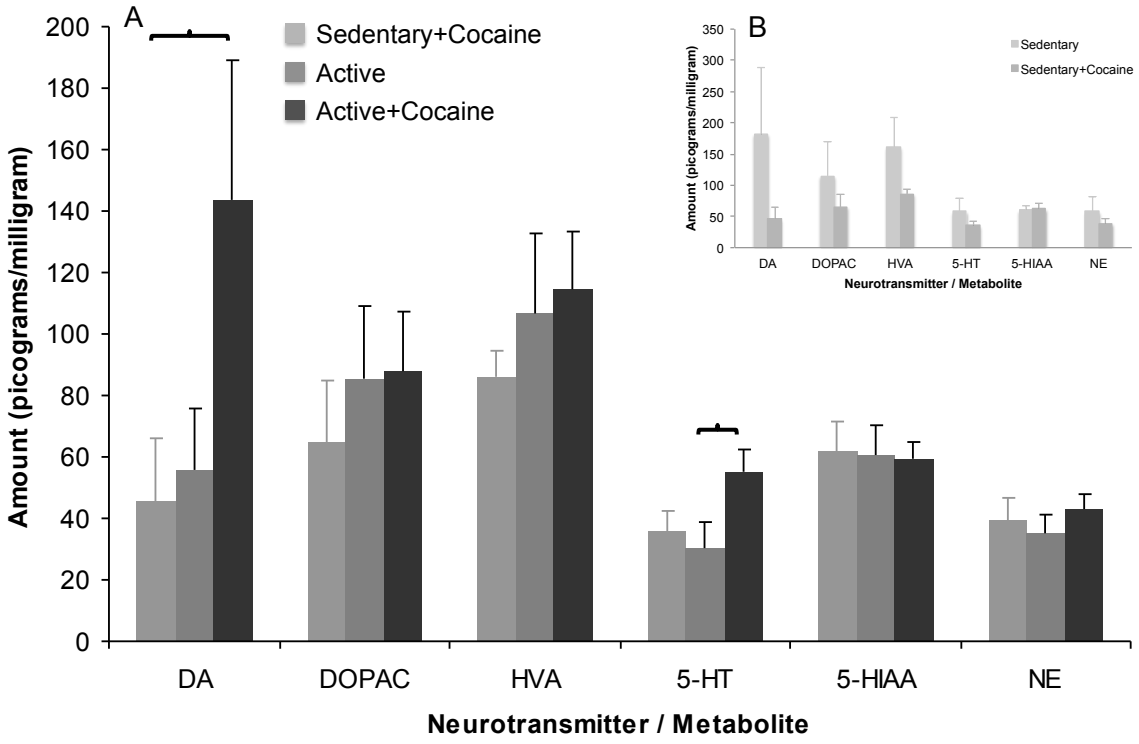

Figure 3 Average amount (±SEM) of neurotransmitter or metabolite content (picograms/milligram of tissue) in the nucleus accumbens shell in (A.) animals reared without a wheel and given an acute dose of 5.0 mg/kg cocaine at adulthood or with a wheel and given saline or cocaine at adulthood and (B.) animals reared without a wheel and given saline or cocaine at adulthood.

FIGURE 4

Acute cocaine administration in active animals decreases neurotransmitter/metabolite content in the medial preoptic area

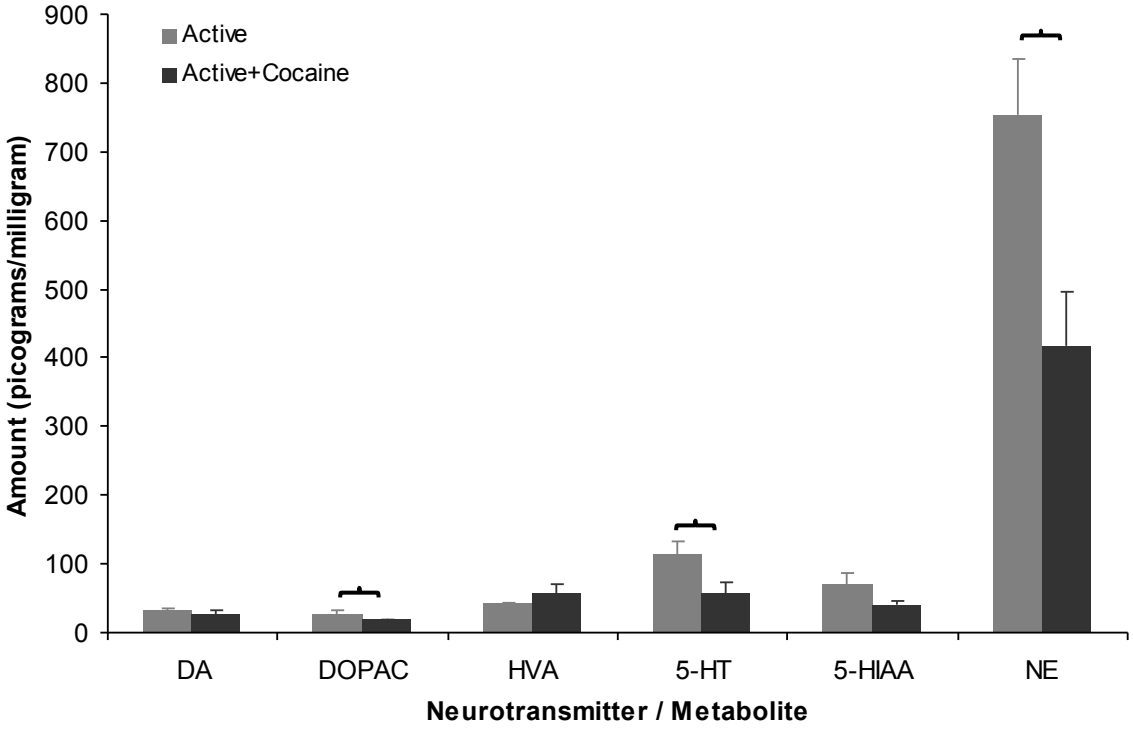

Figure 4 Average amount (±SEM) of neurotransmitter or metabolite content (picograms/milligram of tissue) in the medial preoptic area in animals reared with a wheel and given either saline or an acute dose of 5.0 mg/kg cocaine at adulthood.

FIGURE 5

Rearing in an active environment does not alter metabolism for a 5.0 mg/kg intraperitoneal dose of cocaine

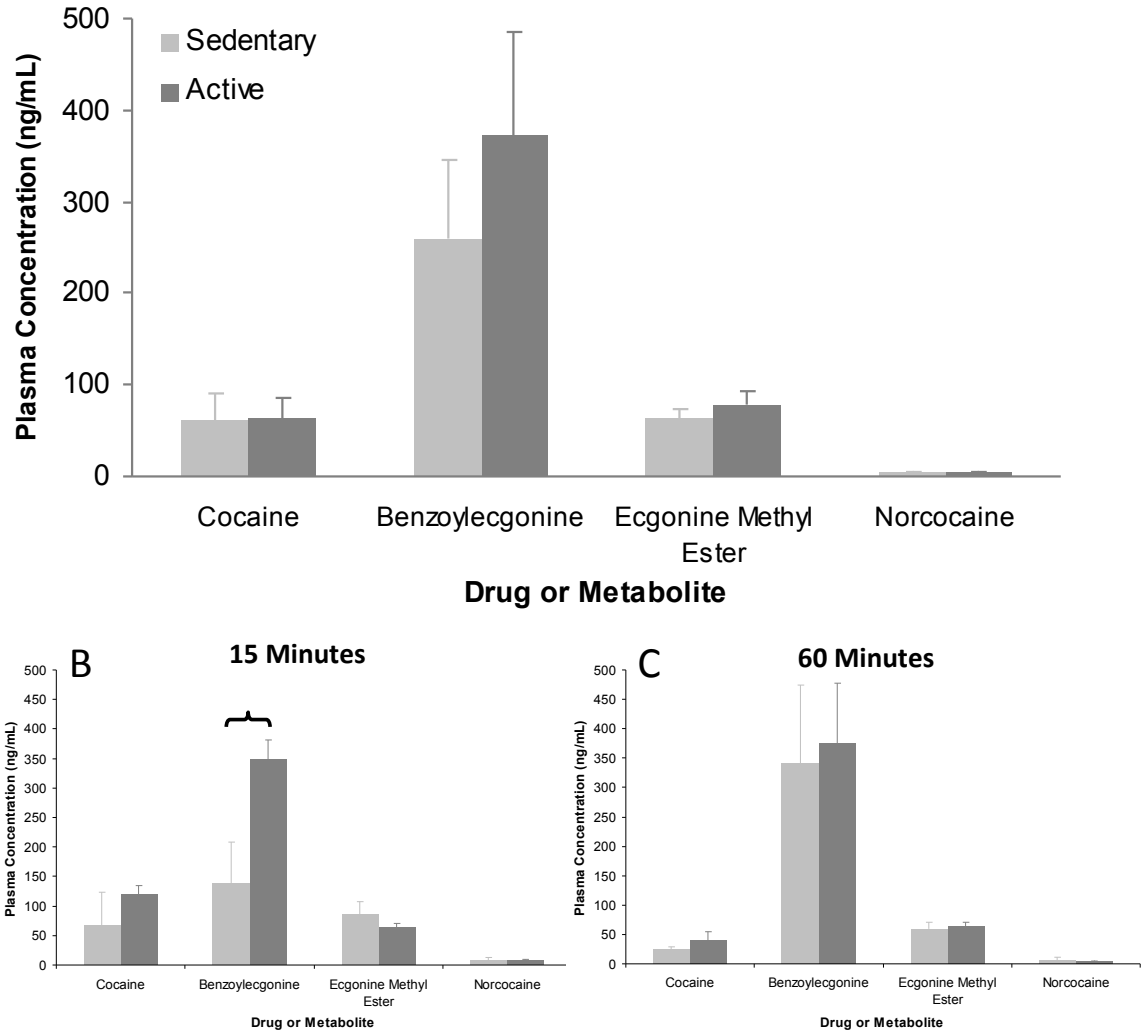

Figure 5 Average (±SEM) plasma concentration (nanogram/millileter) of cocaine and its metabolites, benzoylecgonine, ecgonine methyl ester, and norcocaine (A) 30 minutes, (B) 15 minutes, or (C) 60 minutes after an intraperitoneal injection of 5.0 mg/kg cocaine in animals raised without or with a wheel.

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CHAPTER 4

Motivation for voluntary wheel running across genders: Behavioral analysis using unconditioned and conditioned measures

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Abstract

Considering that wheel running in laboratory rodents may be a useful preclinical model of exercise in humans, this work explored the motivation to engage in voluntary wheel running through a set of behavioral proofs of the incentive salience of wheel running, which were designed to be antecedent to examining the CNS basis for these motivational processes. We hypothesized that rats run because the whole experience of wheel running as well as its aftereffects have positive incentive salience for them. We determined whether gender differences in *ad libitum* running are also reflected in gender differences in the incentive salience of wheel running. We examined the motivation for voluntary wheel running in male and female Sprague Dawley rats using both unconditioned and conditioned procedures, examining both the acquisition and habit running phases of the behavior as well as the aftereffects subsequent to these phases. Conditioned place preference (CPP) was used to examine the incentive salience of the acquisition of the total experience of running as well as the aftereffects of wheel running alone. The incentive salience of stable habitual levels of running was examined by measuring the recovery response of wheel running after a period of forced wheel abstinence (1 or 72 hours). Both genders displayed a strong CPP for the acquisition phase of the total wheel running experience, as well as a strong recovery response to wheel deprivation during the stable habit phase of running. Only males demonstrated a CPP for the aftereffects of habitual running. These data suggest that rats find voluntary wheel running to be a rewarding activity, and the fact that males run less than females does not indicate that they like it any less.

Introduction

The Center for Disease Control (CDC) reports that physical activity promotes both physical and mental health, including weight control, bone, muscle, and joint health, reductions in injuries from falling in older adults, improvements in sleep, and reductions in the risk of diabetes, high blood pressure, cardiovascular disease, depression, and anxiety (www.cdc.gov). However, 74% of adult humans in the U.S. do not attain the recommended 30 minutes a day of physical activity and 25% do not participate in any level of physical activity at all (American Heart Association; CDC; US Department of Health and Human Services), suggesting that a problem in the motivation to engage in activity may be a crucial issue. Based on the idea that wheel running in laboratory rodents may be the basis for a preclinical model of exercise (Eikelboom, 1999), we explore here the motivation to engage in voluntary wheel running in experiments designed to be antecedent to exploring the brain regions that mediate these motivational processes. We further suggest that the CNS circuitry prevalently understood to mediate the motivational processes that underlie responses to natural and pharmacological stimuli also mediates the motivational processes that underlie wheel running, and we test this hypothesis in a subsequent set of experiments.

Rats engage with running wheels spontaneously and robustly, producing their first wheel turns within the initial few minutes of wheel exposure (Stewart, 1898; Richter, 1927; Shirley, 1929; Sherwin, 1998; Basso and Morrell, 2010). The 2 to 3 week acquisition phase of voluntary wheel running transitions into

many months of stable habit running in adulthood (Richter, 1927; Eayrs, 1954; Koh et al., 2000; Basso and Morrell, 2010). Particularly once at this stable habitual stage, voluntary wheel running is extremely robust, with males running on average 4.3 km per day and females, 6.6 km per day (Afonso & Eikelboom, 2003; Basso and Morrell, 2010; Greenwood et al., 2011; Richter, 1927; Basso and Morrell, 2010).

Hypotheses as to why rats engage in this behavior have suggested that voluntary wheel running is a measure of general locomotor activity, a means of exploring the environment for food, water, or other materials, an obsessive-compulsive or dependent behavior, a form of fictive migration or escape, or even play behavior (Barnett, 1958; Ferreira et al., 2006), but data supporting these ideas are not strong (Sherwin, 1998; Albelda and Joel, 2012). On the contrary, there is considerable evidence for the hypothesis that rats engage in wheel running because it has positive incentive salience for them.

While it is commonly thought that the robust routine engagement with a stimulus, for example the rat's vigorous interaction with the wheel, can be interpreted as an indication of its motivation to interact with a stimulus with positive incentive salience, additional approaches have yielded data supporting the hypothesis that rats find wheel running rewarding. These include demonstration of spontaneous recovery, also called rebound running, that is, the unconditioned response of increased running behavior upon return of the wheel after a period of forced wheel abstinence (Hill, 1956; 1961; Sugimoto et al., 1994; Mueller et al., 1997; 1999; Aoyama & McSweeney, 2001), a response that is

similar to the rebound response to natural or pharmacological stimuli after forced abstinence from them (McSweeney et al., 2005). Additionally, rats perform conditioned responses including conditioned place preference (CPP) and operant responses for the opportunity to engage in voluntary wheel running (Kagan & Berkun, 1954; Premack et al., 1964; Collier & Hirsch, 1971; Pierce et al., 1986; Iversen, 1993; Belke & Heyman, 1994; Belke, 1997; Belke & Wagner, 2005; Belke, 2006; Belke & Pierce, 2009). An early study demonstrated that rats prefer a location associated with a running wheel versus other enrichment objects (Hill, 1961), but most studies in rats have focused on a CPP for the aftereffects of wheel running (Lett et al., 2000; 2002; Belke & Wagner, 2005; Greenwood et al., 2011). Although hamsters demonstrate a CPP for the total experience of wheel running (Antoniadis et al., 2000; Ralph et al., 2002), no similar proof is currently present in the rat literature.

While collectively these studies have supported the hypothesis that various aspects of wheel running in rats have positive incentive salience for them, these studies are limited by the sole use of males, as well as certain experimental details, for example, the use of running opportunities limited to the normally inactive period of the light-dark cycle, as well as approaches that include the added complexity of water- and/or food-deprivation, which has been shown to increase voluntary wheel running and cause activity-induced anorexia in some instances (Premack & Premack, 1963; Routtenberg & Kuznesof, 1967; Collier et al., 1969; Levitsky, 1970; Looy & Eikelboom, 1989; Lattanzio & Eikelboom, 2003; Scarpace et al., 2010).

Our examination of the incentive salience of wheel running in rats considers that the process consists of three separable components, interaction with the wheel as a large object suitable for climbing, the wheel running experience itself, and the aftereffects of wheel running or the physical state experienced after running. The experimental design was informed by our prior analysis of the progression of voluntary wheel running that followed wheel-naïve males and females to stabilized habitual running patterns (Basso and Morrell, 2010, 2012). We designed our experiments to test motivation to wheel run in both the acquisition and habitual phase of wheel running, and to determine whether the pattern of greater running and faster speeds in females versus males signified gender differences in motivation for the behavior.

We used an unconditioned measure of motivational response to the wheel suitable for examination of the habit or stabilized running phase of the behavior with a quantitative analysis of response recovery (rebound) effects after periods of forced wheel abstinence. We also used CPP to test the incentive salience of wheel running in the acquisition phase of wheel running behavior, examining the incentive salience of the total experience of wheel running as well as one of its separable components, the aftereffects, in both the acquisition and habit phases of wheel running. The CPP approach was chosen because it allows testing of wheel seeking after only a minimal number of exposures to the stimulus. This approach was informed by our prior extensive work with conditioned place preference for pharmacological and natural stimuli (Mattson et al., 2001; 2003; Seip et al., 2008; Wansaw et al., 2008).

Methods

Subjects

Male and female Sprague Dawley rats (original stock from Charles River Laboratories, Kingston, NY, USA) were bred in our colony at the Rutgers University Laboratory Animal Facility (RAF) (Newark, NJ, USA) (accredited by the American Association for Accreditation of Laboratory Animal Care). All animals were kept on a 12-hour light-dark cycle (lights on at 7:00 am; unless otherwise noted) in a room at 22(±1)°C and given *ad libitum* access to water and rat chow (Lab Diet 5008, PMI Nutrition International, LLC, Brentwood, MO, USA). Daily checks were conducted for health and availability of food and water. Weights were taken once per week and animal husbandry was performed twice to seven days a week depending on the protocol. All animals remained healthy and of normal body weight throughout the experiments. Animal care and experimental procedures performed in this protocol were in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were reviewed and approved by the Rutgers University Animal Care and Facilities Committee. Care was taken to minimize the suffering and curtail the number of animals utilized.

Running wheel apparatuses

Experiments were conducted using either an AccuScan Instruments (Columbus, Ohio, USA) VersaMax Animal Activity Monitor (wheel: 25 cm diameter, stainless steel mesh floor; home cage: 40 cm long x 40 cm wide x 30 cm wide) or a Med Associates Inc. (St. Albans, VT, USA) ENV-046 Activity Wheel with Plastic Home

Cage for Rats (wheel: 35.6 cm diameter, 4.8 mm stainless steel grid rods with a 1.6 cm spacing, 12 gram freewheeling drag; home cage: 48.26 cm long x 26.67 wide cm x 20.32 cm high with a 7.2 cm wide x 10.2 cm high opening to wheel). The resistance of both running wheels was low and equivalent, and no extra weight/resistance was placed upon either of the wheels. Both housing apparatuses were lined with woodchip bedding (Beta chip, Northeaster Products Corp., Warrensburg, NY, USA), except when otherwise noted. Food and water were provided *ad libitum* at all times. For both systems, data was captured automatically. In the AccuScan system, 16 infrared beams lined each axis of the box. Each box was connected through wires to the computer and data was captured through the Windows based software, VersaMax and VersaDat. In the Med Associates system, the LCD digital counter captured wheel turns, the computer was connected through wires to the computer, and data was captured through the Windows based software MedPCIV.

Analysis of reinstatement (rebound) running after forced abstinence from the wheel in stabilized habitual runners

At PND 65, wheel naïve males (n=12) and females (n=34), previously group housed in shoebox cages in the RAF, were individually placed in the AccuScan or Med Associates home cages with running wheels at ~1:00 pm (approximate midpoint light period; lights on at 7:00 am, off 7:00 pm). Animals remained in these home cages for 21 days, except for husbandry. After at least 21 days, and up to 15 weeks, of *ad libitum* access to the wheels, subjects were in the stabilized or habitual phase of wheel running, as seen in these groups and in

running analyses carried out in independent groups of both genders from our rat colony source. Subjects were subsequently given two forced abstinence tests. (1) A one-hour period of forced abstinence from the wheel was conducted, during which husbandry, weighing, and handling-based wellness checks were carried out. Around 1 pm (midpoint normal resting time), wheels were returned for a minimum of three days. (2) A similar but longer forced wheel abstinence period of 72 hours occurred, which also included periodic routine husbandry, weighing, and handling-based wellness checks. Seventy-two hours later, around 1:00 pm (midpoint normal resting time), wheels were returned for at minimum four days of *ad libitum* wheel access. In the AccuScan system, wheels were removed, and in the Med Associates system, manual sliding doors were placed in between the wheels and the home cage to block access to wheel running. Whether wheel access was interrupted for 1 or 72 hours, the wheel was always returned during the midpoint of the light period of the daily cycle to optimally reveal the reinstatement (rebound) running response and so it could be compared to a normal undisrupted baseline of the rest period, that is, when virtually no wheel running occurs.

Conditioned Place Preference for the total experience of wheel running in the acquisition phase of running A two-chambered CPP apparatus was devised within the laboratory informed by our prior CPP work (Mattson et al., 2001; Seip et al., 2008), consisting of two AccuScan Instruments (Columbus, Ohio, USA) boxes of equal size (40 cm long x 40 cm wide x 30 cm wide), placed side by side and connected with a short opaque tunnel (4” diameter, 2” length). Each box

was decorated with unique cues, which consisted of wallpaper in either horizontal or vertical black and white stripes and tactile flooring of small paper squares (ALPHA-dri, Shepherd Specialty Papers, Kalamazoo, MI) or small corn cobs (Bed-o" cobs (1/4") The Andersons, Maumee, OH). All boxes were covered with transparent lids and lit by overhead lights. Luminance (Konica Minolta Luminance Meter LS-100, Japan) was equal in all chambers (220 lumens). Two weeks prior to the pre-conditioning session, wheel naïve males (n=14) and females (n=24) were obtained from the RAF and placed on a 12-hour light-dark cycle (lights on at 12:00 am, off at 12:00 pm).

Pre-conditioning chamber preference baseline At PND 65, subjects were exposed to the two-chambered apparatus for 60 minutes and allowed to roam freely between the chambers. This pre-conditioning session occurred around 10:30 am so that the session ended before the lights turned off. Time spent in each chamber was manually recorded. Based on criterion discussed below, animals were then assigned to receive the wheel in one uniquely cue decorated chamber and no wheel in the alternate uniquely cue decorated chamber.

Conditioning sessions Pre-conditioning boxes were replaced with cue decorated boxes without the entrance hole in the side, one of which had a running wheel and one of which did not, both providing food and water *ad libitum*. Twenty-four hours after the pre-conditioning baseline session, animals were isolated in one of the uniquely cue-decorated chambers for 23 hours (with or without a wheel). The 23 hours of conditioning occurred from ~11:30 am to the following 10:30 am. One hour of the 24 hour cycle was left so that chambers could be cleaned and

animals could be placed in the alternate chamber before the lights turned off at 12:00 pm. During cleaning (1% Liquinox, distilled water, 70% alcohol), animals were placed in a shoebox holding cage with food and water *ad libitum*. The idea behind this timing was that animals were placed in the chambers with lights on to view their environment, but soon thereafter, the lights turned off, which is the most active time period for the rat in terms of wheel running (i.e., highest hourly running rate). Essentially, the change in the light-dark period (lights off) correlated with a change in conditioning environment. The next day, animals were subsequently placed in the alternate chamber environment for 23 hours. This cycle of chamber environment changes continued for 14 days, such that each animal received 7 days of conditioning with the wheel and 7 days of conditioning without the wheel (i.e., an alternate-day running experience). Until their first experience with the wheel (i.e., the first day of conditioning with the wheel), subjects were wheel naïve. At the end of conditioning, each subject had received 7 days of wheel exposure, which our prior data establish is in the acquisition phase of wheel running behavior (see data at the initial section of results; Basso and Morrell, 2010; 2012). At the end of conditioning, animals were returned to shoebox cages with food and water, which served as temporary home cages until the post-conditioning test below.

Post-conditioning test of place preference Twenty-four to ninety-six hours after the final conditioning session, animals were exposed to the two-chambered apparatus, which had been cleaned and cue decorated just as throughout the pre-conditioning and conditioning sessions, but without wheels present. The

chambers were connected to allow exploration of both chambers. Animals were tested at most twice, that is once with 24 or 48 hours of forced wheel abstinence and a second time with 72 or 96 hours of forced wheel abstinence respectively. Previous data from our lab indicates that repeated post-conditioning testing (maximum of 2 times) provides similarly robust results, so this protocol was adapted for the present work (Seip et al., 2008). The subjects were allowed to roam freely between the chambers for 60 minutes, with time spent in each chamber recorded manually by three observers who were naïve to the stimulus-chamber associations learned by the individual animals during conditioning.

Analyses and chamber assignments To understand the preference of each individual animal as well as the group preference as a whole, data was analyzed using two techniques, individual chamber preference and group chamber time (Mattson et al., 2001; 2003). Data were analyzed at both the pre- and post-conditioning sessions. In order to determine if an individual animal showed a preference for a particular chamber, a stringent quantitative criterion was developed. To show a preference, the animal must have spent ≥30 minutes in one chamber, and this time also had to be ≥25% larger than that of the other chamber time. If these two criteria were not met, the animal was categorized as showing no preference. In the case of the two-chambered apparatus, three preference categories were possible (square, corn cobs, no preference). After individual chamber preference from the pre-conditioning session was established, animals were assigned to receive the wheel in their least-preferred side chamber. If an animal showed no preference, then the wheel was assigned

to one of the two chambers at random. Group chamber times were calculated by averaging the time spent in each chamber by all animals.

Conditioned place preference for the aftereffects of wheel running in the acquisition or habitual phase of running Experiments examining the aftereffects of running were conducted by methods as described above, except that this approach used a three-chambered place preference apparatus as most frequently used and described in our prior work (Mattson et al., 2001; Seip et al., 2008). All pre-conditioning, conditioning, and post-conditioning testing and analyses were carried out as described above except for the following technical details. The three-chambered apparatus had three equally sized, uniquely cue-decorated chambers; the two side chambers were used for conditioning of either an association with the after-effects of wheel running or the after-effects of a sedentary experience (no wheel or locked wheel). The middle chamber provided access between the two conditioning chambers only during one pre-conditioning test and one post-conditioning test, and therefore served as a chamber unassociated with either stimulus, an alternative to a forced choice between the two chambers.

Males (n=8) and females (n=8) were naïve to running at the start of the experiment and were kept in home cage environments that were the Med Associates wheel apparatus housing environments in which the wheel could be unlocked and available for running or locked and not available for running. In both cases, subjects could interact with the wheels by sitting in them or touching them. Animals were previously acclimated to a new light-dark cycle for one week

(lights on at 12:00 pm). On the first day of conditioning (12:00 pm, beginning of light cycle), subjects received the running wheel *ad libitum*. At the end of the dark cycle (the normal active running period), upon lights turning on, animals were placed in one uniquely cue-decorated chamber of the three-chambered CPP apparatus described above for 30 minutes. Subjects were then returned to their home cage environment but with a locked wheel. Upon lights turning on the following day, the same conditioning process was repeated, as subjects were returned to the home cage environment with access to the unlocked running wheel provided. This process continued for 40 days, with animals receiving 20 days of total running. Based on our prior work on the acquisition of habitual running, at this point, both males and females completed their acquisition phase of running and were now in the stable or habitual phase of running (see first section of results; Basso and Morrell, 2010; 2012).

After the experiment was complete, with 48 hours of forced wheel abstinence, a probe test was conducted to test the preference for the chamber associated with the aftereffects of running. The post-conditioning test was carried out as described in the prior section.

Additional experiments, using only females, utilized the three-chambered CPP apparatus to test the aftereffects of wheel running as listed in Table 2. Variants to these experiments including the number of wheel access conditioning days, the amount of time per day (30 minutes, 2 or 23 hours) of wheel access, whether animals were wheel naïve or habitual runners at the start of the experiment, and whether the sedentary condition included no wheel or a locked

wheel. All other CPP procedures and analyses were carried out as already described.

Analyses and Statistics All statistical analyses were conducted using the computer software IBM® SPSS® 21.0 (Chicago, IL, USA). A significance value of $p \leq 0.05$ was used for all statistical analyses. Interval data met the tests of normalcy and homogeneity of variance and were analyzed with parametric tests. Categorical data was measured using nonparametric tests. If data did not meet normality (t-test) or sphericity (repeated measures ANOVA), corrections such as Greenhouse-Geisser (repeated measures ANOVA) were used.

Wheel running data were analyzed by examining wheel turns each minute of the day that the animal had access to the wheel. The computer software captured running wheel data in time bins of 1 minute, and in this way, distance, time and rate could be calculated. A one-way repeated-measures analysis of variance (ANOVA) was used to determine statistical significance between measures sampled in the same set of animals on two or more occasions. For post-hoc comparisons, an independent samples *t*-test was used to determine all statistically significant differences between one measure in two separate groups.

CPP data were analyzed as previously described (Seip et al., 2008). Briefly, the interval data, namely the times spent by the groups in each chamber, were analyzed using a one-way or two-way repeated-measures analysis of variance (termed group chamber time). Categorical data, namely the individual chamber preferences, were analyzed with the non-parametric test, the Fisher's exact test (termed individual preference).

Results

Acquisition and stabilization of wheel running Running (i.e., distance, time, and rate) increased for the first two to three weeks of wheel availability, after which running behavior stabilized for up to the 15 week limit of these experiments (Figure 1). During this period, there was no correlation between body weight and the fundamental parameters of running. The acquisition of stabilized or habit running was faster in females, which reached their peak daily distance run by week 2 (week 1 versus 2: $F(1,27)=21.472$, $p<0.001$), than males which did not acquire stabilized habit running until week three of wheel availability (week 1 versus 2 $F(1,27)=6.352$, $p=0.018$; week 2 versus 3 $F(1,27)=26.401$, $p<0.001$). At the end of 3 weeks (Figure 1, day 21 of running), females ran on average 1.5 times farther daily than males (males 4.3 kilometers [2.7 miles]; females 6.6 kilometers [4.1 miles] per day). At each weekly time point, females ran farther than males, with significance during the 1st and 2nd week (week*gender $F(2,104)=5.578$, $p=0.005$, week 1 $t(37.082)=3.665$, $p=0.001$; week 2 $t(30.516)=5.276$, $p<0.001$; week 3 $p>0.05$). Similar to distance run, both males ($F(2,50)=33.149$, $p<0.001$) and females ($F(1.336, 36.075)=23.424$, $p<0.001$) increased their average daily running rate during the first 3 weeks of wheel exposure, with females peaking at week 2 and males at week 3. At all time points, females ran at significantly faster average rates than males (week*gender $F(2,104)=8.253$, $p<0.001$; week 1 $t(35.608)=-5.001$, $p<0.001$; week 2 $t(37.450)=-7.513$, $p<0.001$; week 3 $t(54)=-4.066$, $p<0.001$). At three weeks of wheel

exposure, females ran on average 1.5 times faster (19.2 m/min [0.7mi/hr]) than males (13.5 m/min [0.5mi/hr]).

Motivational measures of the total running experience

Conditioned place preference for the total experience of wheel running during the acquisition phase After a 14 day conditioning paradigm with only 7 days of wheel running experience, both males and females showed a strong preference for a place associated with the total experience of wheel running. At the post-conditioning session, no differences were found in the time spent in the chamber associated with the wheel running experience as a function of the of time before the post-conditioning test, which served as an additional period of forced wheel abstinence, varying from 24 to 96 hours. Therefore, all data were pooled for graphical presentation and statistical comparison (Figure 2).

Females developed a conditioned place preference for a place associated with the total experience of running (Figure 2). They showed a conditioning effect in terms of both individual preference ($p<0.001$) and group chamber time ($F(1,23)=14.375$, $p=0.001$), with 60% of females spending 75% of their time at the post-conditioning session in the chamber associated with the wheel running experience.

Males also developed a conditioned place preference for a place associated with the total experience of running (Figure 2). They showed a conditioning effect in terms of both individual preference ($p<0.001$) and group chamber time ($F(1,13)=8.739$, $p=0.011$), with 64% of males spending 80% of

their time at the post-conditioning session in the chamber associated with the wheel running experience.

No gender differences were found for these conditioned place preference experiments, and as a group, all animals showed a conditioning effect in terms of both individual preference ($p<0.001$) and group chamber time ($F(1,37=22.003$, $p<0.001$), with all animals spending 37% more time in the chamber associated with the wheel running experience at the post-conditioning session compared to the chamber associated with the non-running experience (trend, $F(1,37)=3.155$, $p=0.084$). No correlation was seen between strength of the preference (time spent in the wheel-associated chamber at the post-conditioning test) and running distances achieved for males or females ($R^2=0.19$).

Spontaneous recovery of running (rebound) after forced abstinence in stabilized habitual runners As seen in Figure 3, among established habitual runners, both males and females had a robust and remarkable recovery of running or rebound running response after 72 hours of forced wheel abstinence visible in the light period of their daily cycle when they would normally be resting. When data from male and female groups were pooled, both the rebound running response after one hour of deprivation ($F(1,45)=21.606$, $p<0.001$; time $F(1,37)=58.143$, $p<0.001$; rate $F(1,37)=71.781$, $p<0.001$) and 72 hours of deprivation (distance $F(1,45)=152.555$, $p<0.001$; time $F(1,37)=356.328$, $p<0.001$; rate $F(1,37)=366.837$, $p<0.001$) were statistically significant from the normal undisturbed rest period. The 72-hour deprivation response was also significantly greater than the 1-hour deprivation response (distance $F(1,45)=34.386$, $p<0.001$;

time $F(1,37)=55.245$, $p<0.001$; rate $F(1,37)=31.966$, $p<0.001$). Both genders showed similar responses to the wheel in terms of distance, time, and rate of running after a period of 1 hour of forced wheel abstinence ($p>0.05$); however, females ran farther (distance*gender $F(1,44)=4.682$, $p=0.036$; $t(44)=2.151$, $p=0.037$) and faster (rate*gender $F(1,36)=14.296$, $p=0.001$; $t(36)3.567$, $p=0.001$) than males after 72 hours of forced wheel abstinence. In fact, during this rebound period, females ran 1.5 times farther and faster than males, which is exactly what females normally do in their habitual phase of running. That is, in their 3rd week of running, females run 1.5 times farther and faster than males (Basso & Morrell, 2010).

Females Running behavior was profoundly affected by periods of forced wheel abstinence. Even when females were deprived of the wheel for one hour (Figure 3; Table 1), upon return of the wheel, they ran significantly longer, farther and faster compared to their normal undisturbed rest period running (distance $F(1,33)=17.582$; time $F(1,25)=49.878$, $p<0.001$; rate $F(1,25)=52.352$, $p<0.001$). When females were deprived of the wheel for 72 hours, a much more robust running response was seen upon return of the wheel, which differed significantly from the undisturbed baseline (distance $F(1,33)=120.096$, $p<0.001$; time $F(1,25)=196.555$, $p<0.001$; rate $F(1,25)=346.793$, $p<0.001$). Additionally, the response after 72 hours of forced wheel abstinence was significantly greater in terms of distance ($F(1,33)=22.155$, $p<0.001$), time ($F(1,25)=53.826$, $p<0.001$) and rate of running ($F(1,25)=49.615$, $p<0.001$) than the wheel interactions that occurred after the 1-hour abstinence period. Both bursts of light-cycle running

upon return of the wheel generally subsided within the first hour after disturbance and did not alter 24-hour running distances ($p>0.05$). These rebound running responses after 72-hours of forced wheel abstinence are comparable to distances and times spent running during the first hour of the dark period on a day without any wheel deprivation, which is the time period when rats run the farthest distances, spending the most time running, at the fastest paces (Basso and Morrell, 2010). However, due to non-significantly greater distances run and non-significantly less time spent running in their first hour of the dark cycle, females ran significantly slower during their rebound response hour than the first hour of the dark period (15.5 m/min versus 18.9 m/min $F(1,25)=10.567$, $p=0.003$)

In spite of the dramatic impact of a 72-hour period of forced wheel abstinence on the overall running pattern, during the 24 hours after the wheel was returned, females ran similar overall distances in the 24 hours before and after the 72-hour abstinence ($p>0.05$). That is, the deprivation did not alter the total daily distance run. However, the time spent running in the 24 hours after deprivation was significantly greater (242 versus 307 minutes $F(1,25)=19.279$, $p<0.001$) and the rate of running was significantly less (21.2 versus 17.0 m/min $F(1,25)=12.994$, $p=0.001$) than the day prior to deprivation. Additionally, due to the significant burst of running in the light period, the overall pattern of running significantly differed on the day after deprivation versus the day before, since undisturbed rats conduct ~95% of their running in their active, dark period. However, on the day after the deprivation period, rats conducted significantly

more of their running in the light period, producing on average 27.7% of their total running in the light ($F(1,33)=97.514$, $p<0.001$).

Males After a one-hour wheel deprivation, males showed a significant increase in distance ($F(1,11)=10.271$, $p=0.008$), time ($F(1,11)=15.491$, $p=0.002$) and rate of running ($F(1,11)=18.939$, $p=0.001$) compared to the undisturbed rest period (Table I). After a 72-hour forced wheel abstinence, males showed an even more robust response in terms of distance ($F(1,11)=70.725$, $p<0.001$), time ($F(1,11)=206.250$, $p<0.001$) and rate ($F(1,11)=180.134$, $p<0.001$) of running over the undisturbed baseline (Figure 3; Table 2). In a pattern similar to that seen in females, distance ($F(1,11)=27.630$, $p<0.001$), time ($F(1,11)=8.418$, $p=0.014$) and rate of running (n.s.) was greater after 72-hours of wheel deprivation compared to 1 hour of deprivation.

As with females, even with the dramatic running response upon return of the wheel, males ran the same distance as compared to the day prior to deprivation (4.3 versus 3.9 km, $p>0.05$), but the 24-hour running pattern differed, with significantly more running taking place in the light period compared to a situation without deprivation (3.9 versus 33.2%, $F(1,3)=38.602$, $p=0.008$). Similar to females, in the 24 hours after deprivation, males spent significantly more time (256 versus 297 minutes $F(1,3)=17.740$, $p=0.024$) running at a slower pace (14.7 versus 12.0 m/min, trend $F(1,3)=6.016$, $p=0.091$) compared to the day prior to deprivation.

Motivational measures of the aftereffects of running

A series of additional CPP experiments, which are summarized in Table 2, were conducted to examine the incentive salience of the aftereffects of wheel running. A CPP emerged for the aftereffects of wheel running in male rats that had just emerged from their acquisition of running and were in the first week of the stable or habitual phase of running. Males showed a conditioning effect in terms of group chamber time ($F(2,14)=7.198$, $p=0.007$). Furthermore, a significant gender difference was found in that males developed a CPP under these conditions whereas females did not (Table 2).

We further pursued an analysis of the experimental conditions that might reveal whether females found positive incentive salience in the aftereffects of running at any point in their running experience, including the acquisition phase as well as the habitual phase of running. We based our analysis on the robust running profile of females given only short-term access to wheels in a hypothesized workout model of wheel availability. Given that the basic acquisition period of habitual running of the female with *ad libitum* wheel access is only two weeks, an independent group of females was conditioned in the first seven days of *ad libitum* running acquisition. Two additional groups of females were conditioned either in the acquisition or habitual phases of running during short-term access regimes, which offered profiles of running that suggested these wheel access periods were of potentially high positive incentive salience. Specifically, limited wheel access yields subjects that run immediately for 90-100% of the 30-minute wheel access time (boutless constant running) and 60% of the 2-hour wheel access time. These are remarkably robust running sessions

compared to the fact that *ad libitum* runners use only 30% of their dark period running. (Basso and Morrell, 2010). Regardless of the conditioning process or the running experience, females never showed a CPP for the aftereffects of wheel running (Table 2).

Discussion

Overall, our findings suggest that the total experience of voluntary wheel running is a stimulus with positive incentive salience (i.e., a rewarding stimulus) to both males and females in both the acquisition and habitual phases of running. This is the first report to demonstrate a CPP for the total experience of wheel running in rats. While the acquisition phase appears to be equally salient to both genders, the habitual phase may have somewhat greater salience for females, considering their higher rebound running with longer forced abstinence. Interestingly, while the aftereffects of wheel running appear to have rewarding features to males, we could not demonstrate such salience in females, and we hypothesize that this may be an additional gender difference in the motivation to wheel run.

A r*obust conditioned response to the total experience of the acquisition of wheel running suggests that the initial wheel running experience has rewarding properties H*ere, we demonstrate for the first time that both male and female rats show a strong preference for a place associated with the total experience of wheel running during the acquisition phase of the wheel running experience. As designed, this experience includes three components, the interaction with the

wheel as an enrichment object, the wheel running experience itself, and the aftereffects of running or the physiological state of the animal that has experienced wheel running.

One of the strongest prior running CPP demonstrations shows that voluntary wheel running in rodents has positive incentive salience. Young hamsters (45-60 days old) show a CPP for a chamber associated with only 30 minutes of wheel running per day for 4 days, which is in the acquisition phase of running (Antoniadis et al. 2000; Ralph et al., 2002). Older hamsters (one year old) also show a CPP for this same protocol, however, this is only the case if they still demonstrate healthy, consolidated running patterns as opposed to unhealthy, fragmented locomotor rhythms (Antoniadis et al., 2000). These are the only two studies in any rodent to examine the question of whether the whole experience of wheel running, that is, the availability of the wheel, the running itself, and the aftereffects of the running, has positive incentive salience. Our findings accord with this work, adding the novel information that rats also find the experience of the acquisition of *ad libitum* voluntary wheel running rewarding and that both genders finding it equally salient.

A robust unconditioned rebound response to the wheel after forced wheel abstinence suggests that the habitual phase of running has rewarding properties After 72 hours of forced wheel abstinence, both male and female rats showed a robust rebound response to the wheel, with running distances, times and rates similar to those run during the first hour of the dark period, the time period of the light-dark cycle that rats run the most vigorously. Although the subjects

displayed a rebound response even upon removal of the wheel for only 1 hour, which can be viewed as a more minimal wheel access disruption process, this 1-hour deprivation response was blunted on every quantitative parameter compared to the 72-hour deprivation response in both genders. We posit that forced abstinence from a stimulus with positive incentive salience increases the rewarding properties of the stimulus, as suggested by the greater rebound running response. While the overall pattern of the response was similar in both genders, some subtle gender differences were seen in that females responded with a greater distance and faster running rate after a 72-hour forced abstinence period than did the males.

Our data are novel in that this is the first demonstration of the reinstatement response in both genders. Although prior studies have examined only males, our findings generally accord with those of others that have examined the rebound running response after periods of forced wheel deprivation. Early studies revealed that activity deprivation (i.e., confinement in a small cage) leads to increased running wheel activity (Hill, 1956; 1961), and the 72-hour deprivation period in the present study can similarly be seen as activity deprivation. These early studies may have been confounded by the stressful nature of the confined environment (McGlone et al., 2004); although, one could argue that wheel deprivation in itself is a stress-inducing situation.

Similar to our work, Mueller and colleagues (1999) conducted an in-depth investigation on the effects of short-term wheel deprivation on running behavior upon return of the wheel. They found a positive correlation between amount of

wheel deprivation (0, 1, 3, and 10 hours) and running distance in the first 24 hours after return of the wheel. That is, the longer the period of wheel deprivation, the more drastic the rebound running effect is upon wheel return. Further, they found that the increase in wheel running is actually proportional to the amount of running that would have occurred if animals had *ad libitum* access to the wheel. For example, if wheel deprivation occurs during the day, then no 24-hour increase is seen because animals generally do not run during the resting period of their daily cycle. Mueller et al. (1997; 1999) also found that if the wheel deprivation period was from 24 to 72 hours, then no change occurred in daily distance run following wheel return. This finding accords with our work, and we further show that in this situation, it is the proportion of running that occurs in the light or the 24-hour pattern of running that is altered rather than the overall 24-hour distance (Basso and Morrell, 2010). Additionally, they show that if wheel deprivation occurs early in the night, when rats are most active, then a 24-hour running distance increase is seen reflecting the missing distance run. In order to see a robust effect, we specifically chose to examine the rebound running response during a period of time when rats generally produce no running.

Our work, in combination with others, reveals that voluntary wheel running shows spontaneous recovery, meaning that an increase in consumption or stimulus interaction occurs after a period of deprivation. Not surprisingly, spontaneous recovery occurs for other natural and pharmacological stimuli with positive incentive salience (see McSweeney et al., 2005 for review), suggesting that voluntary wheel running shares similar characteristics to other stimuli with

positive incentive salience for rats. For example, upon re-exposure to drugs of abuse, such as alcohol, cocaine, or heroin, after a forced period of abstinence, rats have been shown to binge or increase usage of the drug (Le & Shaham, 2002; Shalev et al., 2002). Rebound running after three days of forced wheel abstinence can be seen as an analogous process of wheel-running binge behavior.

Is there a gender difference in the incentive salience of the aftereffects of wheel running? We found that males show a CPP for the aftereffects of voluntary wheel running; however, new to the literature, females do not. This is the first report of a gender difference in a CPP for the aftereffects of running. Even with a variety of CPP paradigms for the aftereffects of wheel running, females did not reveal a conditioning effect for the aftereffects or running. Considering that both males and females readily displayed a robust CPP for the total experience of running, but only males displayed a CPP for the aftereffects of running, we speculate that that there may be a gender difference in motivation for this specific feature of the running experience. Considering that in males, the CPP for the total experience of running was much stronger than the CPP for the aftereffects of running, we speculate that this indicates that the running itself adds a significant rewarding component to the experience.

Recognizing that negative data are not conclusive, we can only state that none of the technical approaches and the variations of the running used to establish conditioning were sufficient to produce a CPP for the aftereffects of running in females. In particular, the aftereffects resulting from short-term wheel

access (i.e., up to two hours of running) during the dark period is not a sufficient stimuli to produce a CPP. Additionally, whether or not females have previous running experience with the wheel does not impact a CPP for the aftereffects of running. That is, the aftereffects during a period of running acquisition (i.e., the first seven days) versus during a period of stabilized running (i.e., experienced runners with a history of 21 days of running) did not have different incentive salience or hedonic properties. Perhaps future studies by others may uncover aspects of the procedure crucial to reveal a CPP for aftereffects in females, or possibly, females do not find that the aftereffects of wheel running to have positive incentive salience. Nonetheless in side-by-side comparisons, with strictly comparable conditions, we were able to establish that the aftereffects of habit running in males did have incentive salience for them, while similar conditions did not have incentive salience for females.

Several earlier studies using male rats only have reported a modest CPP for the aftereffects of voluntary wheel running (Lett et al., 2000; 2002; Belke & Wagner, 2005). In one report, food-deprived male rats were given 12 days of alternate-day access to a wheel or no wheel for either 22 hours or 2 hours during the light period and conditioned for 30 minutes after this process (Lett et al., 2000). Though the CPP was somewhat stronger when immediate placement from the running wheel to the conditioning chamber occurred, the CPP was upheld even when a 10 minute delay was interjected between the running experience and conditioning (Lett et al., 2002); however, this was not the case if the delay was 30 minutes, indicating that the rewarding aftereffects may have

dissipated by this time period. Recent work, more similar to our paradigm, used male rats exposed to the wheel for the entirety of the dark period and conditioned for 30 minutes upon lights on for 42 days with alternate-day access to a wheel or locked wheel (Greenwood et al., 2011). Thus, this protocol allowed rats 21 days of wheel exposure, which is an adequate time period for rats to reach habitual, stable running levels (Eayrs, 1954; Koh et al., 2000; Basso and Morrell, 2010). These conditions produced a robust CPP for the aftereffects of the wheel. Interestingly, no significance was seen at an intermittent probe test at two weeks (Greenwood, et al 2011), which might indicate that the aftereffects of wheel running are not salient during periods of running acquisition, as during this first week of running, males and even female rats are still acquiring habitual, stable running levels. In contrast, we found a strong CPP for the total experience of wheel running at this time point, during the acquisition of running in both genders. Together, these findings may indicate that though the experience of wheel running is rewarding during this acquisition period, the aftereffects are only salient once running becomes a habitual behavior.

Running farther or faster does not mean you prefer it more Interestingly, neither distance run nor running rate has been shown to correlate with preference for the aftereffects of the wheel or preference for the total experience of wheel running as measured by a CPP paradigm (Antoniadis et al., 2000; Lett et al., 2002; Belke & Wagner, 2005; Greenwood et al., 2011). That is, rats that run longer distances faster do not show an increased preference for the wheel. Our work accords with these findings, showing that rats that run longer distances

do not show an increased preference for the total experience of wheel running during its acquisition phase. Additionally, neither lever-pressing rates nor post-reinforcement pauses show a correlation to preference for the aftereffects of the wheel (Belke & Wagner, 2005). To these data, we add that gender does not influence the preference for voluntary wheel running. It might be surmised that because females run on average 1.5 farther and faster than males (Basso & Morrell, 2010), that females prefer the running experience more; however, we have found this not to be the case.

Rats harbor a set point for the amount of voluntary wheel running they conduct Though females run farther and faster than males on a daily basis (Basso and Morrell, 2010), we show that both measures of the incentive salience of voluntary wheel running are quite similar. The fact that there is no correlation between the amount run and preference for running may indicate that there may be a motivational set point for running that is unique to each subject. This may be why great running variability exists between individuals, but once a period of habitual running is reached, relatively stable distances are seen from day to day. If we take the position that rats are running because it is rewarding, then we can imagine that a variety of reward-related brain mechanisms are taking place. For example, the involvement of the dopamine and endogenous opioid systems have been implicated in voluntary wheel running (De Castro & Duncan, 1985; Hoffman et al., 1990; Werme et al., 2000; Rhodes & Garland, 2003; Knab & Lightfoot, 2010; Basso et al., 2011), and further, opioid antagonists decrease both running distances and the preference for the aftereffects of running (Boer et al., 1990;

Lett et al., 2001; Sisti & Lewis, 2001). Our data suggests that running serves as a salient stimulus to the brain. The fact that this behavior is stable within the individual seems to suggest that subjects use running as way to acquire a set point amount of neuronal stimulation or neurotransmitter/neuromodulator/protein accumulation within the brain. Additionally, this set point appears to be altered by ovarian hormones, which certainly have been implicated in regulation of other types of rewards (Russo et al., 2003; Dreher et al., 2007; Seip et al., 2008; Parada et al., 2012).

Reflections on the neural circuits that may mediate motivation to engage in voluntary wheel running Based on this work and our earlier work revealing that lifelong running alters the neurochemical response to an acute dose of a salient pharmacological stimuli in a variety of motor and motivational brain regions (Basso et al., 2011), we hypothesize that the neural circuit prevalently understood to mediate motivational responses, including the ventral tegmental area, nucleus accumbens and medial prefrontal cortex, likely including but not limited to the dopaminergic connections among these regions, are involved in regulating the motivation to engage in voluntary wheel running. Studies are in progress to examine brain regions necessary for the motivation to engage in voluntary wheel running as examined in this work from both the unconditioned and conditioned perspectives (Basso & Morrell, 2012). Specifically, we are examining the involvement of the nucleus accumbens core and the prelimbic medial prefrontal cortex, regions involved in the acquisition and reinstatement of drug taking behavior (Kalivas, 2008). We hypothesize that these areas might

also be involved in the motivational processes regulating the rebound running response and the seeking out of the wheel running experience.

A final speculation about the rewarding nature of exercise in humans Our work from this preclinical model suggests that exercise in humans can be an acutely rewarding experience, both during an initial acquisition period and after the exercise routine becomes habitual. Our work also shows that males and females may find different components of their workout routine rewarding, for example, males may find the aftereffects of their workout more satisfying than females. Considering that 74% of the US population does not attain the recommended daily level of physical activity (American Heart Association; Center for Disease Control) and obesity is an epidemic (35.7% of the US population is obese), motivation to exercise is an obvious issue for the American populace. Participation in a daily physical activity regimen is one way to combat these ills; however, understanding the long-term physical and mental health benefits of exercise does not seem to be enough to get us to exercise. Perhaps people would be more motivated to exercise if they obtained an immediate reward from the experience, and this work sheds some light on the idea that physical activity has short-term immediate effects that can be robustly rewarding for both genders.

TABLE 1

Larger rebound responses occur with greater levels of wheel deprivation

		No Deprivation	1 Hour Deprivation	72 Hours Deprivation
Distance (meters)	**Females**	0.07 (±0.05)	**260.57 (±62.12)**	**666.16 (±60.77)***
	Males	1.23 (±1.16)	**111.00 (±32.16)**	**435.06 (±52.26)***
Time (minutes)	**Females**	0.09 (±0.06)	**14.88 (±2.08)**	**40.08 (±2.83)***
	Males	0.50 (±0.42)	**20.75 (±5.08)**	**40.5 (±2.97)***
Rate (m/min)	**Females**	0.04 (±0.03)	**7.5 (±1.02)**	**15.46 (±0.82)***
	Males	0.30 (±0.24)	**8.32 (±1.79)**	**10.67 (±0.85)**

Table 1 Average (±SEM) distance (meters), time (minutes) and rate (meters/minute) run during the 1 hour period after no deprivation, 1 hour of wheel deprivation or 72 hours of wheel deprivation. Bold figures in the 1 or 72 hour deprivation category denotes statistical significance from the no deprivation category. Asterisks in the 72-hour deprivation category denote statistical significance from the 1-hour deprivation category. Enlarged font indicates statistical significance between genders; females ran 1.5 times farther and faster in their response after a 72-hour period of forced wheel abstinence.

TABLE 2

CPP experiments examining the preference for the aftereffects of wheel running

	Gender and Number of Animals	Conditioning Protocol	Other Variables	Result
Aftereffects of *ad libitum* running in habitual runners	Females and males (n=8, both genders)	23 hour alternate-day access to a wheel or locked wheel for 40 days (20 days of running)	Conditioning took place upon lights turning on; naive to the wheel at the start of experiment	Positive CPP in males but not females
Aftereffects of *ad libitum* running in acquisition runners	Females (n=8)	23 hour alternate-day access to a wheel or no wheel for 14 days (7 days of running)	Conditioning took place upon lights turning on; naive to the wheel at the start of experiment	No CPP
Aftereffects of workout running in habitual runners	Females (n=8, each group)	30 minutes or 2 hour alternate-day access to a wheel or locked wheel during the dark period for 14 days	21 days of previous running experience (habitual runners)	No CPP
Aftereffects of workout running in acquisition runners	Females (n=8)	2 hour alternate-day access to a wheel or locked wheel during the dark period for 14 days (7 days of running)	Naïve to the wheel at the start of experiment	No CPP

* Note: All conditioning sessions lasted 30 minutes and took place in a three-chambered conditioned place preference apparatus. All animals were kept on a 12-hour light-dark cycle.

Table 2 Description of methods and results of experiments examining a conditioned place preference for the aftereffects of wheel running.

FIGURE 1

Time course of acquisition of stable running

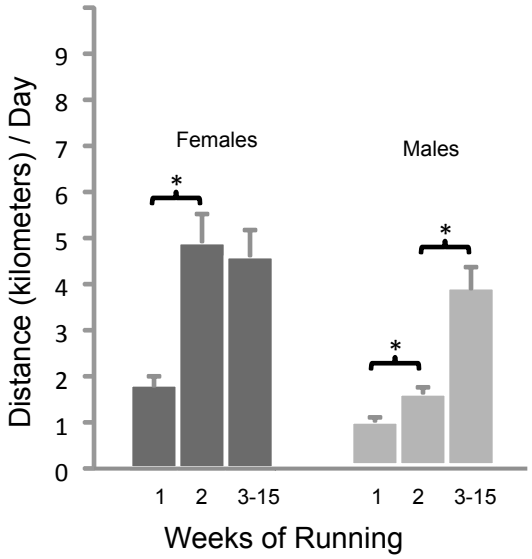

Figure 1 Average (±SEM) distance (kilometers) run in a 24-hour period during the 1st, 2nd and 3rd (to 15th) week of wheel exposure. Each data point represents an average of data from all seven days of the week. Females reach stabilized habitual levels faster than males, that is, during their 2nd week compared to their 3rd.

FIGURE 2

Females and males develop a conditioned preference for a place associated with the experience of wheel running

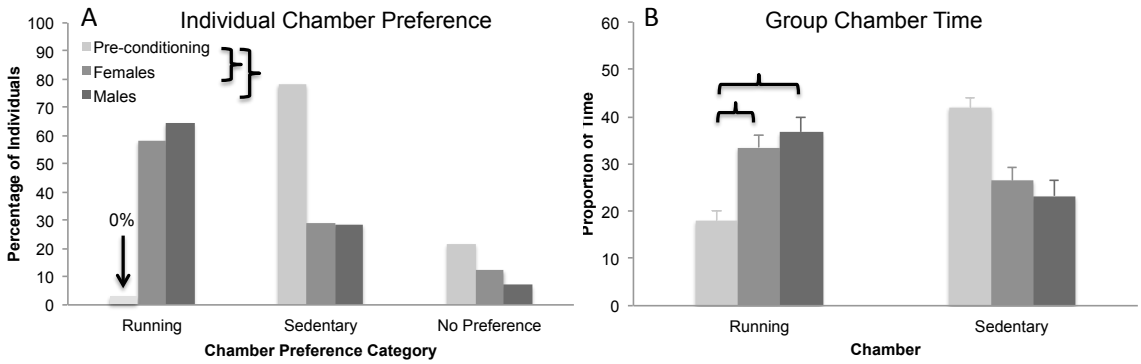

Figure 2 (A). Percentage of females or males demonstrating a preference for a chamber associated with the experience of running, the experience of no running, or no preference at the pre- and post-conditioning sessions. (B). The amount of time spent in the chamber associated with running or no running at the pre- and post-conditioning session. In both A and B, the pre-conditioning session is an average of both genders.

FIGURE 3

Females and males display a rebound running response after 72 hours of forced wheel abstinence

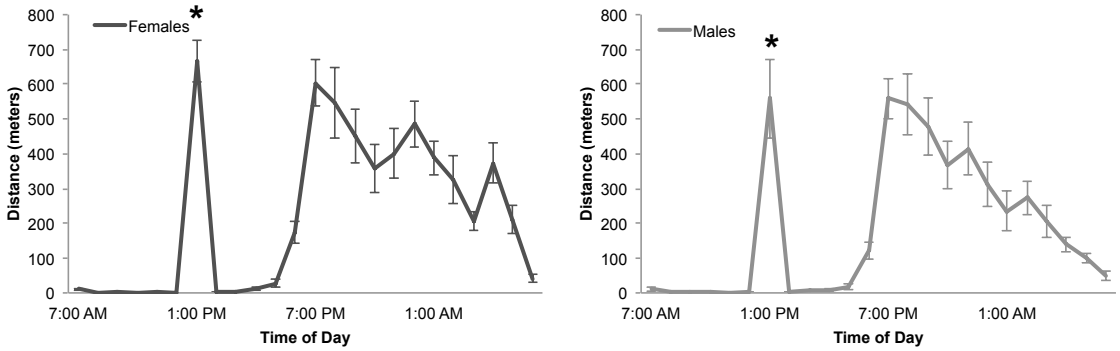

Figure 3 Average (±SEM) distance (meters) run during each hour of the 24-hour light-dark cycle during the 3rd week of running, a time period of stable, habitual running. The time point at 1:00 pm represents the distance run in the first hour after return of the wheel after a period of 72 hours of forced wheel abstinence.

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CHAPTER 5

Motivation for voluntary wheel running across genders: Brain regions mediating unconditioned and conditioned responses to the wheel

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Abstract

Previous work from our laboratory and others shows that voluntary wheel running is a motivated behavior with positive incentive salience for rats. Though females acquire stable levels of the behavior faster than males and run 1.5 times farther and faster, few other gender differences exist within the quantitative measures of voluntary wheel running. Voluntary wheel running has a myriad of effects on certain regions of the brain, but little has been done to investigate what brain regions directly regulate the motivation for voluntary wheel running. The prelimbic medial prefrontal cortex (mPFC) and nucleus accumbens (NA) core have been shown to be involved in the acquisition and reinstatement of self-administration of both natural and pharmacological stimuli. Based on this model, we hypothesized that these regions might be involved in the acquisition and reinstatement of voluntary wheel running as well. In combination with transient inactivation of these areas using bupivacaine and muscimol, we utilized a conditioned place preference (CPP) model to examine the motivation for voluntary wheel running during its acquisition phase and the reinstatement (rebound) of running after a period of forced wheel abstinence. We found that cannulation alone significantly impairs the CPP for the total experience of wheel running during the acquisition phase. Additionally, we found that saline infusion significantly decreases the rebound response after a period of forced wheel abstinence. However, when the prelimbic mPFC and NA core were inactivated, further significant decreases were seen, suggesting that these regions may regulate the motivation for voluntary wheel running. This is the first report to implicate specific brain regions in the motivation for this behavior.

Introduction

Voluntary wheel running is a robust behavior in rodents (Richter, 1927; Shirley, 1929; Sherwin, 1998; Afonso & Eikelboom, 2003; Basso & Morrell, 2010; Greenwood et al., 2011). Recent research indicates that it is a motivated behavior with positive incentive salience (Lett et al., 2000; 2002; Belke & Wagner, 2005; Basso & Morrell, 2010; 2012; Greenwood et al., 2011). Though females run approximately 1.5 times farther and faster than males, quantitative measures of the motivation for this behavior indicates that voluntary wheel running is an equally motivating stimulus for both genders. Though this is understood on a behavioral level, little has been done to investigate what brain regions underlie the motivation for voluntary wheel running. We hypothesize that distinct brain regions, such as the medial prefrontal cortex (mPFC) and nucleus accumbens (NA), which have been implicated in the motivation to seek, obtain and consume other natural and pharmacological stimuli, may be involved in the regulation of the motivation for voluntary wheel running. Here, we examine the motivation for voluntary wheel running during two periods of running, the acquisition phase (days 1 to 7) and the habitual phase (after day 21). For the acquisition phase experiments, we analyzed the conditioned response to the total experience of wheel running using a conditioned place preference (CPP) model. For the habitual phase experiments, we examined the unconditioned response to the wheel after a period of forced wheel abstinence. Through transient inactivation of distinct brain regions, this work seeks to investigate for the first time, brain systems that mediate the motivation for voluntary wheel running.

The mesocorticolimbic pathway is a network of distinct brain regions, including the orbitofrontal and medial prefrontal cortices, the basolateral nucleus of the amygdala, the hippocampus, and the ventral tegmental area (VTA), which have been implicated in the motivational processes involved in goal-directed behaviors. The nucleus accumbens receives excitatory inputs from all of these cortical and subcortical structures as well as modulatory dopaminergic inputs from the VTA (Mogenson et al., 1980; Pennartz et al., 1994; Groenewegen et al., 1996, 1999) and projects to a variety of structures including hypothalamic and brainstem nuclei, the ventral pallidum, substantia nigra pars reticulata, VTA and medial prefrontal cortex (Zahm & Heimer, 1993), which serves as the motor output for numerous behaviors including voluntary wheel running. Though the mesocorticolimbic pathway has traditionally been thought of as a dopaminergic pathway, recent research indicates that this system is more complicated, including glutamatergic and GABAergic components as well (Yamaguchi et al., 2011).

Data indicate that voluntary wheel running, as well as forced treadmill running, has significant impact on the mesocorticolimbic pathway. For example, physical activity produces a variety of alterations in the VTA, NA and mPFC (De Castro & Duncan, 1985; Hattori et al., 1994; Wilson & Marsden, 1995; Meeusen et al., 1997; Werme et al., 2002; Greenwood et al., 2011). We further propose that particular subregions of this pathway regulate the motivation to engage in voluntary wheel running. Recent research indicates that specific components of the mPFC and NA are involved in different aspects of motivation. Participation in

a motivated behavior consists of several stages: the acquisition of the behavior, the habitual phase of the behavior, and in some behavioral protocols, the extinction and reinstatement of the behavior. For example, researchers have revealed that the prelimbic mPFC and NA core are involved in the acquisition and reinstatement of learned behaviors, like fear-conditioning and operant responses for natural and pharmacological stimuli, whereas the infralimbic mPFC and NA shell are involved in the extinction of these behaviors (McFarland & Kalivas, 2001; Quirk et al., 2006; Vidal-Gonzalez et al., 2006; Floresco et al., 2008; Kalivas, 2008; Peters et al., 2008; 2009; LaLumiere et al., 2010; Rocha & Kalivas, 2010). When behaviors become well learned or habitual, the prefrontal circuitry is no longer necessary. Rather, these habitual behaviors engage the cortico-striatal-thalamic circuitry. In this way, subjects can engage in the motor components of the habitual behavior while freeing the prefrontal cortex for cortical processing of other events. This information suggests that the prelimbic mPFC and NA core are necessary for the expression of motivated behaviors whereas the infralimbic mPFC and NA shell are necessary for the suppression of motivated behaviors. Peters and colleagues (2008) suggest that these regions do not play a direct role in the motor component of these behavior, but rather the decisions to engage in (prelimbic mPFC/NA core) or suppress (infralimbic mPFC/NA shell) a conditioned response. Considering this work, we investigated the involvement of these four regions in the motivation to engage in voluntary wheel running. We hypothesized that the prelimbic mPFC and the NA core would be involved in both the decision to seek out a place associated with the

total experience of wheel running (CPP) as well as the reinstatement or increase in running after a period of forced wheel abstinence (rebound response). After the terminology of Kalivas and colleagues (2008), we term this the "GO" subcircuit of the larger motivational circuit. Subsequently, we hypothesized that the infralimbic mPFC and NA shell would be involved in the suppression of voluntary wheel running, as seen through a decreased CPP or rebound response, which we term the "STOP" subcircuit component.

We transiently inactivated these regions with either bupivacaine, a Na^+ channel blocker, or muscimol, a $GABA_A$ agonist. We then used the tests of motivation described in Chapter 4 of this thesis to measure the expression of a CPP for the total experience of wheel running or the recovery response (rebound or reinstatement) of running after a period of forced wheel abstinence. Considering the differences in voluntary wheel running distances and rates between males and females, we examined these quantitative motivational tests and the involvement of these brain regions in the motivation for this behavior in both genders. We hypothesized that transiently inactivating the "GO" subcircuit, (i.e., the prelimbic mPFC and NA core) would decrease both the CPP for the total experience of wheel running as well as the rebound response, whereas transiently inactivating the "STOP" subcircuit (i.e., the infralimbic mPFC and NA shell) would increase these behaviors.

Methods

Subjects Male and female Sprague Dawley rats (original stock from Charles River Laboratories, Kingston, NY, USA) were bred in our colony at the Rutgers

University Laboratory Animal Facility (RAF) (Newark, NJ, USA) (accredited by the American Association for Accreditation of Laboratory Animal Care). All animals were kept on a 12-hour light-dark cycle (lights on at 7:00 am, unless otherwise noted) in a room at 22(±1)°C and given *ad libitum* access to water and rat chow (Lab Diet 5008, PMI Nutrition International, LLC, Brentwood, MO, USA). Daily checks were conducted for health and availability of food and water. Weights were taken once per week and animal husbandry was performed twice to seven days a week depending on the protocol. All animals remained healthy and of normal body weight throughout the experiments. Animal care and experimental procedures performed in this protocol were in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were reviewed and approved by the Rutgers University Animal Care and Facilities Committee. Care was taken to minimize the suffering and curtail the number of animals utilized.

Running wheel apparatuses Experiments were conducted using either an AccuScan Instruments (Columbus, Ohio, USA) VersaMax Animal Activity Monitor (wheel: 25 cm diameter, stainless steel mesh floor; home cage: 40 cm long x 40 cm wide x 30 cm wide) or a Med Associates Inc. (St. Albans, VT, USA) ENV-046 Activity Wheel with Plastic Home Cage for Rats (wheel: 35.6 cm diameter, 4.8 mm stainless steel grid rods with a 1.6 cm spacing, 12 gram freewheeling drag; home cage: 48.26 cm long x 26.67 wide cm x 20.32 cm high with a 7.2 cm wide x 10.2 cm high opening to wheel). The resistance of both running wheels was low and equivalent, and no extra weight/resistance was placed upon either of the

wheels. Both housing apparatuses were lined with woodchip bedding (Beta chip, Northeaster Products Corp., Warrensburg, NY, USA), except when otherwise noted. Food and water were provided *ad libitum* at all times. For both systems, data was captured automatically. In the AccuScan system, 16 infrared beams lined each axis of the box. Each box was connected through wires to the computer and data was captured through the Windows based software, VersaMax and VersaDat. In the Med Associates system, the LCD digital counter captured wheel turns, the computer was connected through wires to the computer, and data was captured through the Windows based software MedPCIV.

Quantitative measures of the motivation for voluntary wheel running

Conditioned place preference (CPP) for the total experience of wheel running in the acquisition phase of running Informed by our prior CPP work, a two-chambered CPP apparatus was devised within the laboratory (Mattson et al., 2001; Seip et al., 2008), consisting of two AccuScan Instruments (Columbus, Ohio, USA) boxes of equal size (40 cm long x 40 cm wide x 30 cm wide), placed side by side and connected with a short opaque tunnel (4" diameter, 2" length). Each box was decorated with unique cues, which consisted of wallpaper in either horizontal or vertical black and white stripes and tactile flooring of small paper squares (ALPHA-dri, Shepherd Specialty Papers, Kalamazoo, MI) or small corn cobs (Bed-o" cobs (1/4") The Andersons, Maumee, OH). All boxes were covered with transparent lids and lit by overhead lights. Luminance (Konica Minolta Luminance Meter LS-100, Japan) was equal in all chambers (220 lumens). Two

weeks prior to the pre-conditioning session, wheel naïve males (n=8) and females (n=8) were obtained from the RAF and placed on a 12-hour light-dark cycle (lights on at 12:00 am).

Pre-conditioning chamber preference baseline At PND 65, subjects were exposed to the two-chambered apparatus for 60 minutes and allowed to roam freely between the chambers. This pre-conditioning session occurred around 10:30 am so that the session ended before the lights turned off. Time spent in each chamber was manually recorded. Based on criterion discussed below, animals were then assigned to receive the wheel in one uniquely cue decorated chamber and no wheel in the alternate uniquely cue decorated chamber.

Conditioning sessions Pre-conditioning boxes were replaced with cue decorated boxes without the entrance hole in the side, one of which had a running wheel and one of which did not, providing both food and water *ad libitum*. Twenty-four hours after the pre-conditioning baseline session, animals were isolated in one of the uniquely cue-decorated chambers for 23 hours (with or without a wheel). The 23 hours of conditioning occurred from ~11:30 am to the following 10:30 am. One hour of the 24 hour cycle was left so that chambers could be cleaned and animals could be placed in the alternate chamber before the lights turned off at 12:00 pm. During cleaning (1% Liquinox, distilled water, 70% alcohol), animals were placed in a shoebox holding cage with food and water *ad libitum*. The idea behind this timing was that animals were placed in the chambers with lights on to view their environment, but soon thereafter, the lights turned off, which is the most active time period for the rat in terms of wheel running (i.e., highest hourly

running rate). Essentially, the change in the light-dark period (lights off) correlated with a change in conditioning environment. Animals were then placed in the alternate chambers for 23 hours. This cycle continued for 14 days, such that each animal received 7 days of conditioning with the wheel and 7 days of conditioning without the wheel (i.e., an alternate-day running experience). At the beginning of the experiment, all animals were wheel naïve. At the end of conditioning, each subject received 7 days of wheel exposure, which our prior data establish is in the acquisition phase of wheel running behavior (Basso and Morrell, 2010; 2012). At the end of conditioning, animals were returned to shoebox cages with *ad libitum* food and water, which served as home cages until the post-conditioning test below.

Post-conditioning test of place preference Twenty-four to ninety-six hours after the final conditioning session, animals were exposed to the two-chambered apparatus, which were cleaned and cue decorated just as throughout the pre-conditioning and conditioning sessions. Animals were tested at most twice, that is once with 24 or 48 hours of forced wheel abstinence and a second time with 72 or 96 hours of forced wheel abstinence respectively. Previous data from our lab indicates that repeated post-conditioning testing (maximum of 2 times) provides similarly robust results, so this protocol was adapted for the present work (Seip et al., 2008). The subjects were allowed to roam freely between the chambers for 60 minutes, with time spent in each chamber recorded manually by three observers who were naïve to the stimulus-chamber associations learned by the individual animals during conditioning.

Analyses and chamber assignments To understand the preference of each individual animal as well as the group preference as a whole, data was analyzed using two techniques, individual chamber preference and group chamber time (Mattson et al., 2001; 2003). Data were analyzed at both the pre- and post-conditioning sessions. In order to determine if an individual animal showed a preference for a particular chamber, a stringent quantitative criterion was developed. To show a preference, the animal must have spent ≥30 minutes in one chamber, and this time also had to be ≥25% larger than that of the other chamber time. If these two criteria were not met, the animal was categorized as showing no preference. In the case of the two-chambered apparatus, three preference categories were possible (square, corn cobs, no preference). After individual chamber preference from the pre-conditioning session was established, animals were assigned to receive the wheel in their least-preferred side chamber. If an animal showed no preference, then the wheel was assigned to one of the two chambers at random. Group chamber times were calculated by averaging the time spent in each chamber by all animals.

Reinstatement of running after a period of forced wheel abstinence in the habitual phase of running At PND 65, wheel naïve males (n=14) and females (n=43), previously group housed in shoebox cages in the RAF, were individually placed in the AccuScan or Med Associates home cages with running wheels at ~1:00 pm (approximate midpoint light period). As previously established through our work, at this point animals were running stable daily distances and were habitual runners. At ~1:00 pm on the 22nd day of running, animals were then

given a period of 72 hours of wheel deprivation. In the AccuScan apparatus, the wheels were removed from the home cage. In the Med Associates apparatus, a manual sliding door was placed in between the home cage and the wheel, thus blocking the rats' access to the wheels. Wheels were subsequently returned (~1:00 pm) for at minimum four days of *ad libitum* wheel access. To optimally reveal the reinstatement or rebound running response, wheels were always returned during the midpoint of the light period. In this way, this response could be compared to a normal undisrupted resting baseline, that is, when virtually no wheel running occurs.

Cannula implantation via stereotaxic surgery At around PND 65, males (n=22) and females (n=51) were randomly assigned to receive bilateral cannulae placement in either the prelimbic (n=33) or infralimbic (n=32) subregions of the medial prefrontal cortex or nucleus accumbens core (n=4) or shell (n=4). Animals were anesthetized with 1 mL/kg of a solution containing ketamine HCl (75.0 mg/mL), xylazine (7.5 mg/mL), and acepromazine maleate (1.5 mg/mL). The incision site was shaved and injected subcutaneously with 0.5% Marcaine. Animals were placed in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA), and incisor bars were placed 3.2 mm below the interaural line so that the skull was flat and Bregma and Lambda were positioned in the same vertical coordinate. An incision was made and the scalp was carefully opened to reveal the skull. Bregma and Lambda were identified and for the prelimbic mPFC, a 22-guage stainless steel guide cannulae (Plastics One, Roanoke, VA, USA) was implanted +2.8 mm AP from bregma, ±0.60 mm ML

from the midline, and -2.0 mm DV from the skull surface. For the infralimbic mPFC, a 22-gauge stainless steel guide cannulae was implanted +2.8 mm AP from bregma, ±0.60 mm ML from the midline, and -3.0 mm DV from the skull surface. For NA core, a 22-gauge stainless steel guide cannula was implanted +1.6 mm AP from bregma, ±1.5 mm from the midline, and -6.0 mm DV from the skull surface. For the NA shell, a 22-gauge stainless steel guide cannula was implanted +1.6 mm from bregma, ±0.75 mm from the midline, and -6.0 mm DV from the skull surface. The guide cannula was secured to the skull using stainless steel screws and cranioplastic cement. To keep the guide cannulae free of tissue or liquids, dummy stylets were inserted. After surgery, animals were placed individually in home cages, and overall health was checked daily. Conditioned place preference experiments commenced approximately one week after surgeries took place. For rebound running experiments, animals were placed with their wheels immediately after surgery, but rebound testing did not take place until at least one week after surgeries.

Intracranial infusions All animals were handled extensively for at least one week before all intracranial infusions. During this time, dummy cannulae were removed periodically and cleaned to ensure that no biological debris accumulated. This also helped the animal habituate to the experience of having the headset touched and manipulated. For infusion purposes, the dummy stylet was removed, and a 28-gauge stainless steel injector was inserted into the guide cannulae. The injector extended 2.0 mm beyond the tip of the guide cannulae and was connected by PE-10 tubing to 10 µL Hamilton syringes. Simultaneous

bilateral infusions occurred via a two-syringe infusion pump (Harvard 22 syringe pump; Harvard Apparatus, Holliston, MA, USA). Bupivacaine (2%), muscimol (50 ng/0.5 µL; Tocris, Ellisville, MO, USA) or saline (0.9%) were injected at a rate of 0.5 µL per minute, with bupivacaine infusions receiving 1.0 µL per side and muscimol infusions receiving 0.5 µL per side. After the total amount was infused, the injectors were left in place for one minute to allow diffusion of the substance.

To avoid a stress response, which would serve as a confound to the infusion outcomes, cannulated subjects were exhaustively habituated to handling, including handling of their headsets. To even further decrease restriction disturbance, the subjects were allowed to freely roam around a holding cage during infusion. While gently restrained, subjects had the dummy cannula removed and the injector cannula inserted, which were attached to the PE tubing and the Hamilton microsyringes in the infusion pump apparatus. Then subjects were released to free roam in a holding cage (without a top) that was adjacent to the infusion pump during the infusion and diffusion processes. During infusions, animals explored the holding cage without any evidence of behavioral discomfort or immediate effect of the infusion process. After this two- to three-minute process, animals were again handled with gentle restraint to remove the injector and replace the dummy cannulae. In the case of bupivacaine, after infusions, animals were placed immediately into the post-conditioning CPP apparatus or home cages with wheel access. In the case of muscimol, after infusions, animals were placed in a holding cage with food and water *ad libitum* for approximately one hour before being placed in the post-conditioning CPP apparatus or home

cages with wheel access. For the conditioned place preference experiments, groups of subjects were infused 1 or 2 days apart. In this way, all post-conditioning testing was conducted with a maximum of 96 hours of wheel deprivation. Previous work indicates that uncannulated animals with post-conditioning sessions from 24 to 96 hours of wheel deprivation show similarly robust responses (Basso & Morrell, 2010). For the rebound response experiments, all injections were conducted at least 7 days apart. Previous work indicates that repeated measures of the rebound response are similar (Basso & Morrell, 2010; 2012), and so this work adapted this protocol for its repeated measures infusion purposes.

Surgical and intact controls Surgical controls consisted of animals whose dummy stylets were immovable or whose cannula placements were off target (i.e., unilateral or dorsal placements). Prior to testing, these animals were handled in a similar manner to all surgical animals, as described above. Intact controls consisted of animals that did not undergo surgery. Prior to testing, these animals were not handled as extensively, as they may have been placed directly into the post-conditioning CPP apparatus or been given wheel access without any handling.

Histology After all testing was complete, subjects were anesthetized with Nembutal (1 mL) and intracardially perfused with 4% formalin. Brains were then removed and placed in formalin for at least 1 day. They were then exposed to a 15% sucrose solution for an additional day. Brains were then blocked, and samples were mounted onto the cryostat's specimen holders with water and dry

ice and placed in the -13° C microtome. Brains were sectioned at 30 μm and mounted on chrom-alum coated slides. At least one week after slicing, all sections were stained with cresyl violet and cover slipped. At least two individuals, naïve to the behavioral results, confirmed cannula locations using a microprojector. The most caudal portion of the cannula placement indicated the anatomical location of the infusion site (based on Paxinos & Watson, 1986).

Tissue past the cannula tips did not show signs of lesion or any form of pathology or ischemia due to the chronic indwelling cannulae or the injected solutions, suggesting that these did not permanently damage the tissue. Any animals that did show signs of lesions of the brain regions or overlying tissues, meninges, bone or skin were removed from all analyses; these were very rare (n=1).

Analyses and Statistics All statistical analyses were conducted using the computer software IBM® SPSS® 21.0 (Chicago, IL, USA). A significance value of $p \leq 0.05$ was used for all statistical analyses. Interval data met the tests of normalcy and homogeneity of variance and were analyzed with parametric tests. Categorical data was measured using nonparametric tests. If data did not meet normality (t-test) or sphericity (repeated measures ANOVA), corrections such as Greenhouse-Geisser (repeated measures ANOVA) were used.

CPP analyses CPP data were analyzed as previously described (Seip et al., 2008). Times spent by the groups in each chamber (interval data, within groups, termed group chamber time) at the pre- and post-conditioning sessions were compared via a two-way repeated measures analysis of variance (ANOVA),

whereas post-conditioning times were compared via a repeated measures one-way ANOVA. Between group times were analyzed using independent-samples t-tests. The individual chamber preference data (categorical data, within groups, termed individual chamber preference) were analyzed using the Fisher's exact test. Between group times were analyzed using a two-tailed test for significance of proportions.

Reinstatement running analyses Running data were analyzed by examining wheel turns each minute of the day that the animal had access to the wheel. The computer software captured running wheel data in time bins of 1 minute, and in this way distance, time and rate could be calculated. A one-way repeated measures ANOVA was used to determine differences between running distances, times and rates in the same group repeatedly measured at different times (e.g., saline, bupivacaine, muscimol infusion). An independent samples *t*-test was used to determine statistically significant differences between one measure in two separate groups.

Results

The data below present methodological proofs and novel data in two large sets of independent groups of subjects, one group designed to examine the CNS regions important for motivational processes in the habit or stable form of wheel running, and the second group designed to examine motivational processes in the acquisition phase of wheel running behavior, prior to achievement of stable running. Within each independent group of subjects, most of the examinations of

running, effects of cannulation, effects of vehicle or inactivating agent (muscimol or bupivacaine) are carried out in a within subjects design, that is with repeated measures testing of subjects. The majority of these data are from intact cycling females, as given the lengthy time course of the running particularly in the habit stage of the experiments, males gained sufficient weight that their baseline running was significantly decreased, which reduced the impact of each subsequent phase of the study. When body weight/baseline low running did not confound and therefore disallow inclusion of males, there were no obvious gender differences, and so male and female data were pooled.

CNS regions necessary for motivational processes in habitual or stabilized running examined by use of recovery (rebound) running response after 72-hour forced wheel abstinence.

Chronic cannulation does not impair the stable habit phase of wheel running When females were allowed to acquire habitual states of running (21 days of wheel exposure) and then were subjected to implantation of cannula, they quickly recovered their daily distances run, such that distances run were the same by the 2nd day after surgery as they were the day prior to surgery (Figure 1, day 21 to 22 $F(1,7)=6.793$, $p=0.035$; all other comparisons for one week after surgery $p>0.05$). Thus, habitual runners that undergo surgical cannulation and are immediately re-exposed to the wheel show robust stable, habitual levels by the day after surgery. In these long-term experiments subjects were running for a total of 4 to 8 weeks after cannulation, and no decrease in baseline running was seen. Additionally, daily distances run on the day after the forced

abstinence period were also similar between uncannulated and chronically cannulated animals (Figure 2B, $p>0.05$).

In fact, upon barely awakening from surgery, most animals enter their wheels to complete some minimal amount of wheel turns. Though they run significantly less in the 24-hours that include the surgery than their absolute maxima, they still accomplish significant distances of 5.3 km on average. This is excellent proof that the process of surgery and the presence of a chronic cannula headset do not impair the processes of running and the baseline motivation for habitual running.

Cannulation does not impair the recovery (rebound) response in running after 72 hours of forced wheel abstinence Whether animals were uncannulated or cannulated, they showed a similarly robust rebound response in the first hour of wheel availability after a period of 72 hours of forced wheel abstinence (Figure 2A, $p>0.05$). Overall, there were no surgical or technical confounds to the chronic presence of cannula for the baseline robust rebound responses after a 72-hour forced wheel abstinence. Thus, the rebound running response is a behaviorally sturdy response, suitable for use in exploration of the motivational processes underlying long-term voluntary wheel running.

Vehicle infusion significantly blunts the recovery (rebound) running response upon wheel return from 72-hours forced abstinence Saline infusion alone significantly blunted the rebound response, reducing it to about half of that found in uncannulated or cannulated uninfused subjects (Figure 2A&B). When the rebound response was compared in cannulated animals that were lightly

handled versus given an infusion of saline before being returned to the wheel, these statically significant decreases in distance, times and rates run in the rebound period (1st hour after wheel return) were seen in animals given the saline infusion (Figure 2A, distance $t(68)=2.757$, $p=0.007$; time $t(66)=3.016$, $p=0.004$; rate ($t(66)=2.077$, $p=0.042$).

Additionally, the total distance run in the day after the forced abstinence period was significantly less in animals infused with saline versus lightly handled (Figure 2B, distance $t(68)=2.061$, $p=0.043$). It should be noted that the saline infused animals were highly habituated to the processes of handling, cannula insert cleaning, re-insertion of internal cannula for infusion, simple placement in the rooms where infusion took place, gentle constraint during infusion, and placement in a holding box while attached to injector apparatus. Thus, it is unlikely that the infusion process without actual vehicle infusion are the explanation for this blunting of the rebound. Whatever the mechanism of this blunting, clearly these data of vehicle infused subjects constitute the only correct baseline measures against which to consider the impact of possible further effects of inactivating agents as seen in the next section.

The prelimbic mPFC and NA core appear necessary for the motivation to engage in the habit or stablized stage of voluntary wheel running Running upon return of the wheel after a period of 72 hours of forced wheel abstinence was significantly affected by inactivation of regions constituting the circuits of interest, compared to the response seen in vehicle infused subjects (Figure 3). In animals where either the prelimbic mPFC or NA core were inactivated, running distances,

times and rates during the 1st hour after wheel return were significantly less as compared to saline infusions (Figure 3, distance $F(1,13)=5.103$, $p=0.042$; time $F(1,13)=4.918$, $p=0.045$; rate $F(1,13)=7.969$, $p=0.014$). These data are presented as pooled data representing the "GO" circuit of the stimulus seeking component of the motivational system, compared to saline infusion controls of these same subjects (i.e., using a within subjects design for comparisons of vehicle versus inactivating agents). Data from muscimol and bupivacaine infusions were pooled, as no differences were seen between these groups ($p>0.05$). This was not the case for the "STOP" components in the theorized circuit (i.e., the infralimbic mPFC or NA shell) ($p>0.05$) or for off target controls ($p>0.05$), which did not result in any significant alteration in rebound running response and hence were pooled for presentation purposes.

Inactivation never affected the total daily distance, time or rate of running regardless of whether target or non-target comparator regions were infused ($p>0.05$). Additionally, it should be noted that when the prelimbic mPFC and NA core were inactivated on the day of wheel return after 72 hours of wheel deprivation, animals spent a lower percentage of their time running in the light period (trend, $F(1,13)=3.801$, $p=0.073$) compared to saline infusion, but nonetheless returned to total daily running distances similar to saline infusions.

Inactivation of the "GO or "STOP" components of the circuit or control regions did not affect rebound running after 1-hour wheel access disruption As demonstrated in Chapter 4, a short burst of running (~185 meters) occurs in subjects in the habit stage of wheel running when subjects are returned to their

wheels after being disturbed for brief periods of time (~ 1 hour) due to cleaning, weighing or feeding. Like in the case of uncannulated animals, cannulated, infused males and females showed identical rebound responses after periods of 1 hour of forced wheel abstinence, and there were no gender differences between them. Therefore, data from cannulated, infused males and females were pooled for these analyses.

Additionally, when our regions of interest were transiently inactivated on habitual, consecutive running days (i.e., without a period of forced wheel abstinence), distances run during the hour after wheel return (distance, time & rate $p>0.05$) and during the entire light-dark cycle (distance, time & rate $p>0.05$) were unaffected. That is, both hourly and daily distances were similar whether the prelimbic mPFC, the immediately adjacent comparator site, the infralimbic mPFC, or all other control infusion sites (dorsal and unilateral controls) were inactivated.

Motivational processes during acquisition phase of wheel running are fragile and easily disrupted by the non-specific aspects of CNS interventions

Cannulation impairs the acquisition of running Females, naïve to the wheel underwent surgical cannulation and were allowed 1 week of recovery before being exposed to the wheel for 21 days. Their running data over this time period were compared to a group of age-matched, uncannulated wheel naïve females that were also given exposure to a running wheel for 21 days. Cannulation dramatically affected the acquisition of running, such that the daily distances run were significantly lower in cannulated than uncannulated females

over the 21 days of running (Figure 4, time*group effect $F(20,280)=2.752$, $p<0.001$). These subjects never attained such high levels of running as their uncannulated counterparts for the 44 days of wheel running that were explored.

Cannulation as well as saline infusion affects the CPP for the total experience of wheel running As seen in Chapter 4 of this thesis, uncannulated males and females show an equally strong CPP for a place associated with the total experience of wheel running during the acquisition phase of running (days 1 to 7). Surprisingly both the process of cannulation and further the process of vehicle infusion both disrupted CPP. Like the subjects from the independent group used to examine the role of the mPFC in motivational processes of stabilized habit running in the prior section of these results, these subjects were normal and healthy after surgery. All standard measures of postoperative health, and indeed even their lower but clear levels of running within the 24 hours after surgery, indicate that they are completely normal, and that the lower running was not due to a health confound of the subjects.

Cannulation did not impair pre-conditioning exploration. That is, uncannulated and cannulated animals showed similar pre-conditioning group chamber times in both the to-be-associated running and sedentary chambers ($p>0.05$). No animals received infusions at this pre-conditioning test.

Cannulation and saline infusion, however, significantly affected the outcome of post-conditioning testing. Subjects that were cannulated and then saline infused showed a trend toward forming a conditioned place preference (Figure 5, individual preference $p=0.011$; group chamber time $F(1,21)=3.505$,

p=0.075), but the preference for the running associated chamber at the post-conditioning session was drastically and significantly less in cannulated versus uncannulated animals (Figure 5, individual preference z-test $p<0.05$; group chamber time $t(58)=1.922$, $p=0.059$). One group in this set of animals did not receive saline infusion before the post-conditioning test. They were lightly handled before the pre-conditioning session to mimic the infusion process. This group of animals did not show any differences from the cannulated, saline infused animals and so all data were grouped. Only 36.4% of cannulated, saline infused animals showed a preference for the running associated chamber compared to 60.5% of uncannulated animals. The location of cannulation did not affect conditioning and times spent in all chambers at both the pre- and post-conditioning sessions were similar for all cannulation placements. Additionally, as seen in the unncannulated condition, no differences were seen between males and females; thus, all data were pooled for these analyses.

Since the cannulation and even vehicle infusion drastically blunted the CPP that forms in the intact subjects for the chamber associated with wheel running, it was not possible to use these conditions to determine if inactivation of the brain regions of interest had a further suppressant effect on the express of the CPP. As see in Figure 5, no significant decrease below the saline infused state of the response is seen with regional inactivation. Overall, it appears that either the process of CPP conditioning or the expression of preference after conditioning during acquisition of wheel running is not a behaviorally sturdy

response suitable for use in exploration of the motivational processes underlying acquisition of voluntary wheel running.

Discussion

Utilizing a transient inactivation model, the present work examined the involvement of specific brain regions, including the prelimbic and infralimbic mPFC and NA core and shell, in the motivation for voluntary wheel running in male and female rats. The prelimbic mPFC and NA core (i.e., the hypothesized "GO" subcircuit) appear to be necessary for the regulation of the motivation for voluntary wheel running in habitual runners. Additionally, these studies indicate that the motivation for voluntary wheel running is a sensitive process requiring completely undisturbed components of the CNS since it can be significantly affected by even the minor intrusion of cannulation or by neutral vehicle infusion. The acquisition of running was dramatically affected by cannulation alone; however, animals that acquired stable levels of running and then cannulated were able to quickly re-achieve high, stable running levels. Though cannulation did not impair the reinstatement response after periods of forced wheel abstinence, saline infusion significantly decreased these responses. Despite this decrease, using the proper baseline comparators with vehicle infused subjects allowed us to determine that the prelimbic mPFC and NA core may be necessary for this reinstatement response seen after periods of forced wheel abstinence, but are not involved in habitualized, routine running. Finally, cannulation and/or saline infusion appears to affect the potent CPP established in male and female rats for the total experience of wheel running. This is the first preclinical report to

establish that any particular subregion of the CNS is necessary for the motivational processes of voluntary wheel running, in this case the prelimbic mPFC and NA core were concluded to directly regulate the motivation for voluntary wheel running.

Cannulation affects running during its acquisition phase but has no affect in animals that have already become habituated to the wheel Animals that were surgically implanted with cannula, allowed to recover for 1 week, and then given *ad libitum* access to the wheel for 21 days (i.e., enough time to become stable, habitual runners) ran significantly less over this time period than their uncannulated counterparts. This was despite whether the placements were in the prelimbic or infralimbic mPFC, NA core or shell, or an off target site. All of these animals were healthy and recovered from surgery, and at post-mortem analysis, all brain tissue was healthy and without obvious lesions outside of cannula placement. Therefore, physical health issues do not seem to contribute to this effect. Though the size and shape of the cannula do not interfere with wheel running, it is possible that animals first exposed to the wheel with cannulation find this motor behavior more difficult or awkward, which prevents them from establishing the behavior in as robust a manner as uncannulated animals. However, when animals are allowed access to the wheel for 21 days with cannula and establish habitual patterns of running before cannulation, a different pattern is seen. Because animals that were cannulated after reaching habitual levels of running re-attained their high, stable running levels by only the day after surgery, there is no reason to believe that the decreased distances

seen in runners cannulated before wheel exposure are due to improper or incomplete surgical recovery.

While it is very unusual for the presence of a fairly minor intrusion on brain tissue such as induced by a cannula to have such measurable effects, the literature does have other such examples. In the case of the medial preoptic area, it has been known for quite some time that even a cannula-sized lesion and other small neurotoxin induced lesions induce dramatic reductions in the full display of maternal behavior in adult rats (Rosenblatt et al 1996; Kalinichev et al 2000; Olazabal, et al 2002). In the case of the medial prefrontal cortex, this effect is surprising as other work in the laboratory demonstrates that neither the CPP for cocaine or pup associated environments, nor the processes of expression of maternal care giving are disrupted by such minor intrusions into either the prelimbic or infralimbic components of the mPFC (Pereira and Morrell, 2011). Nonetheless, the data are clear and we posit that the acquisition of wheel running requires a completely intact mPFC for normal running acquisition and for the clear motivational component associated with it. Further we conclude that once wheel running has moved to the habit or stabilized stage of the process, the mPFC is not so sensitively required for these processes but is certainly involved in the motivational components of wheel running. This transition across brain regions involved in the acquisition and stable self-administration of wheel running has certain similarities to that demonstrated for the onset or reinstatement of drug self-administration, and the shift of which regions are crucial for the

processes as they move into the habit form of the behavior which has been elegantly suggested by Kalivas (2008) and his colleagues.

Saline infusion, but not cannulation, significantly affects the motivational areas involved in the habit stage of voluntary wheel running Cannulated animals showed similarly robust rebound responses as well as daily distances run to their uncannulated counterparts. These data suggest that the lesions from cannulation do not impair either the motor or motivational components of voluntary wheel running. Importantly, this sets the stage for us to be able to determine if inactivation of our regions of interest affects the behavior of voluntary wheel running. However, saline infusion significantly impaired the rebound response as well as the daily distances run. That is, when animals were given an infusion of saline in any of our on target or off target sites, the rebound response after 72 hours of forced wheel abstinence was significantly less than when animals were just given the wheel back after light handling. This light handling mimicked the restraint process that occurred during the saline infusion, but did not have all of its components such as transfer from rooms, noise from the pump, and insertion of infusers. Though infused animals were allowed at least 1 hour to settle before being returned to their wheels, we hypothesize that the saline infusion served as a stressful experience for the animal causing them to significantly decrease their rebound running response. Though others have hypothesized that stressed rats run more, for example, for the purpose of fictive escape (see Sherwin, 1998 for review), here we see a situation where stress actually leads to a decrease in wheel running. If one reason that rats run is for

fictive escape due to stress, this type of running might occur in short bursts (i.e., directly after the stressful incident), with the overall long-term effect being a decrease in running. Because of the saline infusion effect, we realized that this was an essential control, and thus all bupivacaine or muscimol infusions were directly compared to these saline infusions.

Both cannulation and saline infusion affect the conditioned place preference for the total experience of voluntary wheel running in the acquisition phase Both uncannulated males and females show a similarly potent CPP for the total experience of wheel running in its acquisition phase. However, cannulation as well as saline infusion significantly decreased the motivation to seek out the wheel running experience. Due to the nature of this experiment, animals were first cannulated and allowed to recover for 7 days before first being exposed and conditioned to the wheel running experience. As described above, cannulation dramatically impairs daily distances run during the acquisition period, and further, saline infusion causes drastic decreases in the rebound response after period of forced wheel abstinence, most likely due to the stressful nature of the infusion process. One group of animals was included that was cannulated but did not experience an infusion before the post-conditioning session. Their post-conditioning responses did not differ from the cannulated, saline infused animals. Therefore, we can conclusively say that both cannulation and saline infusion affected this CPP. Therefore, we hypothesize that due to these two methodological issues, and perhaps the stressful nature of (1) undergoing surgery and then being exposed to a novel environment with the opportunity to

engage in a completely new behavior and (2) the saline infusion, a decrease in the CPP for the wheel running experience was seen in cannulated, infused animals.

Inactivation of the mPFC does not affect the motor component of wheel running, and the mPFC is not involved in habitual, routine running We examined the involvement of the prelimbic and infralimbic mPFC in voluntary wheel running during its habitual phase without a long-term period of forced wheel abstinence. That is, we inactivated these regions of interest on days when habitual runners were not deprived of the wheel. From our previous work, we knew that briefly interrupting (~ 1 hour) animals from their wheels for purposes of cleaning, weighing or feeding causes a small burst in running that subsides within the hour, so we hypothesized that doing this for purposes of infusion would cause the same type of burst running. This was in fact the case; however, inactivation of any of our on or off target areas did not affect distances run during the hour after wheel return or the entire daily cycle. Importantly, these data suggest that inactivation of the mPFC does not affect the motor component of running. Even when regions of the mPFC are inactivated, rats are still able to conduct robust daily running distances. Additionally, since these inactivation studies were conducted on days when the wheels were freely available (i.e., without long-term deprivation), these findings suggest that the mPFC is not involved in habitual, routine running. This is surely what we hypothesized based on the model by Kalivas (2008), which suggests that it is the structures of the basal ganglia, such as the caudate putamen, globus pallidus, and substantia nigra, that are involved

in habitual behaviors. More likely, daily distances run on habitual, non-deprived days would be decreased by inactivation of these structures. This type of work has yet to be done, but it would be a nice supplement to the present findings.

The prelimbic mPFC and NA core may be necessary for the reinstatement of voluntary wheel running after a period of forced wheel abstinence Inactivation of the prelimbic mPFC and NA core significantly decreased times, distances and rates run during the 1st hour after wheel return from a 72-hour period of forced wheel abstinence. This was not the case for the infralimbic mPFC, NA shell or any other off target sites. These data suggest, that the prelimbic mPFC and NA core may be regulating the reinstatement response that occurs after periods of wheel deprivation. Considering that the reinstatement response is a measure of the motivation for a behavior and is seen after periods of both food and drug abstinence, this implies that the prelimbic mPFC and NA core may be necessary for the motivation to engage in voluntary wheel running.

Though no other studies have examined the direct involvement of these regions in the motivation for voluntary wheel running, other evidence supports our findings that the mPFC and NA are involved in voluntary wheel running. For example, voluntary wheel running induces changes in the nucleus accumbens. Rats given *ad libitum* access to running wheels for 30 days showed increased levels of ΔFosB in the nucleus accumbens core compared to rats given *ad libitum* access to locked wheels (Werme et al., 2002). ΔFosB is a transcription factor that accumulates in areas that have experienced chronic perturbation or stimulation, such as the striatum after repeated administration of drugs of abuse

(Nestler et al., 1999). The increase in ΔFosB predominately occurred in the dynorphin-containing neurons of the nucleus accumbens core, suggesting in combination with our work, that the endogenous opioids might be involved in the motivation for voluntary wheel running. Supporting our work, no differences in ΔFosB levels were seen between these two groups in the nucleus accumbens shell. Additionally, rats that are well habituated to wheel running (6 weeks of *ad libitum* access) show increased ΔFosB/FosB immunoreactivity in the nucleus accumbens, particularly in the mid and caudal core and shell, compared to their sedentary counterparts (Greenwood et al., 2011). These active rats also show decreased levels of D2 dopamine receptor mRNA and increased levels of kappa opioid receptor mRNA in the nucleus accumbens core (Greenwood et al., 2011), suggesting the involvement of dopamine and endogenous opioids in the motivation for voluntary wheel running.

The mPFC has also been implicated in the behavior of voluntary wheel running. For example, hyperactive mice show significantly increased Ritalin-induced cFos changes in the mPFC as compared to controls, suggesting that this area may be involved in the high running behavior characteristic of this hyperactive line of mice (see Rhodes et al., 2005 for review). Additionally, rats that were exercised during a period of forced abstinence from cocaine showed significantly decreased phosphorylated levels of extracellular signal-regulated kinase (pERK) in the medial prefrontal cortex (Lynch et al., 2010). Incidentally, pERK is a marker of neuronal activation that requires both dopamine and

glutamate signaling and is upregulated during periods of cocaine craving (Koya et al., 2009).

Additionally, in humans, acute bouts of moderate exercise increase activation in the left dorsolateral prefrontal cortex, as measured by multichannel functional near-infrared spectroscopy, and enhance performance on the Stroop test, a neurocognitive task that examines executive functioning and is known to be regulated by the prefrontal cortex (Yanagisawa et al., 2009). Two hours of endurance running has also been associated with decreased opioid receptor availability in the orbitofrontal cortices as measured through positron emission tomography (Boecker et al., 2008), another indicator that the endogenous opioid system may be at play in the motivation for physical activity.

Speculations on the neurochemical regulation for the motivation for voluntary wheel running Understanding that these regions as a whole affect the motivation to engage in voluntary wheel running is important and a necessary first step in uncovering the region specific neurochemical modulation of the motivation for voluntary wheel running. The present work does not directly point to any one specific neurotransmitter or neuromodulator that regulates the motivation for voluntary wheel running, but a few conjectures can be made using the information in this work to build hypotheses about the neurochemistry at work. Three particular components of the neurochemistry of the regions that include the “STOP and GO” subcircuits of the motivational processes of voluntary wheel running may be importantly involved in the actions of these circuits. These are dopamine, the endogenous opioids, and the endocannabinoids. All of these,

and their receptors and transports, where that applies, are present in the mPFC and NA, which this work suggests are necessary for the “GO” subcircuit in question.

Dopamine has been implicated in motivation for a variety of behaviors including physical activity. Our previous work as well as that of others has shown that voluntary wheel running increases total and extracellular levels of dopamine and its metabolites in many areas including the NA (De Castro & Duncan, 1985; Hattori et al., 1994; Hull et al., 1995; Wilson & Marsden, 1995; Meeusen et al., 1997; Hasegawa et al., 2000). Additionally, compared to controls, the hyperactive, high running line of mice show higher levels of total dopamine and its metabolites in the NA (Mathes et al., 2010). Additionally, VTA dopamine neurons, which have direct connections to the NA and mPFC, burst fire at both the onset and offset of running (Wang & Tsien, 2011), suggesting that dopamine may be involved in the bout patterns of running (i.e., the start and stop nature of running), and that the controllability of running is what rats find rewarding. In fact, rats will lever press to turn on and off a wheel (Kavanau, 1963) and find a forced, continuous running experience to be stressful (Moraska et al., 2000; Brown et al., 2007).

Endogenous opioids may also regulate the motivation for voluntary wheel running. Along with showing a CPP for the total experience of wheel running, rats also establish a CPP for an environment paired with the aftereffects of wheel running (Lett et al., 2000; 2002; Belke & Wagner, 2005; Greenwood et al., 2011). When the opioid inverse agonist, naloxone, is injected immediately after wheel

running and immediately prior to conditioning, the CPP for the aftereffects of wheel running is suppressed (Lett et al., 2001; Vargas-Perez et al., 2008), suggesting that the availability of endogenous opioids are increased after wheel running and contribute to the positive incentive salience of the aftereffects of wheel running. Additionally, injections of morphine, an opioid agonist, increase daily wheel running, whereas injections of naloxone, an opioid inverse agonist, decreases daily wheel running (Boer et al., 1990; Sisti & Lewis, 2001). Administration of naloxone also abolishes the acquisition of voluntary wheel running mice in D2L receptor-deficient (D2L-/-) mice but not wild-type mice (Vargas-Perez et al., 2004), indicating that it might be a combination of endogenous opioids and dopamine, which are needed to produce the rewarding effects of voluntary wheel running.

Endocannabinoids are another possible neuromodulator that may regulate the motivation for voluntary wheel running (see Fuss & Gass, 2010 for review). For example, CB_1 knockout mice show a 35% decrease in daily wheel running compared to wild-type controls without other apparent locomotor dysfunction (Dubreucq et al., 2010), and when rimonabant, a cannabinoid CB1 receptor antagonist, is administered, rodents significantly decrease running distances (Keeney et al., 2008) as well as operant responses for access to a wheel (Rasmussen & Hillman, 2011), suggesting that the endocannabinoid system may be involved in the regulation of the motivation for voluntary wheel running.

A final conjecture on the involvement of the prelimbic mPFC and NA core in the motivation to exercise in humans The present work is the first

demonstration that the mPFC and NA core may directly regulate the motivation for voluntary wheel running. We hypothesize based on these preclinical studies that the functional homologies of these regions may also be involved in the motivation for exercise in humans. As a nation, only 26% of us get the daily amount of physical activity that is recommended by the American Heart Association. This means that 74% of the US population is putting themselves at risk for diseases and disorders that are directly caused by or enhanced through physical inactivity. Because of this, we need to begin to understand why as a nation we have such an issue with motivating to do something so beneficial for our bodies and minds. This work sheds some light on the idea that the prelimbic mPFC and NA core directly regulate the motivation for physical activity. These regions certainly have involvement in other disorders such as depression, anxiety, obsessive compulsive disorder, and addiction, and this work suggests that these systems may also be dysregulated in individuals that have a severe lack of motivation for voluntary physical activity, such as individuals with obesity. Though at this point, we certainly do not have pharmaceutical agents that directly target brain regions, this is a first step at understanding the particular brain sites and potential neuropharmacological agents that may help to support the motivation for voluntary wheel running.

FIGURE 1

Cannulation does not affect daily wheel running in the stable habit stage

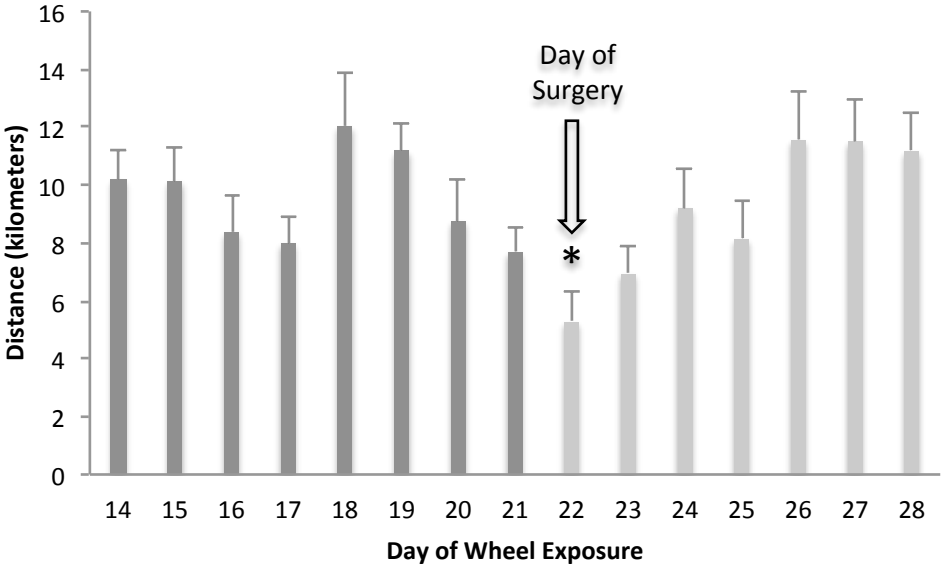

Figure 1 Total daily distance (kilometers) run (± SEM)in females that were habituated to wheel running and then received surgical implantation of cannula on their 22nd day of wheel exposure. Though daily distances run were significantly lower on the day of surgery, running recovered by only the day after surgery.

FIGURE 2

Saline infusion, but not cannulation, impairs the rebound response

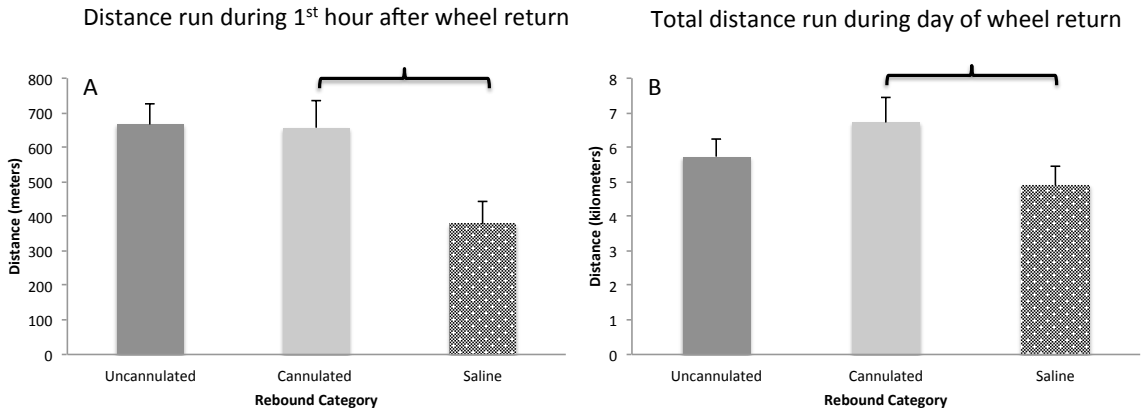

Figure 2 (A) Distance (meters) run (± SEM) in the hour following return of the wheel after a period of 72-hours of forced wheel abstinence in uncannulated, cannulated or saline-infused animals. (B) Total daily distance (kilometers) run (± SEM) during the entire light-dark cycle after a period of 72-hours of forced wheel abstinence in uncannulated, cannulated or saline-infused animals. Saline infusion, but not cannulation significantly affected hourly as well as daily distances run.

FIGURE 3

The prelimbic medial prefrontal cortex and nucleus accumbens core may be necessary for the motivation to engage in voluntary wheel running

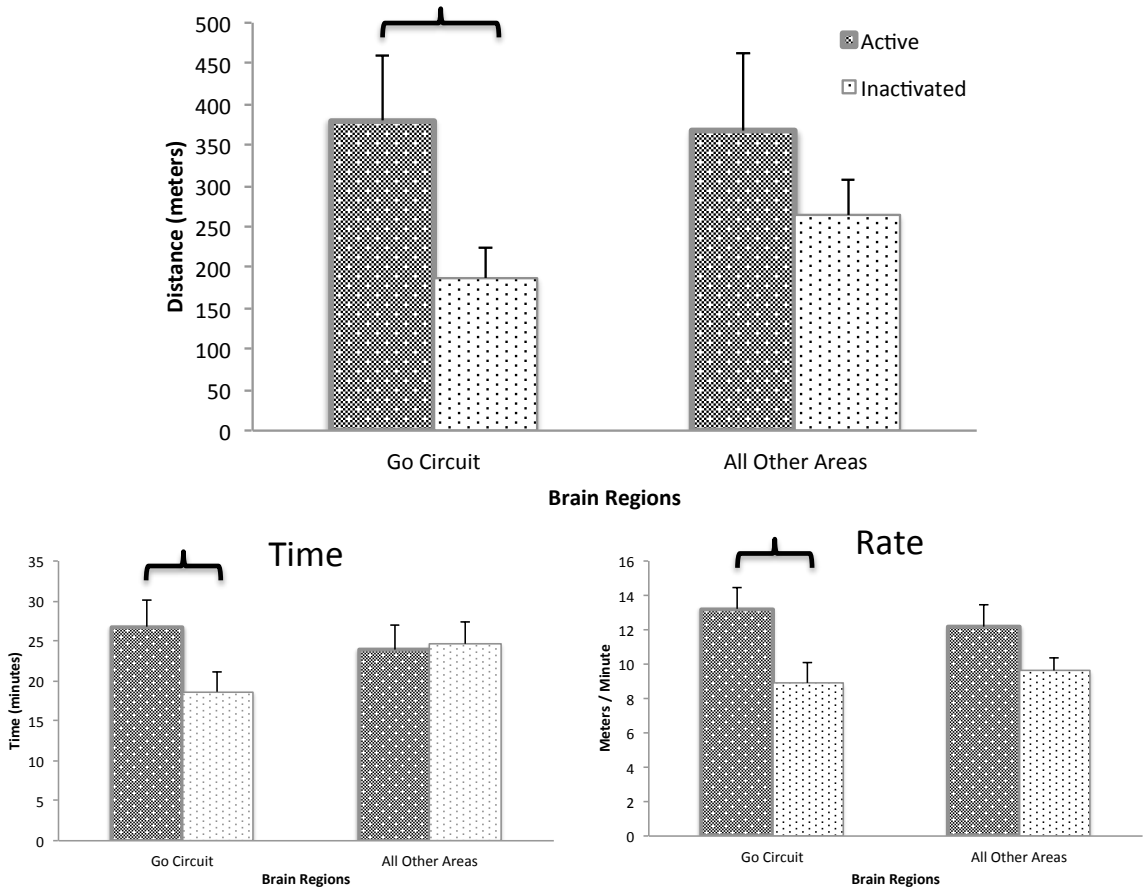

Figure 3 Total distance (meters), time (minutes) and rate (meters/minute) run (± SEM) in the hour following return of the wheel after a period of 72-hours of forced wheel abstinence in saline-infused (active) or bupivacaine- or muscimol-infused (inactivated) animals with cannulation in either the prelimbic mPFC or NA core (Go Circuit) or the infralimbic mPFC, NA shell or off target sites (All Other Areas). Inactivation of the prelimbic mPFC and NA core significantly decreased the distances, times, and rates of the reinstatement running response.

FIGURE 4

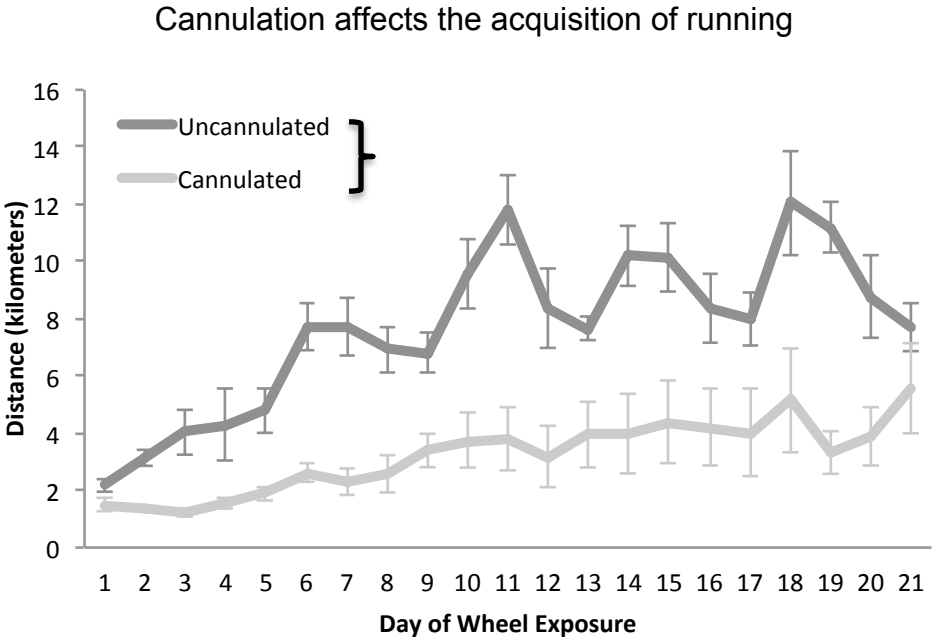

Figure 4 Total daily distance (kilometers) run (± SEM) on days 1 to 21 of wheel exposure in female rats that were uncannulated or received surgical implanation of cannulae in the prelimbic or infralimbic mPFC, NA core or shell, or off target sites 1 week prior to wheel exposure. Daily distances run were significantly lower for cannulated versus uncannulated animals.

FIGURE 5

Cannulation and saline infusion impairs the conditioned place preference for the total experience of wheel running

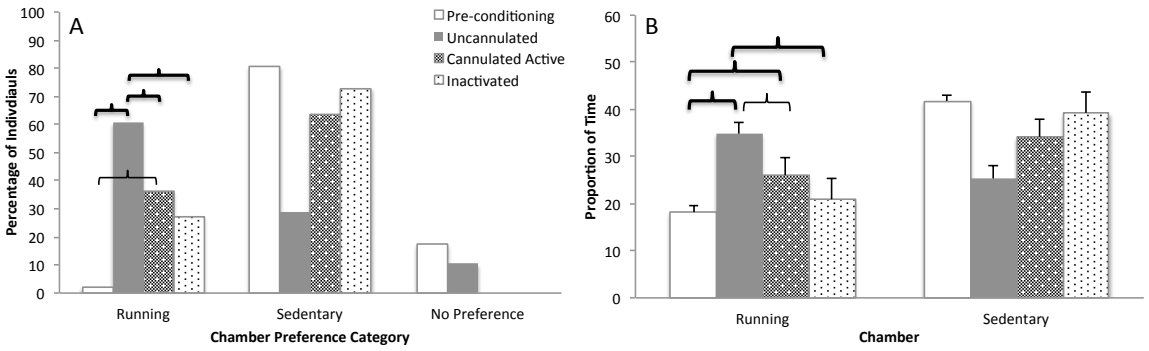

Figure 5 (A) Percentage of individuals that show a preference for a running-associated chamber, a sedentary-associated chamber or no preference during the pre-conditioning session and the post-conditioning session in (1) uncannulated, (2) cannulated, saline-infused (cannulated active), and (3) cannulated, bupivacaine-/muscimol-infused (inactivated) animals. (B) Proportion of time (minutes) spent in the running-associated chamber or the sedentary-associated chamber during the pre-conditioning session and the post-conditioning session in (1) uncannulated, (2) cannulated, saline-infused, and (3) cannulated, bupivacaine-/muscimol-infused animals. Cannulation and saline infusion impaired the conditioned place preference for the total experience of wheel running during the acquisition phase of running.

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CHAPTER 6: DISCUSSION

Voluntary wheel running as a motivated behavior with positive incentive salience may serve as an indicator of a set point for hedonics and speak to the neuroanatomical and neurochemical state of the brain

Julia C. Basso

In essence, my thesis work examined whether voluntary wheel running has positive incentive salience and whether motivation for this behavior is regulated by brain systems that mediate motivated responses to other natural and pharmacological stimuli. In other words, we asked, do rats run because it is rewarding and how does the brain regulate this response? I also investigated whether participating in this behavior throughout life affects these systems and their responsiveness to other stimuli with known incentive salience.

I found that for both genders, *ad libitum* voluntary wheel running has positive incentive salience in both its acquisition (days 1 to 7) as well as its habitual phase (after day 21), which has a natural and persistent maximum in all individuals. That is, rats wheel run because it is rewarding, and there appears to be some natural limit to the total running in rats given *ad libitum* access to the wheel, food and water. Interestingly, though females acquire the habitual form of the behavior faster and run longer distances at faster rates than males, both genders show an equally strong conditioned place preference for the total experience of running during the acquisition phase, indicating that males and females find this experience equally salient during this time period. In addition, in the stable habit phase of running, the behavioral interaction with the wheel also has marked incentive salience as demonstrated by reinstatement (rebound) responses of both genders after forced wheel abstinence. Quantitative analysis of these reinstatement responses demonstrate that they are not simply related to or confounded by spurious running stimulated by disruption of home cage activity due to husbandry or handling, but are responses correlated to the length of time

of the forced wheel abstinence. Although there are some subtle differences in the recovery responses across genders in that females run more and faster in the immediate response period upon return of the wheel, the general response pattern and its robust nature are in principle clear demonstrations of the incentive salience of the wheel for both males and females that are experienced, habitual runners.

Surprisingly, a CPP for the aftereffects of running during the habitual phase could only be found in males, suggesting that gender differences may be inherent in the preference for the various components of wheel running. While I acknowledge the natural limits of any collection of negative data, which cannot be proof that females do not experience the incentive salience of the aftereffects of wheel running, I would also argue that some gender differences in its salience are suggested by the fact that the genders differ in their response to this aspect of wheel running salience.

The most novel aspect of my work has revealed that certain regions known to be involved in the acquisition and reinstatement of other motivated behaviors for natural and pharmacological stimuli are involved in the motivational processes at work in *ad libitum* wheel running in rats. Using the approach of reinstatement (rebound) responses in both genders, I discovered that the prelimbic mPFC and NA core regulate the motivation for voluntary wheel running in its habit or stable phase. Further, participating in this motivated motor behavior throughout life appears to affect motivational circuits such that the behavioral and brain responsiveness to even an acute pharmacological stimulus

with known incentive salience, that is cocaine, at adulthood is altered. Collectively, these data suggest that voluntary wheel running shares characteristics of other motivated behaviors and may inform us generally about the response to hedonic properties of salient stimuli and the CNS components that mediate these responses. This discussion places voluntary wheel running in the larger context of motivated behaviors, making comparisons to both natural and pharmacological stimuli.

A set point for voluntary wheel running and other motivated behaviors

The expression of voluntary wheel running is quite different between males and females. That is, in their habitual phase of running, males run between 1.5 to 8 km per day, whereas females run between 1.5 to 21 km per day (data based on all running experiments Chapters 2 to 5). Essentially, on a given day, females run 1.5 times farther and faster than males. The behavior also differs across individuals of each gender. The distances accomplished each day are quite consistent within a particular individual, to the point of being able to predict how far an individual will run on a given day. However, distances vary greatly between individuals in the same sex. While females run different daily distances across their natural 4-5 day estrus cycle, the distances run within this period in any one individual are still consistent and predictable. Considering this, one might hypothesize that the genders and subsequently the individuals within the genders have a different motivation for engaging in the behavior of voluntary wheel running. However, males and females show an equally strong preference for the total experience of wheel running as measured through CPP.

Additionally, the motivation for voluntary wheel running as measured through CPP does not correlate with distances run (Chapter 4). That is, animals showing a stronger preference for the chamber associated with the total experience of wheel running do no run greater daily distances. Others that have studied the motivation for voluntary wheel running using CPP have also shown this to be the case for the total experience of wheel running in hamsters (Antoniadis et al., 2000) as well as the aftereffects of running in rats (Lett et al., 2000; Belke & Wagner, 2005; Greenwood et al., 2011). Therefore, just because a subject runs more does not indicate that the individual has a stronger preference for or more motivation to engage in the behavior of voluntary wheel running.

I propose that an argument can be made for the hypothesis that each individual subject has a particular set point for voluntary wheel running that is determined by some state in the brain. The interconnection between the body and brain can be considered part of the more theoretical aspect of the work in this thesis, and I suggest that voluntary wheel running is of particular interest because of some of the apparent natural limits and the potential dysfunctions that can occur. The remarkable daily running of rats occurs despite the fact that they learn they are not being rewarded with other stimuli such as food, water, enrichment objects, or cage mates, etc. They also learn that they cannot use the wheel to explore their environment or locomote to some other location (i.e., escape). Additionally, as they learn these things (i.e., that wheel running is not providing some alternate externally apparent reinforcement), they increase the behavior until it becomes robust and stable around the 3rd week of wheel

exposure. This indicates that rats are obtaining something else from the behavior of voluntary wheel running, and the supposition is that it is an achievement of a positive internal state due the interaction with the stimulus. That is, rats are utilizing their bodies to produce, as I hypothesize, some acute alterations in the brain that they find reinforcing or contributes to a positive internal state that is rewarding. Potentially, these acute changes may lead to longer-term, permanent alterations, similar to the mechanistic actions of for example, anti-depressants. Indeed, in Chapter 3, I reveal some neurochemical alterations that result from a lifelong experience of voluntary wheel running.

The neurobiological underpinnings of why rats engage in voluntary wheel running and find the behavior to have positive incentive salience were largely unknown prior to my work and a few others. These data and their attendant neurobiological concepts are discussed below. Before that, I would like to discuss some other literature supporting my idea that motivation, like other homeostatic, regulatory processes, such as energy homeostasis, temperature, and fluid and electrolyte balance, may have a set point. This set point takes into account the summation of all the positive, and potentially negative, salience that a variety of stimuli have for a subject. This interaction, along with certain combinations of these stimuli, can be considered as regulated, to a certain extent, by the summation of this motivational state. More specifically, I would like to suggest that the hedonic state may be regulated by some internal summation of all experience or interaction with stimuli of positive inventive salience. If this were true, then one could imagine that if a subject attained or interacted with a

stimulus with positive incentive salience, then the subject might decrease other behaviors that led to attainment of other stimuli with positive incentive salience. In this way, the subject would be defending a particular set point of this hypothesized hedonic state, in theory achievable with various stimulus interactions. It is this summed hedonic state that may be what varies across individuals and may be achieved by somewhat different levels of interaction with different forms of stimuli. A concrete manifestation of this might be that various individuals run different stable daily maxima because the brain processes needed to achieve this desired hedonic state have a natural variation due to genetic or epigenetic factors, and the CNS requires varied amounts of this form of stimulus interaction to get to its hedonic set point.

Participating in voluntary wheel running affects other motivated behaviors

One major area of research suggesting that voluntary wheel running affects other motivated behaviors is the examination of the relationship between food consumption and physical activity. Voluntary wheel running affects ingestive behaviors in rodents, but in a counterintuitive way. One could hypothesize that because rats with *ad libitum* access to voluntary wheel running expend more energy than sedentary rats, the most straightforward expectation would be that physically active rats would have a higher caloric intake than sedentary rats, but surprisingly this is not the case. Rather, rats that engage in voluntary wheel running actually consume fewer calories than sedentary rats (Scarpace et al., 2010). For example, when *ad libitum* fed rats are given limited or *ad libitum* access to a running wheel, whether forced or voluntary, they markedly decrease

both food consumption and body weight (Premack & Premack, 1963; Collier et al., 1969; Levitsky, 1970; Katch et al., 1979; Looy & Eikelboom, 1989; Lattanzio & Eikelboom, 2003), with high intensity runners showing larger decreases in both categories (Katch et al., 1979). In this paradigm, food consumption restabilizes after several weeks (Afonso & Eikelboom, 2003); however, in other situations, animals will actually decrease food consumption and increase running to the point of starvation. This paradigm is known as activity-induced anorexia (AIA) and serves as a preclinical model for anorexia nervosa, which is a human disorder characterized by a severe decrease in food consumption and increase in physical activity accompanied by weight loss, and in females, disruption of the menstrual cycle.

When animals are concomitantly given *ad libitum* access to a wheel but limited access to food, in comparison to their *ad libitum* fed counterparts, significantly increase running, decrease eating, and lose body weight to the point of self-starvation (Routtenberg & Kuznesof, 1967; Epling & Pierce, 1992; Morse et al., 1995). This phenomenon, though typically conducted with *ad libitum* access to the wheel, has also been shown to occur with only two hours of running wheel exposure (Boakes & Dwyer, 1997; Lett et al., 2000). Rats with the lowest body weights at the onset of the experiment are the most vulnerable to AIA (Boakes & Dwyer, 1997), and in females, AIA is also associated with a disruption in the estrus cycle (Dixon et al., 2003). If both voluntary wheel running and eating are motivated behaviors, then in this situation, as one increases (voluntary wheel running), the other decreases (food consumption). In the

opposite case, rats that are fed a high-fat diet (i.e., higher daily caloric consumption) run less than rats that are fed a normal diet (Judge et al., 2008). Additionally, if rats are given a period of forced wheel abstinence, they increase food consumption (Afonso & Eikelboom, 2003), indicating that they increase a behavior with positive incentive salience (eating) while their other means of attaining a rewarding stimulus (wheel running) are removed.

Another example of the interaction between voluntary wheel running and other motivated behaviors is its interaction with pharmacological stimuli with positive incentive salience. For example, rats that are given the opportunity to consume oral alcohol or amphetamine decrease consumption if they are concomitantly given access to a voluntary running wheel (Kanarek et al., 1995; Ehringer et al., 2009). Additionally, when rats are given periods of forced alcohol deprivation, voluntary wheel running increases (Ozburn et al., 2008). These data suggest that the positive hedonic state, like other regulatory states, may have a particular summed set point that subjects defend. Voluntary wheel running, as one motivated behavior, has the ability to affect participation in other motivated behaviors.

An alternate idea is that when animals engage in such motivated behaviors, the set point may change or escalate. For example, Ahmed & Koob (1998) revealed that in a situation where rats show uncontrolled increases in self-administration of cocaine (due to longer availability of drug), compared to a group that show low, stable levels of self-administration, their preference for all but the lowest cocaine dose increased, as measured by a self-administration paradigm.

The sensitivity to cocaine was unchanged, as both groups of rats responded similarly to the lowest dose given (Ahmed & Koob, 1998). Additionally, after a period of forced drug abstinence, these uncontrolled users showed a reinstatement effect and increased drug intake to levels dramatically higher than prior to self-administration (Ahmed & Koob, 1998). The authors conclude that these data suggest a change (an increase) in the hedonic set point for cocaine. This uncontrolled increase in behavior has not been shown to happen with natural stimuli, such as food (Christensen et al., 2008), and is certainly not happening with voluntary wheel running, as *ad libitum* fed rats come to run high, stable levels after the 3rd week of wheel exposure (Chapter 2). However, this may be what is happening in the situation of the AIA paradigm.

It is useful to note at this point that while both the preclinical and clinical literature notes that there are well known dependent or addictive states to pharmacological stimuli, which can be considered pathological or dysregulated interactions with these stimuli, it is also true that what has been termed behavioral addictions or dysregulations can occur as in the case of gambling, excessive exercise, or even abnormal interactions with natural stimuli, such as in the case of food or sexual addictions. Perhaps excessive interaction with any of these stimuli can be harmful to the system and result in a pathological state or dysregulation of the larger motivational regulatory system, leading to both behavioral and CNS pathology, an addictive-type of disorder. Because the regions involved in the reward circuitry are plastic and susceptible to change, this leaves the system open to alterations, which may not always be beneficial to the

subject. These concepts lead to the idea that healthy behavior may result from participation in these motivated behaviors or engaging with stimuli with positive incentive salience in moderation. In the normal physiological state, this results in achieving, via a summed or global input, a particular set point in the hedonic state, whereas in the dependent or addicted state, a fundamental dysregulation of this universal set point may occur.

Voluntary wheel running is one motivated behavior in a series of many and shares many characteristics of other motivated behaviors In many ways, the rat's interaction with the voluntary running wheel is similar to it's interaction with drugs of abuse. I make this argument based on six points that draw upon different aspects of the literature on both interactions with pharmacological and other forms of stimuli.

First, I would argue that the acquisition phase of wheel running and drug stimulus interaction are quite similar. For example, in a self-administration paradigm, when rats are exposed to cocaine with limited access (1 hour per day), they show low, stable levels of use (lever-pressing) over time, whereas when rats are exposed to cocaine with longer daily access (6 hours per day), they show a steady increase over time (Ahmed & Koob, 1998). When considering the stable or habitual state of stimulus interaction however, there are significant differences between wheel running and drug taking or even electrical self-stimulation of the brain. The prevalent information in the literature and my work shows that habitual or stable runners that are not food deprived or in other abnormal states, have a normal limit on their daily running. That is, they do not run themselves to

death. This is markedly different from mammals that are allowed to self-administer certain drugs of known abuse potential, for example cocaine or morphine, or allowed to self-administer electrical brain stimulation (Olds, 1958). In those cases, animals will self-administer to the point of completely neglecting food, water, and rest, if allowed, to the point of death. In fact, modern scientific protocols of self-administration of these stimuli are only used currently with investigator-imposed limits on subject access to the stimuli. This is not a problem with normal *ad libitum* runners, but I would like to suggest that the dysregulation seen in the prior section discussing AIA reveals that it is possible to access, with experimental manipulations, the limits of running regulation and to generate a pathological state of running that is analogous to the pathology of the unregulated self-administration of drugs or electrical stimulation.

A second set of arguments about the similarities of wheel running and interaction with pharmacological stimuli can be seen in that rats that are habituated to the use of either running wheels or drugs of abuse show similar reinstatement or rebound responses after a period of forced abstinence. After a period of forced drug abstinence, upon re-exposure to alcohol, cocaine, or heroin, rats binge or increase usage of the drug (Ahmed & Koob, 1998; Le & Shaham, 2002; Shalev et al., 2002). Similarly, after a period of forced wheel abstinence, rats dramatically increase running distances (Ferreira et al., 2006; Mueller et al., 1999; Chapter 1 & 3). Therefore, it appears that after animals are deprived of their salient stimulus, they load up on their intoxicant, whether it is running or drugs. This also suggests that during a period of forced wheel

abstinence, similar to forced drug abstinence, subjects may experience withdrawal symptoms. In fact, these exercise withdrawal symptoms have been shown in both rodents (Hoffmann et al., 1987) and humans (Chan & Grossman, 1988; Morris et al., 1990).

A third argument can be made with the fact that, just as cross-tolerance occurs with drugs of abuse, this phenomenon also occurs with wheel running. Though drugs of abuse have various specific anatomical and pharmacological substrates, they ultimately produce similar effects in the mesocorticolimbic dopamine pathway, and animals chronically administered psychostimulants, opiates, or alcohol can show a cross-tolerance effect between these drugs (McSweeney et al., 2005; Robledo et al., 2008). Cross-tolerance also occurs with voluntary wheel running. For example, rats readily establish a CPP for a place associated with a low dose of morphine (1.0 mg/kg), but in rats that have previous exposure to running wheels, morphine CPP is abolished, suggesting that wheel running causes a cross-tolerance to opiates (Lett et al., 2002). This cross-tolerance effect is also demonstrated by the fact that exogenously administered opiates have a decreased antinociceptive effect in rats with three weeks of *ad libitum* voluntary wheel access compared to controls (Mathes & Kanarek, 2001).

A fourth set of arguments can be made based on the fact that cross-sensitization occurs for both drugs of abuse and voluntary wheel running. That is, enhanced behavioral effects, such as locomotion, occur when drugs of abuse are administered over time, and when another drug of abuse is given in a

challenge dose, animals will also show enhanced (locomotor) effects to that drug as well (Segal & Mandell, 1974; Segal et al., 1980; Kalivas & Weber, 1988; see Wise, 1988 for review; see McSweeney et al., 2005 for review; Celik et al., 2006). Cross-sensitization has also been shown to occur with voluntary wheel running. Ferreira et al. (2006) demonstrated that upon a challenge dose of amphetamine, high running rats show higher levels of locomotor responsivity in comparison to low running rats; however, conclusions that can be drawn from this study are limited by the absence of a sedentary comparator group of rats. In one interesting examination of alcohol consumption and wheel running, animals were given access to a voluntary running wheel during a period of forced abstinence from alcohol (Werme et al., 2002). Rodents that had access to the wheel during this period actually consumed more alcohol upon its return than those that were sedentary during this period (Werme et al., 2002), suggesting that the voluntary wheel running experience during alcohol withdrawal actually potentiated the withdrawal experience and thus the motivation to consume alcohol upon its return.

My fifth argument can be made by the fact that after very long-term deprivation, similar effects occur with voluntary wheel running and drug consumption. Even in rats that were habituated to the use of pharmacological stimuli, such as cocaine, a prolonged period of forced drug abstinence returns the subjects to the initial, that is drug-stimulus naïve, state and hence levels of use (Ahmed & Koob, 1998). This is similar to the running response that occurs after long-term wheel deprivation (Chapter 2). That is, after 4 to 6 months of

wheel deprivation, rats that were once habitual runners appear as if they were naïve to the wheel, showing similar patterns of acquisition and stabilization upon return of the wheel. In the case of cocaine, experienced users actually escalate their cocaine use twice as fast than when first given the substance (Ahmed & Koob, 1998), a marked difference between drug and wheel running consumption. This may indicate, as discussed earlier, that in the situation of the pharmacological stimulus, cocaine, the motivational, hedonic set point, and thus the motivational circuitry, has become dysregulated, whereas in the case of *ad libitum* running with *ad libitum* access to food and water, the motivational set point and circuitry remains in a normal physiological state.

Sixth and finally, the similarity of drug and wheel stimulus interaction can be argued based on the fact that conditioned taste aversion (CTA) has been shown to occur for both drugs of abuse as well as voluntary wheel running. Conditioned taste aversion (CTA) is the process whereby when food or a taste (i.e., a flavored solution) is paired with a sensation of illness, consumption or preference for the food/taste decreases (see Davis & Riley, 2010 for review). CTA occurs with drugs of abuse like amphetamine and morphine (Reicher & Holman, 1977; Sherman et al., 1980), and CTA has also been shown to occur when specific tastes are paired with voluntary as well as forced wheel running (Lett & Grant, 1996; Nakajima et al., 2000; Heth et al., 2001; Lett et al., 2001; Salvy et al., 2004; Masaki & Nakajima, 2006; Forristall et al., 2007).

Proof that voluntary wheel running affects the motivational circuitry As discussed extensively in Chapter 3, voluntary wheel running decreases both the

CPP for and the self-administration of a variety of drugs of abuse including cocaine, amphetamine, morphine and heroin (Cosgrove et al., 2002; Hosseini et al., 2009; Xu et al., 2007; Chen et al., 2008; Smith et al., 2008; El Rawas et al., 2009; Solinas et al., 2009; Thanos et al., 2010). These data indicate that voluntary wheel running has the ability to affect drug preference both during the acquisition and dependent phases of drug consumption.

Considering that exercise is generally thought to have positive, beneficial effects and drug use can lead to serious negative consequences, it would be convenient to surmise from these studies that the overall effect of physical activity is to decrease the preference for drugs of abuse; however, my research and that of one other has shown this not precisely to be the case, and that the story is in fact more complex. The majority of the studies above utilized one specific drug dosage, whereas I utilized several low doses of cocaine. Exploring this series of doses revealed that lifelong voluntary wheel running decreased the CPP for some doses of cocaine (0.5 and 1.0 mg/kg), but increased the CPP for a somewhat higher dose (5.0 mg/kg cocaine). Additionally, Smith et al. (2008) showed that lifelong voluntary wheel running increased a CPP for 10.0 mg/kg cocaine, though this was not the case for 5.0 mg/kg, and Solinas et al. (2009) found that rats raised in enriched environments with a wheel showed a decreased CPP for 10.0 and 20.0 mg/kg cocaine. Discrepancies in these studies may be due to the specific environments utilized in these protocols. That is, Smith et al. (2008) isolated their animals during rearing whereas Solinas et al. (2009) reared their animals in an enriched environment with a variety of

interactable objects and cage mate conspecifics. Nonetheless, collectively, these studies suggest that participating in physical activity throughout life alters the motivation to seek out a place associated with the cocaine experience. Voluntary wheel running does not unilaterally and universally increase or decrease preference for drugs of abuse, but rather alters the motivational set point for seeking out these drugs, possibly by shifting the dose response curve of these pharmacological stimuli. In essence, we see a fine-tuning of the motivational set point for pharmacological, and possibly other, stimuli.

Through my thesis work, we know that voluntary wheel running is an extremely robust, motivated behavior with positive incentive salience in both males and females. Subsequently, long-term participation in this motivated behavior alters the motivation to seek out a pharmacological stimulus with positive incentive salience at adulthood. I have also shown that a lifetime of participation in voluntary wheel running causes certain neurochemical alterations in specific areas related to motor and motivational functioning, that these regions are, neurochemically speaking, differentially affected by a pharmacological stimulant with known incentive, and that specific regions like the prelimbic mPFC and NA core are directly involved in the motivation for this behavior. Therefore, I hypothesize that by engaging in this behavior throughout life, the motivational circuits are being stimulated and acute brain changes are occurring that cause long-term alterations in the brain. Presumably, since drug preference changes occur when the running experience happens in adulthood (Cosgrove et al., 2002; Hosseini et al., 2009; Xu et al., 2007; Chen et al., 2008; El Rawas et al., 2009;

Solinas et al., 2009) and a CPP for the total experience as well as the aftereffects of running can occur when the running takes place in adulthood (Lett et al., 2000; Belke & Wagner, 2005; Greenwood et al., 2011; Chapter 4), these acute changes can occur at any time period of the lifespan.

My data suggest that certain discrete regions of the brain are being affected by and are involved in the motivation for voluntary wheel running. I hypothesized that because voluntary wheel running is a motivated behavior to seek out a stimulus with positive incentive salience, like so many other motivated behaviors, it would be regulated/mediated by the mesocorticolimbic system. Through experiments using anatomically site-specific transient neuronal inactivation, I found that the prelimbic mPFC and NA core are necessary for the increase in running that occurs after a period of forced wheel abstinence, suggesting that these areas are involved in the motivation for voluntary wheel running. Additionally, I found that participating in a lifetime of voluntary wheel running altered the neurochemical content of the CP, VTA, mPFC and mPOA as well as the neurochemical responsiveness of brain regions such as the VTA, NA shell, and mPOA to a pharmacological stimulant with positive incentive salience at adulthood. Others have shown that other brain regions are affected by or implicated in the behavior of voluntary wheel running (De Castro & Duncan, 1985; Freed & Yamamoto, 1985; Hattori et al., 1994; Wilson & Marsden, 1995; Liste et al., 1997; Meeusen et al., 1997; Hasegawa et al., 2000; Rhodes et al., 2003; Bronikowski et al., 2004; Marques et al., 2008; Mathes et al., 2010). These include the CP, NA, hippocampus, medial entorhinal cortex, bed nucleus

of the stria terminalis, and hypothalamus, all areas that are connected to or directly involved in the motivational circuitry. Mine are the first studies to directly test the hypothesis that the prelimbic mPFC and NA core may be involved in the regulation for the motivation of this behavior. Having identified certain brain regions that may regulate the motivation for this behavior is important, as this information opens a region specific path for future exploration of the specific neurotransmitters or neuromodulators in these regions that are involved in the motivation for voluntary wheel running. Certain other research indicates three neurochemical substrates that may participate in the motivational processes for this behavior.

Dopamine may regulate the motivation for voluntary wheel running

Dopamine has been implicated in motivation for a variety of behaviors including physical activity. Voluntary and forced wheel running have been shown to increase total and extracellular levels of dopamine and its metabolites in the whole brain, CP, NA, MPOA, and hypothalamus (De Castro & Duncan, 1985; Hattori et al., 1994; Hull et al., 1995; Wilson & Marsden, 1995; Meeusen et al., 1997; Hasegawa et al., 2000; Chapter 3). In comparison to controls, hyperactive mice also show higher levels of total dopamine and its metabolites in the CP and NA (Mathes et al., 2010). Additionally, different genetic lines of mice, which vary in their midbrain dopaminergic anatomy as well as the expression of tyrosine hydroxylase and D1 receptors, display different patterns of running in terms of daily distance, duration, and speed (Lightfoot et al., 2004; Knab et al., 2009; 2010), and show different wheel running responses upon administration of

dopaminergic agonists and antagonists (Rhodes et al., 2001; 2003). Further, Nurr1$^{+/-}$ deficient mice, that lack midbrain dopamine neurons, never develop an increase in running wheel activity over days unlike control mice (Werme et al., 2003). One other piece of evidence shows that VTA dopamine neurons burst fire at both the onset and offset of running (Wang & Tsien, 2011), suggesting that dopamine may be involved in the bout patterns of running (i.e., the start and stop nature of running), and that the controllability of running is what the rats find rewarding. In fact, rats will lever press to turn on and off a wheel (Kavanau, 1963) and find a forced, continuous running experience to be stressful (Moraska et al., 2000; Brown et al., 2007).

In the human literature, DNA sequence variations in the DRD2 gene, a dopamine D2 receptor gene, are associated with physical activity levels in Caucasian women (Simonen et al., 2003); however, no differences were seen in dopamine concentrations in the brain, as measured through positron emission tomography (PET) scans with the D2 radiotracer [^{11}C] raclopride, before and after 30 minutes of treadmill running (Wang et al., 2000).

Endogenous opioids may regulate the motivation for voluntary wheel running The most striking preclinical evidence to suggest that the endogenous opioid system is involved in the motivation for voluntary wheel running comes from a series of CPP experiments. As discussed extensively in Chapter 4, rats establish a CPP for an environment paired with the aftereffects of wheel running (Lett et al., 2000; 2002; Belke & Wagner, 2005; Greenwood et al., 2011). When the opioid inverse agonist, naloxone, is injected immediately after wheel running

and immediately prior to conditioning, the CPP for the aftereffects of wheel running is suppressed (Lett et al., 2001; Vargas-Perez et al., 2008), suggesting that the availability of endogenous opioids are increased after wheel running and contribute to the positive incentive salience of the aftereffects of wheel running.

Rats also establish a CPP for a place associated with a low dose of morphine, but in rats that have previous exposure to running wheels, this morphine CPP is abolished (Lett et al., 2002). These data suggest that wheel running may induce a tolerance to opioids, and perhaps runners may require a higher dosage of morphine to show a CPP. In fact, this is what my data shows in Chapter 3 with a cocaine CPP. This tolerant effect is also demonstrated by the fact that exogenously administered opiates have a decreased antinociceptive effect in habitual runners as compared to their sedentary counterparts (Mathes & Kanarek, 2001).

Other evidence reveals that the endogenous opioid system may be involved in the motivation for voluntary wheel running. For example, voluntary wheel running increases beta-endorphin levels in the cerebral spinal fluid (Hoffmann et al., 1990) and dynorphin and enkephalin mRNA concentrations in the medial caudate putamen, with this effect being blocked with administration of naloxone (Werme et al., 2000). Injections of morphine, an opioid agonist, increase daily wheel running whereas injections of naloxone, an opioid inverse agonist, decreases daily wheel running (Boer et al., 1990; Sisti & Lewis, 2001). Additionally, administration of naloxone abolishes the acquisition of voluntary wheel running mice in D2L receptor-deficient (D2L-/-) mice but not wild-type mice

(Vargas-Perez et al., 2004), indicating that it might be a combination of endogenous opioids and dopamine, which are needed to produce the rewarding effects of voluntary wheel running. Similarly, in a naloxone-induced withdrawal paradigm, where naloxone was administered after a period of running, a strong positive correlation was seen between distances run and withdrawal symptoms, suggesting that those animals that ran more had higher levels of endogenous opioids and thus more potent withdrawal symptoms (Kanarek et al., 2009).

Final evidence comes from human studies. In the popular press, the runner's high is thought to be produced by an increase in endorphins. This popular notion originates in the hypothesis that running produces a subjective state of euphoria, which may be accompanied by changes in central opioidergic transmission (i.e., the endorphin hypothesis) (Morgan, 1985). The endorphin hypothesis originated from data demonstrating that in humans, running increases plasma and cerebral spinal fluid levels of endorphins and naloxone reverses the exercise-induced mood elevation, pain perception, and pupillary miosis (see Boecker et al., 2008 for review). Additionally, Boecker et al. (2008) revealed that two hours of endurance running produces a subjective sense of euphoria as well as decreased opioid receptor availability in the prefrontal/orbitofrontal cortices, dorsolateral prefrontal cortex, anterior and posterior cingulate cortex, insula and parahippocampal gyrus, sensorimotor/parietal regions, cerebellum and basal ganglia as measured by positron emission tomography. Further, euphoria ratings were inversely correlated to opioid binding in the prefrontal/orbitofrontal cortices, the anterior cingulate cortex, bilateral insula, parainsular cortex, and

temporoparietal regions. These findings suggest that intense aerobic exercise increases opioid release in widespread cortical and subcortical regions, and this increase in opioid transmission is associated with an increase in the perceived euphoric state.

Endocannabanoids may regulate the motivation for voluntary wheel running Endocannabanoids may also play a part in the rewarding aspect of voluntary wheel running (see Fuss & Gass, 2010 for review). For example, CB_1 knockout mice show a 35% decrease in daily wheel running compared to wild-type controls without other apparent locomotor dysfunction (Dubreucq et al., 2010). Additionally, voluntary wheel running increases the total content of the endocannabinoid, anandamine, in the hippocampus (Hill et al, 2010). When rimonabant, a cannabinoid CB1 receptor antagonist, is administered, rodents significantly decrease running distances (Keeney et al., 2008) as well as operant responses for access to a wheel (Rasmussen & Hillman, 2011), suggesting that the endocannabinoid system may be involved in the regulation of the motivation for voluntary wheel running. Voluntary wheel running also alters the electrophysiological response in the striatum to the CB_1 receptor agonist, HU210, with runners showing a potentiated GABAergic response compared to their sedentary counterparts. The human literature also reveals that the endocannabinoid system is involved in exercise, with exercise increasing circulating levels of endocannabinoids and the intensity of the exercise showing a positive correlation to endocannabinoid levels (Sparling et al., 2003; Raichlen et al., 2012).

Why such drastic gender and individual differences without difference in the motivation for voluntary wheel running? If these or other neurochemicals produce the reinforcing effects of voluntary wheel running, and an accumulation of these substances is what drives rats to run, then based on individual neuroanatomical differences, it makes sense that the amount of wheel running varies drastically between individuals. The amount of voluntary wheel running that an animal produces per night may serve as an indication for the neuroanatomical/chemical tone of the brain. Therefore, it would be interesting to investigate the impact of certain drugs that activate the aforementioned circuits (dopamine, endogenous opioids, endocannabinoids) on individuals with inherently different running levels. Indeed, hyperactive mice, as compared to controls, show different behavior effects to dopamine agonists and antagonists (Rhodes & Garland, 2003), and high runner as compared to low runner rats show increased levels of cocaine self-administration and cocaine-induced reinstatement (Larson & Carroll, 2005).

The estrus cycle has also been shown to influence reward, with estrogen enhancing the rewarding nature of stimuli (Dreher et al., 2007; Sakaki & Mather, 2012). For example, when estrogen levels are high, self-administration of drug increases and the acquisition, escalation and reinstatement of drug taking are enhanced (Steiner et al., 1981; Bless et al., 1997; Anker & Carroll, 2011). The brain regions considered to constitute the reward system are intensely interconnected with brain regions that contain the vast majority of neurons with gonadal steroid hormone receptors, and therefore, it is understandable that the

estrus cycle influences the hedonic qualities of stimuli with incentive salience and that voluntary wheel running increases during periods of proestrus to estrus. In order to examine the influence of gonadal hormones on the motivation for voluntary wheel running, I would conduct my CPP experiment for the total experience of wheel running in animals that were gonadectomized and given a hormone replacement for example, of estradiol, during all days of the experiment to see if this altered the expression of the CPP. To date, no studies have been conducted to examine the influence of gonadal hormones on the preference for voluntary wheel running.

Implications for the motivation to exercise in humans Educational experience and the intellectual understanding of the long-term body and brain benefits of exercise do not appear to be enough to motivate the majority of humans to exercise. Because of this, we may need some more immediate benefits of exercise to keep us motivated for participation in this physically and mentally beneficial activity. My thesis work suggests that exercise in humans may have positive incentive salience, and that we can utilize our bodies to produce movement that is rewarding. It may take time to develop a habitual exercise routine, the positive aftereffects of physical activity may only develop later in this routine, and the way that males and females interact with their workout routine may be different. For example, the time course for the acquisition of the routine may be faster for females, males and females may find different aspects of their workout routine more rewarding, and females may be more motivated to exercise at different times of their cycle. The key is to keep

the workout routine consistent, without too much of a break (abstinence) in between workouts, otherwise, the motivation for this behavior might diminish.

Additionally, as a society, we like to think of exercise as all good, but in some instances, like use of any other salient stimulus, this behavior can become excessive and thus harmful to the subject. We need not obsessively engage in either over exercising or over dieting; otherwise, these brain circuits regulating our hedonic functioning may become dysfunctional and lead to an unhealthy lifestyle. Further, though participating in physical activity is beneficial for the body and brain in so many ways, it might leave us more susceptible for showing a stronger preference for some, but perhaps more limited, doses of drugs with abuse potential that are either currently legal or illegal. By engaging our motivational circuitry through exercise, we may be fine-tuning these circuits to have stronger preferences for other stimuli with positive incentive salience, and these things may not always be beneficial for us. Inevitably, participating in physical activity is an important aspect of keeping a healthy body and mind, and engaging in this behavior consistently will help keep us motivated to exercise and sustain a healthy lifestyle. Even if the first experience with exercise happens in adulthood, the ability to motivate and sustain that motivation is possible.

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PUBLICATIONS

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