Exercise for the prevention and treatment of adolescent-onset nicotine addiction

Victoria Sanchez
Montgomery Village, MD

B.S., Frostburg State University, 2007

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Wendy J. Lynch, PhD (Advisor)

Ignacio Provencio, PhD (Committee Chair)

Alev Erisir, MD, PhD

Michael M. Scott, PhD

Jill Venton, PhD

ABSTRACT

Cigarette smoking is the leading cause of preventable death in the United States followed closely by obesity. Given that most adult smokers initiate tobacco use during adolescence and find it difficult to quit, preventative measures to curtail adolescent smoking initiation are necessary. Thus, one objective of this dissertation was to determine, using animal models, whether exercise might block the initiation of nicotine use. As the use of pharmacotherapies is controversial in teens due to ongoing neurodevelopment, another objective of this dissertation was to determine whether exercise would prevent nicotine relapse. Vulnerability to nicotine addiction is also apparent in women and adolescent females as they have been found to progress to dependence faster, have more difficulty quitting, a greater vulnerability to relapse, and to be at a higher risk for the smoking-related diseases than their male counterparts despite equal or even lower levels of use. Adolescent and adult female rodents also have a greater propensity for nicotine self-administration, suggesting that these sex differences in vulnerability to nicotine addiction are biological based. Thus, another objective of this dissertation was to determine if the efficacy of exercise as an intervention for nicotine addiction differs between males and females in an adolescent-onset model. Nicotine is known to produce long-lasting changes in the reward pathway, and changes within the nucleus accumbens in particular, are thought to underlie vulnerability to relapse. Given that the mechanisms for the efficacy of exercise are unknown, the final objective of this dissertation was to examine the possibility that its efficacy is related to its ability to modulate these nicotine-induced adaptations. To address these objectives, I examined the

efficacy of voluntary wheel running, an animal model of aerobic exercise, at reducing nicotine use initiation and relapse vulnerabilities in adolescent male and female rats. I used electron microscopy to examine the effect of exercise on nicotine-induced structural plasticity of synapses within the nucleus accumbens. I found that contemporaneous exposure to brief bouts of exercise prevented acquisition of nicotine self-administration in adolescent rats. Exposure to brief bouts of exercise during an abstinence period also significantly attenuated subsequent nicotine-seeking in both male and female rats. However, in contrast to males, females displayed an enhanced sensitivity to the effects of environmental enrichment and/or non-aerobic exercise in a locked running wheel control condition and showed attenuated nicotine-seeking under both locked and unlocked wheel conditions. I also showed that exercise during abstinence normalized nicotine abstinence-induced structural plasticity of excitatory synapses in the nucleus accumbens. Together, the results from this dissertation demonstrate that exercise, by modulating plasticity within the reward pathway, may be an effective prevention and intervention for adolescent-onset nicotine addiction.

CREDITS

Portions of this dissertation were published previously and are republished here with the permission of the publishing groups and co-authors. References for Chapters 3, 4, and the Appendix are listed below.

Chapter 3

Sanchez V, Moore CF, Brunzell DB, Lynch WJ (2013) Effect of wheel-running during abstinence on subsequent nicotine-seeking in rats. *Psychopharmacology* 227: 403-411.

Chapter 4

Sanchez V, Moore CF, Brunzell DB, Lynch WJ. Sex differences in the effect of exercise during an abstinence period on subsequent nicotine-seeking. *Psychopharmacology*. In press. DOI: 10.1007/s00213-013-3359-3

Appendix

Sanchez V, Feinstein SD, Lunardi N, Joksovic PM, Boscolo A, Todorovic SM, Jevtovic-Todorovic V (2011) General anesthesia causes long-term impairment of mitochondrial morphogenesis and synaptic transmission in developing rat brain. *Anesthesiology*. 115(5): 992-1002.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

DEDICATION

For Ana Lydia Rios Medina

CHAPTER 1:
Introduction

Smoking is the leading cause of preventable death in the United States and a

major health concern worldwide, accounting for nearly 5 million deaths each year (World

Health Organization 2009). People continue to smoke despite the risk of disease due to

their developed addiction to nicotine, the primary addictive component of tobacco. Most

smokers initiate use during adolescence. A steady decline in rates of smoking initiation

over the last several decades indicates that prevention strategies have been somewhat

effective (Substance Abuse and Mental Health Services Administration 2013). However,

rates of smoking initiation are still quite high with approximately 700 people under the

age of 18 initiating use per day (SAMHSA 2013), suggesting that the continued

investigation of methods that could improve current prevention strategies or aid in the

development of novel prevention strategies is necessary. Adolescents are believed to be

particularly vulnerable to nicotine addiction following initiation because they display an

accelerated progression to dependence and vulnerability to persistent heavy smoking in

adulthood. Most of the currently available cessation aids for tobacco addiction replace

nicotine or mimic its actions at the nicotinic acetylcholine receptors. However, the use of

such pharmacotherapies is controversial for use in adolescents due to ongoing

neurodevelopment and there are currently no FDA approved cessation aids for people

under the age of 18 years old. Sex is another vulnerability factor with women displaying

a more rapid progression to dependence and greater difficulty quitting as compared to

men (Perkins et al. 1999). Therefore, the identification of novel sex-specific preventions

as well as developmentally safe cessation strategies for adolescent males and females is necessary.

Physical activity, and specifically exercise, has been suggested as a potential non-pharmacological intervention and treatment for nicotine addiction that also has secondary health benefits (i.e., prevention of obesity and related diseases). This is an intriguing option based on promising initial findings and because it is presumably safe for use in adolescents and relatively inexpensive to implement. Clinical and preclinical evidence suggests that exercise may protect against both the initiation of drug use as well as relapse to drug use in abstainers. Epidemiological studies have demonstrated that physical activity in adolescents is associated with lower levels of smoking and other drug use, although direct causative effects are not yet known. Exercise during abstinence from smoking has been shown to acutely reduce cravings for cigarettes as well as alleviate psychological issues that are known to contribute to relapse in adults (Bock et al. 1999; Taylor and Katomeri 2007; Janse Van Rensburg et al. 2009, Haasova et al. 2012). Although, not yet examined for nicotine, preclinical studies using other drugs of abuse, such as cocaine, have demonstrated that wheel and treadmill running, animal models of aerobic exercise, can decrease initiation, use, and drug-seeking in adult male and female rats (see Lynch et al. 2013 for review). There also appears to be a sex differences in the effect of exercise at reducing cocaine self-administration where efficacy appears to be greater in females as compared to males (Cosgrove et al. 2002). Thus, exercise is an intriguing option for the prevention and treatment of adolescent-onset nicotine addiction in both males and females.

The objectives of the research presented in this dissertation are to assess the potential of exercise to prevent the initiation of nicotine use, prevent relapse following abstinence, assess the effectiveness of exercise in females, and identify potential mechanisms by which exercise exerts its beneficial effects in adolescents. In this chapter, rationale for the use of a rodent model of exercise will be presented along with further clinical and preclinical support for adolescent and female vulnerability to nicotine addiction. The available preclinical and clinical data on the use of exercise as an addiction therapy will be reviewed. Lastly, the neurobiology underlying nicotine addiction is discussed and potential mechanisms by which exercise might prevent initiation and facilitate prolonged abstinence are proposed.

Rationale for a rodent model

Although our ultimate goal is to apply these studies to humans, it is difficult in human studies to control for psychosocial influences that motivate smoking behavior. Animal models of nicotine addiction, however, enable experimental control over environmental parameters. Animal studies are particularly valuable in this regard since it is not ethical to study the factors that influence the initiation or resumption of smoking in drug-naïve or drug-abstinent humans except by retrospective reports and long-term epidemiological studies. Initiation of drug use, or acquisition, is a transitional phase of addiction when one goes from initial exposure to regular use. This phase is best modeled in rodents using a self-administration paradigm. During daily sessions animals perform

an operant response to receive an infusion of drug and the amount of time needed to meet a certain level or stability of intake is a measure of acquisition vulnerability. Relapse vulnerability in assessed in rodents using an extinction/reinstatement paradigm. As in humans, many different types of cues can elicit drug-seeking in animals, including stress, the cues formerly associated with the drug, and priming injections of the drug itself (Shaham et al. 2003). Responding during extinction sessions, which are not reinforced by the drug, as well as the rate by which responding becomes extinguished, serve as measures of drug-seeking in the absence of cues. Reinstatement testing is then assessed under various conditions. For example, under a cue-induced paradigm like the one used in these studies, the cues formerly associated with the drug are presented at the beginning of the session and following lever-presses on the drug-associated lever. Total lever presses thus serve as a measure of drug-seeking in response to drug associated cues. These animal models of drug use initiation and relapse vulnerability can be useful for determining the biological basis for the efficacy of novel prevention and intervention strategies without the influence of psychosocial factors.

Several studies have examined the effects of exercise in these acquisition and reinstatement paradigms (e.g., Smith et al. 2011; 2012 Lynch et al. 2010; Zlebnik et al. 2010; Smith and Pitts 2011; Sanchez et al. 2013a; b; Peterson et al. 2013), and like results in humans the initial findings are promising. It is worth briefly discussing the many variations in schedules of exercise that have been used in this field. The first consideration is relationship of the exposure of exercise with respect to drug self-administration which varies with the research question addressed. Most studies have

analyzed the effect of exercise when available contemporaneously, but not concurrently to drug administration, for example, when the exercise condition is available in the home cage but not during drug self-administration session. This design allows for an examination of the effects of contemporaneously available exercise on ongoing drug-taking and seeking behavior. In contrast, other studies have made exercise available concurrently with drug self-administration to examine its effects as a competing nondrug reinforcer. Some studies have examined the effects of exercise prior to or after but not during the self-administration phase to assess potential lasting protective effects of exercise. Another factor is the type exercise made available to the animal whether it is forced exercise on a treadmill or voluntary in a running wheel. Treadmill running is advantageous over voluntary wheel running in that it allows for experimenter control over distance and intensity of exercise. However, this form of exercise can also be stressful which itself can affect drug-taking and seeking behaviors (Piazza and Moal 1998). Voluntary wheel running circumvents the issue of stress; however, the distance and intensity of exercise cannot be controlled and levels of running can be quite variable. The length of exercise exposure has also been manipulated with levels of access varying from 24-hours over a prolonged period of time to less than an hour during brief bouts. Notably, most studies that have used voluntary access to running wheels as a model of aerobic exercise have allowed unlimited access to running wheels. Little information is available on the effects of limited voluntary exercise, which may be a more appropriate model of human exercising behavior.

Adolescent vulnerability to nicotine addiction

The acquisition of many drugs of abuse begins during adolescence and this is particularly true for nicotine. A recent survey of drug use in the United States (SAMHSA, 2013) revealed that an estimated 57.5 million Americans over the age of 12 are current smokers. When current use is compared across ages, an exponential increase is observed is observed between ages 12 and 20 and then these rates remain constant well into late adulthood. Furthermore, of the 2.3 million people who initiated smoking in the past year, 51.4% were under the age of 18 (SAMSHA 2013). Many studies have associated an early age of smoking onset with greater probability of being a heavy smoker and having a greater difficulty successfully quitting later in life (Breslau and Peterson 1996; Chen and Millar 1998; Taioli and Wynder 1991; Hu et al. 2006; Colby et al. 2000). Adolescents also appear to progress more rapidly to addiction after initial tobacco use and display greater levels of dependence despite lower levels of use as compared to adults (Tanski et al. 2004). Thus, it appears that adolescents are more vulnerable to initiating smoking and to succumbing to the addiction than adults.

Similar to humans, adolescent rodent models display increased vulnerability to nicotine addiction-related behaviors. Adolescents have been found to be more sensitive to the rewarding effects of nicotine as assessed under the conditioned place preference (CPP) procedure. In this procedure, which utilizes Pavlovian conditioning, nicotine and vehicle are administered in separate chambers with distinct tactile and sensory cues. In test sessions, animals are permitted to enter either chamber to determine their preference. In comparing adolescent versus adult rodents, adolescents have been found to prefer the

nicotine paired chamber at lower nicotine doses than adults (Vastola et al. 2002; Kota et al. 2007; Torres et al. 2008; Torrella et al. 2004; Shram et al. 2006; 2010). Adolescents also display significant place preference after just one conditioning session while adults do not (Brielmaier et al. 2007) further underscoring their heightened vulnerability. Differences in the rewarding properties of nicotine have also been demonstrated during the adolescent period, with younger adolescent rats displaying increased sensitivity to nicotine as compared to older adolescents under CPP conditions (Belluzi et al. 2004). The use of self-administration paradigms, in which the animal learns to perform an operant response for the delivery of a drug infusion, have been used for many drugs of abuse including nicotine to study their reinforcing properties. Intravenous (IV) nicotine self-administration is used to model the smoke inhalation route used by humans since delivery to the brain is rapid with this method. Several reports have demonstrated that adolescents will self-administer more IV nicotine infusions than adults (Levin 2003; 2007; 2011; Chen et al. 2007; but see Shram et al. 2007; 2008). Thus, as in humans, adolescent rats appear to more vulnerable to nicotine addiction as compared to adults.

Female vulnerability to nicotine addiction

Rates of smoking have historically been higher in males than females, which has led to the impression that men are more vulnerable to nicotine addiction than women. However, substantial evidence with smoking behavior, as well as with other drugs, indicates that females are more vulnerable to certain aspects of addiction than males. For example, although the historically higher rates of smoking in males than females are

maintained in current data among adult populations (33% versus 21%), such differences are absent among adolescent populations (6.8% in males and 6.3% in females; SAMHSA 2013). Evidence of enhanced vulnerability in females is also apparent in studies demonstrating that both adult and adolescent females develop dependence to nicotine more rapidly after initial use as compared to males of their age (DiFranza et al. 2002; Ridenour et al. 2006; Thorner et al. 2007; Pierce and Gilpin 1996). It has also been reported that women are not as responsive as men to currently available cessation aids such as nicotine replacement therapies (Cepeda-Benito et al. 2004; Perkins and Scott 2008) and may be at a greater risk for relapse as compared to men (Japuntich et al. 2011; Piper et al. 2010). Furthermore, women are more vulnerable to tobacco carcinogens then men despite reporting smoking fewer cigarettes (Zang and Wynder 1996). Women also appear to be more vulnerable to myocardial infarction (Prescott et al. 1998) and coronary heart disease (Huxley and Woodward 2011) than men due to smoking. Thus, the development of smoking cessation therapies that are more effective in females is desperately needed.

Although few studies have examined sex differences in nicotine self-administration, the findings available are consistent with the idea of an enhanced sensitivity in females (Donny et al. 2000, Chaudri et al. 2005; Lynch 2009; Li et al. 2012; Levin et al. 2011; Klein et al. 2004). Adult and adolescent females have been found to acquire nicotine self-administration more rapidly than males under low dose conditions, suggesting that females are more sensitive to the positive reinforcing effects of nicotine (Donny et al. 2000; Lynch 2009). Furthermore, females respond at higher levels under a

progressive ratio, where response requirements increase with each subsequent infusion, and to display a shorter latency to first response, suggesting they are more motivated to self-administer nicotine (Donny et al. 2000; Lynch 2009; Chaudri et al. 2005; Li et al. 2012). Similarly, adult female rats have been found to consume more oral nicotine solution as compared to males (Klein et al. 2004). Thus, similar to humans, female rodents appear to be more vulnerable to nicotine addiction than males and as such, may require different prevent/intervention strategies.

Exercise as a prevention for nicotine addiction

Exercise is a promising non-pharmacological prevention for adolescent-onset nicotine addiction. A plethora of epidemiological data exists on the association of physical activity levels and drug use in adolescents collected from surveys, longitudinal studies, and twin studies. Cross-sectional studies examining data collected from in-school surveys consistently report that physically active teens are less likely to smoke cigarettes as compared to less physically active teens (e.g., Pastor et al. 2003; Escobedo et al. 1993; Field et al. 2001; Melnick et al. 2001; Naylor et al. 2001; Pate et al. 1996; 2000; Rainey et al. 1996; Mattila et al. 2012; Castrucci et al. 2004). Similarly, longitudinal studies following a sample population from adolescence to early adulthood report that lower levels of smoking over many years is associated with higher levels of initial and continued physical activity (Nelson and Gordon-Larsen 2006; Kujala et al. 2007; Wichstrøm and Wichstrøm 2009; Korhonen et al. 2009). Results from longitudinal studies in twins that differed in their physical activity levels at baseline show that a

persistently inactive twin is more likely to smoke and use other drugs as compared to their more active twin (Kujala et al. 2007; Korhonen et al. 2009). However, a caveat to these epidemiological findings is that the measure of physical activity or exercise is often associated sports team participation. Thus, it is unclear whether the effect of increased physical activity on smoking behavior is due to a biological effect of exercise or to psychosocial influences of participating in a particular sport (e.g. expectations and influences of teammates, coaches, etc.). In partial support of the latter, some data indicate that participation in team sports may provide no protection or even increased risk of alcohol and smokeless tobacco use (Pate et al. 1996, 2000; Terry-McElrath and O'Malley 2011; Peretti-Watel et al. 2003; Martinsen and Sundgot-Borgen 2012). A direct causal effect of exercise in preventing initiation of drug use is difficult to determine with humans.

Animal studies support a biological basis for an effect of exercise in preventing drug use initiation. As mentioned above, the initiation of drug use is modeled in animals with a self-administration acquisition paradigm. Voluntary wheel running exercise prior to and contemporaneous with acquisition testing has been shown to reduce rates of cocaine and methamphetamine acquisition (Smith and Pitts 2011; Engelmann et al. 2013), suggesting that exercise can reduce vulnerability during the drug acquisition phase. This idea is further supported by studies utilizing CPP paradigms, which have demonstrated that prior exercise, either voluntarily on a running wheel or forced on a treadmill, prevents the development of a preference for an environment paired with MDMA, amphetamine or cocaine (Lett et al. 2002; Rozeske et al. 2011; El Rawas et al.

2009; Solinas et al. 2008; Fontes-Ribeiro et al. 2011; Thanos et al. 2010). The effect of

exercise has also been found to extend beyond initial vulnerability and to decrease intake

during maintenance phases of drug self-administration. When voluntary wheel running is

available concurrently with cocaine self-administration it has been shown to reduce

intake and prevent escalation of use in adults and adolescents (Cosgrove et al. 2002;

Zlebnik et al. 2012). Under contemporaneous conditions, exercise has been found to

decrease heroin, morphine, methamphetamine, and cocaine self-administration (Smith

and Pitts 2012; Hosseini et al. 2009; Engelmann et al. 2013; Smith and Witte 2012). This

effect seems to be independent of whether an extensive exercise-training period occurred

prior to self-administration. That these findings have been reported under both concurrent

and contemporaneous exercise conditions suggests that the beneficial effects of exercise

persist beyond the immediate exercise exposure period. Whether these effects of exercise

also extend to adolescent-onset nicotine self-administration is examined in Chapters 2.

Exercise as an intervention for nicotine addiction

Exercise has been increasingly studied for its ability to treat psychiatric disorders

including nicotine addiction. Recently, a few meta-analyses of the literature found that

brief bouts of exercise in abstinent smokers acutely attenuated cigarette craving (Taylor

et al. 2007; Roberts et al. 2012; Haasova et al. 2013). These effects may be due to

positive effects of exercise on mood, stress, craving and withdrawal symptoms (Bock et

al. 1999; Ussher et al. 2003). However, the long-term effect of exercise in maintaining

abstinence in adults is less clear and requires more rigorous effort and attention in the

field (Ussher et al. 2012). In adolescents, a two pilot studies have found that including an exercise component within a behavioral drug treatment program further decreases rates of smoking with increased success rates observed as far out as 6 months from treatment (Horn et al. 2011; Collingwood et al. 2000). These studies hint that exercise may be an effective intervention for cigarette smoking in adolescents. However, further work is necessary to determine if exercise can prevent relapse in adolescents that are addicted to nicotine and abstaining from use and to determine a causal effect of exercise without other behavioral interventions and/or sociocultural factors.

Although not yet examined for nicotine, preclinical studies indicate that exercise may be effective in reducing relapse vulnerability for other drugs of abuse. As mentioned before, relapse vulnerability is modeled in animals using extinction and reinstatement paradigms. Concurrent access to voluntary wheel running exercise during extinction and reinstatement sessions has been shown to significantly attenuate cocaine-seeking in females (Zlebnik et al. 2010). Similarly, unlimited voluntary wheel running available contemporaneously with extinction and reinstatement sessions resulted in significantly attenuated cue-induced and cocaine-primed reinstatement in both males and females (Smith et al. 2012). When exercise was made available only during abstinence from cocaine self-administration, it was found to significantly attenuate subsequent cocaine-seeking during reinstatement in males (Lynch et al. 2010; Peterson et al. 2013). Importantly, in contrast to the other studies, these latter results were obtained under modest exercise conditions (2 hr/day access) suggesting that even a modest level of exercise can protect against relapse. Taken together, these findings support the idea that

exercise has the ability to reduce relapse vulnerability in both males and females with effects that persist beyond the immediate exercise exposure. Chapter 3 of this dissertation will determine whether these effects of exercise extend to adolescent-onset nicotine self-administration in males and Chapter 4 will examine potential sex differences in these effects.

Neurobiology of nicotine addiction

Addiction is a disease that progresses from initial experimentation to compulsive use and there are often are periods of abstinence followed by resumption of use. Accordingly, the neurobiology underlying addiction changes as one transitions through the phases of the addiction process. The mesolimbic dopamine pathway, commonly referred to as the "reward pathway," is the circuitry believed to mediate motivated behavior for drug rewards. Persistent administration of psychoactive drugs results in modulation of this circuitry. In a simple model, the major components of the reward pathway include the ventral tegmental area, nucleus accumbens, and cortical regions including the prefrontal cortex. Dopamine neurons of the ventral tegmental area send projections to the nucleus accumbens and the prefrontal cortex. The medial prefrontal cortex also sends glutamatergic projections to the nucleus accumbens in a region specific manner (Voorn et al. 2004). Specifically, the ventral prefrontal cortex projects primarily to the ventral medial region of the nucleus accumbens termed the shell. The shell region surrounds the central core region which receives glutamatergic projections from the

dorsal medial prefrontal cortex. Nicotine-induced changes to these connections during the initiation, maintenance of use, abstinence and relapse are discussed in this section.

The initial exposure to drugs of abuse leads to dopamine overflow in the nucleus accumbens, which is believed to mediate their rewarding or reinforcing effects. Microdialysis studies have demonstrated that a single exposure to ethanol, nicotine, cocaine, amphetamine, and other drugs of abuse significantly increases extracellular dopamine concentrations in the nucleus accumbens (Imperato and Di Chiara 1986; Imperato et al. 1986; Di Chiara and Imperato, 1988; Carboni et al. 1989). Nicotine induces dopamine release in the nucleus accumbens via nicotinic acetylcholine receptors on VTA neurons (Corrigall et al. 1994; Nissell et al. 1994; Calabresi et al. 1989). Similar to other drugs of abuse, the initial rewarding effects of nicotine are believed to be due to dopamine release preferentially in the shell versus the core region of the nucleus accumbens. A single non-contingent exposure to cocaine, morphine amphetamine or nicotine results in robust increase in extracellular dopamine in the nucleus accumbens shell region and to a lesser extent in the core region (Pontieri et al. 1995, 1997). Repeated self-administration of nicotine during 1-hour sessions results in sensitized response to dopamine release in both the nucleus accumbens shell and core regions (Lecca et al. 2006). Thus, as self-administration continues, the dopamine response increases in both regions of the nucleus accumbens suggesting that they are both involved in nicotine self-administration. In parallel with the neurochemical effect of drug exposure, the initial reinforcing properties of drugs of abuse including nicotine are blocked by disruption of dopamine projections to the nucleus accumbens (Lyness et al. 1979; Roberts and Koob

1982; Corrigall et al. 1992; Caine and Koob 1994). Taken together, from initial exposure to maintenance of use, there appears to be a gradual increase in dopamine transmission in the nucleus accumbens.

Chronic drug use is often associated with escalated and uncontrolled drug intake in humans, which is best modeled using prolonged self-administration paradigms as these lead to an escalation of intake and the development of dependence over time (Ahmed and Koob 1998; O'Dell and Koob 2007). Although the neurobiological changes associated with extended access self-administration have not been examined with nicotine, studies with cocaine suggest that escalation of cocaine self-administration is associated with dopamine hypofunctionality in the mesolimbic pathway (Ahmed et al. 2003; Ahmed and Koob 2004; for review see, Melis et al. 2005). Escalation of cocaine self-administration under prolonged access conditions has been associated with higher levels of extracellular dopamine (Ahmed et al. 2003) but decreased function or number of dopamine receptors (D1- and D2-like) in the nucleus accumbens as compared to non-escalating short access conditions (Ahmed and Koob 2004). Similarly, during acute withdrawal from chronic high levels of cocaine administration, spontaneous activity of dopamine neurons is decreased in the VTA and basal levels of dopamine are lower within the nucleus accumbens (Koeltzow and White 2003). A significant role of glutamate in the mesolimbic pathway emerges after extended access to cocaine self-administration as well. Following extended but not short access to cocaine self-administration an increase in the autoregulatory presynaptic metabotropic glutamate receptor 2/3 (mGluR2/3) function has been observed (Hao et al. 2010), suggesting enhanced negative modulation

of glutamate release. Furthermore, a significant decrease in the postsynaptic metabotrobic glutamate receptor 5 (mGluR5) receptors is observed in nucleus accumbens after extended access self-administration as compared to short-access self-administration (Hao et al. 2010), suggesting a decrease in the glutamate transmission through this receptor. Basal levels of extracellular glutamate and glutamate function have also been observed in the ventral medial prefrontal cortex following extended access self-administration (Ben-Shahar et al. 2012). Thus, unlike low levels of cocaine use observed earlier in the addiction process, an increase in cocaine use results in a hypofunctionality of dopamine as well as dysregulation of glutamatergic signaling in the mesolimbic pathway. Whether extended nicotine self-administration also results in similar neurobiological changes is not yet known.

In contrast, prolonged abstinence from nicotine is believed to result in an increase in sensitization of dopamine signaling and glutamatergic plasticity within the mesolimbic pathway, and these changes are believed to underlie lasting behavioral vulnerabilities. In particular, glutamate projections from the medial prefrontal cortex to the nucleus accumbens core region are thought to be particularly important for reinstatement drug-seeking for many drugs of abuse including nicotine (Everitt and Robbins 2005; Fischer-Smith et al. 2012; Hotsenpiller et al. 2001; Kalivas and Volkow 2005; McFarland et al. 2003; Gipson et al. 2013). There is evidence that the ventral medial prefrontal cortex and its nucleus accumbens shell target are important for reinstatement of drug-seeking (Koya et al. 2009; Bossert et al. 2013); however, most of these data are from studies with cocaine and not nicotine. Abstinence from cocaine and amphetamine has also been found

to induce long-lasting structural plasticity observed within 24 hours, 2 weeks, 1 month and 3.5 months in the medial prefrontal cortex and nucleus accumbens (Robinson and Kolb 1997; 1999; 2004; Ferrario et al. 2005; Heijtz et al. 2003; Kolb et al. 2003; Crombag et al. 2005; Robinson et al. 2001; Li et al. 2004; Norrholm et al. 2003). Abstinence from experimenter administered nicotine has yielded similar results with observed increases in dendrite length and dendritic spine density in the medial prefrontal cortex and nucleus accumbens shell region (Brown and Kolb 2001; Hamilton and Kolb 2005; McDonald et al. 2005; 2007; Bergstrom et al. 2008). To date, only 1 study has examined the effect abstinence-induced structural plasticity following adult-onset nicotine self-administration, which found an increase in spine head diameter size in the nucleus accumbens core region associated with nicotine-seeking (Gipson et al. 2013). In Chapter 5 of this dissertation, abstinence-induced structural plasticity associated with nicotine-seeking in an adolescent-onset model is examined within the nucleus accumbens core and shell regions.

Finally, the molecular mechanisms underlying the effectiveness of exercise as a prevention and intervention for nicotine addiction are largely unknown. In animal models, exercise has been shown to modulate dopamine and glutamate release within the striatum as well as effect structural plasticity in the hippocampus. It is possible that the behavioral effects of exercise are mediated by exercise serving as a non-drug reward that produces similar neurobiological effects to reduce vulnerability to drug use initiation and relapse vulnerability. In Chapter 5 of this dissertation, the effect of exercise structural plasticity associated with nicotine-seeking is also examined. In Chapter 6, the hypothesis

that the efficacy of exercise as a prevention and intervention is due to its ability to prevent neurobiological changes essential for the development of drug use initiation and relapse vulnerability is discussed further.

In summary, preclinical and clinical evidence indicates exercise may be an effective prevention and intervention for nicotine addiction. However, remaining questions involving the specificity of exercise in preventing acquisition and nicotine-seeking following abstinence in an adolescent-onset model will be addressed in this dissertation. Due to disparities in the effectiveness of current tobacco cessation therapies in women, sex-differences in the effect of exercise as an intervention will also be explored. Furthermore, structural plasticity within the mesolimbic pathway as a mechanism for the efficacy of exercise in attenuating nicotine-seeking will be investigated.

CHAPTER 2:
Wheel running exercise as a prevention for initiation of nicotine use

Introduction

Early smoking initiation is associated with heavy smoking (Taioli and Wynder 1991), nicotine dependence (Breslau et al. 1993; Van De Ven et al. 2010), and difficulty quitting later on in life (Breslau et al. 1996; Khuder et al. 1999) suggesting that the earlier one begins to smoke, the more likely they will die from smoking-related diseases. Although rates of smoking initiation among adolescents have decreased over the years, rates of use are still alarmingly high among adolescents. The Substance Abuse and Mental Health Services Administration (2013) estimated that in the past year, 3 million adolescents (ages 12-17) smoked cigarettes and each day 3,701 adolescents smoked for the first time. Preventing or postponing smoking initiation could greatly reduce smoking-related deaths.

Clinical and preclinical data suggest that physical activity may protect against the initiation of drug use. Epidemiological studies have consistently demonstrated that adolescents involved in some sort of physical activity are less likely to be current smokers as compared to their less active peers (for a review see Lynch et al. 2013). A major confound in this literature is that most physically active teens participate in organized sports. Since some reports have found that participation in specific sports or type of sport may increase risk for use of smokeless tobacco, alcohol, or other drug use (Pate et al 2000; Terry-McElrath et al. 2011; Mattila et al. 2012; Castrucci et al. 2004; Kirkcaldy et al. 2002; Moore and Werch 2005; Rainey et al. 1996; Aaron et al. 1995; for

review see Lisha and Sussman 2010), it is likely that psychosocial influences associated with being an athlete also affect drug use. Further work is necessary to determine if exercise, without social influences, can prevent drug use initiation.

Factors that may delay or prevent the initiation of regular drug use in humans can be difficult to determine in prospective studies since it is unethical to expose drug-naïve individuals, particularly adolescents, to drugs. The use of animal models circumvents this issue and, like in humans, many variables have been shown to influence the risk of drug acquisition, the transition from initial exposure to regular use (Cambell and Carroll 2000). Acquisition is usually modeled in drug and experimentally naïve animals given non-contingent infusions and then allowed to respond for drug in operant sessions. In rats, wheel running, a model of aerobic exercise, has been shown to be effective in reducing acquisition of cocaine and methamphetamine self-administration (Smith and Pitts 2011; Engelmann et al. 2013) suggesting that there is a biological basis for the effect of exercise in preventing drug use. Wheel running exercise during cocaine self-administration has also been shown to decrease intake and drug-seeking in other phases of addiction such as maintenance of use and relapse (Cosgrove et al. 2002; Lynch et al. 2010; Zlebnik et al. 2010; Smith et al. 2012). However, no studies have examined the effect of wheel-running exercise on the acquisition of nicotine self-administration. Thus, the goal of this study was to test the hypothesis that voluntary wheel running exercise would prevent the initiation of nicotine self-administration in adolescent rats. To this end drug naïve adolescent rats were permitted daily bouts of wheel running exercise followed by nicotine self-administration sessions under acquisition testing conditions.

Methods

Animals

Male Sprague-Dawley (Charles River Laboratories, Portage, ME, USA) rats (N = 22) were shipped on day of weaning (postnatal day 21) and arrived at the laboratory on postnatal day 22. Upon arrival, animals were individually housed in self-administration chambers, and were maintained on a 12 hour light/dark cycle with ad libitum access to water and food except during exercise sessions during which only water was available. The Animal Care and Use Committee at the University of Virginia approved all procedures and were in accordance with the guidelines set by the National Institutes of Health.

Apparatus

Individual 31 cm x 24 cm x 21 cm self-administration chambers (ENV-008CT, Med Associates, St. Albans, VT, USA) were equipped with a house light (4.76 W), water bottle holder, food-hopper, retractable active (drug-associated) lever, a light (4.74 W) above the active lever, and a stationary inactive lever. Each chamber was centered within a ventilated sound-attenuating box (ENV-018M, Med Associates, St. Albans, VT, USA) along with a pump (PHM-100, Med Associates, St. Albans, VT, USA). A 10-ml drug syringe was mounted on the pump and connected to Tygon tubing that attached to swivel (Instech Laboratories Inc., Plymouth Meeting, PA, USA) embedded in a counterbalanced metal arm above the chamber. A polyethylene tube encased in a metal spring (C313CS; PlasticsOne, Roanoke, VA, USA) was attached to the swivel and to a 22-gauge guide

(C313G; PlasticsOne, Roanoke, VA, USA) embedded within an infusion harness (CIH95AB; Instech Laboratories Inc., Plymouth Meeting, PA, USA) the animal wore following surgery and thereafter until study completion. The house light was illuminated from 0700 to 1900 daily to maintain a 12 hour light dark cycle. Running wheels (ENV-046; Med Associates, St. Albans, VT, USA) with polycarbonate cage attachment were equipped with a revolutions counter.

Surgery

On postnatal day 28, rats underwent surgery to implant a chronic indwelling silastic catheter (0.51 and 0.94 mm o.d.; Dow Corning Corporation) into the right jugular vein as described previously (Lynch 2008). Specifically, rats were anesthetized with a combination of dexmedetomidine (0.2 mg/kg) and ketamine (40 mg/kg). Approximately 1.6 – 2.0 cm of the catheter was inserted into the vein with the tip resting above the opening of the right atrium and was anchored in place with suture. The other end was led subcutaneously to a small incision between the scapulae and was connected to a metal cannula within a silicone harness that the animal wore for the remainder of the experiment. Rats were given ketoprofen (2-5 mg/kg, subcutaneous) and gentamicin (5.5 mg/kg, i.v.) on the day of surgery and for the 2 subsequent days. Catheter patency was assessed by flushing a small amount of heparinized saline into the catheter and pulling back to check for the presence of blood. This check was conducted prior to self-administration sessions on the 2 days following surgery and every Monday, Wednesday, and Friday thereafter. Rats were given 2 days to recover from surgery.

Drug

Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9%
sterile saline (pH 7.4) and passed through a microfilter; the doses are expressed as the
free base weight. A single moderate dose (10 μg/kg/infusion) of nicotine was selected
based on previous work demonstrating this dose leads to rapid and maximal rates
acquisition of nicotine self-administration in adolescent males (Lynch 2009). Infusion
duration was based on each individual's weight (0.1 ml/sec), which was adjusted 3
times/week. Nicotine solution was stored in the dark at 4°C but was available at room
temperature during self-administration.

Wheel-running exercise

Rats were randomly assigned to either a sedentary (n = 12) or an exercise
condition (n = 10). Sedentary controls included a group that did not have access to a
wheel (no wheel; n = 6) and as a control for environmental enrichment, a group that had
access to a running wheel that was stationary (locked wheel; n = 6). Figure 1 outlines the
experimental timeline. Prior to surgery, on postnatal days 23-24, rats were acclimated to
their assigned wheel condition for 2 hours (0930-1130). Beginning on postnatal day 30,
rats were removed from their self-administration chambers 2.5 hours prior to daily self-
administration sessions (0930) and placed in a polycarbonate cage either with (locked and
unlocked) or without (no wheel) a wheel attachment for 2 hours. Rats were then moved
back to their self-administration chambers (1130) and they were attached for nicotine

self-administration. During the 2 hour access, both wheel groups could move freely

between the wheel and the polycarbonate cage

Acquisition of nicotine self-administration

At noon, 30 minutes after the returning to the chamber, nicotine self-

administration under a fixed ratio (FR) 1 schedule began. Daily sessions initiated with the

presentation of the active lever into the chamber. Responses on the active lever were

reinforced under a fixed ratio (FR) 1 schedule and each infusion was paired with the

illumination of a light above the active lever. Sessions were terminated when the rat

obtained all 20 infusions that were available or after 21.5 hours. Acquisition was defined

as 2 consecutive days of 20 infusions with a 2:1 preference of the active lever over the

inactive lever.
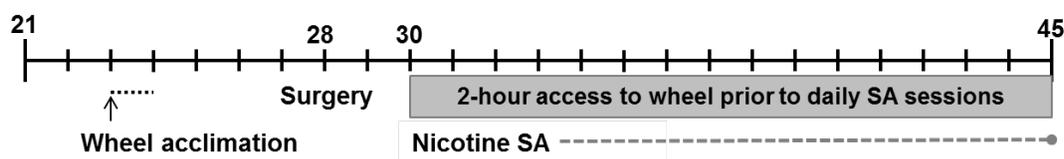
Figure 1 Experimental timeline used to examine the effect of exercise on nicotine self-administration in adolescent rats. Rats were given 2 hour access to their wheel exercise or sedentary condition each day prior to SA beginning on postnatal day 30.

Data analysis

Rate of acquisition and percentage of rats acquiring were compared between wheel condition groups using the Kaplan-Meier survival analysis followed by Mantel-Cox rank statistic. The two groups of sedentary controls (i.e., the no wheel and locked wheel groups) were combined into one sedentary group since no differences were observed between these groups in any measures examined in this study (i.e. body weights and rates of acquisition; also see Figure 2). Weights at the beginning (postnatal day 30) and at the end (postnatal day 44) were compared between groups using an ANOVA and are reported as the mean ± SEM. Statistics were run using SPSS 20 with the alpha defined as 0.05.

Results

No difference in rates of acquisition or the percent of the total group reaching acquisition criteria was observed between the locked and no wheel groups (Fig 2). The percent group reaching the acquisition criteria in each group was 67%. Of the rats that acquired, it took on average 3.0 ± 1.1 days for those in the locked wheel condition and 4.3 ± 1.3 days for those in the no wheel condition to reach the criteria. Weights were also similar between locked and no wheel groups at the beginning (96 ± 3 versus 94 ± 5 g, respectively) and at the end of this experiment (203 ± 6 versus 194 ± 9 g, respectively). Thus these groups were combined (sedentary) for the comparison with the wheel-running exercise condition.
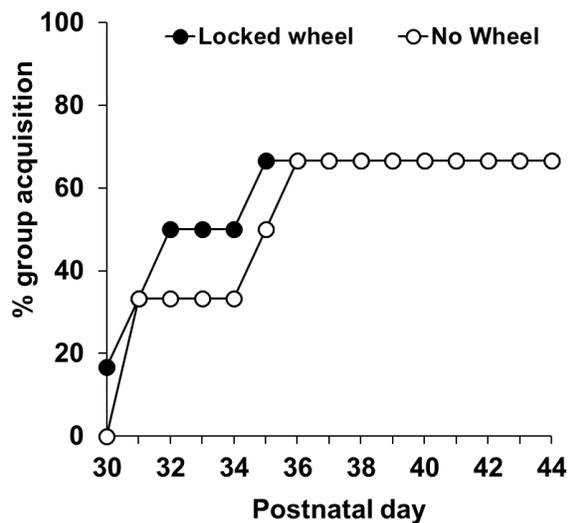
Figure 2 Acquisition of nicotine self-administration in male adolescent rats with exposure to a locked wheel (black circles) or no wheel (white circles) conditions for 2 hours prior to each self-administration session.

A greater percentage of rats in the sedentary group acquired nicotine self-administration as compared to rats in the exercise group (Fig 3). Acquisition of nicotine self-administration was achieved by 8 of 12 (67%) rats in the sedentary group, but only 2 of 10 (20%) rats in the exercise group. A survival analysis revealed a significant effect of exercise condition ($\chi^2 = 4.11$, $p < 0.05$) indicating a significant difference in percent group acquisition. However, the rate of acquisition criteria was similar between the sedentary and exercise groups. Specifically, in the exercise condition, the 2 rats acquired on day 2 and 3 and in the sedentary condition, rats acquired in an average of 3.6 ± 0.8 days. All rats gained weight over the course of the experiment with no differences observed between the exercise or sedentary groups on initial (93 ± 3 versus 94 ± 9 g, respectively) or final (193 ± 10 versus 199 ± 5 g, respectively) weights. Thus the differences observed in acquisition are not likely due to differences in weight.
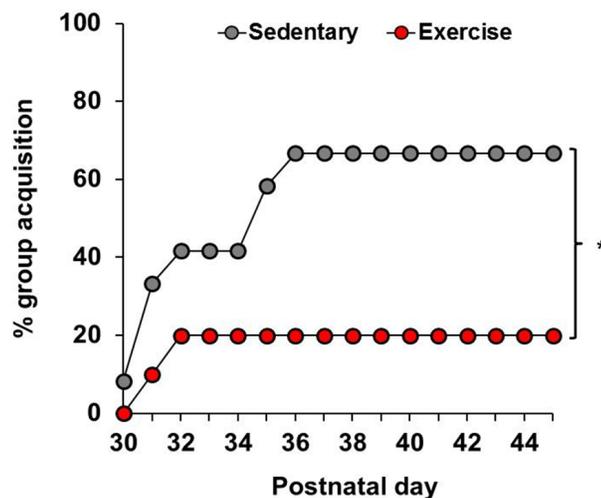
Figure 3 Percent group reaching nicotine self-administration acquisition criteria in male adolescent rats with exposure to sedentary (locked or no wheel condition; gray circles) or exercise (unlocked wheel; red circles) conditions for 2 hours prior to each self-administration session. Asterisk indicates a significant difference between the wheel conditions. The average number of days to reach acquisition criteria for the sedentary animals is represented with the black bar.

Discussion

The goal of this study was to determine if voluntary exercise would prevent the initiation of nicotine self-administration in adolescent rats. In support of our hypothesis, significantly fewer animals in exercise group acquired nicotine self-administration as compared to those in the sedentary group. These results appear to be attributable to exercise and not environmental enrichment since the locked wheel group, which was housed in a similar environment to the exercise group, was not different from the no wheel controls. Taken together, these findings suggest that exercise in the running wheel attenuates the initiation of nicotine self-administration in adolescent rats.

This study is the first to demonstrate a preventative effect of wheel running exercise in adolescents on nicotine self-administration. Our findings are consistent with a

previous report demonstrating that wheel-running exercise prevents acquisition of cocaine self-administration (Smith and Pitts 2011). In this previous study, rats were permitted to run 24-hours/day during a prolonged exercise training phase that spanned adolescence and into adulthood and they were able to run in their home cages following self-administration sessions. Other reports have demonstrated that exercise can decrease cocaine self-administration when the exercise is concurrently available as an alternative reinforce during self-administration sessions (Cosgrove et al. 2002; Zlebnik et al. 2012). The current report suggests that extensive training is not necessary for the beneficial effects of wheel-running exercise on acquisition of drug self-administration to emerge and that it need not be concurrently available with nicotine self-administration. Similarly, Smith and Witte (2012) demonstrated that wheel running exercise was effective in reducing the positive reinforcing effects of cocaine when initiated contemporaneously with self-administration, but that if exposure to wheel running was only available prior to self-administration it was not effective. Although, the current findings suggest that wheel running exercise decreases the positive reinforcing effect of nicotine in adolescent male rats, further is research is necessary using more demanding schedules of reinforcement (e.g. escalating FR or progressive ratio schedule), as has been demonstrated with cocaine (Smith et al 2008; Smith et al. 2011), to prove this mechanism for the effect of wheel running exercise.

In the literature examining the effects of wheel running exercise on drug self-administration there have been inconsistencies on control conditions. Some studies utilized a locked wheel condition (Lynch et al. 2010; Zlebnik et al. 2010, 2012; Sanchez

et al. 2013a) and others used a polycarbonate cage condition without a wheel (Smith et al. 2008, 2011, 2012; Smith and Pitts 2011, 2012; Smith and Witte 2012). The locked wheel condition controls for the presence of the wheel and the environmental enrichment this might provide; however, under these conditions, it is possible that animals may perform "alternative" forms of exercise within the wheel (e.g., climbing and hanging). While the polycarbonate cage condition without a wheel eliminates the "alternative exercise" issue it does not account for potential environmental enrichment effects. Our work is novel in that we used both control groups and found no difference between them in adolescent males. Therefore, the effect observed in the unlocked wheel group was not due to environmental enrichment provided by the wheel. This work also suggests either control (no wheel or locked wheel) is appropriate for examining the effects of wheel running exercise in adolescent males.

In human adolescents it is difficult to determine whether the suppressive effect of physical activity on smoking initiation is due to social influences or biological effects. The robust preventative effect of wheel running exercise on nicotine self-administration that we observed in adolescent male rats supports the notion that the biological effects of exercise may at least in part explain the negative association of physical activity and smoking in human adolescents. Further research is needed to determine if there are sex differences in this preventative effect of exercise and if it extends to other phases of addiction.

CHAPTER 3:
Wheel running during abstinence as an intervention for nicotine relapse

Introduction

Cigarette smoking is the leading cause of preventable death in the United States

and is a major health concern worldwide (Danaei et al. 2009; World Health Organization

2003). Most smokers initiate use during adolescence and those that do find it more

difficult to quit later in life (Breslau and Peterson 1996). Adolescents are also known to

progress to addiction more rapidly despite smoking less than adults (Tanski et al. 2004).

Although rates of smoking have tapered off over recent decades, an alarming number of

individuals begin smoking each year, and these rates are on the rise among adolescents

(i.e., 1.5 million in 2009 compared to 1.3 million in 2002; SAMHSA 2010). Although

there are FDA approved cessation treatments for nicotine addiction, none have been

approved for adolescent populations, and the use of such pharmacotherapies is

controversial in adolescents due to ongoing neurodevelopment (Kaplan and Ivanov

2011).

Exercise appears to be a promising non-pharmacological treatment for nicotine

addiction that may be useful for the vulnerable adolescent population. Clinical studies in

adults have shown that acute bouts of exercise decreases cigarette craving, withdrawal

symptoms, and cue-elicited craving (Bock et al. 1999; Taylor and Katomeri 2007; Janse

Van Rensburg et al. 2009, Haasova et al. 2012). In animals, wheel running, a model of

aerobic exercise, appears to have both and short and long-term beneficial effects.

Specifically, wheel running has been found to effectively reduce the self-administration

of other psychostimulant drugs such as cocaine and methamphetamine and to decrease drug-seeking when contemporaneously available (Miller et al. 2012; Zlebnik et al. 2012; Smith and Pitts 2011; Smith et al. 2011; Zlebnik et al. 2010; Smith et al. 2008; Cosgrove et al. 2002). Wheel running during abstinence has also been shown to decrease subsequent cocaine-seeking even when not contemporaneously available (Lynch et al. 2010). Much less information is available on the effects of wheel running in adolescents and on nicotine self-administration.

The purpose of this study was to determine if wheel running during an abstinence period would prevent subsequent nicotine-seeking in rats that began self-administering nicotine during adolescence. An extended access paradigm was used to approximate human access conditions (Paterson and Markou 2004; O'Dell et al. 2007). Specifically, rats given extended access to nicotine (23-hr/day) have been reported to achieve daily nicotine levels that are comparable to those observed in humans (i.e. 0.18-1.38 versus 0.14-1.14 mg/kg nicotine, in rats and humans, respectively; Valentine et al. 1997). Importantly, such conditions also lead to the development of physical dependence (i.e. withdrawal symptoms; O'Dell and Koob, 2007) and after prolonged abstinence; produce increased subsequent nicotine-seeking (e.g., increased drug-seeking behavior and increased motivation to obtain the drug; Abdolahi et al. 2010). The effects of 2-hr/day access to a running wheel on nicotine-seeking were assessed at a time when levels of nicotine-seeking are known to be high (Abdolhai et al. 2010) using a within-session extinction/cue-induced reinstatement paradigm. Two doses of nicotine were used in this study to model light versus moderate-to-high levels of consumption. Additional groups of

rats were given access to saline infusions in order to determine whether nicotine was functioning as a reinforcer under these extended access conditions. Additionally, given previous findings showing that light/auditory cues have reinforcement value, these saline controls established baseline levels of responding in the presence and absence of cues and allowed us to then determine whether prior nicotine self-administration affects subsequent extinction and reinstatement responding. These saline groups also served as a control for nonspecific effects of wheel running on subsequent lever responding during extinction and reinstatement. We expected to find a differential effect of wheel running in attenuating nicotine-seeking, with the greatest benefit seen in rats that formerly self-administered the lower nicotine dose since these rats were expected to self-administer less nicotine and have lower levels of subsequent nicotine-seeking as compared to rats that formerly self-administered the higher dose.

Materials and Methods

Animals

Male Sprague-Dawley (Charles River Laboratories, Portage, ME, USA) rats (N=55) arrived at the laboratory on postnatal day (PND) 22 with nicotine self-administration taking place from PND 30 to PND 45. Upon arrival, animals were individually housed in self-administration chambers within sound attenuating boxes (Med Associates, St Albans, VT, USA). The self-administration apparatus design was the same as that used in Chapter 2 (pg 21). Rats were maintained on a 12-hour light/dark schedule (lights on at 0700 and off at 1900) and had free access to food and water throughout the

experiment. All procedures were approved by the Animal Care and Use Committee at the University of Virginia and were in accordance with NIH guidelines.

Lever-press training

To ensure rapid acquisition of nicotine self-administration during the narrow window of adolescence, rats were pre-trained to lever-press for sucrose pellets (45 mg) as described previously (Lynch 2008). Briefly, on PND 25, rats were permitted daily 23-hour access to lever press for sucrose pellets under a fixed ratio 1 (FR1) schedule, in which a response on the active lever resulted in the delivery of a sucrose pellet from an automated food hopper. No light or auditory cues were used in these pre-training sessions. Pre-training was terminated after 2 days of responding for 50 or more sucrose pellets. Animals were weighed upon arrival, prior to surgery, for the 2 days following surgery and 3 times a week thereafter.

Drug

Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline (pH 7.4) and passed through a microfilter; the doses are expressed as the free base weight. Two doses of nicotine were used in this study; a low (5 μg/kg/infusion) and a moderate (10 μg/kg/infusion) dose were selected because our pilot studies indicated these doses lead to nicotine intake that were comparable to light and moderate-to-heavy smoking, respectively. Infusions of saline or nicotine (5 μg/kg/infusion and 10 μg/kg/infusion) were delivered at a rate of 0.1 ml/sec. Infusion duration was based on

each individual's weight which was adjusted 3 times/week. Nicotine solution was stored

in the dark at 4°C but was available at room temperature during self-administration.

Surgery

On PND 28, rats were implanted with a chronic indwelling silastic catheter to

allow intravenous self-administration of nicotine or saline as described previously in

Chapter 2 (page #). Rats were given ketoprofen (2-5 mg/kg, subcutaneous) and

gentamicin (5.5 mg/kg, i.v.) on the day of surgery and for the 2 subsequent days. To

ensure the patency of the catheter, it was flushed with heparinized saline every other day

prior to the daily sessions.

Nicotine Self-administration

All self-administration occurred under a FR1 schedule, in which a single response

on the active lever led to an infusion of nicotine. A response on the inactive lever was

recorded but had no programmed consequence. A schematic of behavioral experiments is

depicted in Fig 4. Rats were initially trained to self-administer nicotine (5 or 10

μg/kg/infusion, N=21 or 18, respectively) or saline (N=16) under limited access

conditions. Beginning on PND 30, rats were permitted a maximum of 20 infusions/day

for 5 days (first shaded box in Fig 4). Once 20 infusions had been delivered within a

session, the active lever was retracted from the self-administration chamber. Animals

were said to have acquired nicotine self-administration after 2 consecutive days of

receiving all 20 infusions with a 2:1 preference for the active lever over the inactive

lever. Beginning on PND 35, self-administration access was extended to 23-hours/day

with an unlimited number of infusions available per session. Rats were then switched

back to limited access conditions with a maximum of 20 infusions per session for 2 days

prior to abstinence (second shaded box in Fig 4) in order to normalize nicotine intake

within each dose condition prior to abstinence.
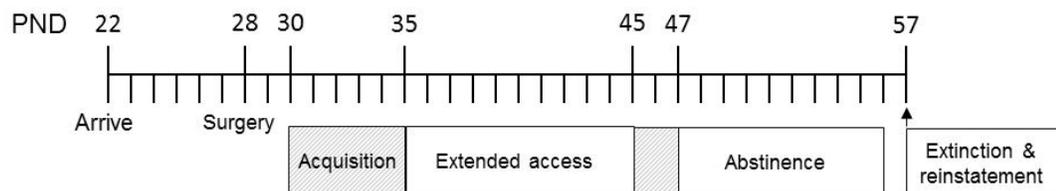
Figure 4 Schematic of behavioral protocol by postnatal day (PND). Self-administration began with a 5-day acquisition period in which rats were limited to 20 infusions/day under a fixed ratio 1 (FR1) schedule. Subsequently, rats were permitted an unlimited number of infusions for 23-hours/day during the 10-day extended access period. Next, infusions were again limited to 20 infusions/day for 2 days prior to forced abstinence. During the 10-day abstinence period rats had 2-hour access to either a locked or unlocked running wheel each day. After the last exercise session rats were returned to self-administration boxes and the following day began a within-session extinction/cue-induced reinstatement paradigm.

Wheel running

On PND 47, rats were moved to polycarbonate cages with a wheel attachment

(diameter: 35.6 cm; Med Associates, St Albans, Vermont, USA) for the 10-day

abstinence period. A prolonged duration of abstinence was selected based on a previous

report demonstrating that nicotine-seeking increases, or incubates, over an abstinence

period with high levels achieved after 7-days (Abdolahi et al. 2010). Rats were randomly

assigned to either 2-hr/day access to a locked (saline n = 8; 5 μg/kg dose n = 11; 10 μg/kg

dose n = 9) or unlocked (saline n = 8; 5 μg/kg dose n = 10; 10 μg/kg dose n = 9) running

wheel condition. In the unlocked wheel condition rats were free to run in the wheel

during the daily access sessions. Rats in the locked wheel condition were able to enter the

wheel, however the wheel was stationary and they were not able to run. Wheel access

sessions occurred between 1000 and 1200, during the light phase of the light/dark cycle,

during which rats were free to move between wheel and their polycarbonate cage. A 2-

hour exercise session was selected based on previous work with cocaine demonstrating

that this level access during abstinence was sufficient to reduce subsequent cocaine-

seeking (Lynch et al. 2010). Revolutions completed during each unlocked wheel session

were recorded daily.

Extinction and cue-induced reinstatement

Following the last wheel session, animals were moved back to their operant

conditioning chambers for the remainder of the day. Nicotine-seeking was then tested the

following day (PND 57) under a within-session extinction and cue-induced reinstatement

paradigm. Extinction responding was examined in at least 5 sessions that were each 1-

hour in duration until responding extinguished (defined as fewer than 15 responses in the

last session). Each extinction session began with the extension of the active lever into the

self-administration chamber. Responses on the active lever were recorded but had no

programmed consequence. At the end of each session there was a 5-minute timeout

period in which the active lever was retracted to separate each extinction sessions. The 1-hour reinstatement session began after the final extinction session and a 5-minute timeout with the introduction of the active lever into the chamber and presentation of the discrete cues formerly associated with nicotine (i.e. the stimulus light and the sound of the pump) for 5 seconds. Each response on the active lever during this session resulted in the presentation of these cues but did not produce an infusion of nicotine or saline.

Data analysis

All data are presented as mean ± standard error of mean (SEM). Repeated measures analysis of variance (ANOVA) was used to analyze group differences in extended access self-administration, wheel-running during the abstinence period, and lever responses during extinction and reinstatement sessions. The main dependent measures included daily number of infusions during extended access, average daily intake during extended access, daily distance run during abstinence, and total responding during the extinction and reinstatement sessions. In addition to these analyses we also further examined rates of extinction by comparing the number of sessions required to meet the extinction criterion using univariate analysis of variance. Specifically, although a minimum of 5 extinction sessions were run some animals met the extinction criterion in fewer than 5 sessions and some required 1(two rats within the 5 µg/kg dose locked wheel condition, two within the 5 µg/kg dose unlocked wheel condition, and one rat within the 10 µg/kg unlocked wheel condition) or more additional sessions (one rat within the 10 µg/kg dose locked wheel condition). All post-hoc analyses were done using the

Bonferroni corrected t-test. One tailed t-tests were used to test all *a priori* predicted comparisons. Specifically, we predicted that nicotine as compared to saline would lead to higher levels of responding under both extended access conditions and extinction/reinstatement testing conditions, that intake during extended access conditions and levels of responding during extinction/reinstatement would be highest in the 10 μg/kg dose group, and that wheel running would decrease levels of extinction/reinstatement responding, particularly in the 5 μg/kg dose group. The Pearson correlation coefficient was used to determine the associations between wheel running and subsequent extinction and reinstatement responding. Statistical analyses were performed using SPSS (version 20) and alpha was set to 0.05 for all tests.

Results

Extended access

During the extended access period, rats that self-administered nicotine at the 5 and 10 μg/kg doses, obtained significantly more infusions than rats that self-administered saline (Fig. 5A; overall effect of group, $F_{2, 52} = 18.2$, $p < 0.001$; 5 μg/kg versus saline, $t_{35} = 5.6$, $p < 0.001$; 10 μg/kg versus saline, $t_{32} = 6.6$, $p < 0.001$). Although the number of infusions obtained did not differ between the 5 and 10 μg/kg groups, average nicotine intake was significantly greater for the 10 μg/kg group as compared to the 5 μg/kg group (effect of dose; $F_{1, 35} = 14.0$, $p < 0.01$; Fig. 5B). Importantly, within each dose, intake did not differ for rats that would later be in the unlocked and locked groups. Thus, any

potential differences found in subsequent nicotine-seeking behavior cannot be attributed to differing levels of nicotine intake.

Inactive lever responding was comparable to the low levels of active lever responding observed among saline controls and did not differ significantly between groups. The average daily inactive lever responses during the extended access period were 22.5 ± 6.6, 31.6 ± 6.9, and 27.6 ± 5.4 for saline, 5 µg/kg dose, and 10 µg/kg dose groups, respectively. Animal weights were the same across all conditions at the onset of self-administration (saline, 102 ± 3 g; 5 µg/kg dose, 97 ± 2 g; 10 µg/kg dose, 98 ± 3 g). At the end of the self-administration period, however, the nicotine groups weighed slightly less than the saline group, although this effect did not reach statistical significance (saline, 225 ± 6 g; 5 µg/kg dose, 204 ± 7 g; 10 µg/kg dose, 213 ± 5 g).

Wheel running during abstinence

Although time-dependent changes in daily levels of running were observed where levels of running increased over time (Fig. 6A), overall levels of running did not differ between doses. A repeated measures ANOVA revealed a significant effect of day ($F_{9,216} = 4.3$, $p < 0.001$), but a non-significant effect of dose and a non-significant interaction of group by day. The similarity between the doses for levels of running is further illustrated in Fig. 6B where data are plotted as average daily distance run for the 0, 5, and 10 µg/kg groups.
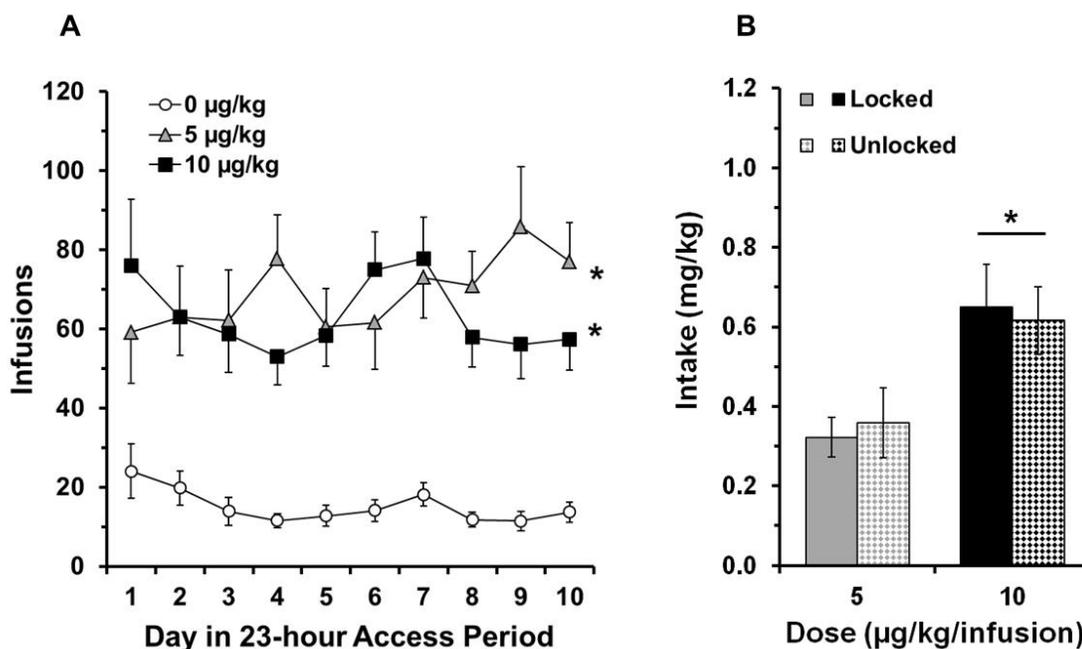
Figure 5 Nicotine is reinforcing in adolescent rats. (a) Number of infusions (mean ± SEM) is plotted for each of the 10 days of extended access period. The open circles represent saline data points (n = 16), gray triangles represent the 5 μg/kg data points (n = 21), and black squares represent the 10 μg/kg data points (n = 18). An * indicates a significant difference from saline (p < 0.05). (b) Average daily intake during extended access (mean ± SEM) is plotted for all nicotine groups. Solid gray bar represents the locked wheel condition in the 5 μg/kg dose (n = 11), gray checkered bar represents the unlocked wheel condition in the 5 μg/kg dose (n = 10), black solid bar represents the locked wheel condition in the 10 μg/kg dose (n = 9) and black checkered bar represents the unlocked wheel condition in the 10 μg/kg dose (n = 9). The bar and * indicates a significant effect of dose (p < 0.05)
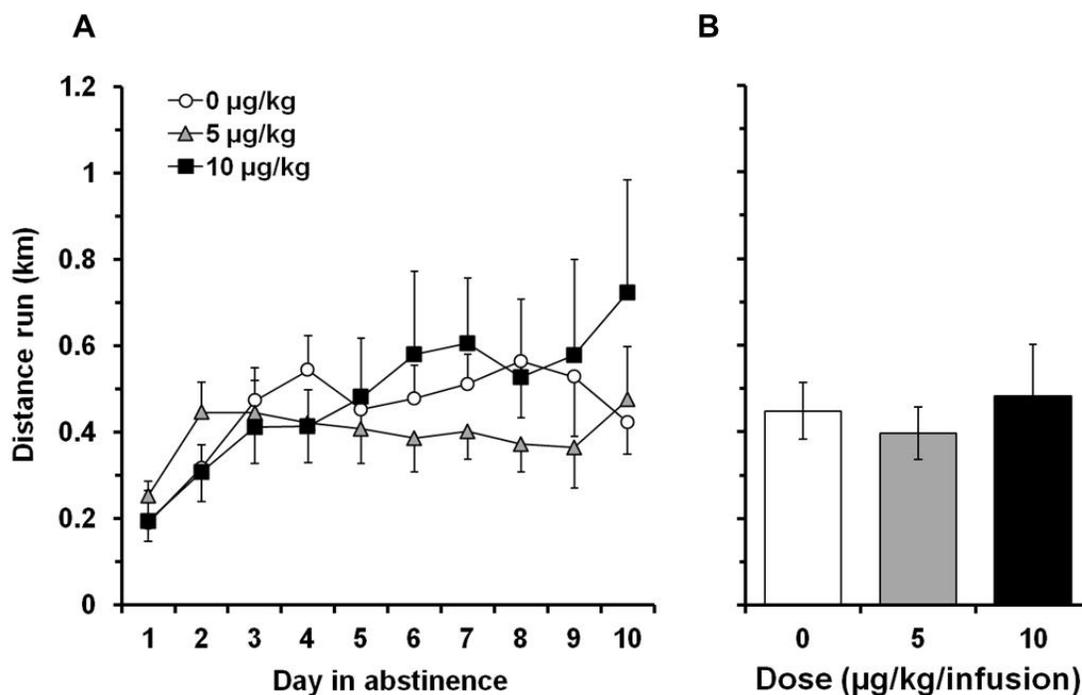
Figure 6 Nicotine self-administration does not affect wheel-running. (a) Number of revolutions (mean ± SEM) is plotted for each of the 10 days during the abstinence period. The open circles represent saline data points (n = 8), gray triangles represent the 5 μg/kg data points (n = 10), and black squares represent the 10 μg/kg data points (n = 9). (b) Average daily distance run in kilometers (mean ± SEM) is plotted between groups. The white bar represents the saline condition, the gray bar represents the 5 μg/kg dose, and the black bar represents the 10 μg/kg dose.

Extinction and cue-induced reinstatement

Levels of extinction responding were highest in rats that self-administered

nicotine and were given access to a locked wheel during abstinence (Fig 7). A repeated

measures ANOVA on responding across the 5 extinction sessions revealed a significant

effect of dose ($F_{2, 49} = 6.5$, $p < 0.01$) and wheel-condition ($F_{1, 49} = 6.2$, $p < 0.05$) (Fig 7).

Subsequent analysis of total extinction responding within each dose, revealed a

significant effect of wheel condition within both the 5 and 10 µg/kg nicotine groups ($t_{19} =$

2.0, $p < 0.05$; $t_{16} = 1.9$, $p < 0.05$, respectively), but not within the saline group. Within the

locked wheel condition, levels of extinction responding were significantly higher within

both nicotine groups as compared to saline controls (effect of dose, $F_{2, 25} = 4.0$, $p < 0.05$;

5 µg/kg versus saline, $t_{17} = 3.1$, $p < 0.01$; 10 µg/kg versus saline, $t_{15} = 3.0$, $p < 0.01$; Fig.

8). Similarly, under unlocked wheel conditions, both nicotine groups had higher levels of

responding during extinction as compared to saline (effect of dose, $F_{2,24} = 3.5$, $p < 0.05$; 5

µg/kg versus saline $t_{16} = 2.7$, $p < 0.05$; 10 µg/kg versus saline $t_{15} = 2.4$, $p < 0.05$)

however, no difference was observed between the two nicotine dose conditions.

Extinction responding was higher in the initial sessions particularly the first session as

compared to later sessions with results from the overall ANOVA revealing a significant

effect of time ($F_{4, 196} = 5.4$, $p < 0.001$). Although no significant interaction of time by

dose was observed when levels of responding were examined across the 5 sessions of

extinction, analysis of the number of sessions required to meet the extinction criteria

revealed a significant effect of dose ($F_{2, 49} = 4.2$, $p < 0.05$), where both nicotine groups

took significantly longer to extinguish responding as compared to saline (5 µg/kg versus

saline, $t_{35} = 2.4$, p < 0.05; 10 µg/kg versus saline, $t_{32} = 3.0$, p < 0.01). However, no

overall effect of wheel condition was observed on rates of extinction. There was also no

association between the levels of wheel running and subsequent extinction responding.

Analysis of the inactive lever responses during extinction revealed no effect of dose or

wheel condition. Inactive lever responding for locked and unlocked wheel conditions

were 8.3 ± 3.2 and 11.0 ± 3.4, 15.9 ± 2.8 and 14.8 ± 4.0, 21.0 ± 6.8 and 6.2 ± 2.5 for

saline, 5 µg/kg dose, and 10 µg/kg dose groups, respectively. Thus, nicotine-seeking as

assessed under extinction conditions was enhanced following abstinence in both nicotine

groups, and wheel running during abstinence attenuated this effect.
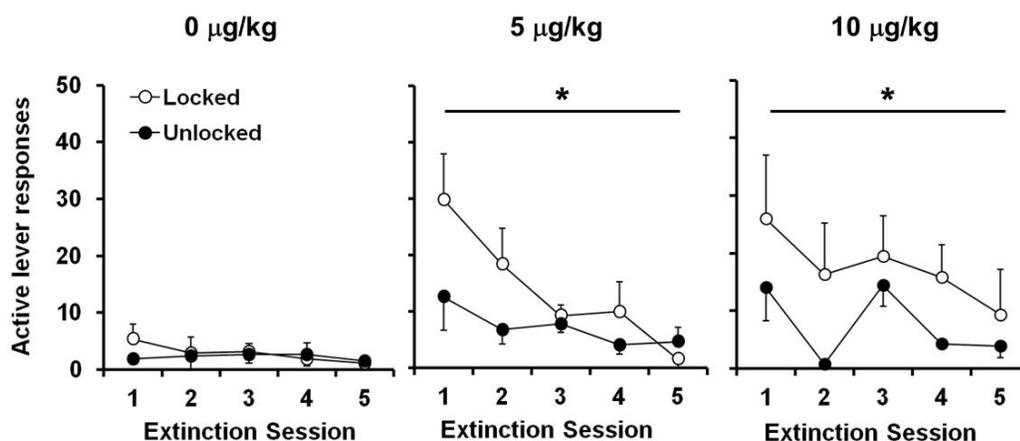
Figure 7 Wheel running during abstinence period significantly attenuates nicotine-seeking during extinction. Number of responses (mean ± SEM) is plotted for each of the first 5 extinction sessions. Open circles represent the locked wheel condition (saline, n = 8; 5 µg/kg dose, n = 11; 10 µg/kg dose n = 9) condition and black circles represent the unlocked wheel condition (saline, n = 8; 5 µg/kg dose, n = 10; 10 µg/kg dose, n = 9). A bar and * indicates a significant effect of exercise condition (p < 0.05).
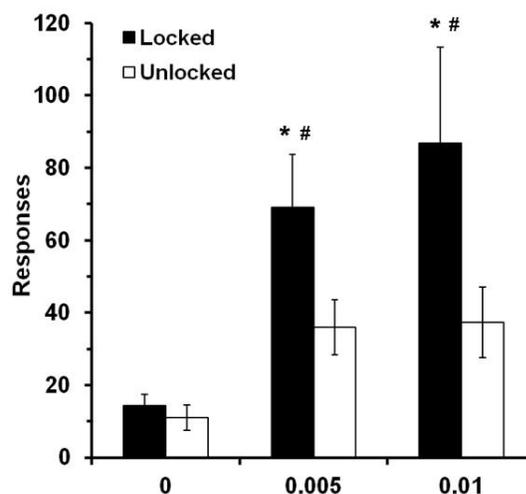
Figure 8 Wheel running effectively reduces during extinction responding in both nicotine doses. Total number of responses during the first 5 extinction sessions (mean ± SEM) is plotted by dose and wheel condition. White bars represent the locked wheel condition and black bars represent the unlocked wheel condition. The * indicates a significant difference from saline counterpart (p < 0.05) and the # indicates a significant difference from unlocked wheel group within dose (p < 0.05).

In contrast to the effects seen in extinction responding, levels of reinstatement responding did not differ by wheel condition. In fact, levels of reinstatement responding were relatively low and although a significant effect of time was observed when comparing levels of responding during the last extinction session to those observed during the reinstatement session ($F_{1, 49} = 13.8$, $p < 0.01$; Table 1), the interactions of time by wheel condition and time by wheel condition by dose were not significantly different. We did, however, observe a trend for a significant time by dose interaction ($p = 0.053$) with subsequent comparisons within each dose revealing significant effect of time in both the 5 and the 10 µg/kg doses ($F_{1, 19} = 19.7$, $p < 0.001$; $F_{1, 16} = 4.7$, $p < 0.05$, respectively) but not the saline, suggesting a tendency for nicotine to induce reinstatement responding.

In order to further explore this possibility we examined responding during just the reinstatement session and found a significant effect of dose ($F_{2, 49}$ = 3.4, $p < 0.05$) with reinstatement responding higher in both nicotine doses as compared to saline (5 µg/kg dose versus saline t_{35} = 3.7, $p < 0.01$; 10 µg/kg dose versus saline t_{32}= 2.2, $p < 0.05$). Importantly, total number of responses during the last extinction session did not differ significantly by dose or wheel conditions. There was no association between the levels of wheel running and subsequent reinstatement responding. Analysis of the inactive lever responses during reinstatement revealed no effect of dose or wheel condition. Inactive lever responding for locked and unlocked wheel conditions were 0 ± 0 and 2.3 ± 1.1, 1.5 ± 0.7 and 2.5 ± 1.5, 0.7 ± 0.3 and 1.0 ± 0.8 for saline, 5 µg/kg dose, and 10 µg/kg dose groups, respectively. Although nicotine induced subsequent reinstatement responding, levels were modest and variable and did not differ by wheel condition.

Table 1. Wheel running does not attenuate cue-induced reinstatement

Group	Last Extinction Responses	Reinstatement Responses	
0 µg/kg			
Locked	1.1 (1.1)	0.5 (0.4)	
Unlocked	1.5 (0.9)	1.0 (0.6)	
5 µg/kg		#	
Locked	0.7 (0.5)	7.1 (2.5)	*
Unlocked	1.5 (0.7)	9.6 (2.8)	
10 µg/kg		#	
Locked	1.4 (0.8)	13.6 (7.7)	*
Unlocked	3.9 (1.8)	7.8 (4.2)	

The mean (± SEM) number of active lever responses
* indicates a significant increase in responding from last extinction (p < 0.05)
# indicates a significant increase from saline (p < 0.05)

Discussion

The goal of this study was to determine if wheel-running during an abstinence period would attenuate subsequent nicotine-seeking in an adolescent-onset model of nicotine addiction. Consistent with our hypothesis, wheel running during abstinence significantly attenuated nicotine-seeking. However, in contrast to our hypothesis, levels of nicotine-seeking were comparable between the two dose conditions, and wheel running produced a similar decrease under both dose conditions. The effects of wheel running were also apparent under extinction conditions, but not under cue-induced reinstatement conditions where levels of responding were relatively low and variable. The present study is the first to show that wheel running effectively reduces drug-seeking in an adolescent-onset model, as well as the first to demonstrate its efficacy for nicotine.

Wheel running during abstinence attenuated subsequent extinction responding in animals that had previously self-administered nicotine. This finding is consistent with previous work in which wheel running during an abstinence period significantly attenuated subsequent cocaine-seeking in adult rats (Lynch et al. 2010). Furthermore, as was found in Lynch et al. (2010), in this study there was no association between levels of wheel running and drug-seeking. It is also important to note that the rats were permitted to run during the light phase of their light/dark cycle where the levels of wheel running are expected to be lower as compared to the dark phase. Despite the time of day and modest levels of running in the wheel, we observed a significant attenuation of extinction responding 10 days after the last self-administration session and 1 day after the last wheel running session. Taken together, these data suggest that even modest levels of exercise

may produce a beneficial effect on nicotine-seeking that persists beyond the acute bout of exercise.

Surprisingly, levels of cue-induced reinstatement were not affected by prior wheel running. These findings are in contrast to studies with cocaine, where wheel and treadmill running have been found to decrease subsequent cocaine-seeking under both extinction and reinstatement conditions (Lynch et al. 2010; Smith et al. 2012; Thanos et al. 2013). One possible explanation for these discrepant results is that exercise differentially affects drug-seeking during extinction versus reinstatement in rodents that previously self-administered cocaine versus nicotine. However, a more likely explanation is that the effects of wheel running during reinstatement were obscured in the present study by a floor effect. Specifically, levels of reinstatement responding under control locked wheel conditions were relatively low (mean, 9 ± 4) and variable (0-74 responses). Indeed, within the locked wheel condition, levels of reinstatement responding were roughly one third of the average observed in these same animals during the first hour of extinction, and comparable to the wheel running-attenuated levels observed during hour one of extinction within the unlocked wheel condition. The levels of reinstatement responding observed here are also much lower than those observed previously for cue-induced reinstatement following cocaine self-administration. In addition to differences in the levels of reinstatement responding induced following cocaine versus nicotine self-administration, age may account for this floor effect. Specifically, recent findings with cocaine, show that while a cocaine prime or stressor led to robust reinstatement in adolescents, the presentation of cues did not reinstate cocaine-seeking in this age group.

However, in the adult comparison group, cues, stress, and cocaine prime all reinstated cocaine-seeking (Anker and Carroll 2010). These findings suggest that cue-induced reinstatement is not sufficiently robust in adolescents to reveal an effect of exercise. Cue-induced reinstatement is known to rely on the prefrontal cortex (Koya et al. 2008), and given that this brain region continues to develop throughout adolescence (Counotte et al. 2011; Geidd 2004), it is possible that adolescents are less vulnerable than adults to drug-seeking in response to cues. Further research is needed to address this possibility.

During the self-administration period, nicotine maintained significantly higher levels of responding than did saline, indicating that nicotine was functioning as a reinforcer in these adolescent rats. As has been previously reported with extended access conditions (O'Dell et al. 2007; Valentine et al. 1997), the higher (10 μg/kg) dose of nicotine maintained significantly higher levels of intake than did the lower (5 μg/kg) dose of nicotine. However, despite the differences in levels of intake and in contrast to our hypothesis, we found that levels of nicotine-seeking were similar between rats that had self-administered either the higher or lower dose of nicotine, and that wheel running during abstinence was equally effective at reducing nicotine-seeking during extinction between both nicotine groups. These results suggest that exercise may be equally effective in both light and moderate to heavy smokers. It should be noted, however, that because rats metabolize nicotine faster than humans, both doses of nicotine may have resulted in lower levels of nicotine in the brain as compared to those achieved in human smokers (Matta et al. 2007). Although exercise can also affect drug metabolism (Døssing.

1985), this would not have been a contributing factor in this study because wheel running did not overlap with nicotine self-administration.

There are currently no FDA-approved drug therapies for smoking cessation in adolescent smokers. The findings from this study showing that wheel running during abstinence attenuates subsequent nicotine-seeking in an adolescent-onset model suggests that exercise may effectively prevent smoking relapse in teens. Our current findings in rats are also consistent with recent findings in adolescent humans showing that high school students enrolled in an afterschool program with an exercise component were more likely than other high school students to abstain from smoking (Horn et al. 2011). One factor to consider is that while wheel running is highly reinforcing in rats, humans have different affinities for various types of exercise (Ekkekakis et al. 2008) and may benefit most from exercise that they "like" to do. In the present study, rats that ran in the wheel showed a significant benefit as compared to rats that could only sit or climb on the wheel and thus appear to benefit from aerobic-like exercise. Future studies should explore the benefits of aerobic and non-aerobic exercise on adolescent smoking behavior.

CHAPTER 4:
Sex differences in wheel running as an intervention for nicotine relapse

Introduction

Disease brought on by cigarette smoking is a major health concern worldwide accounting for approximately 5 million deaths each year (World Health Organization, 2009). Although the rate of cigarette smoking has decreased over the last decade, rates have remained high in younger populations. In the United States, approximately 10% of youth (age 12-17) and 34% of young adults (age 18-25) are current smokers (Substance Abuse and Mental Health Services, 2011). This is concerning because people who begin smoking early in life have greater difficulty with quit attempts and are more likely to be heavy smokers in adulthood (Breslau and Peterson 1996). Despite the fact that more men than women are current smokers, smoking rates among adolescent boys and girls do not differ (SAMHSA, 2011). Female smokers are at a greater risk for smoking-related diseases and have more difficulty quitting smoking as compared to male smokers (Perkins et al. 1999). Physiological predispositions to nicotine dependence and inability to quit may be further impacted by psychosocial factors to control appetite in teens (Klesges et al. 1997; Boles & Johnson 2001; Cavallo et al. 2010). Preclinical studies also suggest an enhanced vulnerability to nicotine addiction in females and adolescent animals (Donny et al. 2000; Lynch 2009; Torres et al. 2008; Torres et al. 2009), suggesting that their use may be suitable for addressing the biological basis of these vulnerabilities without the influence of social factors, as well as for determining potential sex-specific interventions.

Since nicotine is the primary addictive component in tobacco products, most currently available therapies either replace nicotine or partially mimic its actions on nicotinic acetylcholine receptors. The use of such pharmacological interventions in adolescents is controversial due to ongoing neurodevelopment and there are currently no FDA approved treatments for people under the age of 18 (Kaplan and Ivanov 2011). Thus, non-pharmacological therapies, such as exercise, would be ideal for treatment of nicotine addiction in the vulnerable adolescent population. Previous work in both humans and animal models suggests that exercise may be an effective cessation aid. For example, in adult smokers, exercise during abstinence has been found to acutely reduce craving, desire to smoke, and withdrawal symptoms (Taylor et al. 2007; for review see Roberts et al. 2012; Haasova et al. 2013). Similarly, in animal models, wheel running has been found to slow the initiation of cocaine use, decrease intake, and attenuate cocaine-seeking (Cosgrove et al. 2002; Smith et al. 2008; Lynch et al. 2010; Zlebnik et al. 2010; Miller et al. 2011; Smith et al. 2011; Smith and Pitts 2011; Zlebnik et al. 2012). We recently demonstrated that voluntary wheel running during an abstinence period effectively reduced subsequent nicotine-seeking in males that began nicotine self-administration during adolescence (Sanchez et al. 2013a). Further work is necessary to determine potential sex differences in the effect of exercise during abstinence in preventing subsequent drug relapse, particularly for nicotine addiction.

The purpose of this study was to determine if wheel running during abstinence would attenuate subsequent drug-seeking in an adolescent-onset model of nicotine addiction in male and female rats and determine if its efficacy varies between the sexes.

An extended access (23-hour) self-administration paradigm was used to model human nicotine consumption and addiction. This self-administration paradigm has previously been found to result in nicotine intake that is comparable to the average daily intake observed in humans and lead to nicotine dependence (Valentine et al. 1997; O'Dell and Koob 2007). In addition, this paradigm has been found to induce robust nicotine-seeking in adolescent and adult male rats following a prolonged (i.e. 7 or 10 days), but not short (i.e. 1 hour), abstinence period (Abdolahi et al. 2010; Sanchez et al. 2013a). To assess the potential of exercise to prevent nicotine-seeking, rats were permitted to exercise in a running wheel during a prolonged abstinence period and their behavior was subsequently examined under a within-session extinction/reinstatement paradigm to test the effects of exercise on relapse vulnerability. Adolescent females have been shown to acquire nicotine self-administration faster and to be more motivated to self-administer nicotine than adolescent males under low nicotine dose conditions (i.e. 5 μg/kg; Lynch 2009). Thus, we hypothesized that females would self-administer more nicotine subsequently leading to greater levels of nicotine-seeking as compared to males. Based on previous data collected from our lab on the effect of wheel running in adolescent males (Sanchez et al. 2013a) and others demonstrating heightened female sensitivity to the effects of wheel running as an alternative nondrug reward (Cosgrove et al. 2002), we hypothesized that exercise would attenuate subsequent nicotine-seeking in both sexes with a greater treatment effect in females.

Materials and Methods

Subjects

Male (N = *49*) and female (N = 43) Sprague-Dawley rats (Charles River Laboratories, Portage, ME, USA) were weaned and shipped on postnatal day 21 and arrived at the laboratory on postnatal day 22. Upon arrival, rats were housed individually in self-administration chambers with ad libitum access to food and water and were maintained on a 12-hour light/dark cycle. The self-administration apparatus design was the same as those used in Chapter 2 (page 21). Results from a subset of the male rats (N = 29) were published as part of another study (Sanchez et al. 2013a) and presented in Chapter 3 with an additional 20 males added for the present study. No differences were observed between the two cohorts of male rats in any of the measures examined (rates of acquisition and levels of nicotine self-administration, wheel running, and nicotine-seeking). Rats were weighed every other day and general health was monitored daily. Prior to surgery, all rats were trained on the active lever under an FR1 schedule for sucrose pellets (45 mg) as described previously in Chapter 3. All procedures were approved by The Animal Care and Use Committee at the University of Virginia and were in accordance with NIH guidelines.

Drug

Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline (pH 7.4) and delivered at a concentration of 5 μg/kg/infusion at a rate of 0.1 ml/sec. The dose is expressed as the free base weight. This dose of nicotine was selected

based on previous work demonstrating robust nicotine-seeking following extended access

self-administration in males (Sanchez et al. 2013a; Chapter 3) and sex differences in

acquisition and motivation for nicotine self-administration in adolescents at this dose

(Lynch 2009). Infusion duration was based on each individual's weight, which was

adjusted 3 times/week. Nicotine solution was stored in the dark at 4°C but was available

at room temperature during self-administration.

Surgery

Rats underwent surgery on postnatal day 28 to allow intravenous (i.v.) self-

administration of nicotine or saline as described previously in Chapter 2. Rats were given

ketoprofen (2-5 mg/kg, subcutaneous) and gentamicin (5.5 mg/kg, i.v.) on the day of

surgery and for the 2 subsequent days. To assess and help maintain patency, the catheter

was flushed every other day with heparinized saline prior to daily sessions. This method

allows the experimenter to easily monitor patency of the catheter without significantly

impairing rats during prolonged self-administration sessions.

Nicotine self-administration

The self-administration methods were described in detail previously in Chapter 3

(page 34). Briefly, rats were initially trained to self-administer nicotine (male, n=20;

female, n=17) or saline (male, n=21; female, n=18) under restricted access conditions (20

infusions maximum/day) to reduce variability in intake prior to extended access self-

administration beginning on postnatal day 30. These sessions were almost 24-hr in

duration (sessions began at 1200 and ended at 1150 the next day) and were conducted

over 5 daily sessions beginning on postnatal day 30. At the beginning of each session, the

active lever was presented into the chamber and responses on it were reinforced under a

fixed ratio 1 schedule. Infusions were paired with illumination of the active lever light

and the sound of the pump for the duration, and thus served as discrete cues associated

with drug delivery. The active lever was retracted from the self-administration chamber

at either 1150 or once a maximum of 20 infusions had been obtained. Acquisition of

nicotine self-administration was defined as 2 consecutive days of 20 infusions with a 2:1

preference of the active lever over the inactive lever. A 10-day extended access self-

administration period began following the fifth training session (on postnatal day 35).

There was no limit on number of infusions obtained during these extended access 23-hr

fixed ratio 1 sessions (sessions began at 1200). Access was again restricted on postnatal

days 45 and 46 to 20 infusions/session to equate nicotine intake between groups prior to

abstinence.

Experiment 1:

Wheel running during abstinence

After the last day of self-administration (postnatal day 47), rats were randomly

assigned to either an unlocked wheel that could freely rotate (male: saline, n = 11;

nicotine, n =10; female: saline, n = 9; nicotine, n = 9) or a locked wheel that was

stationary (male: saline, n = 11; nicotine, n =10; female: saline, n = 9; nicotine, n = 9). A

metal gate, that separated the polycarbonate cage from the wheel, regulated access to the

wheel. The gate was manually lifted by the experimenter each day at 1000 and lowered

following 2 hours (1200). A 2-hour exercise session was selected based on our previous

results with males demonstrating that this level access during abstinence was sufficient to

reduce subsequent nicotine-seeking (see Chapter 3; Sanchez et al. 2013a). During the 2-

hour sessions, rats were free to move between the wheel and their polycarbonate cage.

Revolutions completed during each unlocked wheel session were recorded daily.

Extinction/reinstatement

Following the last wheel session, rats were placed back into their previous self-

administration chambers to allow for habituation prior to extinction/reinstatement testing.

Testing began the next morning (postnatal day 57) under a within-session

extinction/reinstatement paradigm using methods described previously in Chapter 3 (pg

36). Briefly, animals underwent a minimum of 5 extinction sessions, each lasting 1-hour

and separated by a 5-minute timeout period, in which a response on the active lever had

no programmed consequence. Most animals met the extinction criterion (i.e. fewer than

15 responses) within 5 sessions, although 5 out of 76 animals did require a 6th session to

extinguish responding. Following the last extinction session a 1-hour reinstatement

session began with the presentation of the cues formerly associated with an infusion and

all subsequent responses on the active lever resulted in the presentation of these cues.

Experiment 2:

In order to examine the potential effects of the presence of the wheel itself on subsequent nicotine-seeking, we included additional groups of males (n = 8) and females (n = 8) housed in polycarbonate cages without access to running wheels during abstinence. These groups were included based on results from pilot studies demonstrating that in females, but not males, levels of responding during extinction and reinstatement were low in both wheel-condition groups. This finding was unexpected since females have been shown to exhibit higher levels of motivation to obtain nicotine infusions as compared to males (Donny et al. 2000), and acquire nicotine self-administration more readily than males (Lynch 2009). Females have also been found to display more robust drug-seeking behavior than males following abstinence from other drugs of abuse (Kerstetter et al. 2008). Thus, we hypothesized that females are sensitive to the attenuating effects provided by the presence of the wheel itself. To test this hypothesis, additional males and females were tested under the same self-administration procedure as described above in Experiment 1; however, following self-administration, they were moved to polycarbonate cages without wheel attachments for the 10-day abstinence period. Nicotine-seeking was assessed under the same within-session extinction/reinstatement paradigm as described above.

Data analysis

All data are presented as mean ± standard error of mean (SEM). Data analysis for Experiment 1 was performed in locked and unlocked wheel groups for males and

females. For Experiment 2 data analysis, the no wheel condition was compared to the locked and unlocked wheel conditions. Repeated measures analysis of variance (ANOVA) was used to analyze group and sex differences in extended access self-administration (Fig 9), lever responses during extinction sessions (Fig 11), and to compare responding during the last extinction session to responding during the reinstatement session (Fig 13) with sex, drug, and wheel-condition as between subject factors and day or session as the within subject factors. Repeated measure ANOVA was also used to analyze wheel running during abstinence (Fig 10) and the average distance run during the first 3 days as compared to the last 3 days (Fig 11) with sex and drug as between subject factors and day as the within subject factor. Subsequent repeated measures ANOVAs within each drug condition (Fig 9 and 11) and sex (Fig 11 and 13) were used to further elucidate the sex difference in self-administration and extinction responding and the effect of wheel condition on extinction and reinstatement responding. Univariate analyses were used to analyze total responding during extinction (Fig 12), number of sessions to reach extinction criteria, and inactive lever responding during extinction and reinstatement with sex, drug, and wheel condition as between subject factors. Due to our predicted hypothesis that females would self-administer more nicotine than males, a one-tailed t-test was used to compare nicotine intake between male and female no wheel groups during extended access. Post-hoc comparisons were performed using one-tailed t-tests to compare total extinction responding between wheel groups based on our predicted hypothesis that the locked wheel condition and/or no wheel access would lead to greater nicotine-seeking than the unlocked wheel condition. Pearson

correlations were used to determine associations between distance run during abstinence and nicotine intake, total extinction responding, and responding during reinstatement. Statistical analyses were performed using SPSS (version 20) and alpha was set to 0.05 for all tests.

Results

Experiment 1

Sex differences in nicotine self-administration

Under 23-hour extended access conditions, both male and female rats self-administered significantly more nicotine infusions than saline infusions (overall effect of drug, $F_{1,67} = 71.21$, $p < 0.001$; Fig. 9A). Although no differences were observed among saline controls, within nicotine groups there was a significant effect of sex ($F_{1, 33} = 7.650$, $p < 0.05$), but a non-significant trend for the effect of day ($p = 0.07$) and no interaction of day by sex, indicating that while females took more nicotine than males, the pattern of intake across sessions was similar between the sexes particularly during the first 7 days of extended access. Interestingly, however, there was a notable decrease in responding for nicotine during the last 3 days of access (postnatal days 42-44) in the female group. Overall, no significant effect of wheel-condition was observed in nicotine or saline infusions (Fig. 9B) indicating that prior to abstinence and subsequent extinction/reinstatement testing, intake was similar between the locked and unlocked wheel groups.
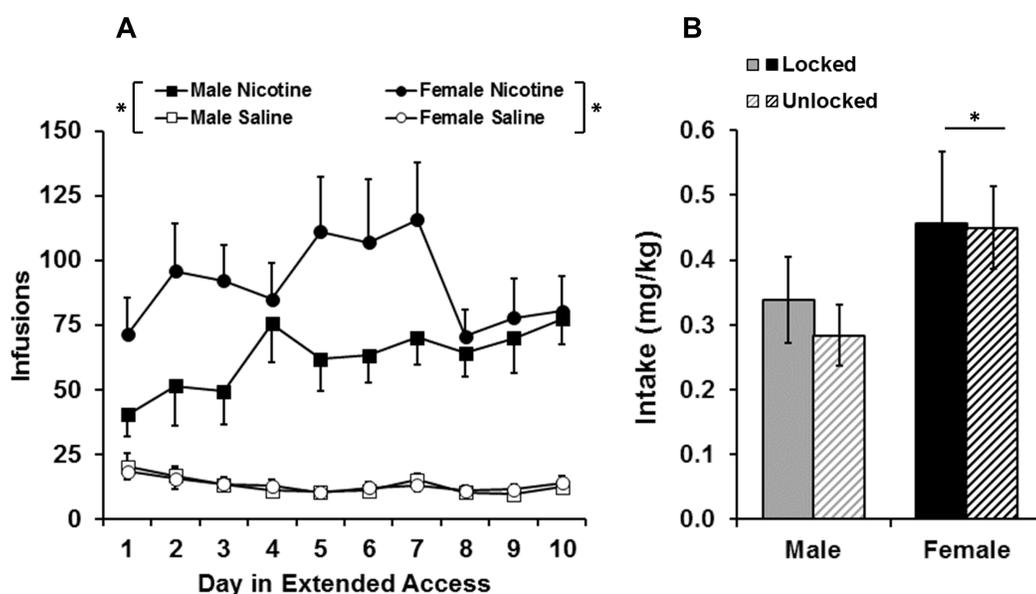
Figure 9. Adolescent females self-administer more nicotine than males under extended access conditions. (A) Average (mean ± SEM) number of infusions self-administered during 10-day extended access sessions for nicotine (black symbols) and saline (white symbols) in male (square symbols) and female (circle symbols) groups. Asterisk indicates significant (*, $p < 0.05$) difference in number of infusions between nicotine (male, n = 20; female, n=17) and saline (male, n=21; female, n = 18) groups. (B) Average daily nicotine intake (mean ± SEM) for male (locked, n = 10; unlocked n = 10) and female (locked, n = 8; unlocked, n= 9) groups. Asterisk indicates significant difference in average daily intake between males and females (*, $p < 0.05$).

Sex differences in wheel running during abstinence

Female rats ran more than their male counterparts during the daily 2-hour sessions (overall effect of sex, $F_{1, 35} = 40.35$, $p < 0.001$; Fig. 10). We also observed significant effects of day ($F_{9, 315} = 8.73$, $p < 0.001$) and day by sex ($F_{9, 315} = 3.14$, $p < 0.01$) indicating that wheel running increased differently between males and females over time. While analysis within each sex revealed a significant effect of day for both males and females, this effect in males appeared to be due to a difference from the first day to the last day rather than a consistent increase over time. In fact, when this day was excluded the day effect was no longer significant in males but was retained in females. Furthermore, an analysis of the first 3 days to the last 3 days revealed significant effects of sex ($F_{1,35}= 44.20$, $p < 0.001$), day ($F_{1,35}= 17.90$, $p < 0.001$), and day by sex ($F_{1,35}= 7.70$, $p < 0.01$) indicating that females increased their running ($F_{1,16}= 15.15$, $p < 0.01$) while males did not. Although levels of wheel running appeared higher in the nicotine groups as compared to saline groups, particularly within females, the level of variability was relatively high, and no significant effects of drug or drug by sex were observed. No correlation was found between distance run during abstinence and infusions during extended access.
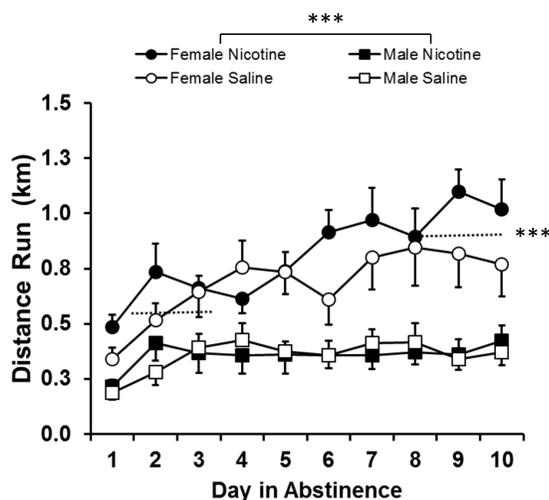
Figure 10 Females run further and escalate their running to a greater extent than males. Average (mean ± SEM) daily distance run during abstinence for females (saline, n = 9; nicotine, n = 9) and males (saline, n = 11; nicotine, n =10) in the unlocked wheel condition is plotted. Asterisks indicate significant difference in distance run between males and females and significant increase in average daily distance run during the last 3 days as compared to the first 3 days in females (***, p < 0.001).

Sex differences in the effect of wheel running during abstinence on subsequent nicotine-seeking during extinction and reinstatement

Prior nicotine self-administration led to significantly more active lever responding during extinction sessions than did prior saline self-administration (effect of drug, $F_{1, 68} = 48.08$, p < 0.001; Fig. 11) indicating that adolescent-onset nicotine self-administration led to robust drug-seeking. Within the saline controls, there was a significant effect of session ($F_{4, 140} = 3.53$, p < 0.01), with the greatest number of responses in the first session as compared to later sessions. There was also a trend for an

effect of sex (p = 0.058), with females tending to have higher levels of responding as compared to males. Within the nicotine animals, higher responding was again observed during the first session as compared to later sessions (effect of session, $F_{4, 132} = 7.58$, p < 0.001), but there was a similar pattern of responding in males and females. Although an overall effect of sex was not found in the nicotine animals, a significant sex by wheel condition was observed ($F_{1,33} = 5.414$, p < 0.05). Within the nicotine males there was a significant effect of wheel condition ($F_{1,18} = 9.21$, p < 0.01) with those in the locked wheel condition displaying higher levels of responding than those in the unlocked wheel condition (Figs 11 and 12). However, in the nicotine females, there was no difference in responses between the wheel conditions; both groups showed low levels of responding which were statistically similar to males in the wheel condition. There were no associations between the distance run during abstinence and the number of active lever responses during extinction. Inactive lever responding during extinction sessions was significantly higher in nicotine groups ($F_{1,68} = 15.92$, p < 0.001) as compared to saline groups; however, no effects of sex or wheel-condition were observed. Inactive lever responding in saline males under the locked and unlocked wheel condition was 6.00 ± 3.05 and 6.27± 3.07, respectively, and in saline females in these same conditions was 2.5 ± 1.15 and 4.67 ± 2.09. Inactive responding in nicotine males under the locked and unlocked wheel condition was 15.9 ± 3.14 and 13.7 ± 4.59, respectively, and in nicotine females in these same conditions was 14.5 ± 3.84 and 8.33 ± 2.30.

Nicotine animals also took significantly longer to extinguish responding as compared to saline controls ($F_{1,68} = 19.615$, p < 0.001). Specifically, the average number

of sessions to reach the extinction criterion (i.e. fewer than 15 active lever responses) for nicotine males in the locked and unlocked wheel condition was 3.67 ± 0.62 and 3.13 ± 0.74, respectively as compared to 1.3 ± 0.24 and 1.55 ± 0.44 for saline males in these same conditions. The average number of sessions to reach the extinction criterion for nicotine females was 3.00 ± 0.74 and 2.22 ± 0.28 in the locked and unlocked wheel conditions, respectively as compared to 1.67 ± 0.33 and 1.56 ± 0.44 for saline females in these same conditions. No effects of wheel condition or sex were observed on rates of extinction. However, the average number of extinction sessions run did not differ between groups as the majority of animals (71 out of 76) in all groups met the extinction criterion by the 5th session.
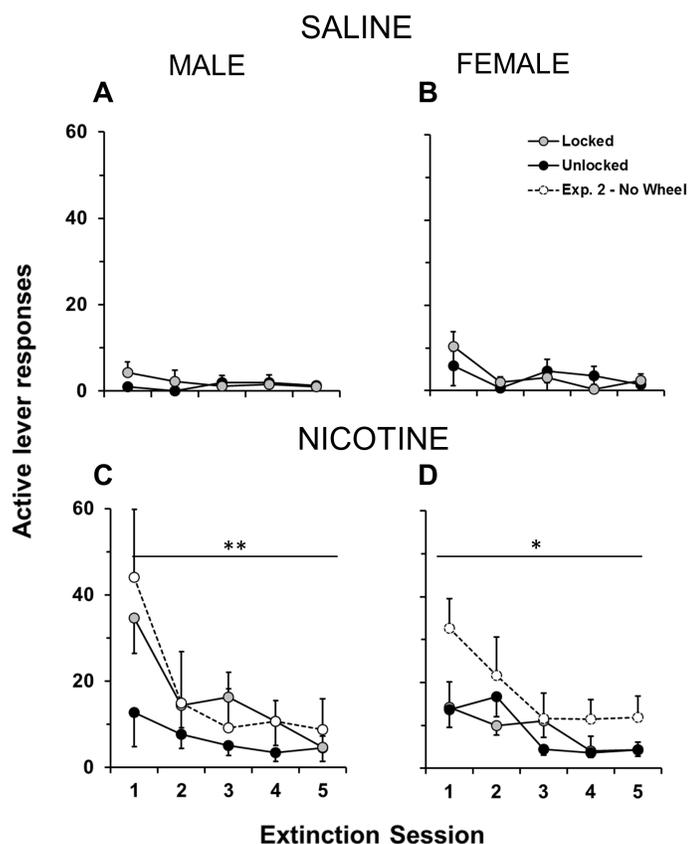
Figure 11. Nicotine self-administration during adolescence followed by an abstinence period leads to robust nicotine-seeking during extinction. Average active lever responses during each of the 5 extinction sessions are plotted for males (saline, A; nicotine, C) and females (saline, B; nicotine, D) and for the locked (gray circles) and unlocked (black circles) wheel conditions. In panels C and D, the extinction responding for the no wheel groups (white circles) from experiment 2 are also plotted for comparison. Asterisks indicate significantly more active lever responding during extinction in nicotine males (C) and females (D) as compared to saline controls (A and B, respectively; **, $p < 0.01$; *, $p < 0.01$).
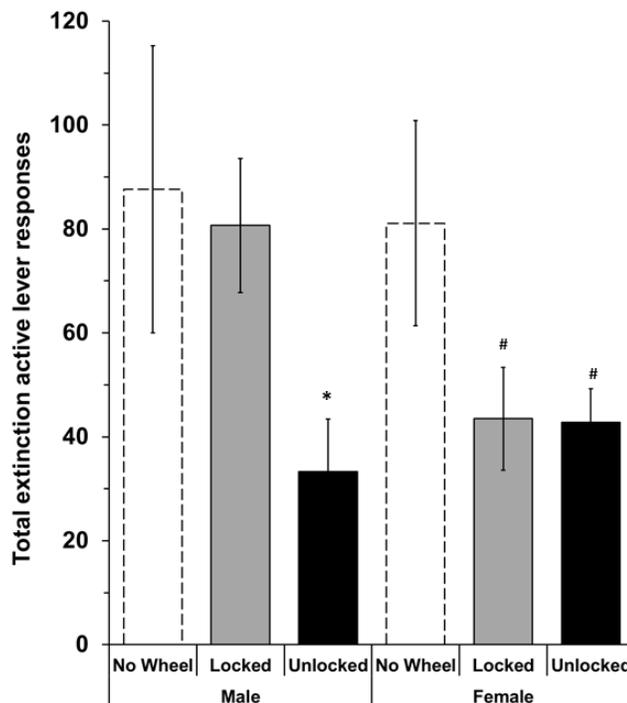
Figure 12. Total active lever responses (mean ± SEM) during the first 5 extinction sessions for males (n = 8, n = 10, n = 10) and females (n = 8, n = 8, n = 9) for the no wheel, locked, and unlocked groups are displayed. Asterisk indicates significant difference from no wheel and locked wheel groups in males (*, $p < 0.05$) and number sign indicates significant difference from no wheel group in females (#, $p < 0.05$).

Prior nicotine self-administration also led to significantly higher levels of cue-induced reinstatement than did saline self-administration (overall effect of drug, $F_{1,68} = 23.52$, $p < 0.001$). Within nicotine animals, there was a significant session effect ($F_{1,33} = 27.14$, $p < 0.001$) with all groups significantly increasing their responding during reinstatement from the last extinction session; however, no effect of wheel condition or sex was observed. Within saline controls, a sex difference was observed due to higher

levels of reinstatement responding within females (effect of sex, $F_{1,35} = 5.75$, $p < 0.05$) but only a trend for a difference was observed between the last extinction session and the reinstatement session, and again there was no effect of wheel condition. There were no associations between distance run during abstinence and active lever responses during reinstatement. Similar to inactive lever responding during extinction, inactive lever responding during reinstatement was significantly higher in nicotine as compared to saline groups ($F_{1,68} = 7.65$, $p < 0.01$) and there were no effects of sex or wheel-condition. Inactive lever responding during reinstatement in saline males were 0 ± 0 and 1.82 ± 0.96 and in females 0.44 ± 0.34 and 0.78 ± 0.57 in the locked and unlocked wheel groups, respectively. Inactive lever responding during reinstatement in nicotine males were 1.1 ± 0.64 and 2.9 ± 1.91 and in females were 4.38 ± 1.46 and 4.44 ± 2.68 in the locked and unlocked wheel groups, respectively.

Initially, weights were similar among males and females (88.9 g ± 1.7 versus 84.9 g ± 1.3 at the start of the experiment, respectively); however, by the end of the experiment males weighed significantly more than females (285.5 g ± 5.1 versus 212.4 g ± 3.2, respectively; sex effect $F_{1,57} = 150.789$, $p < 0.001$). No effect of wheel condition was observed for either sex, but by the end of the experiment, saline males weighed significantly more than nicotine males (297.8 ± 5.9 g versus 276.4 ± 7.2g, respectively; drug effect, $F_{1,28} = 7.468$, $p < 0.05$).
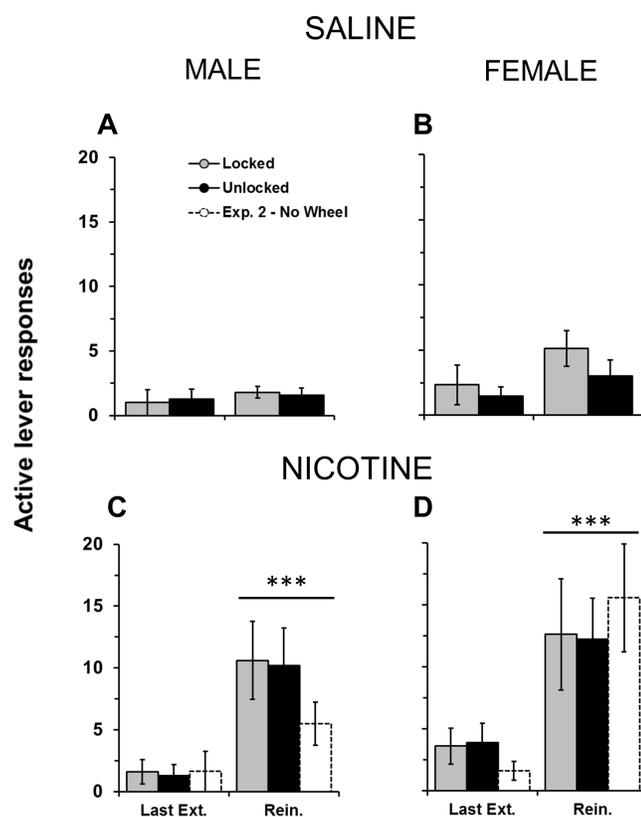
Figure 13 Total active lever responses (mean ± SEM) during the last extinction session (Last Ext.) and reinstatement session (Rein.) are plotted for saline males (A) and females (B) and nicotine males (C) and females (D). Locked, unlocked, and no wheel (Experiment 2) groups are portrayed by gray, black, and white bars, respectively. Asterisk indicates a significantly greater responding during reinstatement as compared to the last extinction session (***, p < 0.001).

Experiment 2

Nicotine-seeking during extinction is attenuated by both wheel conditions in females but not males

Prior to abstinence, males and females without access to a running wheel during abstinence (no wheel) did not differ from their locked and unlocked wheel counterparts in nicotine intake during extended access (males, 0.26 ± 0.05 mg/kg; females, 0.47 ± 0.08 mg/kg) and again a significant effect of sex was observed ($F_{1,47} = 8.385$, $p < 0.01$). Average daily intake was significantly greater in females in the no wheel condition as compared to males ($t_{14} = 4.854$, $p < 0.05$). A significant effect of wheel condition was observed in total extinction responding ($F_{1,47} = 5.317$, $p < 0.01$; Fig 12). Although significant effects of sex and sex by wheel condition were not observed overall, planned analysis within each sex revealed interesting differences in the effects of wheel condition. Within males, a significant effect of wheel condition was again observed for total active lever responding during extinction sessions ($F_{2,25} = 6.418$, $p < 0.05$). Males in the no wheel condition did not differ significantly from those in the locked wheel condition in active lever responding during extinction and they displayed significantly greater responding than males in the locked wheel condition ($t_{16} = 4.112$, $p < 0.05$). The females in the no wheel group did, however, show significantly elevated nicotine-seeking during extinction sessions (effect of wheel condition, $F_{2,22} = 3.88$, $p < 0.05$; Fig 12) as compared to the locked or unlocked wheel condition female groups (locked versus no wheel $t_{14} = 3.968$, $p < 0.05$; unlocked versus no wheel, $t_{15} = 4.482$, $p < 0.05$; Fig 12). In fact, levels of extinction responding were not different from those observed in males that were in the

locked wheel condition during abstinence. As in Experiment 1, a significant increase in responding was observed in the reinstatement session as compared to the last extinction session (session effect, $F_{1,47}= 37.240$, $p < 0.001$) and no significant effect of wheel condition was observed. However, with the addition of the no wheel groups, a significant effect of sex emerged ($F_{1,47}= 4.633$, $p < 0.05$) due to higher levels of reinstatement in the no wheel females compared to the no wheel males ($t_{14}= 2.177$, $p < 0.05$).

Discussion

The goal of the present study was to determine if there are sex differences in the effect of wheel running during abstinence on subsequent nicotine-seeking. As was anticipated based on our previous findings in Chapter 3 (Sanchez et al. 2013a), we observed robust levels of nicotine-seeking during extinction in males under the locked and no wheel conditions, and these levels were markedly reduced by running under the unlocked wheel condition. Surprisingly, in females, levels of nicotine-seeking during extinction were low under both wheel conditions, locked and unlocked, with levels comparable to those observed in males tested under the unlocked wheel condition. Females in the no wheel condition, however, showed high levels of nicotine-seeking during extinction that were similar to males without access to wheel or with a locked wheel access. Levels of nicotine-seeking in these females were also higher than either of the female wheel groups. Thus, while wheel running during abstinence effectively reduced subsequent nicotine-seeking in males, the presence of the wheel was sufficient to reduce nicotine-seeking in females.

Although environmental enrichment is one possible mechanism to account for the lower levels of nicotine-seeking during extinction in females under both wheel conditions (Chauvet et al. 2009), another possibility is that the locked wheel condition permits physical activity within the wheel and thus also functions as an exercise condition. While the locked wheel condition is an important control for environmental enrichment, animals are still able to be quite active within the wheel (e.g., climbing and hanging; Koteja et al. 1999). Females have been shown to be more active than males (Hitchcock 1925; Elkelboom and Mills 1988; Smith et al. 2011, 2012), and here we also showed that females ran greater distances in the unlocked wheel condition as compared to males. As such, females may have been more active in the locked wheel as well and thus may have been more sensitive to this alternative form of exercise within the wheel as compared to males. This finding may also suggest that non-aerobic exercise could benefit females but not males. Further studies using photocells or video tracking to directly monitor activity within wheels are needed to dissociate the effects of environmental enrichment versus exercise on nicotine-seeking in females. It is also possible that the sucrose active lever training used in this study differentially affected subsequent active lever responding for nicotine between males and females. Further research investigating the impact of sucrose pre-training on subsequent drug-seeking in adolescents and potential sex differences for this effect is needed.

Consistent with our hypothesis, adolescent females self-administered significantly more nicotine under 23-hour unlimited access conditions as compared to males. Although the present study is the first preclinical study to demonstrate sex differences in nicotine

self-administration under extended access conditions, previous work from our lab has demonstrated an increased sensitivity to the positive reinforcing effects of nicotine in adolescent females with higher rates of initiation and levels of motivation to self-administer nicotine as compared to adolescent males under the same low dose condition used in the present study (Lynch 2009). Interestingly, this increased motivation was found beginning on postnatal day 43 under a progressive ratio schedule and in the present study we found that as a group, females decreased the number of responses for nicotine under a fixed ratio schedule during the last 3 days of self-administration corresponding to postnatal days 42-44. Thus, the decrease in responding observed here might have been due to an increased sensitivity to the positive reinforcing effects of nicotine at this later stage of adolescence. Further research is needed to directly examine this possibility. Similar to findings in adolescents, adult female rats have also been shown *t*o acquire nicotine self-administration faster (at low doses), achieve higher breakpoints under progressive ratio conditions, and have shorter latencies to first response during both fixed ratio and progressive ratio conditions as compared to males (Donny et al. 2000). Nonetheless, some studies have failed to find a sex difference in adolescent and adult nicotine self-administration under fixed ratio conditions (Chen et al. 2007; Levin et al. 2011; Feltenstein et al. 2012; Li et al. 2012). However, these studies used short-access paradigms (1-2 hours/session) and/or higher doses of nicotine than used in the present study, which may account for the discrepancy with our findings. Notably, Chen et al. (2007) used the same extended access paradigm in adolescent rats and failed to observe sex differences likely due to the use of higher nicotine doses (i.e. 7.5, 15, 30, and 60

µg/kg), which may obscure individual differences (Donny et al. 2000, Lynch 2006, 2009). Li et al. (2012) did, however, find that under progressive ratio conditions adolescent female rats responded significantly more than adolescent male rats. Also, Levin et al. (2011) found that although females did not self-administer more initially, their responding for nicotine persisted longer than that of males. Thus, it appears that adolescent and adult females may be more vulnerable than males to the positive reinforcing effects of nicotine.

In contrast to our hypothesis, females did not display greater nicotine-seeking following a prolonged abstinence period as compared to males. Psychostimulant drugs such as nicotine and cocaine are known to incubate over an abstinence period (i.e. drug-seeking increases with longer abstinence periods; Grimm et al. 2001; Lu et al. 2004; Abdolahi et al. 2010) and there is evidence that there are sex differences in this incubation effect for cocaine with females displaying greater levels of cocaine-seeking than males following prolonged abstinence (Kerstetter et al. 2008). Although, no studies have directly examined sex differences in the incubation effect of nicotine-seeking, our findings here as well as recent findings from others suggests that sex differences in drug-seeking following a prolonged drug abstinence period do not extend to nicotine (Feltenstein et al. 2012).

Also in contrast to our hypothesis, we did not find an effect of exercise on cue-induced reinstatement in either males or females. Although nicotine self-administration followed by prolonged abstinence did lead to a significant increase in responding during cue-induced reinstatement, this effect was modest and, as was reported previously in

males (Sanchez et al. 2013a), highly variable. The modest levels of cue-induced nicotine-seeking observed here are likely due to the age of the animals in this study. Compared to adults, adolescent rodents show attenuated neurochemical adaptations during withdrawal from nicotine (Natividad et al. 2010, 2012; Tejeda et al. 2012). Adolescent animals also do not have complete development of critical brain regions, such as the prefrontal cortex (Geidd 2004; Counotte et al. 2011), that are known to be important for cue-induced reinstatement (Koya et al. 2008). Others have similarly reported modest levels of cue-induced cocaine-seeking in adolescent rats as compared to adults and other types of reinstatement within adolescents (i.e. stressor led or cocaine primed; Anker and Carroll 2010). Thus, the low level of reinstatement observed here in this adolescent-onset model of nicotine addiction may have created a floor effect for which the effects of exercise could not be determined. Further studies are needed to determine if other reinstatement paradigms (e.g. nicotine or stress induced reinstatement) would be more robust and thus a better tool to examine the effects of exercise.

Our findings are consistent with previous reports that demonstrate a significant attenuation of drug-seeking when wheel-running was available concurrently with cocaine extinction and reinstatement procedures (Zlebnik et al. 2010) or during abstinence from cocaine or nicotine prior to extinction and reinstatement (Lynch et al. 2010; Sanchez et al. 2013a). Although in humans, the data are equivocal for its long-term efficacy (Ussher et al. 2012), studies suggest that exercise may be an effective therapeutic for smoking cessation. Two recent meta-analyses of the literature found that exercise acutely attenuates the desire to smoke as well as tobacco withdrawal symptoms (Roberts et al.

2012; Haasova et al. 2013). There is also some evidence that the effects of exercise extend to adolescents with results from a few studies demonstrating that the addition of an exercise component to behavioral treatment programs effectively reduces rates of smoking in adolescents (Werch et al. 2005; Horn et al. 2011; Horn et al. 2013). In an interesting parallel to our rodent studies, girls benefitted from after-school enrichment programs whereas boys only showed a reduction in smoking behavior when the program had an exercise component (Horn et al. 2011). Our preclinical findings suggest that these differences may be due in part to biological mechanisms.

In summary, wheel running during abstinence attenuated subsequent nicotine-seeking in male and female rats that began self-administration during adolescence. Given that most adult smokers initiate use during adolescence our results support the notion that exercise may be an effective smoking cessation aid in this vulnerable population. Sex differences were observed in sensitivity to the locked wheel condition either by functioning as an environmental enrichment or an alternative form of exercise. Thus, future studies in humans considering the use of exercise as a treatment for human smoking cessation, should explore the use of aerobic versus non-aerobic exercise with varying intensities in males and females.

Chapter 5:
Structural plasticity associated with
exercise as an intervention for adolescent-onset nicotine addiction

Introduction

Drugs of abuse including nicotine, cocaine, and amphetamine are known to have long-lasting effects on the structure of dendrites and dendritic spine density in key areas of the mesolimbic reward pathway (for review see, Robinson and Kolb 2004; Russo et al. 2010). For example, chronic intermittent exposure to non-contingent administration of nicotine followed by an abstinence period has been shown to increase dendrite length and dendritic spine density in the medial prefrontal cortex and nucleus accumbens shell in adult and adolescent male rats (Brown and Kolb 2001; McDonald et al. 2005; 2007). Only one study has examined structural plasticity following abstinence from self-administered nicotine (Gipson et al. 2013). In this study performed in adult rats, they observed an increase in spine head diameter within the core region (Gipson et al. 2013). However, structural plasticity following abstinence from adolescent nicotine self-administration has yet to be examined in either the core or the shell region of the nucleus accumbens. Furthermore, a direct comparison of structural plasticity within the core and shell regions remains to be investigated. Thus, one goal of this study was to determine if adolescent-onset nicotine self-administration followed by a prolonged abstinence period and subsequent nicotine-seeking, induces long-term plastic changes within the nucleus accumbens core and shell regions.

Abstinence from drugs of abuse including nicotine is associated with long-lasting behavioral effects such as a progressive increase in vulnerability to relapse over a protracted period of abstinence (i.e., "incubation of craving"; Pickens et al. 2011; Abdolahi et al. 2010; Bedi et al. 2011). Abstinence from nicotine self-administration has been shown to induce nicotine-seeking in extinction and reinstatement procedures (Abdolahi et al. 2010; Sanchez et al. 2013a; b), an effect that has been associated with structural plasticity within the nucleus accumbens core in adult rats (Gipson et al. 2013). In previous chapters of this dissertation, voluntary wheel running exercise during abstinence was shown to attenuate subsequent nicotine-seeking in an adolescent-onset model (Sanchez et al.2013). Although the effect of exercise on neuronal structural plasticity within the nucleus accumbens is not known, exercise has been found to be rewarding and to affect dopamine signaling in the nucleus accumbens core and shell regions (Greenwood et al. 2011) as well as glutamate signaling in other regions of the brain (Meeusen et al. 1997; Guezennec et al. 1998). Furthermore, exercise has been found to increase dendrite length and spine density within the hippocampus (Stranahan et al. 2007; Eadie at al. 2005; Redila and Christie 2006), suggesting that exercise can modulate structural plasticity of dendrites. Thus, the second goal of this study was to determine if voluntary wheel-running exercise, that has previously been shown to attenuate nicotine-seeking, also attenuates nicotine-induced structural plasticity within the nucleus accumbens.

To address the goals of this study, rats were given voluntary access during a 10-day abstinence period to either an unlocked (exercise) or a locked (sedentary) running

wheel and then tested for nicotine-seeking under an extinction and reinstatement paradigm. To assess the structural plasticity associated with nicotine-seeking and its modulation by exercise, histological examination of synapses within the nucleus accumbens core and shell regions were performed using electron microscopy. We focused specifically on asymmetric synapses onto dendritic spines as these are the most common type of synapses found on spine heads and are widely accepted as excitatory synapses (Nimchinsky et al. 2002; Gray 1959). Furthermore, abstinence from nicotine has been shown to induce increases in spine head diameter and glutamate signaling within the nucleus accumbens (Gipson et al. 2013; Russo et al. 2010). It was hypothesized that increased nicotine-seeking following abstinence would result in an increase in synapse length in the nucleus accumbens core and shell regions and that exercise during abstinence would attenuate this effect.

Methods

Animals

Adolescent male Sprague-Dawley (Charles River Laboratories, Portage, ME, USA) rats (N = 14) arrived on postnatal day 22 and were singly housed in self-administration chambers (see Chapter 2 for details) under a 12-hour light/dark cycle (lights on 0700). Food and water were available *ad libitum* throughout the experiment. The health of the animals was monitored daily and they were weighed every Monday, Wednesday, and Friday. All procedures were in accordance with NIH guidelines and approved by the University of Virginia Animal Care and Use Committee.

Drug

Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9%

sterile saline (pH 7.4) and passed through a microfilter. A single low dose (5 μg/kg) of

nicotine was selected based on previous work demonstrating that under the same

experimental parameters robust acquisition and maintenance of self-administration as

well as subsequent nicotine-seeking following abstinence were observed. The dose is

expressed as the free base weight and infusions were delivered at a rate of 0.1 ml/sec

based on the animal's weight. Nicotine solution was stored at 4 °C but was available at

room temperature during self-administration sessions.

Behavioral paradigm

All behavioral methods (i.e. sucrose pre-training, surgery, nicotine self-

administration, abstinence, wheel-running, and extinction/reinstatement testing) were

performed as described in Chapter 3. Briefly, prior to surgery, animals were food pre-

trained to respond on the active lever for sucrose pellets under an FR1 schedule until 2

days of 50 pellet deliveries. On postnatal day 28, rats underwent surgery to implant a

chronic indwelling catheter into the right jugular vein to allow intravenous nicotine (n =

8) or saline (n = 6) self-administration. Self-administration training (5 days) began on

postnatal day 30, under an FR1 schedule with a maximum of 20 infusions. Acquisition

was defined as 2 consecutive days of 20 infusions with a 2:1 preference of the active

lever over the inactive lever. The 10-day extended access self-administration phase began

on postnatal day 35 during which animals could receive an unlimited number of infusions

(FR1 schedule) during 23-hour sessions. Abstinence began after a 2-day equilibration phase during which infusions were again limited to 20/session. On the first day of abstinence (postnatal day 47), rats were moved from their self-administration chambers to a polycarbonate cage with either a locked (saline, n = 3; nicotine, n = 4) or unlocked wheel (saline, n = 3; nicotine, n = 4) attachment to live for the 10-day abstinence period. Each day, the experimenter removed a metal gate separating the polycarbonate cage from the wheel and rats were permitted to move freely between they cage and the wheel for 2 hours. Revolutions performed within the unlocked wheel were recorded daily. After the last wheel access session, rats were moved back to their self-administration boxes. The following morning, drug-seeking was tested under a within-session extinction and cue-induced reinstatement paradigm. During each of the 5 1-hour extinction sessions responses on the active lever had no programmed consequences. In this study, all animals met the extinction criterion (i.e. fewer than 15 responses in the last session) within 5 sessions. A 1-hour reinstatement session began following the last extinction session with the presentation of the cues formerly associated with a nicotine infusion (i.e. the light and the sound of the pump) and each subsequent response on the active lever resulted in the presentation of cues, however no infusions were delivered.

Tissue preparation for electron microscopy

Immediately following the end of the reinstatement session, rats were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with Tyrode's solution until the vasculature was clear (< 2 minutes) and then with a 4%

paraformaldehyde with 0.5% gluteraldehyde fixative solution in 0.1 M phosphate buffer (PB, pH 7.4) for 20-30 minutes. Brains were removed and placed in the same fixative solution overnight at 4 °C. The following day, 80 μm coronal sections including the nucleus accumbens were cut on a vibratome and stored in 0.1% sodium azide in PB at 4 °C. Sections were washed in 0.1 M PB and then postfixed in 1% osmium tetroxide in 0.1 M PB for 1-hour. Sections were subsequently washed with 0.1 M PB three times (each wash 3 minutes) followed by 50% ethanol (ETOH) for 3-minutes and then stained with 4% uranyl acetate for 1-hour. The sections were then serially dehydrated in 70% ETOH for 1 minutes, 90% ETOH for 5 minutes, 100% ETOH for 5 minutes twice, and acetone for 2 minutes 3 times. The last acetone wash was replaced with a 1:1 dilution of EPON in acetone in which the sections remained overnight. The following day the EPON-acetone mixture was replaced with EPON for 2-4 hours. Tissue was then transferred to an aclar sheet with EPON and covered with a another aclar and placed in an oven at 60 °C overnight. Camera lucida was used to trace landmarks of the tissue for localization of the nucleus accumbens core and shell regions. These regions were then cut from the flat embedded tissue and placed in a capsule BEEM that was subsequently filled with EPON and allowed to polymerize in the 60 °C oven for 3 days. Using a diamond knife, 70 nm sections of tissue were cut and collected on grids for visualization with a Joel 1010 transmission electron microscope (TEM). Images were captured using a digital camera.

Quantitative analysis of electron micrographs

For each rat, 1 section was used to analyze the nucleus accumbens core and shell regions. Sections were selected at 1.92 mm Bregma based on a standard neuroanatomical atlas (Paxinos and Watson 2005). From each region (core and shell), 3 areas were selected parallel to the midline of the brain in the middle of the shell region and 3 corresponding areas were selected in the core region (blue boxes in Fig 14). Areas in the core were selected so that they were adjacent to areas that contained the anterior commissure (green boxes in Fig 14). Within each area 15 non-overlapping pictures were taken of neuropil at 10,000 times magnification with a 16M-pixel camera (2.75 nm pixel size resolution, for a total of 45 pictures from each core and shell.
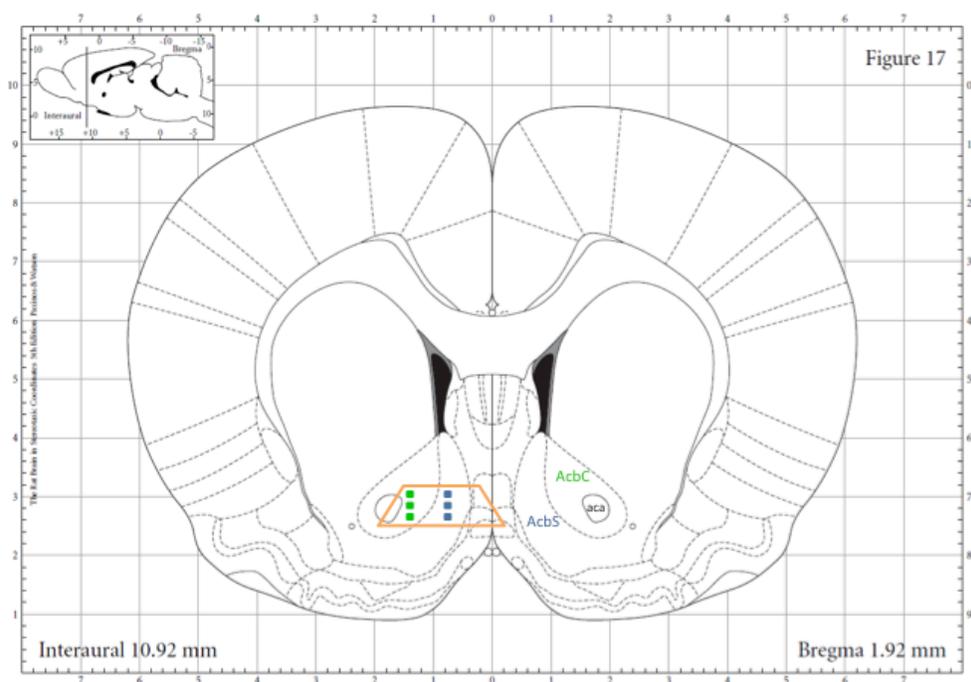

Figure 13 Schematic of areas within the nucleus accumbens core (AcbC; green boxes) and shell (AcbS; blue boxes) regions analyzed. The orange trapezoid represents the area from each section that used to make ultrathin sections. Not to scale. Modified from Paxinos and Watson 2005.

Each photograph was examined by an experimenter blinded to the condition at a final resolution of 363 pixels/μm, and each synapse encountered was marked, its synapse length was measured. Total area of each photograph, excluding myelinated axons, blood vessels and cell bodies was also measured. Measurements of synapse length and neuropil area were taken using Image Pro Plus (Media Cybernetics, Silver Spring, MD). Synapses were defined by the presence of at least 3 synaptic vesicles in the presynaptic terminal and the parallel alignment of the pre- and post-synaptic membranes creating a synaptic cleft. Synapses were identified as either asymmetric (highlighted in blue in Fig 15) or symmetric (highlighted in green in Fig 15 Aii, Bii) based on the presence or absence of a postsynaptic density, respectively. Postsynaptic profiles were classified as spines if mitochondria and microtubules were absent (red arrows in Fig 15 Ai). The presence of a spine apparatus (black arrow in Fig 15 Ai) was also used for classifying postsynaptic profiles as a spine, but was not necessary. Other postsynaptic profiles were also identified as (1) dendritic shafts if microtubules and mitochondria were present (star in Fig 15 Ai) (2) cell bodies by the presence of endoplasmic reticulum, Golgi apparatus, or a nucleus or (3) an axon terminal if vesicles were present.
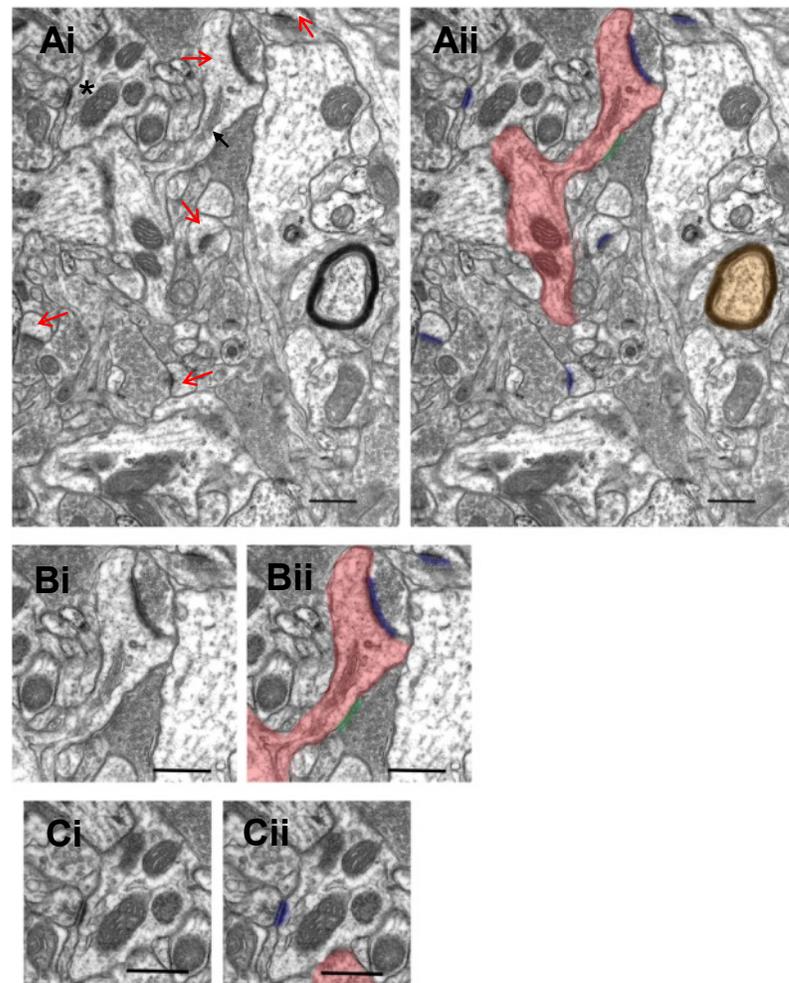
Figure 15 An example electron micrograph of neuropil from which synapses were measured. In panel **Ai**, red arrows mark dendritic spines, the black arrow marks a spine apparatus. In panel **Aii**, a dendritic spine that connects back to the dendritic shaft is highlighted in red, asymmetric synapses are highlighted in blue, symmetric synapse is highlighted in green, and a myelinated axon is highlighted in orange. Panels **Bi** and **Bii** show an enlarged image of a dendritic spine with asymmetric (blue) and symmetric (green) synapses. Panels **Ci** and **Cii** show an enlarged image of an asymmetric synapse (blue) onto a dendrite with a clearly defined synaptic cleft. Scale bar represents 0.5 μm.

Statistical analysis

Behavioral data are presented as the mean ± SEM. Self-administration was analyzed using a repeated measures ANOVA with drug and exercise condition as the between subject factors and day as the within subject factor. T-tests were used to compare average daily intake between nicotine groups and average daily distance run between exercise groups. A univariate analysis as used to analyze differences in active lever responding during extinction and subsequent post hoc t-tests were performed. A repeated measures ANOVA of the last extinction session and the reinstatement session was used to determine differences in reinstatement of drug-seeking. Differences in the distribution of synapse length were analyzed using the Mann-Whitney U-test. The vast majority of synapses observed were in asymmetric synapses onto spines (approximately 90%) thus all analyses were performed on measurements taken from this type of synapse. Statistical analyses were performed using SPSS (20) and alpha was set at 0.05 for behavioral data and 0.01 for histology data.

Results

Behavioral results

During extended access, animals that self-administered nicotine responded for significantly more infusions than those that self-administered saline with an overall effect of drug ($F_{1,10} = 10.825$, $p < 0.01$; Fig 16A) and no effect of day. However, no significant effects of exercise condition or interaction of drug by exercise condition were observed. Furthermore, within the nicotine condition, no difference in nicotine intake was observed

between the sedentary and exercise groups (Fig 16B). During abstinence, average daily

distances run were similar between the saline and nicotine exercise groups (Fig 16C).

During extinction, active lever responses were highest in the nicotine groups (effect of

drug, $F_{1,10} = 6.962$, $p < 0.05$; Fig 16D). A non-significant trend for a wheel by drug

interaction was observed ($p = 0.062$) and subsequent analyses revealed that the nicotine

sedentary group had significantly higher responding as compared to both saline

conditions (versus saline sedentary, $t_5 = 4.366$, $p < 0.05$; versus saline exercise, $t_5 =$

4.340, $p < 0.05$) and the nicotine exercise group ($t_6 = 3.996$, $p < 0.05$). No significant

difference in extinction responding was observed between the saline groups but the

nicotine exercise group did have significantly higher responding than both the saline

sedentary ($t_5 = 11.558$, $p < 0.001$) and the saline exercise ($t_5 = 12.708$, $p < 0.001$) groups.

Reinstatement responding was low and not significantly higher than responding during

last extinction session and no effect of drug or wheel condition was observed (Fig 16D).
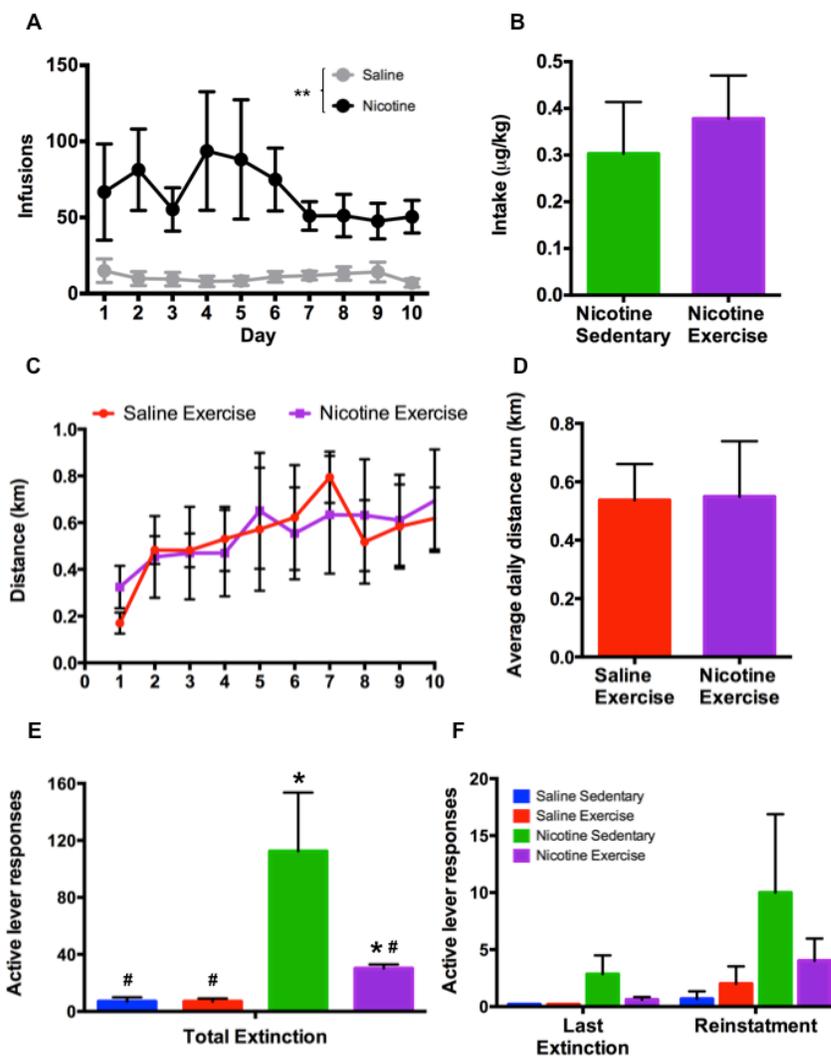
Figure 16 Behavioral data for extended access self-administration (A and B), distance traveled during abstinence (C and D), and during extinction (E) and reinstatement test (F). In panel A, the number of infusions is plotted for saline (gray circles; n = 6) and nicotine (black circles; n = 8) during the 10-day extended access self-administration period. Asterisks indicate a significant difference (p < 0.01) between saline and nicotine groups. In panel B, average daily nicotine intake is plotted for the nicotine sedentary (green bar; n = 4) and exercise (magenta bar; n = 4) groups. In panel C, the daily distance run in kilometers (km) is plotted for each exercise session during the 10-day abstinence period. In panel D, the average daily distance run is plotted for the saline (red bar; n = 3) and nicotine (magenta bar; n = 4) exercise groups. In panels E and F, the active lever responses during all extinction sessions (Total Extinction; E), the last extinction session (F), and the reinstatement session (F) are plotted for the saline sedentary (blue; n = 3), saline exercise (red), nicotine sedentary (green), and nicotine exercise (magenta) groups. Asterisks indicate a significant difference from saline sedentary group and the number signs indicate a significant difference from nicotine sedentary.

Synapse length distribution in the nucleus accumbens core

Nicotine self-administration followed by abstinence (nicotine sedentary group) lead to a significant rightward shift in the distribution of synapse length as compared to the saline sedentary group ($p < 0.01$; Fig 17 Ai and Aii). The nicotine exercise group displayed a significant leftward shift in the distribution of synapse size as compared to the nicotine sedentary group ($p < 0.001$; Fig 17 Bi and Bii). Indeed, the synapse length distribution of the nicotine exercise group did not differ from the saline sedentary group (Fig 17 Ci and Cii). Thus, abstinence from nicotine self-administration led to increased synapse length while the exercise during abstinence normalized synapse length back to saline levels in the core region of adolescent rats.
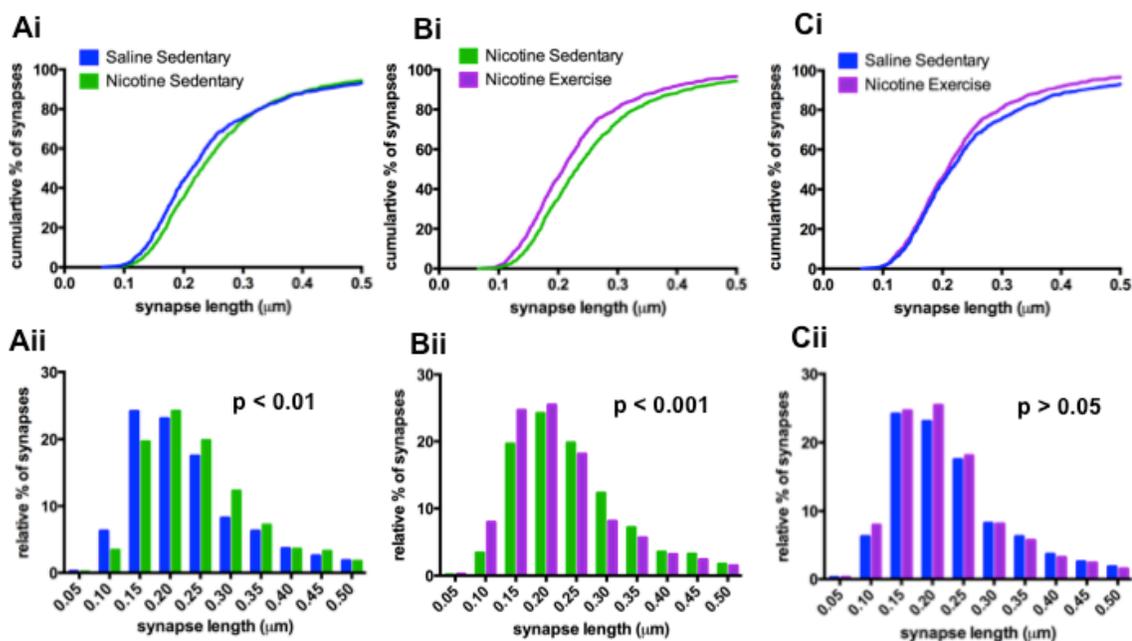
Figure 17 Cumulative (Ai, Bi, and Ci) and relative (Aii, Bii, and Cii) freqency plots for asymmetric synapses onto spines in the nucleus accumbens core region.

The saline exercise group showed a similar synapse length distribution as the saline sedentary however a non-significant trend for larger synapse length was observed (p = 0.045; Fig 18 Ai and Aii) and nicotine exercise (Fig 18 Ci and Cii) groups and was also significantly different from the nicotine sedentary group (p < 0.001; Fig 18 Bi and Bii). These results indicate that exercise in the saline group did not have a significant effect on synapse length in the nucleus accumbens core.
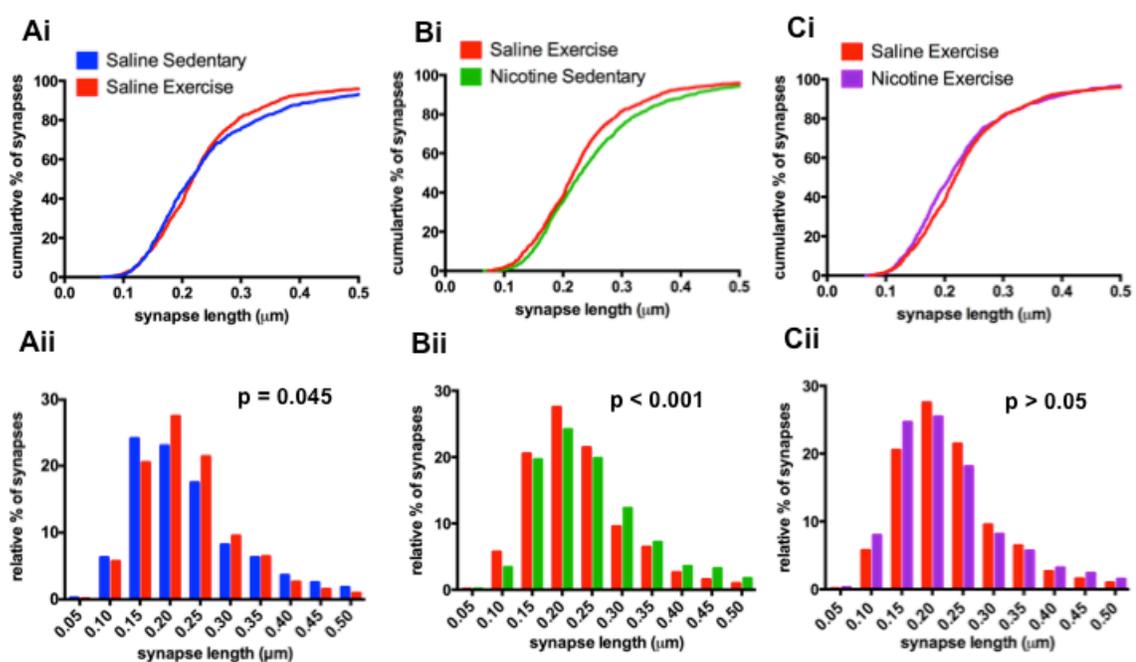
Figure 18 Cumulative (Ai, Bi, and Ci) and relative (Aii, Bii, and Cii) freqency plots for asymmetric synapses onto spines in the nucleus accumbens core region.

Synapse length distribution in the nucleus accumbens shell

The nicotine sedentary group displayed a significant rightward shift in the distribution of synapse length as compared to the saline sedentary group ($p < 0.001$; Fig 19 Ai and Aii). A significant leftward shift in synapse length was observed in the nicotine exercise group as compare to the nicotine sedentary group ($p < 0.001$; Fig 19 Bi and Bii). As seen in the core, the synapse length distribution of the nicotine exercise did not differ from the saline sedentary condition (Fig 19 Ci and Cii). Thus, abstinence from nicotine self-administration led to increased synapse length and exercise during abstinence normalized synapse length back to saline control levels in the shell region.
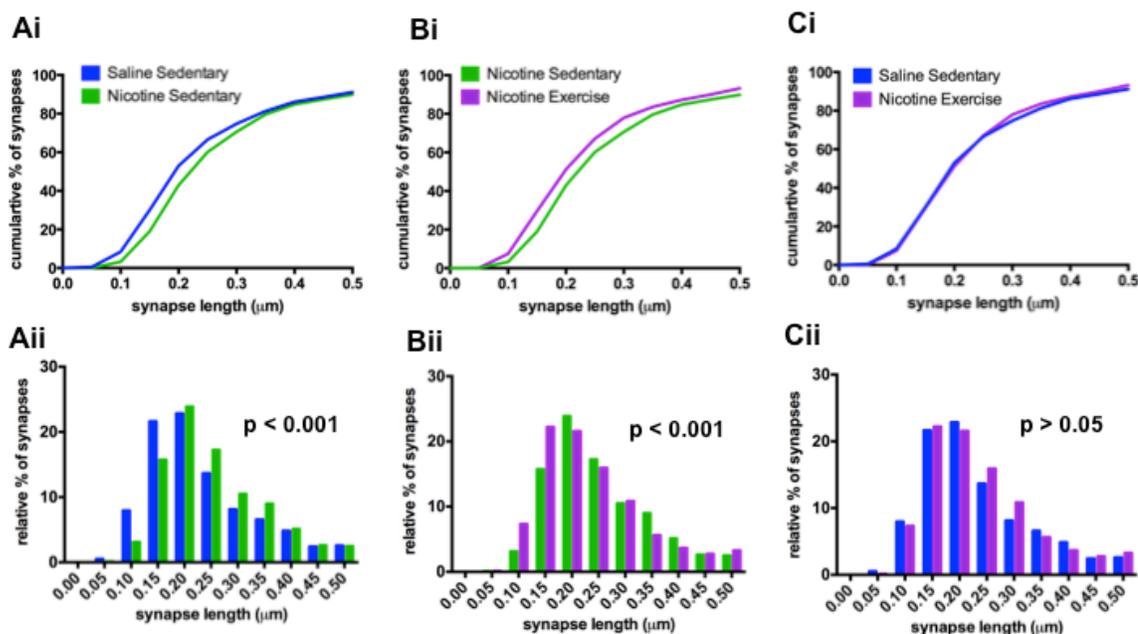

Figure 19 Cumulative (Ai, Bi, and Ci) and relative (Aii, Bii, and Cii) freqency plots for asymmetric synapses onto spines in the nucleus accumbens shell region.

In contrast to findings in the core, a significant rightward shift in the synapse length distribution of the saline exercise was observed as compared to the saline sedentary and nicotine exercise groups (ps < 0.01; Fig 20 Ai and Aii; Fig 20 Ci and Cii). The saline exercise group synapse length distribution was similar to that of the nicotine sedentary group (Fig 20 Bi and Bii). These results suggest that exercise in the saline control group increased synapse length as compared to the sedentary saline group in a manner similar to that of the nicotine.
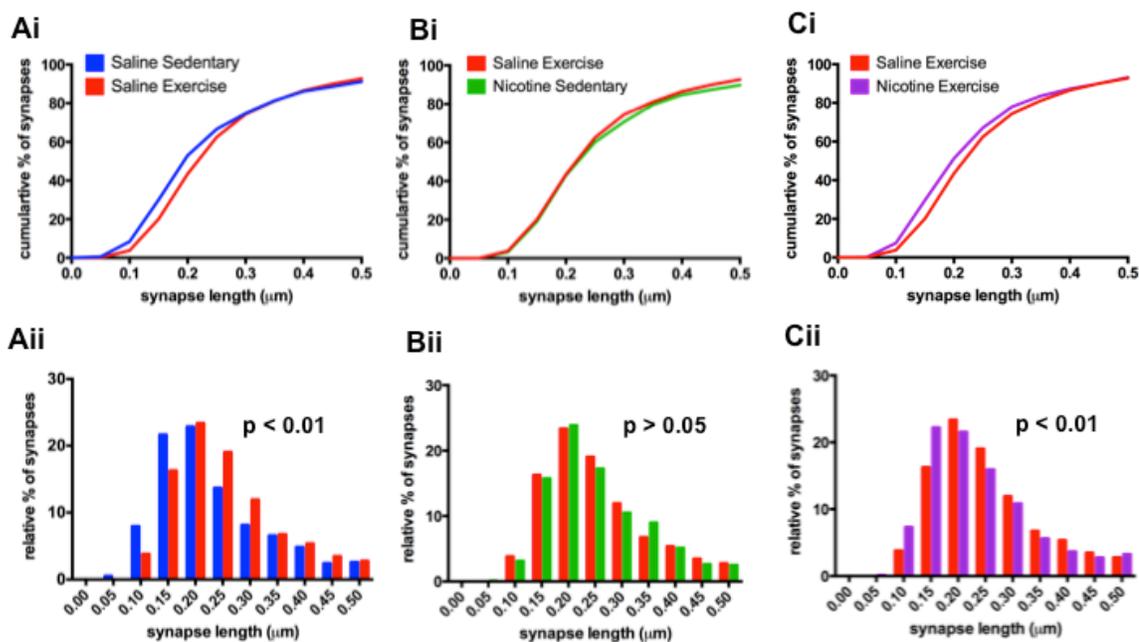

Figure 20 Cumulative (Ai, Bi, and Ci) and relative (Aii, Bii, and Cii) freqency plots for asymmetric synapses onto spines in the nucleus accumbens shell region.

Discussion

The main finding of the present study is that abstinence from nicotine self-administration and subsequent nicotine-seeking was associated with an increase in asymmetric synapse length onto spines in the nucleus accumbens core and shell regions. Exercise during abstinence not only significantly attenuated subsequent nicotine-seeking but also normalized synapse length in both regions. These results indicate that the effectiveness of exercise may be associated with its ability to prevent abstinence-induced structural plasticity within the nucleus accumbens.

In this study, we demonstrated that similar to self-administration paradigms in adults (Gipson et al. 2013), there is a long-lasting structural plasticity of dendritic spines within the nucleus accumbens core region of adolescents. Gipson et al. (2013) found an increase in spine head diameter in the nucleus accumbens core following abstinence that was associated with greater nicotine-seeking. The increase in synapse length observed in this study is likely associated with an increase in spine head size as these two measures are directly correlated (i.e., larger spines contain longer synapses; Harris and Stevens 1989). Furthermore, the increased length of the postsynaptic density is likely associated with an increase in AMPA receptor surface expression (Baude et al. 1995; Kharazia and Weinberg 1999; Takumi et al. 1999). Indeed, along with abstinence-induced enlargement of spine head diameter following abstinence, Gipson et al. (2013) observed evidence of increased surface expression of calcium permeable AMPA receptors. In this study we demonstrate that asymmetric synapse length on dendritic spines is also associated with nicotine-seeking, in that groups with higher levels of nicotine-seeking under extinction

conditions also had longer synapses. Taken together, these results suggest that an increase in larger synapses not only represents an increase in structural plasticity and functional plasticity, but also may underlie the long-term behavioral changes.

Aberrant glutamatergic signaling is believed to be a key component of drug-seeking in rodent models (Kalivas and Volkow 2005). An important piece of evidence to support this idea is that the incubation effect of cocaine is associated with a significant increase in the insertion of highly conductive calcium permeable AMPA receptors in the postsynaptic membrane of nucleus accumbens neurons (Conrad et al. 2008; Loweth et al. 2013). Drug-seeking is believed to be mediated by nucleus accumbens core region specifically (Kalivas and Volkow 2005; Everitt and Robbins 2005). However, the nucleus accumbens shell has also been implicated in drug-seeking following abstinence. Specifically, with nicotine there is evidence that glutamate projections to both the core and shell regions of the nucleus accumbens mediates nicotine-seeking (Tessari et al 2004; Liechti et al. 2007; Bespalov et al. 2008; Palmatier et al. 2007; Gipson et al. 2013), and in studies with cocaine, activation of the ventral medial prefrontal cortex, which sends projections to the shell region (Voorn et al. 2004), has been shown to be important for cue-induced reinstatement. Furthermore, changes in calcium permeable AMPA receptor surface expression associated with the incubation of craving for cocaine has also been observed in the shell region following prolonged abstinence but to a lesser extent than in core (Conrad et al. 2008). In the present study we found increased glutamatergic synaptic plasticity in both the core and shell regions. Since exercise attenuated this effect in both regions it is unclear which region is associated with nicotine-seeking in adolescents.

The most interesting finding in the current study was that exercise appeared to normalize abstinence-induced changes of synapse length within the nucleus accumbens. This effect may be due to the ability of exercise to mimic nicotine's actions within the brain and thus prevent abstinence-induced plasticity. Although, glutamatergic signaling induced by exercise within the nucleus accumbens shell region specifically has not yet been examined, some evidence indicates that exercise can modulate glutamate and dopamine neurotransmission within the striatum. Specifically, a microdialysis study has demonstrated that during exercise, extracellular glutamate and dopamine levels are increased in the striatum (Meeusen et al. 1997). Given that exercise has been shown to be rewarding (Lett et al. 2000, 2001; Greenwood et al. 2011; Belke 1997; 2000; Belke and Wagner 2005; Brene et al. 2007; Iversen 1993), it is possible that exercise could also affect glutamate and dopamine release within the nucleus accumbens as well. One caveat to these findings is that exercise induced an increase in synapse length in the nucleus accumbens shell of saline control rats in a manner that was similar to the effect of abstinence from nicotine. There is also some precedence within other brain regions to indicate that running can affect structural plasticity. Within the hippocampus, exercise has previously been shown to increase dendrite length and complexity as well as increase spine density (Stranahan et al. 2007; Eadie at al. 2005; Redila and Christie 2006). The functional significance of this increased synapse length 1-day after the last exercise session within the shell is not clear. Interestingly, prolonged access to wheel running followed by 25 days without running did not have an affect of spine density in the nucleus accumbens shell (Robinson and Kolb 1999), suggesting that the increase in

synapse length observed in the present study may not be a persistent alteration in plasticity.

In summary, the effectiveness of exercise in reducing nicotine-seeking may involve its ability to prevent abstinence-induced plasticity of glutamatergic connectivity within the nucleus accumbens. Further work is necessary to determine if this effect of exercise is due to an alteration of glutamatergic projections to the core or shell regions or both. Future studies should also aim to elucidate the circuitry involved in both abstinence-induced plasticity and the efficacy of exercise.

Chapter 6
General Discussion

The work presented in this dissertation was designed to examine the efficacy of exercise as a prevention and intervention in an adolescent-onset model of nicotine addiction. In addition, this work was the first attempt to identify structural plasticity associated with nicotine-seeking and its modulation by exercise. In the following sections, I will summarize findings from my dissertation and discuss their potential implications. Future directions are also suggested.

Behavioral effects of exercise

In Chapter 2, the behavioral effect of exercise on the initiation of nicotine self-administration was examined. This study demonstrated that specifically exercise, and not the environmental enrichment provided by the presence of a wheel, robustly attenuated vulnerability to initiate nicotine self-administration in adolescent rats. This finding supports a growing body of literature suggesting that exercise can prevent the initiation of drug use (Smith and Pitts 2011; Engelmann et al. 2013), and extends this previous work to nicotine self-administration and to adolescence, a developmental stage known to be critical in drug use initiation. In light of epidemiological findings of lower smoking rates in active adolescents as compared to their less active peers (e.g., Pastor et al. 2003; Escobedo et al. 1993; Field et al. 2001; Melnick et al. 2001; Naylor et al. 2001; Pate et al. 1996, 2000; Rainey et al. 1996; Mattila et al. 2012; Castrucci et al. 2004), this work suggests that the effects of exercise are not solely due to social influences and norms but

are also biologically based. The implications of our findings are important and suggest that future tobacco prevention programs should include an exercise component.

In Chapters 3, 4, and 5 of this dissertation, the behavioral effect of exercise on the relapse vulnerability was examined. Results from these experiments consistently demonstrated that exercise during an abstinence period attenuated subsequent nicotine-seeking under extinction conditions. These studies also demonstrated that the use of an adolescent-onset nicotine self-administration paradigm leads to robust nicotine-seeking in both males and females. Similar to findings in Chapter 2, in males, the protective effect of wheel-running was due to exercise, and not the environmental enrichment provided by the wheel itself. In contrast, females displayed a reduced sensitivity to nicotine-seeking under both the unlocked exercise condition and the locked wheel control condition. There are several interpretations of these results. First, females may use the locked wheel condition in a manner that provides a non-aerobic form of exercise and are thus more sensitive to the effects of exercise than males. Second, females may be more sensitive than males to the environmental enrichment provided by a wheel and this effect is similar to the effectiveness of exercise in females. Third, exercise may not account for the attenuated nicotine-seeking in females and this effect may be due to environmental enrichment alone. However, given evidence showing that females are more active (Hitchcock 1925; Eikelboom and Mills 1988; Smith et al. 2011; 2012) and given results with other drugs of abuse indicating that wheel running can reduce cocaine self-administration as well as cocaine-seeking following abstinence in females (Cosgrove et al. 2002; Zlebnik et al. 2010; 2012), the most parsimonious explanation is that adolescent

females are more sensitive to alternative forms of exercise within locked running wheel. Future preclinical research should directly compare the effects of nonaerobic models of exercise between adolescent males and females to examine this possibility. Future clinical research may consider including various types of exercise, aerobic and non-aerobic forms, as part of cessation treatment in adolescent boys and girls.

Neurobiological effects of exercise

In Chapter 5, structural plasticity within the nucleus accumbens following abstinence from adolescent-onset nicotine self-administration was analyzed with and without exercise. Nicotine self-administration followed by abstinence resulted in an enlargement of synapse length and exercise normalized these levels in both the nucleus accumbens core and shell regions. These results were observed in asymmetric synapses onto spines, which are known to be excitatory synapses (Nimchinsky et al. 2002; Gray 1959), suggesting that the behavioral attenuation of nicotine-seeking may be due to structural attenuation of glutamatergic plasticity.

An interesting finding in the experiment described in Chapter 5 was the observed increase in plasticity within both regions of the nucleus accumbens. These findings can be reconciled in a couple of ways. First, enhanced glutamatergic plasticity in both regions may be important for the drug-seeking behavior. Second, one or both of the regions may express an increase in plasticity that is not related to drug-seeking behavior. In support of the latter, Li et al. (2004) found that cocaine-induced behavioral sensitization following an abstinence period from experimenter administered cocaine was associated with

increased spine density in the core region specifically. An increase in spine density occurred in the absence of behavioral sensitization within the nucleus accumbens shell region suggesting that structural plasticity within the shell is a consequence of the history with the drug and not related to the behavior. However, although it is popularly believed that medial prefrontal cortex projections to the nucleus accumbens core region underlie drug-seeking (Everitt and Robbins 2005; Fischer-Smith et al. 2012; Hotsenpiller et al. 2001; Kalivas and Volkow 2005; McFarland et al. 2003; Gipson et al. 2013), there is evidence that projections to the shell region are involved as well (Koya et al. 2009; Bossert et al. 2013). The result from this study support the idea that at least the glutamatergic plasticity associated with late abstinence is present in both the core and shell regions of the nucleus accumbens.

The neurobiological mechanism of exercise that led to a behavioral attenuation of relapse to vulnerability and the observed normalization of synapse length are likely a result of its effects on the mesolimbic reward pathway. As discussed in the Chapter 1, extended access drug self-administration and early withdrawal are associated with lower glutamate and dopamine receptor function in the nucleus accumbens (Ahmed and Koob 2004; Koeltzow and White 2003; Hao et al. 2010). The persistent absence of drug during abstinence leads to neuroadaptations that that render neurons within the nucleus accumbens more sensitive to dopamine and glutamate (Schmidt and Pierce 2010). In animal models, exercise has been shown to increase glutamate and dopamine overflow in the striatum (Meeusen et al. 1997). Thus, the addition of exercise may maintain dopamine and glutamate levels and thus prevent the induction of plasticity associated with the

absence of drug. Therefore, exercise during abstinence likely prevented the increase in glutamatergic plasticity observed in Chapter 5 rather than reduced nicotine induced glutamatergic plasticity.

Although not examined in this dissertation, I would like to speculate on the neurobiological mechanism underlying behavioral attenuation of acquisition to nicotine self-administration. In animal models, exercise has been found to be rewarding with animals developing a conditioned place preference for environments paired with the after effects of exercise (Lett et al. 2000, 2001; Greenwood et al. 2011) and performing an operant response to gain access to a running wheel (Belke 1997; 2000; Belke and Wagner 2005; Brene et al. 2007; Iversen 1993). Similarly, as discussed above exercise increases dopamine release in the striatum with increased levels observed for at least several hours following exercise (Meeusen et al. 1997). It is possible that exercise prevented a further elevation in nucleus accumbens dopamine levels induced by nicotine self-administration and thus blunted the reinforcing effects of nicotine. Indeed, it has previously been shown that exercise blunts MDMA-induced increases in dopamine overflow in the nucleus accumbens along with MDMA-induced conditioned place preference (Chen et al. 2008). Further work is necessary to determine if and how exercise might affect dopamine signaling following nicotine self-administration in adolescent rats.

One caveat to the prevention and plasticity studies of this dissertation is that they were conducted in males only. As discussed in Chapters 1 and 4 of this dissertation, males and females differ on many different aspects of nicotine addiction including vulnerability to acquire nicotine self-administration (Lynch et al. 2002; Donny et al.

2000; Lynch 2009; Torres et al. 2008; Torres et al. 2009). Given that females differed from males in their sensitivity to the effects of exercise in preventing nicotine-seeking, I would hypothesize that similar sex-differences would be observed in preventing acquisition of nicotine self-administration. Specifically, females would show slowed and reduced rates under exercise unlocked wheel and control locked wheel conditions as compared to females that did not have access to a running wheel prior to acquisition of nicotine self-administration testing. Incredibly, to date there are no published studies on sex differences in drug abstinence-induced structural plasticity. Given that females have been shown to have a greater number of dendritic spines and larger spines within the nucleus accumbens as compared to males (Forlano and Woolley 2009; Wissman et al. 2012), it is possible that structural plasticity induced by abstinence may also be sexually dimorphic.

In summary, voluntary exercise prior to drug self-administration during adolescence reduces acquisition vulnerability suggesting that exercise *per se* may impart protection for smoking initiation in human adolescents. Exercise during abstinence also attenuated nicotine-seeking in an adolescent-onset model of nicotine addiction, suggesting that exercise may help adolescent smokers maintain smoking abstinence by reducing craving. However, since the efficacy of exercise in attenuating nicotine-seeking was not dissociable from the environmental enrichment effect in females, further preclinical and clinic work is needed to elucidate sex-differences in sensitivity to exercise as a prevention and intervention for nicotine addiction. Finally, exercise during abstinence was found to normalize nicotine-induced structural plasticity of excitatory

synapses in the nucleus accumbens, suggesting that the mechanism underlying the efficacy of exercise may be its ability to modulate abstinence-induced plasticity. Together, the results from this dissertation demonstrate that exercise, by modulating plasticity within the reward pathway, may be an effective prevention and intervention for adolescent-onset nicotine addiction.

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APPENDIX

General anesthesia causes long-term impairment of mitochondrial morphogenesis and synaptic transmission in developing rat brain

Abstract

Background: Clinically used general anesthetics, alone or in combination, are damaging to the developing mammalian brain. In addition to causing widespread apoptotic neurodegeneration in vulnerable brain regions, exposure to general anesthesia at the peak of synaptogenesis causes learning and memory deficiencies later in life. Our in-vivo rodent studies have suggested that activation of the intrinsic (mitochondria-dependent) apoptotic pathway is the earliest warning sign of neuronal damage, suggesting that a disturbance in mitochondrial integrity and function could be the earliest triggering events.

Methods: Since proper and timely mitochondrial morphogenesis is critical for brain development, we examined the long-term effects of a commonly used anesthesia combination (isoflurane, nitrous oxide, and midazolam) on the regional distribution, ultrastructural properties, and electron transport chain function of mitochondria, as well as synaptic neurotransmission, in the subiculum of rat pups.

Results: We found that this anesthesia, administered at the peak of synaptogenesis, causes protracted injury to mitochondria, including significant

enlargement of mitochondria, impairment of their structural integrity, increase in their complex IV activity and decrease in their regional distribution in presynaptic neuronal profiles where their presence is crucially important for the normal development and functioning of synapses. Consequently, we showed that impaired mitochondrial morphogenesis is accompanied by heightened autophagic activity, decreased mitochondrial density and long-lasting disturbances in inhibitory synaptic neurotransmission. The interrelation of these phenomena remains to be established.

Conclusion: We suggest that developing mitochondria are exquisitely vulnerable to general anesthesia and, accordingly, may be important early target of anesthesia-induced developmental neurodegeneration.

Introduction

Over the last decade, a myriad of studies have presented evidence that various mammalian species are susceptible to significant neurotoxicity when exposed to general anesthesia during early stages of their brain development. Anesthesia-induced neurotoxicity is described as apoptotic and its severity is age-dependent; i.e., the peak of susceptibility coincides with the peak of synaptogenesis.[1]

Since it has been suggested that general anesthetics used in clinical practice are not as innocuous as they were previously thought to be,[2] recent research has been focused on deciphering the earliest cellular targets and mechanistic pathways of anesthesia-induced developmental neurotoxicity so that a method of effective and timely prevention can be devised. This is essential because the use of general anesthetics often cannot be avoided when a child's life is in danger.

Based on presently available evidence, it appears that very early events in anesthesia-induced apoptotic neurodegeneration involve the activation of a mitochondria-dependent apoptotic cascade,[1, 3] which leads to effector caspase activation and DNA fragmentation. Thus, mitochondria may be the initial and most vulnerable target of anesthesia-induced impairment of neuronal development. Since mitochondria are crucially important organelles for the formation, maintenance, and function of developing synapses, which can be permanently impaired by a single exposure to anesthesia,[4,5] we did a series of

experiments on the effects of general anesthesia on morphogenesis and regional distribution of mitochondria in the developing subiculum.

We found that early exposure to general anesthesia significantly modulates mitochondrial morphogenesis and the function of mitochondria electron transport chain enzyme activity. Regional distribution of mitochondria in presynaptic neuronal profiles is also significantly reduced, where their presence is crucially important for the normal development and function of synapses. We also found that impaired mitochondrial morphogenesis is accompanied by heightened autophagic activity, protracted neuropil destruction, and long-lasting disturbances in inhibitory synaptic neurotransmission.

Materials and Methods

Animals. We used Sprague-Dawley rat pups (Harlan Laboratories, Indianapolis, IN) at postnatal day (P) 7 for all experiments, since this is when they are most vulnerable to anesthesia-induced neuronal damage[1]. Experimental rat pups were exposed to 6 hours of anesthesia; controls were exposed to 6 hours of mock anesthesia (vehicle). After the administration of anesthesia, rats were allowed to recover and were reunited with their mothers. Each day, we weighed them and noted their general appearance. On P21, these rats were randomly divided into three groups, one for ultrastructural analysis of the subiculum, one for measuring electron transport chain enzyme activity, and the other for functional studies of synaptic activity (patch-clamp recordings of excitatory and inhibitory synaptic neurotransmission). Rats were used at P21 for ultrastructural and enzyme activity examination and at P21-P28 for electrophysiological studies, since synaptic maturation is generally completed at this age.

All experiments were approved by the Animal Use and Care Committee of the University of Virginia Health System, Charlottesville, VA and were done in accordance with the Public Health Service's Policy on Human Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used.

Anesthesia. Nitrous oxide and oxygen were delivered using a calibrated flowmeter. To administer a specific concentration of nitrous oxide/oxygen and isoflurane in a highly controlled environment, an anesthesia chamber was

used.[1,3,6] Isoflurane was administered using an agent-specific vaporizer that delivers a set percentage of anesthetic into the anesthesia chamber. Midazolam (Sigma-Aldrich Chemical, St. Louis, MO) was dissolved in 0.1 % dimethyl sulfoxide just before administration. For control animals, 0.1 % dimethyl sulfoxide was used alone. To administer a specific concentration of nitrous oxide/oxygen and isoflurane in a highly controlled environment, an anesthesia chamber was used [1,3,6]. Rats were kept normothermic throughout the experiment, as previously described.[7] For control experiments, air was substituted for the gas mixture. After initial equilibration of the nitrous oxide /oxygen/isoflurane or air atmosphere inside the chamber, the composition of the chamber gas was analyzed by infrared analyzer (Datex Ohmeda, Madison, WI) to establish the concentrations of nitrous oxide or nitrogen, isoflurane, carbon dioxide, and oxygen. We used our standard general anesthesia protocol, giving P7 rat pups a single injection of midazolam (9 mg/kg, intraperitoneally) followed by 6 hours of nitrous oxide (75%), isoflurane (0.75%), and oxygen (approximately 24%). Several studies have shown that this protocol causes severe neurodegenerative damage to developing neurons[1,3,5,6].

Histopathological studies. On P21, each pup was deeply anesthetized with phenobarbital (65 mg/kg, intraperitoneally) (University of Virginia Pharmacy, Charlottesville, VA). After cannulating the left ventricle, we clamped the descending aorta and did an initial flush with Tyrodes solution (30-40 ml) (Sigma-Aldrich Chemical). For morphometric analyses of the neuropil, this was followed

by 10 min of continuous perfusion with freshly prepared paraformaldehyde (4%) and glutaraldehyde (0.5%).[1,3,6,8] For morphometric analyses of pyramidal neurons, perfusion was done using paraformaldehyde (2%) and glutaraldehyde (2%). After the perfusion, we removed the rats' brains and stored them in the same fixative overnight. Both control and experimental pups were perfused by an experienced experimenter on the same day, using the same solution to assure uniform tissue fixation. Any brains considered to have been inadequately perfused were not processed for electron microscopy (EM) analysis. Fixed brains were coronally sectioned (50-75 µm thick) with a DTK-1000 microslicer (Ted Pella, Tools for Science and Industry, Redding CA). The subiculum was localized as described in anatomical maps,[9] fixed in 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA), stained with 4% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA), and embedded in aclar sheets using epon-araldite resins. The subiculum was then dissected from the aclar sheets and embedded in BEEM™ capsules (Electron Microscopy Sciences, Hatfield, PA). To prepare capsules for microtome cutting (Sorvall MT-2 microtome, Ivan Sorvall, Norwalk, CT) the tips were manually trimmed so that ultrathin slices (silver interference color, 600-900 Å) could be cut using a diamond knife (Diatome, Hatfield, PA). Ultrathin sections were placed on grids and examined using a 1230 TEM electron microscope (Carl Zeiss, Oberkochen, Germany). Using a 16M-pixel digital camera (SIA-12C digital cameras, Scientific Instruments and Applications, Duluth, GA), we took 12 random, nonoverlapping

electron micrographs (12,000X magnification) of each subicular layer (pyramidal, polymorphic, and molecular). Our electron micrographs depicted neuropils and large pyramidal neurons, depending on the type of analysis needed (see Results). The investigator analyzing electron micrographs was blinded to the experimental conditions.

Morphometric analyses. To do morphometric analyses of mitochondria in the cytoplasmic soma of pyramidal neurons in their entirety which is not possible to perform with a single photo frame at such high magnification (12,000x) we took multiple sequential pictures and tiled them seamlessly together to make a mosaic of one whole cell body (n=15 neurons in control group and anesthesia-treated group each). From these mosaic pictures, the cytoplasm area and mitochondrial area were measured using Image-Pro Plus 6.1 computer software (MediaCybernetics, Bethesda, MD). The number of animals used for detailed ultrastructural histological studies was sufficiently powered to make proper conclusions.[5]

For morphometric analyses of mitochondria in presynaptic neuronal profiles, we first identified the synapses, using the following criteria: the presence of a postsynaptic density; the presence of more than one synaptic vesicle closely apposed to the presynaptic membrane; and the presence of a synaptic cleft delineated by parallel pre- and postsynaptic membranes.[5,10] Both excitatory and inhibitory synapse-bearing presynaptic neuronal profiles were examined using the criteria specified by Crain et al.[11] Once identified, the area of the presynaptic

neuronal profile was measured and the presence or absence of mitochondria was noted. Using Image-Pro Plus 6.1 computer software, the area of mitochondria, where present, were measured so that the mitochondrial index could be calculated; the mitohondrial index is defined as the ratio between mitochondrial and presynaptic neuronal profile areas.

Spectrophotometric analyses of mitochondrial electron transport chain activity. After homogenizing and centrifuging subicular tissue, we measured mitochondrial electron transport chain (ETC) and citrate synthase (CS) activity using a Genesis 10-UV spectrophotometer (Thermo, Rochester, NY). The incubation temperatures for complexes I, II, and CS were 30°C and, for complex IV, 38°C. Electron transport chain (ETC) complex activities were determined in supernatants as described by Perez-Carreras et al.[12] and expressed as a percentage of the specific activity of CS to correct for the neuronal content of mitochondria. ETC assays were performed in triplicates.

Statistical analysis: Single comparisons among groups were made using unpaired two-tailed t-test. When ANOVAs with repeated measures were needed, the Bonferroni correction was used to help maintain prescribed alpha levels (e.g. 0.05). Using the standard version of GraphPad Prism 5.01 software (Media Cybernetics, Inc., Bethesda, MD) we considered $p < 0.05$ to be statistically significant. All the data are presented as mean ± standard error of mean (SEM).

Electrophysiology studies: All experiments were done on 300-μm-thick transverse rat brain slices from 21-28 day-old animals using procedures

described previously.[13] The subiculum was localized as described in anatomical maps.[9]

Rats were briefly anesthetized with isoflurane and decapitated. Their brains were rapidly removed and placed in chilled (4° C) cutting solution consisting, in mM, of 2 $CaCl_2$, 260 sucrose, 26 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 NaH_2PO_4, and 2 $MgCl_2$ equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. A block of tissue containing the subiculum and hippocampus was glued to the chuck of a vibratome (World Precision Instruments, Sarasota, FL) and slices were cut in a transverse plane. The slices were incubated at 36° C in oxygenated saline for 1 hour, then placed in a recording chamber that was perfused with extracellular saline at a rate of 1.5 ml/min. Incubating saline consisted, in mM, of 124 NaCl, 4 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $MgCl_2$, 10 glucose, and 2 $CaCl_2$ equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. Slices were maintained in the recording chamber at room temperature and remained viable for at least 1 hour. Since the half-life of halogenated volatile anesthetics in nerve tissue after the induction of anesthesia is only about 10 min,[14] it is unlikely that the isoflurane used to euthanize the rats could have interfered with the results of our experiments, which were performed at least 2 hours later.

Recording procedures: Whole-cell recordings were obtained from pyramidal subiculum neurons visualized with an IR DIC camera (Hamamatsu

C2400, Bridgewater, NJ) on a Zeiss 2 FS Axioscope (Carl Zeiss Jena, Thuringia, Germany) with a 40 X lens.

The standard extracellular saline for recording of evoked inhibitory postsynaptic currents (eIPSCs) and evoked excitatory postsynaptic currents (eEPSCs) consisted, in mM, of 2 $CaCl_2$, 130 NaCl, 1 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 2 mM KCl. This solution was equilibrated with a mixture of 95% oxygen and 5% carbon dioxide for at least 30 min and had a resulting pH of about 7.4. For recording eIPSCs, we used an internal solution containing, in mM, 130 KCl, 4 NaCl, 0.5 $CaCl_2$, 5 ethylene glycol tetraacetic acid (EGTA), 10 4-(2-hydroxyethil)-1-piperazineethanesulfonic acid (HEPES), 2 $MgATP_2$, 0.5 Tris-GTP, and 5 lidocaine N-ethyl bromide. pH was adjusted with KOH to 7.25. For recordings of eEPSCS, this solution was modified by replacing KCl with equimolar K-gluconate. To eliminate glutamatergic excitatory currents, all recordings of eIPSCs were done in the presence of 5 μM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), 50 μM d-APV (2R)-amino-5-phosphonovaleric acid; AP5, (2R)-amino-5 phosphonopentanoate) in a bath solution. To eliminate inhibitory currents, all recordings of eEPSCs were done in the presence of 20 μM picrotoxin [a noncompetitive gamma-amino butyric acid (GABA)$_A$ antagonist] in the bath solution.

Synaptic stimulation of pyramidal subiculum neurons was achieved with a Constant Current Isolated Stimulator DS3 (Digitimer, Welwyn Garden City Hertfordshire, England). Electrical field stimulation was achieved by placing a

stimulating electrode within the hippocampal CA1 soma layer with stimulation intervals of at least 20 sec to allow recovery of synaptic responses. In all recordings, we first determined the current-output relationship, and then used current intensities in the stimulating electrode corresponding to the maximal amplitudes of eIPSCs and eEPSCs. Recordings were made with the standard whole-cell voltage clamp technique. Electrodes having final resistances of 2-4 MΩ were fabricated from thin-walled microcapillary glass. Membrane currents were recorded with an Axoclamp 200B amplifier (Molecular Devices, Foster City, CA). Voltage commands and digitization of membrane currents were done with Clampex 8.2 of the pClamp software package (Molecular Devices) running on an IBM-compatible computer (Dell, Inc., Round Rock, TX). Neurons were typically held at -70 mV.

Analysis of current: Current waveforms or extracted data were fitted using Clampfit 8.2 (Molecular Devices) and Origin 7.0 (OriginLab, Northhampton, MA). The decay of eIPSCs was estimated by a single-exponential term. All salts and chemicals were obtained from Sigma-Aldrich Chemical.

<u>Results</u>

I. <u>General anesthesia disturbs the fine ultrastructural balance of developing</u>

<u>neuronal mitochondria</u>

Since mitochondrial ultrastructure dictates their function,[15] we studied the

ultrastructural appearance of mitochondria with special emphasis on changes

indicative of distorted mitochondrial integrity. Our work has been focused on the

subiculum because this brain region is highly vulnerable to anesthesia-induced

developmental neurodegeneration, as shown by substantial acute neuroapoptotic

damage after the administration of anesthesia[8,16] and chronic changes marked

by substantial neuronal loss.[16,17] The subiculum is part of the hippocampus

proper and part of Papez's circuit; it is intertwined with the hippocampal CA1

region, anterior thalamic nuclei, and both the entorhinal and cingulate cortices.

Accordingly, the subiculum is important in cognitive development, especially the

development of learning and memory.[18]

For ultrastuctural analysis of mitochondria, we examined the perikaryon of

pyramidal subicular neurons (Fig. 1A, B) and nerve terminals in subicular

neuropil (Fig. 1C, D) two weeks after exposure to anesthesia (at P21, see

Materials and Methods). We noted that in control subiculi (Fig. 1 A and C), the

mitochondria appear normal with no evidence of swelling or injury; there was a

typical homogeneous staining pattern of the matrix without excessive pallor. In

contrast, in the experimental subiculum (Fig. 1 B and D) many mitochondria

displayed structural disorganization of cristae (see asterisks), including dilated intracristal spaces with vacuoles and overall swelling, which made the mitochondria appear substantially larger than normal. In addition, multiple mitochondrial profiles were suggestive of severe degenerative changes (Fig. 1 D, black arrows).

Since degenerated organelles are removed by autophagy, we investigated whether the use of general anesthesia leads to heightened autophagic activity. We examined pyramidal neurons in control and experimental subiculi for ultrastructural signs of autophagosomes, lysosomes, and autophagic vacuoles. Using electron microscopy as the gold standard to examine the formation of autophagic profiles, we found that experimental neurons displayed many autophagic profiles (Fig. 2A and 2B). Dispersed throughout the cytoplasm were many lysosomes (Fig. 2A, double asterisks) and autophagic vacuoles. These single-membrane structures are formed by the fusion of lysosomes and autophagosomes to allow the digestion of biological "garbage"; they are also referred to as autolysosomes (Fig. 2A, arrowheads). The morphological hallmark of autophagy is the formation of double-layered membrane structures called autophagosomes. Experimental neurons frequently contained multiple autophagosomes where parts of cannibalized mitochondria could be detected (Fig. 2B, single asterisk). The presence of numerous autophagic profiles suggests that general anesthesia increases the autophagic load in immature neurons.

Since general anesthesia appears to cause mitochondrial enlargement (Fig. 1 B and D), we did a detailed morphometric analysis of mitochondria in the somas of pyramidal subicular neurons. When measured as a percent of the cytoplasmic area of pyramidal neurons, we found that mitochondria in the experimental neurons occupied approximately twice as much area of the cytoplasmic soma area than did controls ($22.5 \pm 3.1\%$ versus $13.44 \pm 1.2\%$,* $p <$ 0.05) (Fig. 3A, n = 15 neurons per group from 3 control and 3 experimental pups). This did not appear to be due to an increase in mitochondrial number, since mitochondrial density, presented as the number of mitochondria per unit area (μm^2) of cytoplasmic soma, was significantly lower in experimental pyramidal neurons as compared to control pyramidal neurons (Fig. 3B).

We confirmed that anesthesia causes mitochondrial enlargement when we categorized mitochondria as small (up to $0.05 \ \mu m^2$), medium (0.06 to $0.25 \ \mu m^2$), large (0.26 to $0.65 \ \mu m^2$), and extra-large (above $0.65 \ \mu m^2$) (Fig. 4). Indeed, we found a complete reversal of the ratio between small and large mitochondria in control and experimental animals. For example, about 15% of the total mitochondria in control pyramidal neurons were small ones, whereas only 5% of mitochondria in experimental neurons were small (***, $p < 0.001$) (Fig. 4A). Large mitochondria constituted only 5% of the total mitochondria in control animals, while more than 15% of the mitochondria in experimental pyramidal neurons were large (*, $p < 0.05$) (Fig. 4C). Interestingly, although extra-large mitochondria represent less than 1% of the total number of mitochondria, they

appeared to be two-fold more prevalent in experimental pyramidal neurons (Fig.

4D) than they were in control pyramidal neurons, suggesting that anesthesia

causes a substantial imbalance in mitochondrial size, with a clear tendency

toward mitochondrial enlargement (n = 15 neurons per group from 3 control and

3experimental pups).

II. *General anesthesia disturbs regional distribution of developing neuronal*

mitochondria

        The proper development and function of synapses depends on

mitochondrial support, since synaptogenesis has high metabolic requirements.[19-22]

Therefore, regional distribution of mitochondria in presynaptic nerve terminals

and their strategic placement in the vicinity of developing synapses is crucially

important. Having demonstrated that general anesthesia causes substantial

enlargement of mitochondria in neuronal somas, as shown in Figures 3 and 4,

we then examined whether similar morphometric changes could be detected in

presynaptic neuronal terminals. To compare regional distribution of mitochondria

in presynaptic neuronal profiles in experimental and control subiculi, we

quantified the number of presynaptic neuronal profiles, out of all those present in

any given electron microscopy photo frame of subicular neuropils (at 12,000x

magnification), that contained mitochondrial profiles. We expressed the findings

as a percentage of presynaptic profiles containing mitochondria. We found a

significantly higher (*, $p < 0.05$) percentage of mitochondria-containing

presynaptic profiles in control as compared to experimental subiculi (Fig. 5) (n =

28 photo frames/group from 4 control and 4 experimental pups from two different litters).

To further assess our ultrastructural observation suggesting substantial swelling of mitochondrial profiles in presynaptic neuron terminals (Fig. 1D), we did detailed morphometric analysis of the area of mitochondrial profiles (in μm^2). We found that experimental mitochondrial profiles were, on average, 38% larger than control mitochondrial profiles (*, $p < 0.05$) (Fig. 6A) (n = 30 photo frames obtained from 5 control pups; n = 45 photo frames obtained from 5 experimental pups; control and experimental pups were litter-matched; total of three different litters were used). When the areas of mitochondria-containing presynaptic nerve terminals were measured, we found no difference between control and experimental animals (Fig. 6B) (n = 30 photo frames obtained from 5 control pups; n = 45 photo frames obtained from 5 experimental pups; control and experimental pups were litter-matched; total of three different litters were used). Consequently, when we calculated the mitochondrial index, which is the ratio between mitochondrial area and mitochondria-containing presynaptic area, we found that this index was significantly higher in experimental subicular neuropils (*, $p < 0.05$) indicating that terminally distributed mitochondria display morphometric changes that are similar to those in mitochondria located in the soma (Fig. 6C).

III. _General anesthesia acutely disturbs functional balance in developing neuronal_
mitochondria

Since we have previously reported that general anesthesia causes acute

disturbances of cytochrome c homeostasis within the first 4 hours of anesthesia

exposure,[1] we question whether anesthesia modulates the function of electron

transport chain (ETC) complexes, particularly complex IV (cytochrome c

oxidoreductase). Complex IV is of interest for three reasons: it depends on the

availability of cytochrome c, since it transfers electrons from cytochrome c to

oxygen, thus controlling the final steps of electron transport and adenosine

triphosphate (ATP) synthesis; it is encoded in part by mitochondrial

deoxyribonucleic acid, which makes it particularly vulnerable to mitochondrial

dysfunction; modulation of complex IV activity has been shown to cause elevated

free oxygen radical production[23] thus making neurons vulnerable to excessive

lipid peroxidation and protein oxidation. We measured complex IV activity in

mitochondrial homogenate prepared from fresh subicular tissue of rat pups on

P8, 24 hours after anesthesia treatment. Since citrate synthase (CS) activity is

directly proportional to mitochondrial content, the activity of complex IV was

expressed as a ratio (per the activity of citrate synthase). As shown in Figure 7A,

there was a significant increase in complex IV activity 24 hours after anesthesia

treatment (*, $p < 0.05$) (n = 8 pups in control group; n = 6 pups in experimental

group). When we measured the activity of complexes I and II/III, we found no

change in experimental groups as compared to controls (Fig. 7B, C, respectively)

(n = 5 pups in control group; n = 5 pups in experimental group for complex I activity; n=3 in control group; n = 4 pups in experimental group for complex II/III activity).

IV. <u>*General anesthesia impairs developmental synaptic transmission*</u>

Given the fact that general anesthesia impairs synapse formation during early brain development,[4,5] as well as our findings suggesting that general anesthetics impair mitochondrial morphogenesis and decrease the numbers of mitochondria in presynaptic neuronal profiles, we ask whether the diminished presence of mitochondria in anesthesia-treated subiculum has any bearing on the functional integrity of its synapses. To address this question, we examined inhibitory (eIPSC) and excitatory (eEPSC) synaptic transmission by recording from the pyramidal layer of control and anesthesia-treated rat subicular slices (see Methods). The traces in Figure 8A show representative eIPSCs from the control (black line) and experimental group (gray line), both of which received anesthesia at P7. Paired stimulation of afferent fibers resulted in pair-pulse depression, a highly characteristic finding for subicular neurons (Fig. 8A). This test is done by analyzing changes in the ratio of eIPSCs elicited by two identical presynaptic stimuli delivered in rapid succession. This paired-pulse depression of test eIPSCs (P2) relative to conditioning eIPSCs (P1) is thought to be due to depletion of a fraction of readily available synaptic sites.

In comparison to the control group, the experimental group had ~ 49% decrease in net charge transfer of eIPSCs measured as the area under the curve (p < 0.05); a decreased decay time constant (tau) from 58 ± 9 ms (n = 14) to 34 ± 4ms (n = 12, p < 0.05); and a significantly altered paired-pulse ratio P2/P1 (amplitude of eIPSC-2: amplitude of eIPSC-1) from 0.81 ± 0.02 (n = 14) to 0.87 ± 0.01 (n = 12, p < 0.05). The data are summarized in the histogram in Figure 8B.

It is generally accepted that presynaptic depressants that change the probability of transmitter release from presynaptic terminals cause a smaller fraction of the readily releasable pool of vesicles to undergo exocytosis and therefore decrease pair-pulse depression. [24] This means that in the presence of presynaptic modulators, the ratio P2:P1 becomes larger. On the other hand, if a modulator acts on postsynaptic sites, pair-pulse depression should remain unchanged while current amplitude and/or decay time might be changed. Changes in decay time constant, as well as decreased current amplitudes and alterations in the P2:P1 ratio strongly suggest that both postsynaptic and presynaptic mechanisms contribute to the decreased synaptic strength of inhibitory transmission in the experimental group. In contrast, we found no significant difference in the synaptic strength (net charge transfer) of eEPSCs between the control (n = 20 cells) and experimental groups (n = 10 cells, data not shown).

<u>Discussion</u>

General anesthesia administered to 7-day-old rat pups causes long-lasting alterations in mitochondrial morphogenesis and regional distribution, heightened autophagocytic activity and ongoing neuropil destruction, as well as significant disturbances in synaptic neurotransmission in the subiculum.

Mitochondrial regeneration in neurons depends on balancing two opposing processes, mitochondrial fusion and fission.[25] Deranged fusion leads to mitochondrial fragmentation; deranged fission leads to mitochondrial enlargement. Since our ultrastructural analyses indicate that general anesthesia causes significant enlargement of mitochondria, general anesthetics may modulate the fine equilibrium between fusion and fission. Disturbances in fusion and fission can lead to disturbances in mitochondrial functioning, particularly normal respiratory function. This may not be well tolerated by immature and functionally busy mammalian neurons, which, during development, are in need of adequate metabolic support. Indeed, impairment of mitochondrial morphogenesis may, at least in part, be the cause of reported anesthesia-induced developmental neurodegeneration,[1,6,8,26-28], especially since an imbalance between fission and fusion appears to have a causal role in initiating several neurodegenerative diseases.[29,30] Interestingly, large (and "giant") mitochondria are often described in aging neurons.[31] Although it is tempting to draw a parallel between certain elements of neurodegeneration unique to aging and anesthesia-induced neurodegeneration unique to developmental brain it

remains to be determined whether they share similar cellular pathways.   In particular, it remains to be determined how anesthesia affects developmental fusion and fission. Although not within the scope of this study, future mechanistic studies using an easy-to-manipulate in-vitro system should focus on various GTPase proteins (e.g., Drp1, fis 1, OPA 1, mitofusin 1 and 2) that are crucial for proper pathway activation.[32-34]

It is possible that fusion and fission are modulated by general anesthesia and thus are the main causes of mitochondrial enlargement. However, our ultrastructural observations indicate that mitochondria are swollen and plagued by deranged, fragmented cristae and inner membranes. This suggests that the impairment of mitochondrial membrane integrity may be the main cause of their "leakiness," allowing the indiscriminate entry of colloids and water. In support of this notion is our previous finding that general anesthesia causes significant downregulation of bcl-x_L proteins, which are important in maintaining mitochondrial membrane integrity. This downregulation leads to significant cytochrome c leakage suggestive of increased mitochondrial permeability.[1]

Mitochondria have been classified by size in chronic neurodegenerative diseases such as Parkinson's and Alzheimer's, in which large mitochondria predominate, while the population of medium-sized mitochondria remains unchanged.[35]   We observed a similar tendency toward mitochondrial enlargement with a seemingly stable population of medium-sized mitochondria. Although medium-sized mitochondria may show a lower propensity for swelling

than small ones, a more likely explanation is that the observed phenomenon is due to the shift in mitochondrial size distribution toward medium and large size category caused by the swelling of small and medium-sized mitochondria, respectively rather than a focal increase in mitochondria of any one size. Therefore, a detailed ultrastructural analysis of mitochondria should always accompany the size analysis.

Mitochondria are generated in the soma and move within the cytoplasm, which allows them to distribute within cells.[36] Since neurons have multiple compartments (e.g., dendrites, axons, and synapses) that are located far from the cell body, they depend heavily on proper mitochondrial distribution.[19] Since mitochondria are the main regulators of adenosine triphosphate (ATP) production, they are frequently found in the vicinity of active growth cones of developing neurons[19] and in terminals with active synapses.[20,21] We report that significantly fewer mitochondria are located in presynaptic neuronal profiles in anesthesia-treated subiculi than are in controls. Also, the mitochondrial profiles in presynaptic neuronal profiles are significantly larger than those in controls, suggesting that anesthesia-induced morphological changes shift the regional distribution of mitochondria away from very distant, thin, and highly arborized dendritic branches at a time when their presence is crucial for normal synapse formation and development. It remains to be determined whether general anesthesia impairs proper mitochondrial trafficking which may explain

anesthesia-induced impairment of the morphogenesis and plasticity of dendritic spines and synapses.[4, 5]

We report a decrease in mitochondrial density in both neuronal soma and presynaptic terminals which may suggest mitochondrial "dropout" due to mitochondrial degeneration and removal via autophagy. Having said that a decrease in mitochondrial density could be relative, caused by the fact that large mitochondria represent a bigger fraction of cytoplasmic and presynaptic terminal areas due to mitochondrial swelling or, perhaps, improper fission/fusion.

We show that the activity of the terminal enzyme of the respiratory chain, complex IV, is significantly up-regulated when measured 24 hours after anesthesia exposure, while the activity of complexes I and II/III remains unchanged. It is possible that an increase in complex IV activity provides anesthesia-treated neurons with increased ATP levels,[37] which would result in a higher energy state in the neuron. Although it would be tempting to consider higher ATP levels to be beneficial for developing neurons, there is a drawback to the increased neuronal energy level based on an isolated increase in complex IV activity. Instead of leading to decreased oxidative stress by decreasing reactive oxygen species production, as previously thought,[38] a recent report suggests that acute elevation of complex IV activity is associated with increased oxygen radical (ROS) production.[23] In other words, a higher complex IV electron transfer rate onto oxygen, in view of intact activity of complexes I, II, and III, results in a higher degree of oxidation of the ubiquinone pool. This allows complexes II and III to

transfer electrons to oxygen, causing an increase in ROS production. If general anesthesia leads to elevated ROS production, protein oxidation, and lipid peroxidation, this may, at least in part, explain the ongoing neuropil destruction and prominent neurite degradation. [5]

Here we focus on subiculum. It is noteworthy that the effects of general anesthesia on complex IV activity could be brain- region specific. For example, in a neurotoxic model of Parkinson's disease it has been shown that striatal, but not cortical neurons demonstrate elevated complex IV activity, resulting in ROS upregulation and neuronal cell death.[37] Hence, it remains to be determined how general anesthesia affects the activity of complex IV and other ETC proteins in other brain regions that are vulnerable to anesthesia-induced developmental neurodegeneration.

We question whether our patch-clamp results showing that a single exposure to general anesthesia leads to lasting depression of inhibitory transmission in subicular neurons could be due to impaired regional distribution of mitochondria. This notion is based on the fact that defective synaptic transmission is associated with the loss of mitochondria from axon terminals.[22] Interestingly, although inhibitory neurotransmission was impaired, excitatory neurotransmission was spared. Our earlier study using hippocampal slices of rats exposed to general anesthesia at age P7 demonstrated that excitatory synaptic transmission was not affected.[8] This is intriguing considering that our morphometric studies of the subiculum show nonselective synapse loss[5] and

nonselective changes in mitochondrial regional distribution/ morphometry when excitatory and inhibitory synapses were examined (data not shown). The obvious reason for this selective functional synaptic plasticity is not known. However, it is of interest that functioning mitochondria and their ATP production are essential for maintaining normal synaptic physiology.[34,39,40]

Acute application of isoflurane or midazolam potentiates the inhibitory drive by heightening inhibitory synaptic activity mediated by gamma amino-butyric acid$_A$ receptors.[41] In contrast, nitrous oxide silences excitatory (N-methyl-D-aspartate-mediated) synaptic transmission.[42] Thus, it is possible that, as a consequence of an inadequate metabolic ratio of supply to demand, anesthesia-induced degenerative changes in mitochondria and a potential decrease in ATP production preferentially impair highly activated inhibitory synaptic function. Other studies have indicated that free radical signaling may be important in modulating synaptic transmission. For example, acute applications of hydrogen peroxide, the common donor of ROS, preferentially reduced inhibitory over excitatory synaptic transmission in thalamocortical,[43] hippocampal, [44,45] cortical, and striatal slices[45] by both pre- and post-synaptic mechanisms. Since the strength of eIPSCs but not eEPSCs in subiculi is greatly diminished in slices of rats exposed to clinical anesthesia early in life, it is tempting to speculate that this can be at least in part a result of the production of ROS in response to anesthetic-induced mitochondrial dysfunction. However, the precise mechanism for the selective homeostatic changes in neuronal function under extensive

GABAergic stimulation associated with various physiological and pathological conditions remains to be examined.[46]

Because damaged mitochondria could become an uncontrollable source of free oxygen radicals and therefore would have to be degraded to ensure neuronal survival, it came as no surprise that general anesthesia created a substantial amount of biological "garbage" and heightened autophagy. Autophagy, a multistep process, is initiated by the formation of autophagosomes, double-membrane-bound cellular structures that enter lysosomes, acidic vacuolar compartments containing various lytic enzymes that have pH optima in the acidic range.[47-49] Lysosomes may slowly leak enzymes, which, in turn, can induce apoptosis via activation of a variety of pro-caspases. Our observation of a substantial number of autophagic bodies, in addition to impaired mitochondrial morphogenesis, neuropil damage, and synapse loss[5] raises the important possibility that general anesthesia kills developing neurons simply by overwhelming natural autophagy with a massive production of defective mitochondria. Further studies will be necessary to test this possibility.

Although a reliable model for studying developmental neurodegeneration our anesthesia protocol is based on the use of anesthetics in combination. As such it prevents us from deciphering the relative contribution of each agent. Further studies focused on individual anesthetics will help us decipher their relative importance in inducing mitochondrial morphological impairments and dysfunction.

We show that general anesthesia causes significant impairment in mitochondrial morphogenesis and their regional distribution in developing subiculi of rat pups. Hence, mitochondria may be the most vulnerable initial target of anesthesia-induced developmental neurotoxicity.

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<u>Figure Legends:</u>

Figure 1:   Anesthesia causes long-lasting ultrastructural changes in mitochondria in subiculi of 21-day-old rats. A, C) The pyramidal neuron (A) and neuropil (C; synaptic contacts are noted with arrows) in a subiculum from a control rat show abundant small mitochondria with no evidence of swelling or injury. B, D) Mitochondria in the perikarion of a pyramidal neuron (B) and nerve terminals in a neuropil (D) of subiculum from experimental rats display structural disorganization of cristae (asterisks), as well as dilated intracristal spaces with vacuoles and overall swelling. Note the presence of dark, condensed mitochondria in late stages of degeneration (arrows) (magnification 12,000x). N - nucleus.

Figure 2: Anesthesia promotes autophagic activity, as shown in subicular pyramidal neurons of 21-day-old rats.   A) In experimental pyramidal neurons, numerous lysosomes (double asterisks) and autophagic vacuoles (arrowheads) were dispersed throughout the cytoplasm. B) Autophagosomes, double-layered membrane structures, were frequently noted in experimental neurons where parts of cannibalized mitochondria could be detected (single asterisk).

Figure 3: Morphometric analysis of mitochondria in the perikaryon of pyramidal

subicular neurons of 21-day-old rats. A) Mitochondria in the experimental

neurons occupy significantly more cytoplasmic soma than do those in controls

(22.5% vs. 13.44%,* $p < 0.05$) (n = 15 neurons per group from 3 animals each).

B) In experiental animals, mitochondrial density, presented as the number of

mitochondria per unit area (μm^2) of cytoplasmic soma, is significantly lower in

pyramidal neurons than that in controls (*, $p < 0.05$, n = 15 neurons per group

from 3 control and 3 experimental pups; control and experimental pups were

litter-matched).

Figure 4:   Mitochondrial size classification in the perikaryon of pyramidal

subicular neurons of 21-day-old rats. A) A small fraction of mitochondria (up to

0.05 μm^2) constitutes about 15% of total mitochondria in control pyramidal

neurons, but only 5% in the experimental pyramidal neurons (***, $p < 0.001$). B)

A medium-sized fraction of mitochondria (0.06 to 0.25 μm^2) represents the

largest population of mitochondria in subicular pyramidal neurons. This fraction

remains unchanged after anesthesia treatment. C) A large fraction of

mitochondria (0.26 to 0.65 μm^2) constitutes only about 5% of total mitochondria

in control pyramidal neurons, but 15% of those in experimental pyramidal

neurons (*, $p < 0.05$) . D) An extra-large fraction of mitochondria (more than 0.65

μm^2) represents less than 1% of the total number of mitochondria in

experimental pyramidal neurons and shows over two-fold higher prevalence in these neurons (Fig. 3D) although it did not reach statistical significance (n = 15 neurons per group from 3 control and 3 experimental pups; control and experimental pups were litter-matched).

Figure 5: Anesthesia decreases mitochondrial density in presynaptic neuronal terminal subicular neuropils of 21-day-old rats. Compared to controls, fewer presynaptic neuronal terminals in experimental subicular neuropils contain mitochondrial profiles. When the findings are presented as a percentage of presynaptic profiles containing mitochondria, a significantly (about two-fold) higher percentage of mitochondria-containing presynaptic profiles occurs in control subiculi than in experimental subiculi (*, $p < 0.05$) (n = 28 photo frames/group from 4 control and 4 experimental pups from two different litters; control and experimental pups were litter-matched).

Figure 6: Morphometric analysis of mitochondria in presynaptic neuronal terminals in subicular neuropils of 21-day-old rats. A) Morphometric analysis of the area of mitochondrial profiles shows that experimental mitochondrial profiles were approximately 30% larger than that of controls (*, $p < 0.05$)) (n = 30 photo frames obtained from 5 control pups; n = 45 photo frames obtained from 5 experimental pups; control and experimental pups were litter-matched; total of three different litters were used). B) There is no difference between control and experimental subiculi with regard to the areas of mitochondria-containing

presynaptic nerve terminals (n = 30 photo frames obtained from 5 control pups; n = 45 photo frames obtained from 5 experimental pups; control and experimental pups were litter-matched; total of three different litters were used). C) The calculated mitochondrial index (ratio between mitochondrial area and mitochondria-containing presynaptic area) was significantly higher in experimental subicular neuropils (*, $p < 0.05$) than in control neuropils.

Figure 7: Anesthesia differentially modulates the activity of mitochondrial respiratory chain proteins. A) Compared to control subiculi, the activity of complex IV in experimental subiculi is significantly increased at 24 h after anesthesia (*$p < 0.05$) (n = 8 pups in control group; n = 6 pups in experimental group).   B) The activity of complex I was unchanged in the anesthesia-treated group as compared to sham controls (n = 5 pups per group). C) The activity of complex II was unchanged in the anesthesia-treated group as compared to sham controls (n = 3 pups in control group; n = 4 pups in experimental group).   The activity of complexes I, II, and IV were expressed as ratios [per the activity of citrate synthase (CS)] since citrate synthase activity is directly proportional to mitochondrial content.

Figure 8: Alterations occurred in inhibitory synaptic transmission in pyramidal cells of subiculi after exposure to anesthesia early in life.

A) Representative eIPSCs obtained using a paired-pulse protocol to record from two pyramidal cells in the subiculi of rats in the control (black trace) and experimental groups (gray trace). Note that the experimental group had decreased current amplitude and faster decay. Arrows indicate the time of paired-pulse stimulus application (interval 1.1 sec). Stimulus transients have been removed for clarity of the current traces. B) Histograms showing average data from control cells (n = 14) and experimental cells (n = 12). Black solid bars indicate control cells; gray bars represent experimental cells; vertical lines indicate the SEM of multiple determinations. All data are normalized to 100% of average responses in the control group. Left panel shows a decrease in net charge transfer of eIPSCs from 100 ± 18% to 51 ± 9% ($p < 0.05$) in the experimental group; middle panels show a decrease in decay of tau from 100 ± 16% to 59 ± 7%; right panels show a small but significant increase in P2/P1 from 100 ± 2% to 107 ± 1% ($p < 0.05$).

Fig. 1

Sham controls   **Anesthesia-treated**

<u>SOMA</u>

<u>NEUROPIL</u>

Fig. 2

Fig. 3

A **Mitochondrial area**

B **Mitochondrial density**

Fig. 4

A

Small

B

Medium

C

Large

D

Extra Large

Fig. 5

Fig. 6

A Complex IV

Fig. 7

B Complex I

C Complex II/III

Figure 8

A

eIPSCs

—— sham controls
—— anesthesia-treated

P1   P2

1 nA

200 ms

B

Percent of Control (mean + SEM)

control
experimental

120

80

40

0

charge
transfer

decay tau

P2/P1

*

*

*