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An Analysis of the Interaction of Methylphenidate and Nicotine in Adolescent Rats: Effects on
BDNF

A dissertation
presented to
the faculty of the Department of Psychology
East Tennessee State University
In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Psychology

by

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August 2015

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Keywords: Methylphenidate, Ritalin, Nicotine, Sensitization, Conditioned Place Preference,
BDNF, Adolescence

ABSTRACT

An Analysis of the Interaction of Methylphenidate and Nicotine in Adolescent Rats: Effects on

BDNF

by

Elizabeth D. Freeman

This investigation was an analysis of the interaction of adolescent exposure to methylphenidate (MPH; trade name: Ritalin) on nicotine sensitization and conditioned place preference (CPP) in a rodent model and underlying mechanisms of this effect. Animals were treated IP with 1 mg/kg MPH or saline using a “school day” regimen of five days on, two days off, from postnatal day (P) 28-50. During the final two weeks of MPH treatment, animals were either behaviorally sensitized to nicotine (0.5 mg/kg free base) or saline for 10 days, or conditioned to nicotine or saline using the CPP behavioral paradigm. In addition, three days after behavioral sensitization was complete, animals were analyzed for stress behavior using the forced swim stress behavioral test. In addition, 24 hours after post-test conditioning animals were analyzed for the effect of a clinically relevant dose of pre-exposed MPH (1 mg/kg) and nicotine treatment on the expression of BDNF in the nucleus accumbens and dorsal hippocampus. Behavioral results revealed that adolescent pre-exposure to MPH blunted nicotine behavioral sensitization in both male and female rats during the first week of testing. However, MPH enhanced nicotine CPP in both adolescent male and female rats. Interestingly, animals administered MPH demonstrated a significantly decreased latency to immobility in the forced swim stress behavioral test. In addition, pre-exposure to a 1 mg/kg dose of MPH appears to have sensitized the BDNF response to nicotine in females as compared to all other groups.

DEDICATION

This dissertation is dedicated to my mentor Dr. Russ Brown. I would like to thank him for his advice and support throughout my graduate studies. Dr. Brown introduced me to seven attributes when I joined his lab; critical thinking, problem solving, time management, commitment, desire, precision and the same “uni” policy. Thankfully, he not only taught me the value of these attributes but had the patience for me to learn them. In addition, he has been an extraordinary role model in both my scientific and personal life. Dr. Brown has inspired me to not only formulate good scientific questions but to approach them in the most thoughtful manner. I am forever grateful for his devotion to mentoring, his energy for science, and his passion to keep discovering. This work has been a joint endeavor and a true honor.

“Working hard for something we don’t care about is called stress; working hard for something we love is called passion.”

-Simon Sinek

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TABLE OF CONTENTS

	Page
ABSTRACT	2
DEDICATION	3
ACKNOWLEDGEMENTS	4
Chapter	
1. INTRODUCTION	10
Mechanisms of MPH	11
Mechanisms of Nicotine	11
ADHD & Smoking	12
MPH & Nicotine	13
The Adolescent Brain	17
The Dopamine System.....	20
Sex Differences in the Dopaminergic System	22
Sex Differences and Response to Psychostimulants.....	23
Neurotrophic Factors and Drug Use	24
The Adolescent Rat.....	25
Behavioral Sensitization	27
Forced Swim Test	29
Conditioned Place Preference	30
Research Questions Addressed in this Dissertation.....	32
2. METHODS	35

Subjects	35
Drug Dosage	35
Drug Treatment	35
Pre-exposure	35
Adolescent.....	36
Apparatus	36
Behavioral Sensitization Apparatus.....	36
Conditioned Place Preference (CPP) Apparatus.....	36
Procedure	38
Adolescent MPH Pre-Exposure	38
Behavioral Sensitization	38
Statistical Analysis.....	39
Forced Swim Stress Test.....	40
Statistical Analysis.....	40
Experiment 2, Conditioned Place Preference	41
Statistical Analysis.....	41
BDNF ELISA Procedure	43
3. RESULTS	45
Behavioral Sensitization: Experiment 1.....	45
Forced Swim Experiment: 1	47
Conditioned Place Preference: Experiment 2	49
BDNF Analysis: Experiment 2	51
4. DISCUSSION.....	53

DAT	53
BDNF	54
MPH Pre-Exposure	54
MPH Pre-Exposure & Nicotine	58
Adolescence & BDNF	59
Sex Differences	61
Sex Differences and the Response to Psychostimulants	61
Translational Relevance	63
Conclusions	65
REFERENCES	67
VITA	96

LIST OF TABLES

Table	Page
1. Design: Behavioral Sensitization.....	39
2. Design Forced Swim.....	41
3. Design Conditioned Place Preference.....	42

LIST OF FIGURES

Figure	Page
1. Conditioned Place Preference Apparatus Pre-Test	37
2. Conditioned Place Preference Apparatus Post-Test	38
3. Behavioral Sensitization Experiment 1	46
4. Behavioral Sensitization Difference Score	47
4. Latency to Immobility Test Experiment 1	48
5. Total Immobility Test Experiment 1	49
6. Conditioned Place Preference: Experiment 2	50
7. Nucleus Accumbens BDNF	52
8. Hippocampal BDNF	52

CHAPTER 1

INTRODUCTION

Methylphenidate (MPH; Trade name Ritalin®) is the most often prescribed psychostimulant to treat the symptomology of attention-hyperactivity disorder (ADHD) (Lakhan & Kirchgessner, 2012; Pliska, 2007). ADHD is one of the more commonly diagnosed disorders in children, with approximately 3-10% of adolescents in the United States affected by this disorder (Buitelaar, 2002). To date, there is no known laboratory test to confirm diagnosis; therefore, diagnosis is often reliant on parent and teacher observation of behavior (Rowland, Lesesne, & Abramowitz, 2002). Furthermore, ADHD also has an increased rate of comorbid diagnosis such as opposition defiant disorder, anxiety /depressive disorder, and learning disability (Rowland et al., 2002).

These disorders have very similar behavioral symptoms as ADHD and can complicate diagnosis and contribute to mis-diagnosis. Consequently, a significant population of children who are diagnosed may not have ADHD (Biederman, 2008), yet, a significant portion of this population is prescribed MPH to treat ADHD (Cox, Motheral, Henderson & Mager, 2003; Greenhill, Findling & Swanson, 2006; Olfson, Marcus & Wan, 2009; Rowe & Hermens, 2006; Safer, Zito & Fine, 1996). Furthermore, prescription rates of MPH tripled during the early 1990s (Safer et al., 1996; Zito et al., 2000), resulting in increased availability of MPH. There are reports that MPH has elicited tolerance, sensitization, withdrawal and possible abuse liability, appearing to coincide with increases in prescription rate of MPH (Dafny & Yang, 2006; Fone & Nutt, 2005). Interestingly, this could suggest that MPH has a potential for abuse when taken alone and can elicit dependence.

Mechanisms of MPH

The primary mechanism of MPH is to bind and block the dopamine transporter (DAT) and to a lesser extent, the NE transporter (Schweri et al., 1985; Solanto, 1998). The blockade of both the DAT and NE transporter reduces synaptic clearance of these neurotransmitters, leaving behind high levels of monoamines in the synaptic cleft. In addition, MPH is a stimulant of the central nervous system that shares similarities in its structure and neuropharmacological profile with amphetamine, methamphetamine, and cocaine, which are known drugs that have a high potential for abuse (Teo, Stirling, Thomas & Khetani, 2003; Volkow et al., 1999). Cocaine is considered one of the most commonly abused drugs, and like amphetamine as well as MPH, causes increases of extracellular dopamine in the brain (Schweri, 1985). MPH produces an increase of dopamine within the nucleus accumbens, which is believed to underlie the rewarding effects of drugs of abuse (Di Chiara & Imperato, 1988). In addition, MPH induced increases of dopamine presumably underlies the reinforcing aspects of this drug, although its rewarding effects are dose-dependent (Volkow et al., 1999; Volkow et al., 2001).

Mechanisms of Nicotine

Most adult smokers start smoking during their adolescence (Breslau, Fenn & Peterson 1993; Taioli & Wynder, 1991). In addition, dependence appears to develop to tobacco after minimal tobacco exposure in adolescents as compared to adults (Kandel & Chen, 2000). The development of one dependent symptom in adolescence can foretell continued use (DiFranza et al., 2002). Indeed, adolescence appears to have a biological basis for enhanced sensitivity to tobacco, but it is not well understood. It has been suggested that nicotine, the main psychoactive component of tobacco, mediates reinforcement via activation of the dopamine system (Corrigall et al., 1992). Nicotine reaches the brain within 10-20s via rapid pulmonary venous circulation, and readily

diffuses into brain tissue where it binds to nicotine acetylcholine receptors (nAChRs; Benowitz et al., 1988). Drugs of abuse and the reinforcing effects of nicotine are in part mediated by the activation of the mesocorticolimbic dopamine system (Koob & Le Moal, 2008). The mesocorticolimbic dopamine system includes dopaminergic neurons that originate in the ventral tegmental area (VTA) and project to the nucleus accumbens, hippocampus, amygdala, and prefrontal cortex. Thus, nicotine stimulates acetylcholine receptors located presynaptically on dopaminergic projections from the VTA to the nucleus accumbens and increases dopamine transmission (Di Chiara, 2000).

ADHD & Smoking

ADHD is a well-documented risk factor for cigarette smoking in adolescence (Hammerness et al., 2013). For example, current research demonstrated that a predominant proportion of adolescents with ADHD smoke, quitting smoking is more problematic compared to non-ADHD counterparts, and progression to nicotine dependence is often heightened (Humfleet et al., 2005; McClernon & Kollins, 2008). In fact, ADHD has been shown to be one of the more prominent risk factors for later nicotine dependence in adulthood (Glantz et al., 2009). Several studies have shown that psychostimulant drugs, such as nicotine, which are readily available, are often used to manage symptomology of behavioral disorders, and ADHD is not withstanding (Gehricke et al., 2007; Khantzian, 1997; Millberger et al., 1997).

The self-medication theory posits that nicotine is a stimulant that acts on the central nervous system and therefore can help improve attentional and cognitive processes. In fact, it is well known that nicotine, as a cholinergic agonist, can enhance attentional and cognitive processes (Levin, McClemon, Rezvani, 2006; Rezvani & Levin, 2001). In agreement with this notion, it has been demonstrated that the nicotinic agonist ABT-418 improved focus and cognitive

function in adults with ADHD (Wilens et al., 1999), and nicotine improved simple reaction time, delayed matching to sample and spatial mental rotation (continuous performance test; CPT) in ADHD diagnosed adults (Levin et al., 2001). Interestingly, the combination of MPH and nicotine provided more robust effects as compared to nicotine independently, however, these effects were limited to the clinical global impressions (CGI) scale in which MPH attenuated the effects of nicotine (Levin et al., 2001). These data appear to suggest that the combination of MPH and nicotine enhances the behavioral effects of nicotine.

Importantly, it should be understood that whereas ADHD adolescents are at increased risk of cigarette smoking as compared to non-ADHD adolescents (Glantz et al., 2009), cigarette smoking most often begins in adolescence (Sims, 2009). In fact, it is known that substance abuse in adolescents is likely to begin with tobacco smoking, followed by marijuana and/or alcohol, psychostimulants and opiate drugs (Johnston, O'Malley, Bachman & Schulenberg, 2011). Furthermore, adolescents who are prescribed MPH are likely engaging in recreational use of other drugs of abuse such as nicotine (Brandon, Marinelli, Baker & White, 2001; Lambert & Hartsough, 1998). Thus, both ADHD adolescents and non-ADHD adolescents are at increased risk for nicotine dependence.

MPH & Nicotine

Research has demonstrated that adolescents who were being treated for ADHD exhibited lower rates of cigarette smoking than adolescents with ADHD who were not being treated with stimulants (Wilens et al., 2003). Specifically, individuals who had been treated for ADHD with stimulants smoked less than those individuals with ADHD who received no treatment. In other words, it may be that medication for ADHD lessens the symptomology of this disorder and therefore provides a smoking cessation treatment (Wilens et al., 2008). However, these findings

did not directly examine the effect of nicotine on self-reported symptoms of ADHD; therefore, this implies indirect evidence for the self-medication theory previously mentioned. Of course, this only applies to the diagnosed ADHD population, and little is known as to the prevalence of nicotine use in the mis-diagnosed population (for review, Charach, Yeung, Climans & Lillie, 2010; Wilens et al., 2008).

Consistent with this, Biederman et al (2008) found that ADHD adolescents who were medicated reduced the risk of later substance use disorders than non-medicated ADHD adolescents, although, the prevalence of mis-diagnosis of ADHD among participants in this study could not be assessed. In contrast, Lambert & Hartsough (1998) analyzed long-term use of MPH and found a significant increase in overall tobacco consumption in ADHD treated adolescents as compared to non-treated ADHD adolescents. In addition, it has also been demonstrated that MPH treatment in individuals diagnosed with ADHD and without ADHD increased overall total number of cigarettes smoked, number of puffs taken while smoking, and carbon monoxide levels (Rush et al., 2005; Vansickel et al., 2011).

The contrast between these studies could be related to acute and chronic administration of MPH. Specifically, Vansickel and colleagues administered MPH acutely, whereas, Biederman and colleagues administered MPH chronically, and in a clinical setting chronic dosing is appropriate (Biederman et al., 2008; Lambert & Hartsough, 1998). In addition, repeated administration of stimulants has been demonstrated to change the function and morphology of neuronal systems, which suggests that an interaction between MPH and cigarette smoking and/or nicotine may be altered following repeated treatment (Russo et al., 2009). Thus, the reinforcing efficacy of cigarettes following MPH treatment might likely be enhanced due to an additive or synergistic interaction of MPH and nicotine (Vansickel et al., 2000). Indeed, increased

extracellular dopamine concentrations in areas of the brain known to be involved in reward processes have been demonstrated following MPH treatment and acute nicotine administration (Gerasimov et al., 2000;). This is especially relevant to this study, because we analyzed the treatment of MPH pre-exposure on tasks that measure behaviors related to addiction after nicotine treatment.

Certainly, there is evidence that MPH may increase the risk of smoking. This theory suggests an increase in sensitization of the dopamine system produced by ADHD stimulant medication may enhance the reinforcing effects of nicotine, cocaine and other drugs of abuse (Monuteaux et al., 2007; Vansickel et al., 2011). First, it should be understood that during adolescence, multiple studies have shown that nicotine addiction is a process that proceeds much faster than in adults (Belluzzi, Lee, Oliff & Leslie, 2004; Shram, Funk, Zhaoxia & Li, 2006; Whalen et al., 2003). From a pharmacological perspective, nicotine and MPH in combination may increase dopamine levels together more than either drug alone, and this is presumably the mechanism mediating the enhanced rewarding effects. For example, Gerasimov et al. (2000) demonstrated that simultaneous administration of nicotine and methylphenidate increased levels of extracellular dopamine in the nucleus accumbens (NAc) by 350% as compared to baseline when measured via the microdialysis technique. Research has suggested that the increases in cigarette smoking during MPH treatment may due to the combination of these drugs and their effect on dopamine levels (Rush et al., 2005), but plasticity in the dopamine system may also be related to these effects.

Interestingly, there is some evidence suggesting that the combination of nicotine and MPH alters the pharmacokinetic effects of each drug independently (Wheeler et al., 2013). Specifically, additive effects were demonstrated in long-term behavioral tolerance, as well as

alterations of D3 receptor mRNA in the ventral striatum. Although the exact mechanism(s) underlying this effect is not known, it has been suggested that mesolimbic dopamine (DA) function is likely involved. Similarly, Gerasimov et al., 2000 demonstrated that nucleus accumbens (NAc) dopamine (DA) levels increased when nicotine and MPH were combined suggesting an additive or synergistic effect, although this was dose dependent on the dose of MPH administered (5-10 mg/kg). Consistent with these findings, adolescents who recreationally abuse MPH frequently report the motivation for combining substances is because the combination enhances the intensity of the perceived high and stimulant effect (Doremus-Fitzwater, Varlinskaya & Spear, 2010). Therefore, the fact that the combination of MPH and nicotine increased accumbal dopamine levels suggests that MPH may enhance the rewarding effects of nicotine, and thus presumably MPH could enhance the addictive properties of nicotine. This suggests that the combination of MPH and nicotine use in adolescents could increase the risk of later adult substance use disorder into adulthood.

However, there are a number of conflicting findings relating MPH treatment use in adolescence to later substance use disorder in adulthood. Clearly, recreational use of stimulants such as MPH or Adderall have been reported to increase risk of later adult substance use disorder (Lambert & Hartsough, 1998; Monuteaux et al., 2007; Vansickel et al., 2011). In contrast, some findings have reported that children who receive stimulants as a medication to manage symptoms of ADHD are not at increased risk for substance use disorder in adulthood (Mannuzza, Klein & Moulton, 2003); however, this appears to be age dependent. Specifically, research has demonstrated that the later in adolescence MPH treatment begins, the higher the risk for developing substance use disorder in adulthood (Manuzza et al., 2008). It is during this later developmental period of adolescence that experiences of reduced self-control, enhanced novelty

seeking and risk-taking behavior has been reported to increase (Spear, 2011). A primary focus of the present study was to analyze whether adolescent exposure to a clinically relevant dose (1 mg/kg) of MPH results in changes in the behavioral response to nicotine, and neurobiological mechanisms of these changes. Certainly, developmental differences in the brain, could contribute to MPH behavioral and neurobiological changes.

The Adolescent Brain

Many of the behavioral characteristics of adolescence such as; hyperphagia, egocentrism, reduced self-control, enhanced novelty-seeking and risk-taking behaviors have been shown to increase the probability of substance use and abuse. (Doremus-Fitzwater, Varlinskaya, & Spear, 2010; Sturman & Moghaddam, 2011;). Further, most of the characteristic behaviors displayed in adolescence can be explained in neurobiological terms (Brenhouse & Andersen, 2011; Casey, Jones & Somerville, 2011; Sturman & Moghaddam, 2011). During adolescence, hormonal fluctuations trigger numerous structural brain circuit remodeling such as; dendritic spine remodeling, apoptosis, myelination, and neuronal pruning. Further, there are numerous differences in the adolescent neurotransmitter systems as compared to the adult neurotransmitter systems.

There is an over-expression of dopaminergic, adrenergic, serotonergic and endocannabinoid receptors across many regions in the adolescent brain (Anderson, Thompson, Krenzel & Teichner, 2002; Brenhouse, & Andersen, 2011), as well as expression of D1 and D2 dopamine receptors at increased levels in the subcortical areas such as; dorsal striatum and nucleus accumbens as compared to adult expression in these regions (Lidow, Goldman-Rakic & Rakic, 1991; Tarazi & Baldessarini, 2000; Tarazi et al., 1999; Teicher et al., 1995). However, as the

development of adolescence progresses, the density of many of these receptors systems (dopamine, serotonin, acetylcholine, and GABA) decrease as adulthood approaches.

Particularly important for the present study, dopamine production and dopamine turn over experience change, and there is evidence that change occurs in downstream effects of receptor ligand binding (Tarazi & Baldessarini, 2000). Evidence has demonstrated that spontaneous activity of midbrain dopamine neurons peaks during adolescence and then decreases in adulthood (McCutcheon & Marinelli, 2009). The expression of D1 and D2 dopamine receptors are at higher levels in subcortical targets, such as the nucleus accumbens and dorsal striatum during adolescence, and there are sex differences in dopamine receptor density that is brain region dependent. Males have higher number of dopamine D1 receptors throughout adolescence, and there are sex-dependent changes in the dopamine D2 receptors throughout adolescence, with males demonstrating higher striatal D2 receptor in early and mid-adolescence, but female have higher striatal D2 receptor in mid-adolescence (Andersen & Teicher, 2000). These sex-dependent changes in dopamine receptor density may be especially important to the behavioral and neurobiological response to dopaminergic drugs during this time period. Activation of dopamine D1 receptors consistently increase behavioral activation (Vezina, 2004) whereas activation of dopamine D2 receptors produce less behavioral activation and have more inhibitory control. In contrast, there has been research demonstrating no reduced expression in the nucleus accumbens in adults as compared to adolescents (Tarazi & Baldessarini, 2000; Teicher, Andersen & Hostetter, 1995).

The anatomy and function of the adolescent brain systems may provide vital information about the increase in impulsivity, substance use and later adult substance abuse (Chambers, Taylor & Potenza, 2003). For example, during adolescence alterations in the primary

motivational brain circuitry have been shown to play a vital role in novelty-seeking behavior as well as augment incentive motivational processes (Casey, Jones & Somerville, 2011). In addition, the imbalance that exists between increased sensitivity to motivational cues and immature cognitive control may explain the adolescent bias toward changes in behavior (risky choices, impulsiveness, drug taking, etc.) (Sturman & Moghaddam, 2011).

Consistent with this, magnetic resonance imaging (MRI) in humans have demonstrated a change in cortical gray matter volume during adolescence, with pre-adolescent increases followed by post-adolescent decreases (Adriani & Laviola, 2003). This study combined both cross-sectional and longitudinal data so that individual neuronal growth patterns could be easily detected in the presence of large interindividual variation. The changes in cortical grey matter volume during adolescence correlate with changes that occur at the cellular level during adolescence (Badanich, Adler & Kirstein, 2006). There are changes in overproduction of axons and synapses in early puberty, and rapid pruning in later adolescence. While it is understood that these changes occur, less is known about the exact mechanism of such synaptic changes. However, research has suggested that this is part of the biological basis in which neurological circuitry is efficiently adapted to environmental needs, thus facilitating more adult behavior including alcohol and drugs which may become significant environmental factors (Adriani & Laviola, 2003).

Moreover, research has suggested that the remodeling of dopamine neurotransmission during adolescence may significantly contribute to a stabilization of adolescent behaviors (Bolanos, Glatt & Jackson, 1998; Teicher et al., 1993). Dopamine transmission plays a role in movement, hormonal regulation, attention, and reward (Schramm-Sapyta et al., 2009). Interestingly, during adolescence reorganization of dopaminergic neurotransmission is receptor

subtype and brain region specific. For example, one study in rats showed that dopamine D1, D2 and D4 receptors rise significantly from P7 to P35, which is equivalent to human adolescence, and as adulthood is reached stabilization of these receptors occurs (Anderson & Teichner, 2000). This study further showed that dopamine receptors that are overproduced in the nucleus accumbens (NAc) and striatum undergo subsequent pruning as adolescence progresses suggesting maturational remodeling of both motor and reward pathways. Consistent with this study, another study in rats has demonstrated that dopamine D3 receptors do not reach peak levels until adulthood (PN 60) in the olfactory tubercle, nucleus accumbens (NAc), and striatum (Monti et al., 2005).

The Dopamine System

The mesolimbic dopaminergic system is formed by dopaminergic cell bodies within the ventral tegmental area (VTA)/substantia nigra and terminates in the olfactory tubercle, nucleus accumbens (NAc), amygdala, hippocampus, septum, and the medial prefrontal cortex. The VTA sends a major axonal projection to both the nucleus accumbens (NAc) and frontal cortex, and this system forms the brain's reward system. These brain areas also send reciprocal projections back to the VTA, and this pathway is also known as the medial forebrain bundle. Dopamine has been shown to be vital in the rewarding properties of psychostimulants, and the reward and reinforcing effects of drugs of abuse (George, Moal & Koob, 2012). Moreover, all addictive drugs have been shown to activate the mesolimbic dopamine pathway, including cocaine, amphetamine, and methamphetamine. All drugs of abuse increase dopamine release within this pathway of the brain's reward system (Volkow et al., 1997).

Dopamine binds to two families of receptors: the D1, which has two receptor subtypes: the D1 and D5. Dopamine also binds to the D2 receptor, and the D2 receptor family has three

receptor subtypes: the D2, D3, and D4. Both the D1 and D2 receptor families are metabotropic G-protein coupled dopamine receptors that negotiate the physiological functions of dopamine. Dopamine plays a role in hormonal regulation, hypertension, voluntary movement and reward (Beaulieu & Gainetdinov, 2011). Consequently, many different drugs that target dopaminergic neurotransmission have been clinically prescribed for the management of several neurodegenerative and behavioral disorders such as Parkinson's disease, schizophrenia, bipolar disorder, Huntington's disease, Tourette's syndrome as well as ADHD.

Moreover, dopamine D1 receptors are involved in the development of sensitization to the rewarding properties of psychostimulants (Meririnne, Kankaanpaa & Seppala, 2001). For example, SCH 23390 (D1 antagonist) prevented amphetamine self-administration if given prior to the animal having access to the drug (Pierre & Vezina, 1998). Further, it was demonstrated that the same D1 antagonist (SCH 23390) also prevented cocaine induced conditioned place preference (CPP) (Shippendberg, Heidbreder & Lefevour, 1996). However, D2 receptors may be involved in the rewarding properties of particular stimulants, as it has been demonstrated that the D2-antagonist raclopride (RAC) was not effective in blocking conditioned place preference (CPP) to cocaine, and prevented development of sensitization to the locomotor-stimulating effects of amphetamine and methamphetamine (Kuribara & Uchihashi, 1993; Meng, Feldpaush & Merchant, 1998). Further, past work in our laboratory has demonstrated that MPH administration in adolescent rats produced an increase in brain derived neurotrophic factor (BDNF) in both the nucleus accumbens (NAc) and dorsal striatum, that was reduced by the D2 antagonist eticlopride (Cummins et al., 2014). Moreover, MPH administration in adolescent rats resulted in a decrease of the dopamine transporter (DAT) in the both the nucleus accumbens (NAc) and the dorsal striatum (Cummins et al., 2013).

Sex Differences in the Dopaminergic System

It is known that there are sex differences in dopamine receptor density and this may be a contributing factor in sex differences that not only exist in the epidemiology of ADHD but also may play a role in adolescent exposure to drugs of abuse (Mannuzza et al., 2003). Much attention has been given to sex differences in the density of dopamine receptors during adolescence. Anderson and Teicher (2000) have shown that adolescent male rats demonstrated significantly higher levels of accumbal D1 receptors as compared to females that persisted throughout adolescence, as well as increases in D1 receptors in the striatum at P40. Interestingly, the dopamine D1 receptor has been shown to play a more important role in the behavioral activating effects of psychostimulants (Kalivas & Stewart, 1991).

Moreover, male rats demonstrated lower levels of striatal D2 receptors as compared to female rats on P25, but the density of D2 receptor rapidly increase in such that males demonstrate a significant increase in striatal D2 receptors at P40, which is greater than females (Anderson & Teicher, 2000). Interestingly, both male rats and female rats demonstrated equivalent numbers of D2 receptor levels in early adulthood at P60. However, females have a higher density of dopamine transporter mRNA in the striatum (Bosse, Rivest, & Di Paolo, 1997). Anderson and Teichner (2000) explain that this sex difference in dopamine receptor density may be related to the increase of ADHD incidence in males as compared to females. However, it also may have important implications relative to sex differences in the response to psychostimulants as well.

In addition, women suffer significantly more adverse health consequences to psychotropic drugs than do men (Simoni-Wastila, 2000). For instance, research demonstrated that women are 48% more likely than men to abuse prescription drugs. In fact, gender may moderate the effects

of alcohol on prefrontal cortex morphometry in adolescents with alcohol use disorder (AUD) (Lisdahl et al., 2013). Specifically, adolescent females with AUD demonstrated smaller prefrontal cortex volumes while adolescent males with AUD had larger prefrontal cortex volumes. Consistent with gender variation moderating drug effects, it has been demonstrated that adolescent males are more likely than adolescent females to combine substances for recreational use (Earlywine & Newcomb, 1997). Consequently, sex differences exist in response to psychostimulants and have important relevance to vulnerability of drug use, dependence and abuse.

Sex Differences and the Response to Psychostimulants

A substantial body of data support that there are sex differences in the acute neurobiological response to psychomotor stimulants and in sensitization of psychomotor behavior induced by amphetamine (Becker, 1999), cocaine (Bowman et al., 1999; Van Haaren & Meyer, 1991), methamphetamine (Schindler, Bross, & Thorndike, 2002), and nicotine (Fallon et al., 2005; McClernon, Kozink & Rose, 2008). It appears that dopamine function in females may be highly related to the fluctuation of ovarian hormones during the estrous cycle. For example, during behavioral estrous in female rats, amphetamine-induced striatal dopamine release and amphetamine-induced behaviors are greater than on other days of the estrous cycle (Becker, 1999; Becker, Robinson & Lorenz, 1982), and ovarian hormone fluctuations induce variation in behavioral and neurochemical responses to psychostimulant drugs.

In addition, studies have shown sex differences in dopamine receptor sensitivity manifested in changes in locomotor behavior, and differences in D₂ receptor sensitivity and manifestation in behavioral changes may be related to the age of the animal. Further, sensitivity of the D₂

receptor changes as both males and females age and this change is also likely influenced by the estrous cycle in females (Frantz & Hartesveldt, 1999). Animals were given an acute injection of several different doses of quinpirole over a range of ages from P10-P60 demonstrated gender differences at P40, 50, and 60 with females demonstrating increased locomotor activity after quinpirole administration as compared to males. These results appear to show that females demonstrate increases of sensitivity to locomotor stimulatory effects to dopamine agonists, especially during late adolescence and early adulthood.

Neurotrophic Factors and Drug Use

The development, maintenance and survival of dopaminergic neurons in the central nervous system (CNS) depend on several neurotrophic factors (Kril, Halliday, Svoboda & Cartwright, 1997). Neurotrophic factors are proteins that essentially promote the development, maintenance and survival of the neurons. It has been shown that brain derived neurotrophic factor (BDNF) is regulated by several drugs of abuse (Russo, Mazei-Robison, Ables & Nestler, 2009) and in general, psychostimulants significantly increase BDNF protein in the brain. Research is ongoing to characterize the cellular and molecular changes that occur during drug use and eventual abuse.

It has been shown that changes in BDNF and signaling pathways change neuronal functioning within the ventral tegmental area (VTA) –nucleus accumbens (NAc) reward regions that regulate motivation to take drugs (Bolanos & Nestler, 2004). For example, several studies have shown increased levels of BDNF in several brain regions after chronic psychostimulant administration (Brown et al., 2012; Correll et al., 2009; Grimm et al., 2003; Meredith, Callen & Scheuer, 2002; Thomas, Kalivas & Shaham, 2008) Changes were demonstrated in nucleus accumbens (NAc), prefrontal cortex (PFC), ventral tegmental area (VTA), and the amygdala.

Specific to MPH, our lab has demonstrated a significant increase in BDNF in both the NAc and striatum following MPH administration (Cummins et al., 2013). In addition, across several studies we and others have reported that nicotine also results in increases of BDNF in the striatum and NAc (Brown et al., 2004; 2006; French et al., 1999; Kenny et al., 2000;).

It has been demonstrated that differing classes of chronically administered addictive substances can alter the brain's reward circuitry (Russo, Mazei-Robison, Ables & Nestler, 2009). It has been suggested that structural and behavioral plasticity that is associated with drugs of abuse are a result of changes in growth factor signaling. For example, increases in BDNF have been observed in humans that were addicted to psychostimulants (Lang et al., 2007; Shim et al., 2008). Unfortunately, human studies examining the signaling pathways in BDNF are limited, and thus the relevant changes that happen in drug addiction remain unclear. However, evidence of BDNF on various phases of the addiction process has been established in animal models (Grimm et al., 2003; Lu et al., 2004; Meredith, Callen & Scheuer, 2002; McGough et al., 2004). Several studies have demonstrated that nicotine administration increases levels of BDNF in both the nucleus accumbens and striatum (Kenny, File, & Rattray, 2000; Maggio et al., 2002). Importantly, the level of BDNF in the VTA, as well as the nucleus accumbens, gradually increases during drug abstinence (Grimm et al., 2003), and this gradual increase has been hypothesized to underlie the progressive increase in drug-seeking behavior that occurs during withdrawal (Le Foll et al., 2005).

The Adolescent Rat

The adolescent age in a rat must be defined when discussing models of drug abuse. Unfortunately, the precise age of what delineates an adolescent rat has been difficult to ascertain.

For example, in the review by Smith (2003), the time between the earliest detection of mature diurnal gonadotropin cycling (around P28 to P30; Ojeda & Urbanski, 1994), and the achievement of reproductive maturity, which is as early as P38 in the female, as late as P60 in the male, it could be concluded that for dosing to include adolescence, it should include the P28-60 range. In agreement with this age range, Faraday, Elliot, and Grunberg (2001) and Yang, Swann, and Dafney (2006) defined adolescence as the period spanning from approximately P30 to P60, as this period spans presexual maturation as well as sexual maturity.

Clearly, this includes a rather expansive developmental period; however, it does include neurobiological changes as well as behaviors that are associated with adolescence and the transition from adolescence to adulthood. When discussing the adolescent developmental period neurobiologically, the developmental period of P30-60 can be characterized by stable increases in serotonergic transporter levels and striatal dopamine (Tarazi, Tomasini & Baldessarini, 1998), increases in cholinergic innervation of the prefrontal cortex (Gould, Woolf & Butcher, 1991), and biphasic changes in striatal dopamine receptors (Tarazi et al., 1998; Teicher, Anderson & Hostetter, 1995). Rats also display peak adrenocorticotrophic hormone and corticosterone responses to stress during the adolescent period (Tarazi et al., 1998). Behaviorally, social play is most often displayed in adolescence as compared to adulthood, with play behavior peaks between P30 and 40 (Varlinskaya, Spear & Spear, 1999).

In contrast, Spear and colleagues have more narrowly defined adolescence in the rat as between the ages of P28 to P42, however, this age span does not extend into postsexual maturation that occurs in adolescence and only defines the age of pre-sexual maturation (Spear, 2000). The definition of adolescence in humans and dosing in some studies in animals extend past the point of reproductive maturity. This age range was originally derived by considering

age-specific behavioral discontinuities (Spear & Brake, 1983) but was also supported by measures including the timing of the growth spurt (Meaney & Stewart, 1981), the loss of excitatory amino acid overshoot to prefrontal cortex (Insel et al., 1990), and the timing of emergence of rats from the protected nest burrow in the wild, which begins at P28 (Galef et al., 1981). On the other hand, as Spear (2000) mentioned, use of this narrow age range was not meant to imply that animals slightly younger or older than this prototypic age range might not also be undergoing adolescent transitions.

Indeed, some developmental changes signaling the early onset of adolescence in female rats may begin to emerge as early as P20 with later changes lasting until P55 or so in males (O'dell, 2006; Ojeda & Urbanski, 1994). Regardless, it is clear that there are critical developmental neurobiological and behavioral changes during the period between weaning and adulthood that are consistent with adolescence in rat. In the current study we focus on the synergistic effects of MPH on the behavioral effects of nicotine during an approximate period between the ages of P28-60, which spans the entire developmental period, also referred to as the preadolescent and adolescent period. This is especially important translationally because drug use and abuse typically initiates during adolescence.

Behavioral Sensitization

Behavioral sensitization is an augmented locomotor behavioral response as a consequence of repeated exposure to a drug (Fanous, Lacagnina, Nikulina & Hammer, 2011; Justo et al., 2010; Kelley & Rowan, 2004). The psychomotor sensitization expresses behaviorally through horizontal movement and vertical rearing defined as locomotor activity (Fanous, Lacagnina, Nikulina & Hammer, 2011; Tirelli, Laviola & Adriani, 2003). This effect is likely the consequence of enhanced dopamine release and activation of mesolimbic dopamine projections

after repeated psychostimulant drug administration (Boileau et al., 2006). Locomotor sensitization has been shown to be produced by psychostimulants such as; nicotine, cocaine and amphetamine, as well as direct dopamine agonists (apomorphine, quinpirole) (Kosowski & Lijequist, 2005; Perna & Brown, 2013; Roeding et al., 2014; Tenn, Kapur & Fletcher, 2005; Tirelli, Lavioloa & Adriani, 2003).

It has also been shown that a dopamine D1 or D2 antagonist such as SCH23390 and eticlopride block sensitization to psychostimulants (Schindler & Carmona, 2002). Enhancing neural plasticity in the medial prefrontal cortex (mPFC) by repeated psychostimulant exposure increases spine density and dendritic branching (Morshedi et al., 2009). The mPFC sends glutamatergic projections to the ventral tegmental area (VTA) and it has been demonstrated that by enhancing dopamine release in the MPFC-VTA projection results in enhanced neural plasticity thus enhancing sensitization. Therefore, the dopamine system plays a crucial role in behavioral locomotor sensitization to psychostimulants.

Specific to this proposal, Wheeler et al., (2013) demonstrated an increase in overall distance in adolescent male rats pre-exposed to nicotine and MPH (P35-56), and a decrease in overall locomotor activity suggesting tolerance to the hypoactive effects of nicotine. However, the decrease in activity was recorded at 5 minutes and later an increase in activity was observed at 15 minutes. Consistent with this, locomotor activity in adult male rats pre-exposed to nicotine and then given a challenge to MPH, showed an initial decrease in locomotor activity followed by an increase in locomotor activity across repeated injections (Wooters, Neuebauer, Rush & Bardo, 2008).

To date there is no evidence to support cross-sensitization between nicotine exposure and repeated MPH injections. In contrast, some studies have demonstrated cross-sensitization

between nicotine and other stimulant drugs such as; amphetamine (Birrell & Balfour, 1998), bupropion (Wilkinson, Palmatier & Bevins, 2006), cocaine (Collins & Izenwasser, 2004), and methamphetamine (Kuribara, 1999). There is no current research to discern the effects and the differences among stimulants, it is suggested that the initial nicotine-induced augmentation in locomotor sensitization demonstrated with repeated injections of MPH is indicative of a possible overlap in the mechanisms that underlie nicotine and MPH sensitization (Wooters, Neubeauer, Rush & Bardo, 2008).

Forced Swim Test

The forced swim test has rapidly developed into the most commonly used model for assessing antidepressant-like activity in rats (Borsoi et al., 2015; Cryan & Holmes, 2005; Petit-Demouliere, Chenu & Bourin, 2005). This is primarily due to the consequence of the ability of specificity, inter-laboratory reliability and the simplicity of the test itself. Moreover, it has the ability of being applied to other species such as; mice, gerbils, and sand rats (Einat, Kronfeld-Schor & Eilam, 2006; Slattery & Cryan, 2012). There are many advantages to using the forced swim test to access anxiety/depressant-like behavior in a rodent.

In a typical forced swim test, swimming is used as an experimental protocol that induces stress in rodent models (Barros & Ferigolo, 1998; Marcondes et al., 1996; Tanno et al., 2002). It is an optimal task for evaluating an animal's physiological response to an acute stressful situation (Tanno et al., 2002). This is accomplished when the swimming session begins and the rats will actively swim in the beginning of the session, and then become less active and immobility is observed (Calil & Marcondes, 2006). It has been shown that immobility is an adaptive behavioral response that has the propensity to increase a rat's chance of survival (Binik & Sullivan, 1983; Bruner & Vargas, 1994). Consequently, the observed immobility behavior is

understood to reflect a failure to persist in escape-directed behavior after stress (i.e., behavioral despair) or the passive behavior that extricates the animal from more active forms of stress coping (Cryan, Markou & Lucki, 2002).

To date, there have only been two studies that have analyzed the effects of long-term MPH use on the effects of stress using the forced swim test. Most recently, Brookshire & Jones (2012) administered 20 mg/kg of MPH to adult C57B1/6J mice for 14 days before commencing behavioral testing. This study demonstrated that chronic MPH treatment produced depressive-like effects, with decreased latency to first immobility and a trend toward increased immobility. In the second study, Carlezon, Mague & Anderson (2003) examined the effects of early exposure to MPH and subsequent enduring behavioral effects. In this study, MPH (2 mg/kg) was administered twice a day from P20-35 in male pups, no treatment was given from P36-60, at which time forced swim testing initiated. This study demonstrated that early exposure to MPH during development caused depressive-like effects, with increases in total immobility observed in both days of the forced swim test.

Conditioned Place Preference

Conditioned place preference (CPP) is a behavioral paradigm that is used to assess the conditioned rewarding effects of a variety of drugs of abuse (Bardo & Bevins, 2000; Tzschentke, 2007). CPP is a commonly used behavioral task in rodents that uses classical Pavlovian conditioning principles to analyze the behavioral effects of rewarding drugs. This is accomplished with the presentation of a previously neutral stimulus, such as a particular environment that can acquire rewarding properties of a drug such as; craving, withdrawal, and drug seeking behavior through learned associations.

In a typical CPP paradigm, animals are injected with a drug that is temporally contiguous with a previously neutral environmental context, and in the same group of animals saline is temporally paired with a different context over several days of conditioning. These contexts are separated by removable dividers and distinct in terms of color and tactile surface. A drug-free test is given at the end of conditioning, with dividers removed, to test for preference. Several studies have demonstrated that animals conditioned with a drug of reward typically spend an increase in time in the drug paired context as compared to saline controls on the post conditioning test (for review, Bardo & Bevins, 2000).

It has been demonstrated that rats that are exposed to MPH early in adolescence will alter the behavioral and neurobiological effects in response to psychostimulants lasting into adulthood. For example, Andersen et al., 2002 demonstrated that early (P20-35) MPH exposure alters sensitivity to the psychostimulant cocaine in adulthood (P60). Specifically, MPH-exposed rats failed to demonstrate CPP when administered a dose (10 mg/kg) of cocaine as compared to saline rats, and interestingly, a higher dose (20 mg/kg) of cocaine failed to produce CPP in MPH-exposed animals. This is consistent with clinical studies that have demonstrated that therapeutic doses of MPH in children with ADHD decrease later risk of substance use (Biederman et al., 1999; Mannuzza et al., 2008; Wilens et al., 2003;), but are in contrast to other studies suggesting a larger risk of substance abuse due to MPH exposure during early adolescence (Bolanos et al., 2003; Brandon et al., 2001; Vendruscolo et al., 2008;).

Finally, research has demonstrated that nicotine induced conditioned place preference is dose and age dependent (Belluzzi et al., 2004; Torrella et al., 2004). While nicotine has induced CPP, it has been shown more prevalently and consistently in adolescence as compared to adulthood. For example, some studies have demonstrated a preference (Calcagnetti & Schechter,

1994; Fudala et al., 1985; Le Foll & Goldberg, 2005), an aversion (Clarke & Fibiger, 1987; Laviolette & van der Kooy, 2004), or no preference (Belluzzi et al., 2004; Torrella et al., 2004; Vastola et al., 2002) to nicotine CPP in adult rats. In addition, Shram et al., 2006 demonstrated nicotine (.8mg/kg) induced conditioned place preference in adolescent rats, but failed to demonstrate nicotine (.8 mg/kg) conditioned place preference in adult rats. Thus, it appears that adolescent rats are more sensitive to the rewarding effects of nicotine than adults.

Research Questions Addressed in this Dissertation

The aim of this study was to examine the following:

1) Analyze the effects of a clinically relevant dose of MPH (1 mg/kg) pre-exposure on the locomotor activating effects to nicotine in both male and female adolescent rats with a focus on sex differences.

It was expected that both male and adolescent female rats pre-exposed to MPH (PN28-P41) and treated with nicotine (P45-62) would demonstrate enhanced behavioral sensitization as compared to saline animals. Further, it was hypothesized that MPH pre-exposed adolescent female rats would demonstrate an enhanced locomotor activation to nicotine as compared to MPH pre-exposed adolescent male rats. This was based on previous research demonstrating that females show an enhanced behavioral response to psychostimulants as compared to males (Becker, 1999; Brown et al., 2012; 2014).

2) Analyze the effects of a clinically relevant dose of MPH (1 mg/kg) pre-exposure and the behavioral effects of nicotine in male and female adolescent rats for stress behavior using the forced swim test.

It was expected that both adolescent male and female rats pre-exposed to MPH will demonstrate a significantly decreased latency to immobility, a behavioral manifestation of learned helplessness (Cryan, Markou & Lucki, 2002). Long-term treatment of MPH has been shown to increase sensitivity to stressful situations (e.g. elevated T-maze) and increase anxiety and depressive-like behaviors (e.g. forced swim test) (Bolanos et al., 2003; Carlezon et al., 2003).

3) Analyze the effects of a clinically relevant dose of MPH (1 mg/kg) adolescent pre-exposure on the behavioral response to nicotine in adolescent male and female rats tested on the conditioned place preference (CPP) behavioral task.

It was expected that male and female adolescent rats that have been pre-exposed to a clinically relevant dose of MPH (1 mg/kg) would demonstrate enhanced nicotine CPP (P41-48) as compared to saline animals. Further, it was expected that MPH pre-exposed adolescent female rats would demonstrate an enhanced CPP as compared to MPH pre-exposed adolescent male rats. It is understood that the nucleus accumbens plays a primary role in CPP, adolescent MPH treatment has been shown to be rewarding (Cummins et al., 2012), resulting in synaptic plasticity changes increasing sensitivity to nicotine presumably through increases in the development of dopaminergic synaptic activity, thus, increasing the behavioral rewarding effects of nicotine (Di Chiara et al., 2004). In addition, it is known that females show an enhanced behavioral response to psychostimulants as compared to males (Becker, 1999).

4) Analyze the effect of MPH (1 mg/kg) adolescent pre-exposure and nicotine treatment on the expression of BDNF in the nucleus accumbens and dorsal hippocampus.

It was expected that both adolescent male and female rats that were pre-exposed to MPH and saline, and MPH and nicotine would demonstrate a significant increase in overall BDNF as compared to controls. MPH decreases DAT and results in less dopamine clearance, and dopamine would be able to continue to bind to postsynaptic dopamine receptors, increasing overall dopaminergic activity. BDNF is a cell activity dependent neurotrophin (Boulle et al., 2012), and increases in dopaminergic activity lead to increases in BDNF, and past work has demonstrated BDNF's involvement in the processes of addiction, craving, and withdrawal (Grimm et al., 2003; Horger et al., 1999; Pu et al., 2006). In addition, across several studies we and others have reported that nicotine also results in increases of BDNF in the striatum, nucleus accumbens (Brown et al., 2011; Kenny et al., 2000; Klein-Schwartz & McGrath, 2003) and hippocampus of male rats administered chronic nicotine (.5 mg/kg i.p.) (Kenny et al., 2000).

CHAPTER 2

METHODS

Subjects

A total of 128 offspring of Sprague-Dawley rats ordered from Harlan Inc. (Indianapolis, IN) were used in all experiments. The day of birth was counted as postnatal day zero (P0) and pups were weaned from the dam at P2. All animals were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC) facility with food and water available ad libitum, and all animals were maintained on a 12:12 on/off light/dark cycle. All behavioral testing occurred during the light cycle. All procedures were approved by the East Tennessee State University Committee on Animal Care which is consistent with the NIH Guide on Care and Use of Animals.

Drug Dosage

Animals were administered 1 mg/kg of methylphenidate based on findings by Berridge et al (2006) that this dose when given IP, produced brain concentrations that are similar to therapeutic doses of methylphenidate. Methylphenidate HCL (Sigma-Aldrich, St. Louis, MO) was diluted in saline to .1 mg/kg. Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) was diluted in saline to 0.5 mg/kg free base (pH ~ 7.0).

Drug Treatment

Pre-exposure. For Experiment 1 and 2, on postnatal days 28 to 63 (P28-63), animals were given a single daily intraperitoneal (i.p.) injection of either methylphenidate (1 mg/kg) or saline (0.9%). For Experiment 3, on postnatal days 28 to 51 (P28-51), animals were given a single daily intraperitoneal (i.p.) injection of either methylphenidate (1 mg/kg) or saline (0.9%). Animals

were randomly assigned to treatment group in order to maintain relative sameness in group size. All animals gained weight normally during drug treatment.

Adolescent. Animals were randomly assigned to treatment groups on P28. Animals were drawn from 13 litters and were assigned to the following treatment group with the first drug representing pre-exposure treatment and the second representing adolescent treatment (or Saline if control group): Saline-Saline (S-S), Saline-Nicotine (SN), Methylphenidate-Saline (MPH-S), or Methylphenidate-Nicotine (MPH-N).

Apparatus

Behavioral Sensitization Apparatus. The locomotor arenas for the behavioral sensitization were Plexiglas boxes painted black and measuring 72 cm on all sides. All activity was monitored by an automated behavioral scanning system (Any Maze, Stoelting Co., Wood Dale, IL). A digital grid was superimposed on the floor of the locomotor arena using the Any-Maze scanning system. The number of lines crossed served as the dependent variable of horizontal activity. Four locomotor arenas were used because the behavioral scanning system allowed for simultaneous data collection of more than one subject.

Conditioned Place Preference (CPP) Apparatus. A three-chambered CPP box was employed. All chambers within the box were equal in size (90 cm on each side), separated by removable wooden doors and distinct in terms of visual and tactile stimuli. The middle chamber of the CPP box is painted solid grey, while each chamber on the end has either black or white vertical or horizontal stripes. The middle compartment is painted solid grey. In addition, each CPP chamber also has different tactile surfaces along the floors of the boxes that help to make regions more distinct. One of the compartments features wire-mesh flooring, while the other has

metal dowel rod flooring. The grey chamber has wooden flooring and the entire box is covered in plexus-glass.

All behavioral testing was recorded using an automated computer program (Any Maze, Stoeling, Wood Dale, IL). The computer program superimposes a grid of lines on the arena, and every movement of the animal is recorded for time spent in each context, overall locomotor activity in each context and the animal traversed into each context. A photograph of the CPP apparatus without dividers is presented in Figure 1.

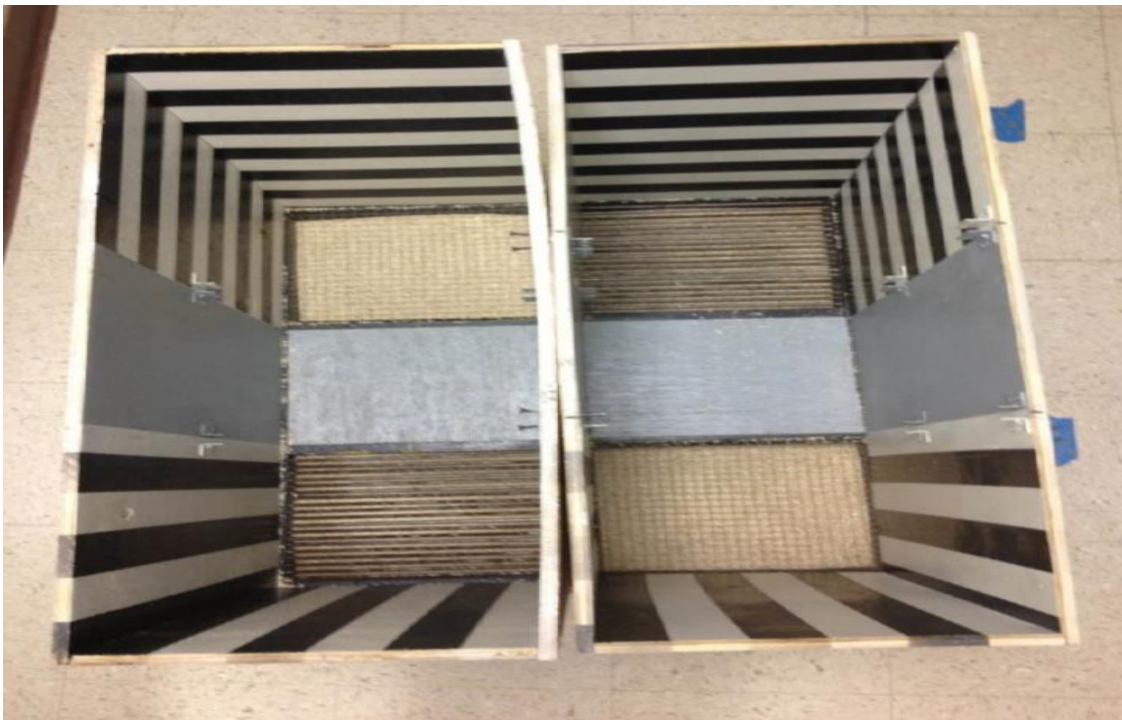


Figure 1. Conditioned Place Preference Apparatus Pre-Test. Note. The above picture shows the conditioned place preference boxes without the wooden dividers in.

A photograph of the CPP apparatus with the dividers is presented in Figure 2.



Figure 2. Conditioning Place Preference Apparatus Conditioning. *Note.* The above picture shows the dividers in the CPP apparatus for the 8 days of conditioning.

Procedure

Adolescent MPH Pre-Exposure. Beginning on P28, animals were IP administered to either MPH (1 mg/kg) or saline 5 days a week with two days off for a period of 14 days. Thus, animals were given MPH from P28-33, given P34 and P35 off with no drug given, followed by another five days of MPH or saline from P36-41. All animals were randomly assigned to each drug condition.

Behavioral Sensitization. Beginning on P42, animals were habituated to the locomotor arena for three consecutive days. On each day of habituation (P42-44), all animals were given an IP injection of saline and placed into the locomotor arena 10 minutes after the IP injection of saline. Each habituation session lasted for 10 minutes. This allowed animals to adapt to the locomotor apparatus and to the injection procedure. Beginning on P45, animals were injected IP with either nicotine (0.5 mg/kg free base) or saline. Ten minutes after either nicotine (0.5 mg/kg) or saline, the animals were placed into the locomotor arena for a 10-minute session. For horizontal activity measures, each time an animal crossed a line on the grid; this was counted as a horizontal

activity count. The total number of activity counts for each 10 minute trial provided the total horizontal activity count for that particular day. This procedure was repeated every other day for 20 days resulting in a total of 10 days of behavioral sensitization testing, and animals were tested from P45-63. All animal behavior was recorded using Any Maze (Stoeling, Wood Dale, IL) software.

Statistical Analysis. An initial 2 (sex) x 2 (pre-exposure drug treatment) x 2 (adolescent drug treatment) x 4 (repeated measure) four-way analysis of variance (ANOVA) was performed for Experiment 1, and a 2 (sex) x 2 (pre-exposure drug treatment) x 2 (adolescent drug treatment) x 3 (repeated measure) three-way ANOVA. Two dependent measures were analyzed: Activity counts on days 1 and 10, and the differences between activity counts on days 1 and 10. The rationale for analyzing two behavioral measures was to provide an overall analysis of locomotor activity as well as a difference in behavioral responding on the final day of nicotine as compared to the first, analyzing the change in response to nicotine over days. However, the primary measure for behavioral sensitization is the analysis of locomotor activity on days 1 and 10. The grouping for behavioral sensitization is presented in Table 1.

Table 1

Design: Behavioral Sensitization

Pre-Exposure P28-63	Nicotine P45-63	Saline P45-63
MPH	M 10 F 10	M 10 F 10
SAL	M 10 F 10	M 10 F 10

Forced Swim Stress Test. Three days after sensitization (P66), all animals began behavioral testing in the forced swim test. All animals were tested in black cylinders measuring 36 cm in diameter, and these cylinders were filled with water of 23-25 C following previous procedures. All animals were given a pre-swim exposure test on P66, 24 h before the swim test session on P67. On the first day of testing, animals were exposed to the water for 15 min, and on the second day, it was a 5 min trial. All animal behavior was recorded using Any Maze (Stoeling, Wood Dale, IL) software.

The latency to first immobility episode (immobility lasting > 5 s) and the total immobility over the 5 minute period were recorded on day two, and data was analyzed. It should be noted, however, that latency to immobility is the primary measure for analyzing learned helplessness in the forced swim stress task (West, 1990). We also decided to analyze total immobility time to provide a more thorough analysis of this particular task.

Statistical Analysis. An initial 2 (pre-exposure drug treatment) x 2 (adolescent drug treatment) analysis of variance (ANOVA) was performed for latency to immobility, and a 2 (sex) x 2 (pre-exposure drug treatment) x 2 (adolescent drug treatment) three-way ANOVA was performed for total immobility time. Two dependent measures were analyzed: latency to immobility and total immobility time. The rationale for analyzing two behavioral measures was to provide an overall analysis of immobility time as well as sex differences in behavioral responding. The grouping for forced swim is presented in Table 2.

Table 2

Design: Forced Swim

Pre-Exposure P28-63	Nicotine P45-63	Saline P45-63
MPH	M 8 F 8	M 11 F 8
SAL	M 7 F 7	M 8 F 11

Experiment 2, Conditioned Place Preference. A separate set of animals were behaviorally tested using the CPP paradigm. Animals were pre-exposed to MPH or saline identically as in Experiment 1. Beginning on P42 and 43, all animals were given an initial preference test for a period of ten minutes each. For initial preference testing, all animals were administered an IP injection of saline 10 minutes prior to being placed into the CPP chamber with dividers removed and allowed freely to explore the apparatus. The purpose of this injection was to control for the stress of the injection and to provide consistency in injection before each placement into the CPP apparatus.

Statistical Analysis. An initial 2 (sex) x 2 (pre-exposure drug treatment) x 2 (adolescent drug treatment) x 3 (repeated measure) three-way analysis of variance (ANOVA) was performed for Experiment 2. One dependent measures was analyzed: The time the animal spent in the conditioning (paired) context on the initial preference (percent pre) minus the time the animal spent in the conditioning (paired) context on the posttest (percent post). The grouping for conditioned place preference is presented in Table 3.

Table 3

Design: Conditioned Place Preference

Pre-Exposure P28-51	Nicotine P44-51	Saline P44-51
MPH	M 10 F 10	M 8 F 10
SAL	M 9 F 9	M 7 F 9

Conditioning began the day after the initial preference test on P44, and removable dividers were placed into the apparatus. The assignment of each context to be paired with nicotine was based on mean performance of the initial preference test, and selection of the “paired” context in the control group was simply balanced across animals administered saline. In animals given nicotine, the paired context was the context that was paired in time with nicotine against their natural preference, although no was no significant preference differences. Controls were given saline in both contexts. In the morning session on conditioning days, all animals were give an IP injection of saline, and 10 minutes later placed into their assigned context for 10 minutes and behavior was recorded. In the afternoon session (approximately 4 h later), animals in the nicotine group were given an IP injection of nicotine (.5 mg/kg) and 10 minutes later, placed into the paired context for a 10 minute trial. The choice of the paired context was also balanced across animals, but this choice was based on an analysis of the initial preference test such that there were no significant differences in initial context preference across groups. Conditioning occurred ever consecutive day from P44-51.

The post-conditioning test was conducted the day after conditioning was complete on P52. This test was identical to the initial preference test, with dividers removed all animals were IP administered saline to control for the stress of the injection, and allowed to freely explore the apparatus. For the post-conditioning preference test, the dependent measure was the percent of

time spent in the paired context on the initial preference subtracted away from the percent time spent in the paired context on the post-conditioning preference test. Thus, performance on each of these tests was taken into account for the final calculation.

The day after post-conditioning (P53), all animals were sacrificed, using a rapid decapitation, brain tissue removed and rapidly frozen in cold (-20) isopentane. Nucleus accumbens and dorsal hippocampus were later dissected away and analyzed for the effects on BDNF. The rationale for analyzing BDNF protein only in the animals that were in the CPP group was because those animals that were in the behavioral sensitization group also were in the forced swim stress test, and the acute stress of the forced swim test has been shown to effect BDNF levels (Borsoi et al., 2015). All tissue was stored in an ultra-low -80°C freezer (So Low Inc, Cincinnati, OH). An Elisa analysis was used to determine BDNF amounts in the nucleus accumbens and dorsal hippocampus.

BDNF ELISA Procedure

Twenty-four hours after the last saline or MPH IP injection, animals were rapidly decapitated. The brains were immediately frozen with isopentane and stored in a -80°C freezer. The nucleus accumbens and dorsal striatum were dissected from each individual brain and stored at -80°C. In brief, 250 µl of RIPA cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1.0% NP-40, 0.5 % Sodium deoxycholate and 0.1% SDS) plus protease and phosphatase inhibitors (P5726, P8340, P0044, Sigma-Aldrich, St. Louis, MO) was added to each tissue sample. Brain regions were homogenized on ice with 10 passes of a Teflon pestle homogenizer. Homogenates were centrifuged at 14,000g for 20 min at 4°C, and the resulting supernatants were removed and stored at -80°C until use. All samples were analyzed according to manufactures instructions using a BDNF assay, anti-BDNF sandwich ELISA kit purchased from Promega, WI. For the

BDNF assay, anti-BDNF monoclonal antibody (mAb) was added to a carbonate coating buffer (pH 9.7, per specifications included with the Promega protocol for BDNF), and 100 μ l of the coating buffer was added to each well of a 96-well polystyrene ELISA plate (MaxiSorb, Nalge Nunc International, Rochester, NY) and incubated overnight at 4°C. All wells were washed using a TBST wash buffer, incubated at room temperature for 1 hour. The BDNF standard curve was prepared using the BDNF standard supplied from the manufacture (1 μ g/ml). The standard was diluted in Block & Sample 1 \times buffer to achieve a concentration range of 0 to 500 pg/ml. Tissue samples were further diluted 1:2 before being assayed. The standards and samples were incubated with shaking at room temperature for 2 hours. Anti-human BDNF pAB was then added to each well plate, incubated at room temperature (2 h), which was followed by incubation (1 h) with anti-IgY horseradish peroxidase (HRP) conjugate. Visualization was achieved by adding TMB one solution to each well followed by an incubation period of 10 minutes at room temperature. The reaction was stopped by adding 1N hydrochloric acid to each well and plates were read within 30 minutes of stopping reaction. Optical density was measured using a Bio-Tek ELx 800 microplate reader (Winooski, VT).

CHAPTER 3

RESULTS

Behavioral Sensitization: Experiment 1

Horizontal activity counts are presented as a function of day of testing and drug condition in Figure 3. We were unable to find any sex differences, so we collapsed across the factor of sex. A three-way ANOVA (pre-exposure drug treatment, adolescent drug treatment, day of testing) revealed a significant main effect of adolescent drug pre-exposure $F(1,83)= 22.64, p < .001$, a significant interaction of pre-exposure drug treatment x adolescent drug treatment $F(1, 83)=11.73, p < .001$ and a significant interaction of adolescent drug treatment x day of testing $F(1, 83)= F(91.61), p < .011$. On day 1, animals given nicotine demonstrated a significant hypoactive response compared to animals given saline, regardless of adolescent pre-exposure condition. On day 10, animals in the SAL-NIC group demonstrated significantly higher activity counts than all other groups, and there were no significant differences between any of the other groups. Therefore it appears that MPH blunted nicotine sensitization in both males and females.

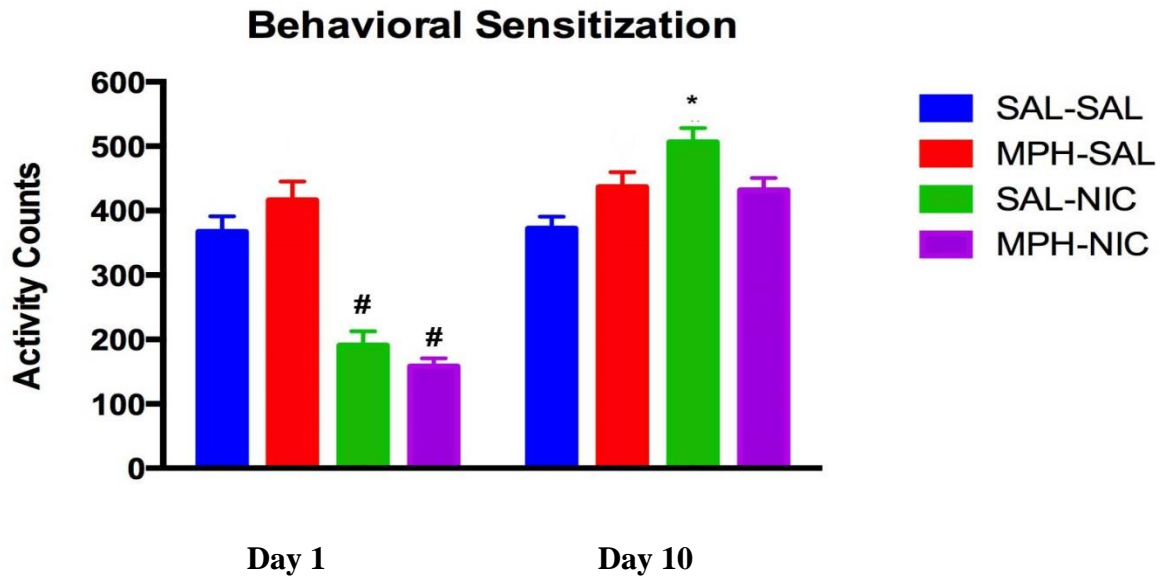


Figure 3. Behavioral Sensitization Experiment 1. Horizontal activity counts are presented as a function of day of testing and drug condition in Experiment 1. The MPH-SAL group demonstrated an increase in overall activity counts as compared to all other groups except the SAL-NIC group. Asterisk (*) indicates group mean was significantly greater than MPH-NIC and saline controls (SAL-SAL), and (#) indicates group mean was significantly lower than as compared to all other groups. All significant effects, $p < .05$.

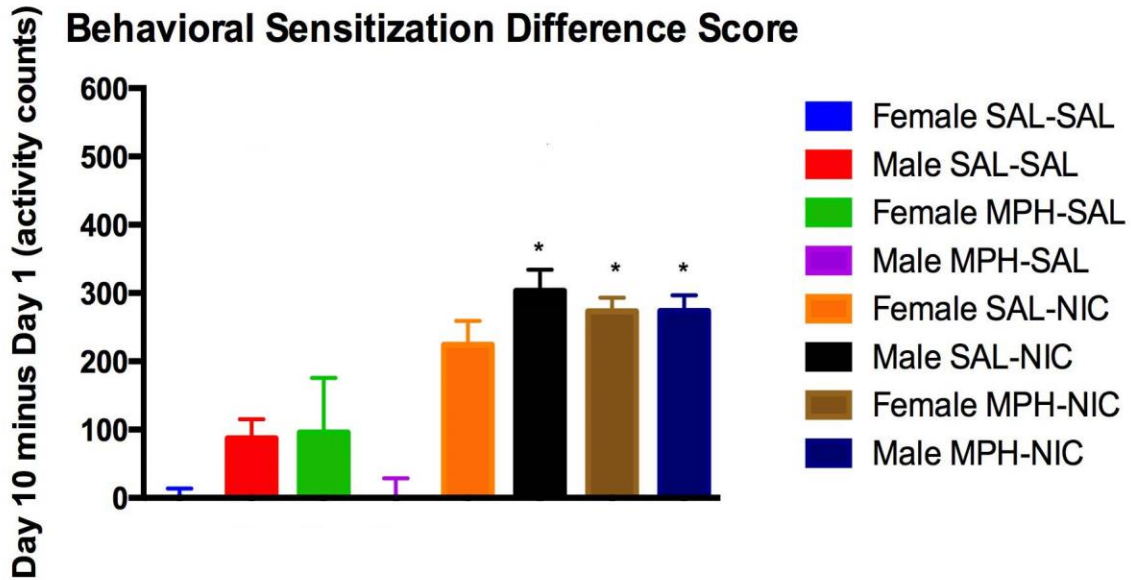


Figure 4. Behavioral Sensitization Difference Score Experiment 1. For the behavioral sensitization difference score, a three-way ANOVA revealed a significant main effect of adolescent drug treatment $F(1,93) = 44.12, p < .001$ and a significant interaction of sex x adolescent drug treatment $F(1,93) = 6.02, p < .016$. Overall, males and females in the MPH-NIC group as well as males in the SAL-NIC group demonstrated significantly higher differences in activity counts between days 1 and 10 than all other groups.

Forced Swim: Experiment 1

Latency to immobility is presented as a function of condition in Figure 5. Again, there was no effect of sex, so this was dropped from the analysis. A two-way ANOVA (pre-exposure adolescent drug treatment, adolescent drug treatment) revealed a significant main effect for adolescent drug pre-exposure $F(1,62)=6.25, p = .015$ and a significant interaction of drug pre-exposure and adolescent drug treatment $F(1,62)=7.47, p = .008$. Animals in the MPH-SAL group demonstrated a significant decrease in latency to immobility as compared to all other groups. This effect appears to show that adolescent pre-exposure to MPH results in an increased learned helplessness response in the forced swim stress response, and this was alleviated by nicotine.

Total immobility time for the Forced Swim Stress test is presented in Figure 6. For total immobility time, a three-way ANOVA (sex, pre-exposure drug treatment, adolescent drug treatment) revealed a significant main effect of sex $F(1, 67)=7.22, p=.009$, a significant two-way interaction of adolescent pre-exposure x adolescent drug treatment $F(1, 67)=5.08, p=.028$, and a significant three way interaction of sex x pre-exposure drug treatment and adolescent drug treatment $F(1, 67)=7.22, p=.009$. The male MPH-SAL group and SAL-NIC were equivalent but demonstrated significantly more immobility time than all other groups. The males pre-exposed to 1 mg/kg of MPH and subsequently treated with nicotine (.5 mg/kg, free base) demonstrated significant decreased latency to immobility as compared to all other groups except the MPH-SAL group. Interestingly, it appears that nicotine appears to have attenuated the stress response to MPH in the male MPH-NIC group.

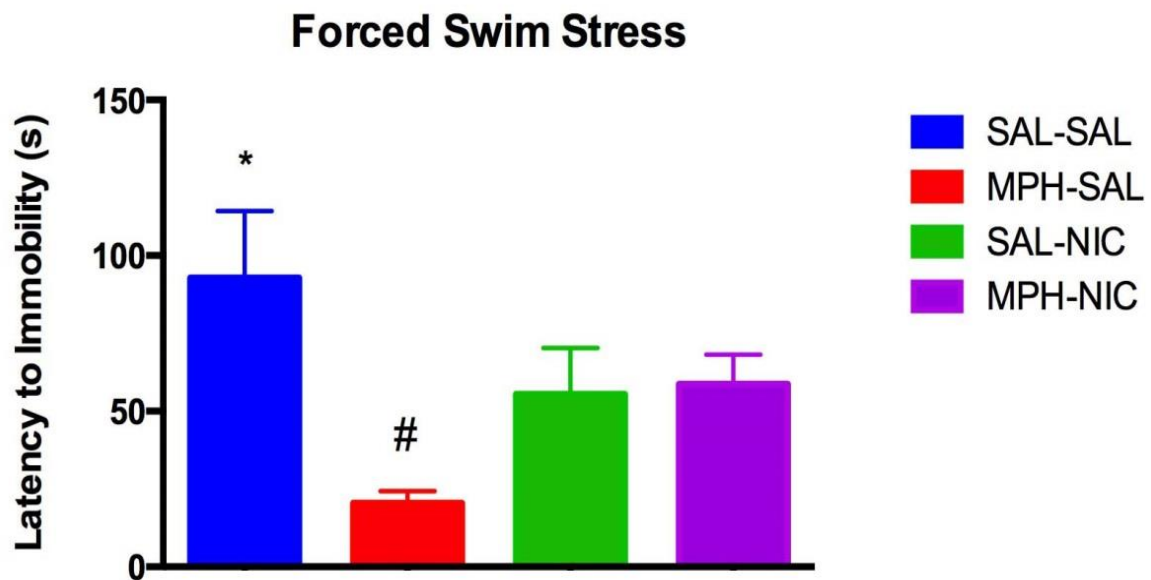


Figure 5. Forced Swim Stress Experiment 1. Latency to immobility is presented as a function of a group for the latency to immobility test in Experiment 2. The animals that were pre-exposed to MPH (1 mg/kg) and subsequent saline treatment demonstrated a significant decrease in latency to immobility as compared to all other groups. Asterisk (*) indicates group mean was significantly greater than all other groups. All significant effects, $p < .05$.

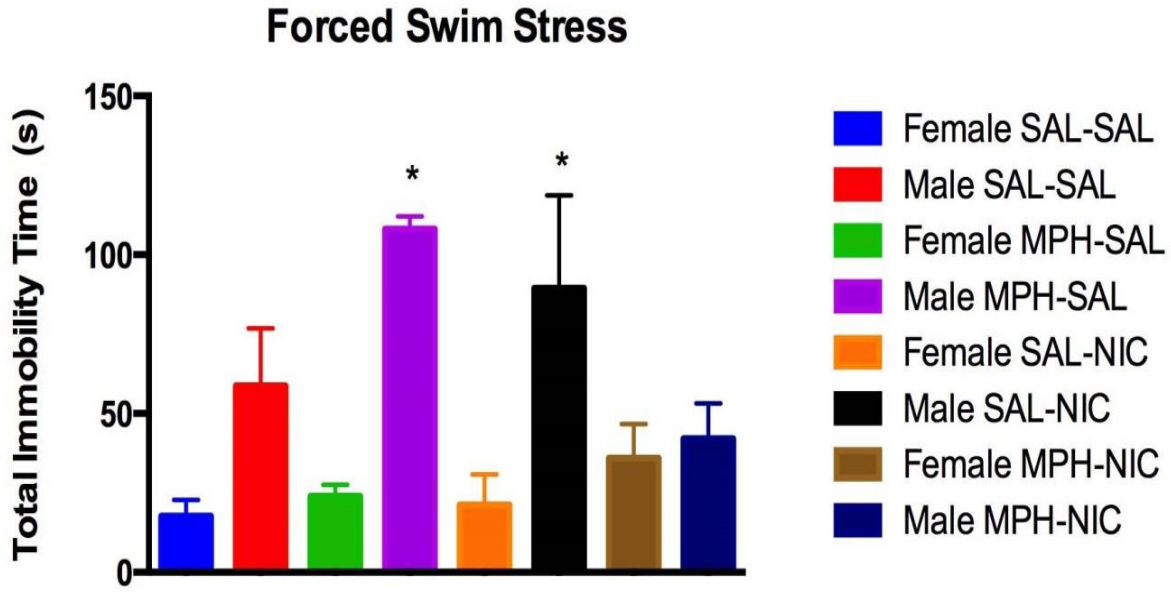


Figure 6. Forced Swim Stress Experiment 1. Forced swim test is presented as a function of condition in Experiment 2. Adolescent males in the MPH-SAL group significantly decreased latency to immobility than all other groups. The males in the MPH-NIC group demonstrated a significant decreased latency to immobility than all other groups except the males in the MPH – SAL group. Asterisk (*) indicates group mean was significantly greater than all other groups. All significant effects, $p < .05$.

Conditioned Place Preference: Experiment 2

The difference in percentage time spent in the paired context on the pre-and post-conditioning test is presented as a function of condition of Figure 7. A three-way ANOVA (sex, pre-exposure adolescent drug treatment, adolescent drug treatment) of the ratio of time spent in the post- versus pre-conditioned preference test revealed a significant main effect of adolescent drug treatment $F(1, 71)=5.21, p < .001$ and a significant interaction of drug pre-exposure x adolescent drug treatment x sex $F(1, 71)=5.12, p < .05$. Females in the MPH-NIC group demonstrated a significantly higher CPP than all other groups. Males in the SAL-NIC group demonstrated a significantly higher CPP than all other groups except females in the MPH-NIC

group, and males that were pre-exposed to 1 mg/kg of MPH and adolescent nicotine treatment demonstrated a significantly higher preference ratio than all other groups except females in the MPH-NIC group and males in the SAL-NIC group. These results demonstrate that a clinically relevant dose of MPH (1 mg/kg) and adolescent nicotine (.5 mg/kg, free base) treatment was sufficient to increase preference in both adolescent male and female rats, as well as an increase in preference in the male SAL-NIC group.

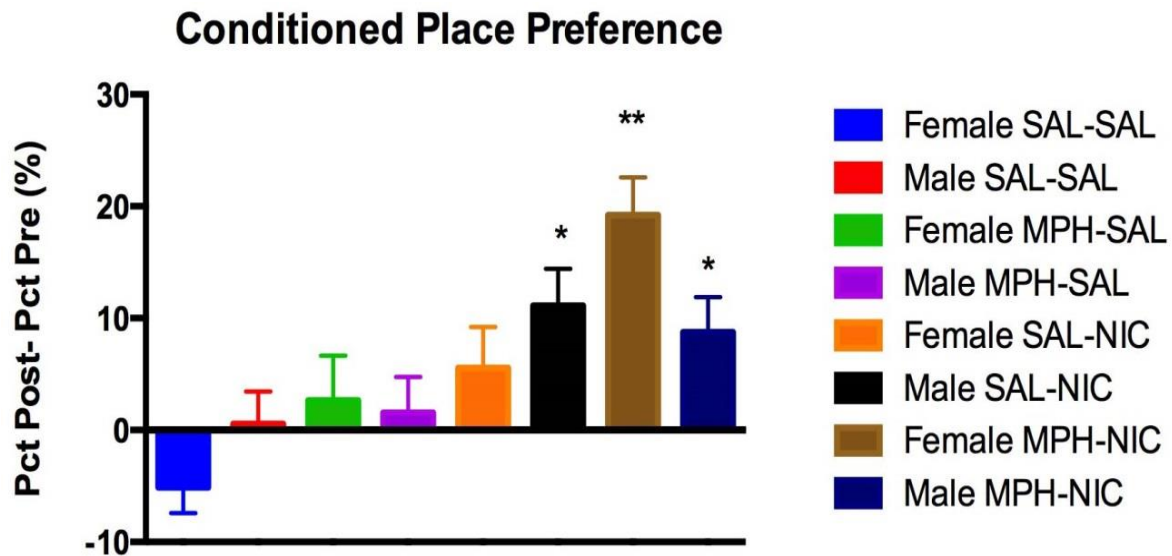


Figure 7. Conditioned Place Preference Experiment 2. The difference in percent time spent in the paired context on the pre-and post-conditioning test is presented as a function of a group for the post-conditioning preference test in Experiment 3. Males that were pre-exposed to saline and adolescent nicotine (.5 mg/kg, free base) treatment demonstrated an enhanced preference for the paired context compared to all groups except the females in the MPH-NIC group. The males that were pre-exposed to 1 mg/kg of MPH and adolescent nicotine (.5 mg/kg) treatment demonstrated an enhanced preference for the paired context compared to all other groups except the females in the MPH-NIC group and males in the SAL-NIC group. Females in the pre-exposed to MPH (1 mg/kg) group and adolescent nicotine (.5 mg/kg, free base) treatment demonstrated an enhanced preference for the paired context compared to all other groups. Asterisk (*) indicates group mean was significantly greater all other groups.

BDNF Analysis: Experiment 2

BDNF protein content is presented as a function of drug treatment group for the nucleus accumbens (Figure 8) and the dorsal hippocampus (Figure 9). We randomly selected 5-6 tissue samples per group for these analyses because we have observed in past analyses that this number per group provides sufficient statistical power. For the nucleus accumbens, a three-way ANOVA (sex, pre-exposure drug treatment, adolescent drug treatment) revealed a significant main effect of adolescent drug treatment $F(1, 35)=7.42, p < .01$, and a significant three-way interaction of pre-exposure drug treatment x adolescent drug treatment x sex $F(1, 67)=6.9, p < .01$. MPH enhanced the accumbal BDNF response to nicotine, and in the female MPH-NIC group relative to all other groups. In addition, nicotine also produced an increase in BDNF in both females and males compared to their saline controls. It appears that MPH adolescence pre-exposure sensitizes the BDNF response to nicotine. For the dorsal hippocampus, a three-way ANOVA (sex, pre-exposure drug treatment, adolescent drug treatment) revealed no significant interactions between groups.

Nucleus Accumbens BDNF

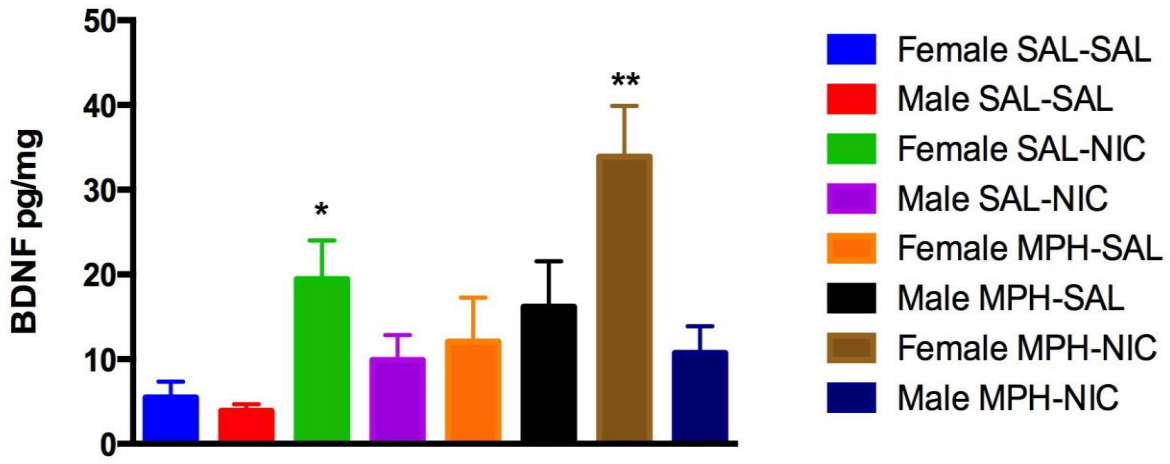


Figure 8. Nucleus Accumbens BDNF Experiment 2. BDNF is presented as a function of drug condition in the nucleus accumbens. Females in the SAL-NIC group demonstrated a significant increase in acumbal BDNF (indicated by *) compared to all other groups except the female MPH-NIC group. Females in the pre-exposed drug treatment and adolescent drug treatment group demonstrated a significant increase in accumbal BDNF (indicated by *) as compared to all other groups.

Hippocampal BDNF

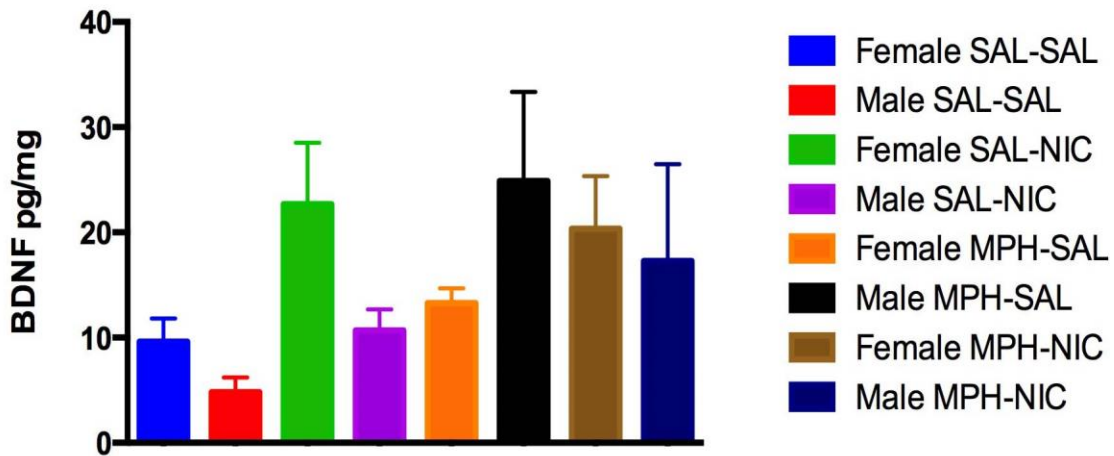


Figure 9. Hippocampal BDNF Experiment 2. BDNF is presented as a function of drug condition in the dorsal hippocampus. There were no significant differences in hippocampal BDNF.

CHAPTER 4

DISCUSSION

The results of the present study report several important findings relative to adolescent MPH treatment and its synergistic effects with nicotine, and are consistent with past work on the long-term behavioral and neurobiological adaptations of MPH. First, all three experiments support existing research that MPH results in changes in the brain's reward system that are likely manifested in changes of locomotor behavior, reward, and increased anxiety/depressive-like behavior (Anderson et al., 2002; Brandon et al., 2001). Second, this study demonstrated that MPH (1 mg/kg) pre-exposure during adolescence blunted nicotine (.5 mg/kg free base) sensitization in both males and females, and resulted in a slight but significant increase in locomotor behavior that persisted throughout testing. Regarding CPP, the effects were more robust, and MPH enhanced the conditioned rewarding effects of nicotine in adolescent females, but not in adolescent males. Further, MPH given through the adolescent period appears to have sensitized the brain-derived neurotrophic factor (BDNF) response to nicotine in the nucleus accumbens in females but had no effect on dorsal hippocampal BDNF. Most interestingly, in females, MPH appears to have sensitized the BDNF response to nicotine, as females administered MPH and nicotine demonstrated a robust increase of accumbal BDNF compared to all other groups.

DAT

Relevant to this study, MPH (2 mg/kg) has been shown to produce a significant decrease of dopamine transporter (DAT) protein in both younger animals (Moll et al., 2001) and in adults (Izenwasser, 1999) and reverse the increase in striatal DAT in an animal model of ADHD (Roessner et al., 2010). Moreover, our laboratory has demonstrated that MPH (5 mg/kg) resulted

in a significant decrease of striatal and accumbal (DAT) density in both adolescent male and female rats (Cummins et al., 2013). Thus, the reduction of availability of DAT in those brain areas that are associated with reward may contribute to the behavioral effects observed in the present study. Nicotine results in an increase of dopamine release in the nucleus accumbens via agonist action at presynaptically located nicotinic receptors (NACHRs). Therefore, the decrease of DAT produced by MPH would result in less dopamine clearance from the synapse, and in combination with increased dopamine release from nicotine, dopamine would be able to continue to bind to postsynaptic dopamine receptors, increasing overall dopaminergic activity.

BDNF

In addition, we observed a significant increase in BDNF in the nucleus accumbens. The nucleus accumbens is the location of dopamine terminals projecting from the midbrain ventral tegmental area (VTA) and BDNF is a cell activity dependent neurotrophin (Boulle et al., 2012). These results suggest increased dopaminergic activity leading to increases in BDNF, and increases in BDNF presumably results in increased synaptic connectivity. Past work has demonstrated BDNF's involvement in the processes of addiction, craving, and withdrawal (Grimm et al., 2003; Horger et al., 1999; Pu et al., 2006). Thus, in the present study, it appears that MPH adolescent exposure likely augments nicotine induced increases in dopamine in the nucleus accumbens that is manifested in an increased rewarding associative response to nicotine in CPP. Future work will hopefully verify this hypothesis.

MPH Pre-Exposure

This study analyzed the effects of a clinically relevant dose of MPH (1 mg/kg) treatment to adolescent male and female rats, a full 14 days before any behavioral testing began. This research design is unique in this literature. Studies in adult rats that had been exposed to repeated

MPH administration during development are inconsistent (see review, Kuczenski & Segal, 2005). For example, Brandon et al. (2001) reported that MPH (2 mg/kg, ip) administration to rats from P35-42 facilitated acquisition of intravenous cocaine self-administration and enhanced psychomotor responses. Similarly, Crawford et al. (2007) have shown that rats treated with MPH in an earlier period (P11-20) displayed increased rewarding effects for morphine or sucrose. In the Crawford et al (2007) study, rats were treated with MPH (2, or 5 mg/kg) once daily from P11-20, and on P60 morphine-induced conditioned place preference or sucrose-reinforced lever pressing was assessed. Those animals that were administered the 5 mg/kg of MPH demonstrated a robust morphine-induced CPP as compared to the other groups, and early MPH exposure also altered nondrug-reinforced behavior. The animals that were administered the 2 and 5 mg/kg of MPH had higher breakpoints than controls when trained on a progressive ratio sucrose-reinforced lever-press task. These findings suggest that MPH exposure affects the neural plasticity of general brain reward circuitry, not just drug responding, and that adolescent MPH treatment may change the reward system in such a way as to be more sensitive to appetitive stimuli.

In contrast, other studies have reported that rats given MPH earlier in life during the weanling period (P11-20) reduced the rewarding effects of cocaine using a conditioned place preference paradigm (Adriani et al., 2006; Andersen et al., 2002; Carlezon et al., 2003) as well as reduced intracranial self-administration of cocaine (Mague et al., 2005). Interestingly, MPH-treated rats (P20-35) have been reported to be less sensitive to natural rewards (e.g. sucrose, novelty and sexual behavior), more sensitive to stressful situations (e.g. elevated t-maze) and more prone to anxiety and depressive-like behaviors (e.g. forced swim test) compare with controls (Bolanos et al., 2003; Carlezon et al., 2003). Therefore, it appears that the

developmental time period in which MPH is administered is critical as to its effects on the plasticity of the brain's reward system and behavioral responses to rewarding stimuli.

Interestingly, we demonstrated that a clinically relevant dose (1 mg/kg) of MPH appears to blunt the locomotor response to nicotine during sensitization induction as well as significant increases in motor activation in the behavioral sensitization test (see figure 3). Therefore, it appears that MPH administered at a clinically relevant dose (1 mg/kg), reduced behavioral sensitization to nicotine in adolescent rats, and no sex differences were discovered. To date, very little studies have examined the interactive effects of MPH and nicotine on behavioral sensitization, and there are no data on sex differences in this effect. This is not consistent with our hypothesis that MPH leads to an increased dopamine response to nicotine, however, it could be that animals demonstrating a blunted sensitization response to nicotine may be exhibiting stereotypic behavior, which is due to an increased dopamine response and often supplants changes in locomotor behavior. Conversely, past findings have shown that rats pre-treated with MPH significantly increased sensitivity to the locomotor activating effects of cocaine (Brandon et al., 2001), and cocaine and nicotine are both psychostimulants and increase dopaminergic activity. It is well-known that nicotine increases dopamine release in the nucleus accumbens and striatum via agonist action at nicotinic receptors located in these regions, and the nucleus accumbens and striatum via agonist action at nicotinic receptors located in these regions, and the nucleus accumbens and striatum have been shown to play a role in psychostimulant behavioral sensitization (Dani & Biasi, 2001; Wonnacott, Sidhpura & Balfour, 2005).

There has been at least one study demonstrating that low-dose MPH (1.5 mg/kg) enhanced the motor activating effects of nicotine (2 mg/kg/d). Wheeler et al (2013) demonstrated an increase in overall distance in adolescent male rats pre-exposed to MPH and nicotine (P35-56).

In contrast, the present study revealed that adolescent pre-exposure to MPH reduced nicotine behavioral sensitization. Importantly, Wheeler et al (2013) administered MPH and nicotine orally, while the present study utilized an ip route of administration. The ip route of administration is similar to the intranasal route in humans, in that it avoids first pass metabolism. Thus, differences in locomotor activity could be attributed to differing routes of administration. In addition, the present study began behavioral testing a full 14 days after animals had already begun adolescent MPH treatment and testing was conducted 6 hours after MPH treatment. This is equivalent to six drug half-lives, since MPH has a half-life in rats of approximately 1 hour. This is important relevant to the present study because repeated administration of MPH will cause more extracellular dopamine to become available at the synaptic cleft and thereby enhance the motor-activation effects of MPH. Thus, increased locomotor activity can be observed in animals that have been chronically administered MPH as demonstrated in the present study.

We also report that adolescent MPH pre-exposure significantly decreased latency to immobility in the forced swim stress behavioral test. This is consistent with studies that have shown that long-term treatment of MPH induces increases in anxiety/depressive-like behaviors (Carlezon et al., 2003). Early (P20-35) exposure of MPH (2 mg/kg) was demonstrated to decrease the latency to immobility in male rats that were pre-exposed to MPH. Specifically; adolescent male rats were ip administered MPH (2 mg/kg) from P20-35, and behavioral testing began at P60. In addition, Warren et al. (2011) demonstrated that low dose MPH (2 mg/kg) administered from P20-34 enhanced susceptibility to swimming stress. Interestingly, rates of anxiety disorders are increased in individuals with ADHD and alcohol/drug addiction as compared with individuals with ADHD only (Wolraich et al., 2005). These results suggest the possibility that long-term MPH treatment in adolescence may lead to co-occurring anxiety

disorders and/or later adult substance use disorder in ADHD adults. Further, ADHD is a disorder that is commonly comorbid with anxiety disorders (Kessler et al., 2006).

This is important because adolescence is a time when more vulnerability exists to external insults (Adriani & Laviola, 2004; for review, Andersen, 2003; Crews et al., 2007). Repeated exposure to MPH, like other psychostimulants, has been shown to induce long-lasting molecular and cellular changes (Adriani et al., 2006; Andersen et al., 2002; Hyman et al., 2006; Torres-Reveron & Dow-Edwards, 2005; Yang et al., 2003) in brain dopaminergic structures. It would seem, a reasonable argument would be that alterations in the mesolimbic dopamine system may be involved in the persistent effects of MPH observed in the present study. Certainly, additional studies are needed to facilitate a better understanding of the cellular and molecular alterations caused by long-term treatment of MPH in adolescents.

MPH Pre-Exposure & Nicotine

There is convincing evidence in the literature that adolescents and adults display differential sensitivity to nicotine, and typically, studies have shown that adolescent rats demonstrate enhanced sensitivity to the behavioral and rewarding effects of nicotine (Adriani et al., 2002; Vastola et al., 2002). In addition, whereas nicotine CPP is typically not achieved in adult rats (Belluzzi et al., 2004; Varlinskaya, & Spear, 2002; Vostola, Douglas), nicotine CPP has been reported several times in adolescent rats (Adriani et al., 2002; Belluzzi, Lee, Oliff, & Leslie, 2004; Vastola et al., 2002). In the current study, results showed that adolescent MPH pre-exposure enhanced the conditioned rewarding effects of nicotine in both males and females. In regards to long-term MPH treatment and reward, Experiment 3 demonstrated that pre-exposure to MPH enhanced nicotine CPP in adolescent male and female rats as compared to controls, and suggests that pre-exposure to MPH enhances the rewarding properties of nicotine. To date, the

use of conditioned place preference to demonstrate the rewarding effects of ip administered nicotine in rodents has yielded ambiguous results.

There has been very little success in demonstrating preference in adult animals using a counterbalanced method for preference of nicotine-paired environments (Calcagnetti & Schechter, 1994; Clarke & Fibiger, 1987; Jorenby et al., 1990), whereas preference for nicotine-paired environments has been reliably demonstrated using a “biased” experimental design (Calcagnetti & Schechter, 1994; Martin & Itzhak, 2000). Here, the current study used a conditioned place preference paradigm in which animals were conditioned against their natural preference, albeit in a three chamber conditioned place apparatus, in which the animal was randomly assigned a context with no initial preference shown. What was particularly interesting was the robustness of nicotine CPP in MPH pre-exposed rats, is that females demonstrated a 20% increase in time spent in the paired context on the post-conditioning test as compared to the paired context on the pre-conditioning test, and this group was significantly greater than all other groups (see Figure 6). Females pre-exposed to MPH and conditioned with nicotine demonstrated a robust increase of BDNF in the nucleus accumbens, consistent with this effect (see Figure 6). The nucleus accumbens, which plays a primary role in CPP (Di Chiara et al., 2004) may be sensitized in its response to nicotine presumably through increases in the development of dopaminergic synaptic activity in this region produced by MPH.

Adolescence & BDNF

Of importance during adolescence, many aspects of development, including stimulation of growth, differentiation of neuronal stems cells into neurons and promotion of newly generated cells and synaptogenesis survivability are influenced by BDNF (Mattson et al., 2004; Tapiarancibia et al., 2004). BDNF, during the aging process, has been suggested to play a protective

role by preventing neurodegeneration, stimulating sprouting and synaptic reorganization in the hippocampus (Smith, 1996; Tapia-Arancibia et al., 2004), or by encouraging neuronal repair (Smith et al., 1995). Interestingly, MPH has also been used as a treatment in traumatic brain injury (Klein et al., 2000), and possibly through increases in BDNF this could be a positive effect.

However, BDNF within the mesocorticolimbic dopamine system is a positive modulator of psychostimulant reward (for review, Ghitza et al., 2009). Most relevant to the present study, Fumagalli et al (2010) demonstrated an increase in BDNF mRNA in the nucleus accumbens and striatum when MPH (1 mg/kg) was administered twice daily from P29-42 and, after an 18 hour drug washout. We demonstrated a significant increase of BDNF in the nucleus accumbens in adolescent female rats pre-exposed to MPH. Multiple lines of evidence suggest that BDNF is involved in processes of addiction, craving and withdrawal, as most psychostimulant drugs including cocaine, amphetamine, methamphetamine, and nicotine all increase BDNF in brain areas of drug reward (Grimm et al., 2003; Horger et al., 1999; Pu et al., 2006). Specific to MPH, our lab has demonstrated a significant increase in BDNF in both the nucleus accumbens and dorsal striatum following a 5 mg/kg MPH administration (Brown et al., 2011). In addition, across several studies we and others have reported that nicotine also results in increases in BDNF in the striatum and nucleus accumbens (Brown et al., 2011; Kenny et al., 2000; Klein-Schwartz & McGrath, 2003; Kosowski & Liljequist, 2005). Thus, there appears to be an association between multiple exposures to nicotine and alterations in BDNF expression level (Andersen et al., 2009; Bhang et al., 2010; Kenny et al., 2000).

Regarding the hippocampus, Scherer et al (2010) as well as Fumagalli et al (2010) reported that BDNF levels in the hippocampus were unchanged after ip administration of MPH (1 or 2

mg/kg), compared to controls which is consistent with the present work. However, Kim et al (2011) showed that BDNF levels decreased in the hippocampus of the spontaneously hypertensive rat, and that 1 mg/kg MPH treatment for 28 consecutive days restored the reduction of BDNF. Past studies have demonstrated increases in BDNF in the hippocampus and pre-frontal cortex of male rats administered chronic nicotine (.5 mg/kg i.p.) (Kenny et al., 2000), and long-term nicotine (2. mg/kg) administration in rodents has been shown to lead to increased protein expression of BDNF in the cortex and hippocampus in adult male rats (Czubak et al., 2009). Moreover and important to the present study, several studies have shown that alterations in BDNF expression are indicated in anxiety and depressive-like disorders (for review, Duman, 2014). To date, no study has examined the interaction of long-term MPH treatment and subsequent nicotine administration on the effects of BDNF expression in brain reward areas. Thus, the results of the present study suggest that increases in BDNF reinforce the behavioral activating effects of nicotine and affect the response to an acute stressor via activation of the dopamine system. Specifically, the interaction of the different mechanism of MPH and nicotine increase dopamine, and highly elevated dopamine levels are associated with addiction, stress, and paranoia (Gerasimov et al., 2000).

Sex Differences

Sex Differences and the Response to Psychostimulants

A considerable number of studies have reported sex differences in the acute behavioral and neurobiological response to psychostimulants, amphetamine (Becker, 1999), cocaine (Bowman et al., 1999; van Haaren & Meyer, 1991), and methamphetamine (Schindler et al., 2002). Several studies have demonstrated that a 3 mg/kg sub-chronic dose of MPH in adult females increased conditioned hyperactivity as compared to adult males (Berridge et al., 2006 Devilbiss &

Berridge, 2006; Wooters et al., 2006). Consistent with this, previous research in our laboratory has demonstrated a more robust sensitization to a 5 mg/kg dose of MPH in adolescent female rats as compared to adolescent male rats (Brown et al., 2012; Brown et al., 2014). In addition, it has been demonstrated that a 2.5 and 10 mg/kg dose of MPH (P1-11) in female adolescent rats induced significantly higher locomotor activity counts than adolescent males (Chelaru, Yang, & Dafney, 2012).

Relevant to the current study, it has been demonstrated in adolescent rats that a clinically relevant dose (1 mg/kg) of MPH (Berridge, et al., 2006; Devilbiss & Berridge, 2006; Klein-Schwartz & McGrath, 2003), results in locomotor suppression in both males and females (Brown et al., 2012; Chelaru, Yang & Dafny, 2012). Further, our laboratory has demonstrated that a clinically relevant dose (1 mg/kg) of MPH failed to produce conditioned place preference (CPP) in both male and female adolescent rats (Cummins et al., 2013). Thus, clinically relevant doses of MPH have not been found to be rewarding in animals or in humans (Farone & Buitelaar, 2010; Greenhill, Muniz, & Ball, 2006; Pliska, 2007;), and given support to the notion that MPH, if dosed properly, is not an addictive drug (for review, Leonard, McCartan, White, & King, 2004).

In the present study no sex differences were revealed in behavioral sensitization. However, there were robust sex differences discovered in the effects of MPH pre-exposure on nicotine CPP. Interestingly, the present study demonstrated that females increased time (20%) spent in the paired context as compared to all other groups. However, whereas sex differences have been reported in response to acute and chronic dosing of MPH, it is important to point out that sensitization and conditioned place preference are two different behavioral tasks, with behavioral sensitization testing the activating effects of psychostimulants, whereas CPP is a test of the

associative value tests of drugs. Here, we show that MPH adolescent exposure robustly increased the associative effects of nicotine, and suggests adolescent exposure to MPH could alter the brain's response to nicotine. This is consistent with several studies from the clinical literature (Gehricke et al., 2006; Lambert & Hartsough, 1998; Rush et al., 2005).

Translational Relevance

The findings of the current study demonstrate that pre-exposure to a clinically relevant dose (1 mg/kg) of MPH followed by administering a rewarding dose (.5 mg/kg free base) of nicotine produces changes in the dopamine system during adolescence, which is a critical developmental period. This has the potential to increase sensitivity of the reward system and create vulnerability for substance use disorder in adulthood. Consistent with the present study, there is evidence that MPH may increase the risk of smoking in adolescence (Rush et al., 2005). An increase in sensitization of the dopamine system produced by MPH stimulant medication may enhance the reinforcing effects of nicotine (Brenhouse & Andersen, 2011). From a pharmacological perspective, nicotine and MPH in combination may increase dopamine levels more than either drug alone, and this is presumably the mechanism mediating the enhanced rewarding effects. However, another key element of this synergistic effect is how MPH may be affecting neural plasticity of the brain's reward system to increase its vulnerability.

It is not well understood the mechanism(s) underlying the cellular and molecular changes that occur during the transition from initial drug use to dependence. Among many types of drug-induced adaptations, changes in BDNF are known to alter the function of neurons within the VTA-NAc pathway and other reward regions to regulate the motivation to take drugs (Bolanos & Nestler, 2004). Consistent with this, several studies have reported increases in BDNF in the NAc, VTA, and pre-frontal cortex following chronic stimulant administration (Grimm et al., 2003; Le

Foll et al., 2005). In the present study, MPH induced increases in BDNF were observed in the nucleus accumbens and dorsal hippocampus. Indeed, the present findings suggest that alterations in BDNF as demonstrated in the present study, during a critical developmental period of adolescence, may contribute to behavioral and molecular adaptations that reflect dramatic changes in the function of the brain reward systems that endure into adulthood, and some of those changes appear to be consistent with increases in anxiety and depressive-like behaviors.

A number of studies have shown that a combination of drugs changes the neurochemical behavioral profile observed as compared to one drug administered on its own. For example, combining psychostimulants and other drugs of abuse has been shown to result in enhanced locomotor activating and rewarding effects in rodents. Therefore, MPH pre-exposure leading to enhancing the rewarding value of nicotine in CPP suggests that adolescent MPH pre-exposure could enhance the addictive properties of nicotine. While the mechanism(s) underlying MPH and nicotine in combination remains unknown, the current study adds critical information to the profile of MPH treatment and changes in the dopamine system and mechanisms of neuroplasticity underlying changes that have the potential to increase vulnerability for later substance use disorder in adulthood. Furthermore, results show here that adolescent pre-exposure to MPH resulted in an increased stress response in the forced swim task that was alleviated by nicotine. This is consistent with the idea that nicotine and other psychostimulants are often comorbid with behavioral disorders, and can be used as a form of “self-medication”. The fact that adolescent pre-exposure to MPH is enhancing the rewarding properties of nicotine certainly implies that MPH may have deleterious effects on the reward system and lead to smoking addiction in cases of comorbidity.

Conclusions

The aim of this study was to determine the effects of pre-exposure of a clinically relevant dose (1 mg/kg) of MPH on the behavioral effects of nicotine (.5 mg/kg free base) using a behavioral sensitization, forced swim test, conditioned place paradigm in adolescent rats, and analyze the effects on the interaction of MPH and nicotine on the expression of BDNF. Results generally found that adolescent pre-exposure to MPH (1 mg/kg) resulted in a reduction of nicotine behavioral sensitization, significantly decreased latency to immobility, and enhanced nicotine conditioned place preference in both adolescent male and female rats. Adolescent pre-exposure to MPH (1 mg/kg) also resulted in a significant increased BDNF response to nicotine in the nucleus accumbens in adolescent females.

Importantly, findings from this series of studies have important implications relative to long term MPH use and increased risk of tobacco dependence in both ADHD and non-ADHD adolescents. The fact that MPH pre-exposure in adolescence may lead to increased accumbal dopamine response to nicotine suggests that MPH may enhance the rewarding effects of nicotine, and thus presumably MPH could enhance the addictive properties of nicotine. However, MPH adolescent pre-exposure did not enhance behavioral sensitization, demonstrating this effect is only specific to certain behavioral properties of nicotine, and this result, although interesting, is difficult to explain but may be related to very specific effects of MPH on the plasticity of the dopamine reward system. MPH is the most commonly prescribed stimulant to treat the symptoms of ADHD in adolescents in clinical settings and adolescents diagnosed with ADHD report smoking cigarettes at a far greater rate than non-ADHD adolescents, and are at increased risk for subsequent substance use disorder (Mannuzza et al., 2003, 2008). Further, many adolescents are misdiagnosed with ADHD and subsequently prescribed stimulant medication to

treat the symptomology of ADHD (Mannuzza et al., 2008). These findings support further studies designed to gain understanding of chronic MPH treatment during the very critical developmental period of adolescence. In addition, the present may offer a crucial step towards the development of therapies with fewer adverse side effects and possible implication towards teen smoking in both ADHD and non-ADHD adolescents.

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