Methylphenidate Conditioned Place Preference in Juvenile and Adolescent Male and Female Rats

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Methylphenidate Conditioned Place Preference in Juvenile and Adolescent Male and Female Rats

A thesis

presented to

the faculty of the Department of Psychology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Masters of Arts in Psychology with a concentration in Experimental Psychology

by

Elizabeth D. Freeman

December 2013

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Keywords: Methylphenidate, Ritalin, Sex Differences, Conditioned Place Preference, Rat, Dopamine Transporter
ABSTRACT

Methylphenidate Conditioned Place Preference in Juvenile and Adolescent Male and Female Rats

by

Elizabeth D. Freeman

This investigation was an analysis of the effects of methylphenidate (MPH; trade name: Ritalin) on drug reward using the conditioned place preference (CPP) behavioral paradigm in a rodent model and underlying mechanisms of this effect. Animals were conditioned in adolescence from postnatal day (P)33-39 or P44-49 with saline, 1 or 5 mg/kg MPH. Rats administered 5 mg/kg but not 1 mg/kg MPH, resulted in a significant preference that was more robust in younger male adolescent rats. The 5 mg/kg dose of MPH also resulted in a significant decrease of the dopamine transporter in both the nucleus accumbens and striatum, revealing dopamine clearance is decreased by MPH in brain areas that mediate reward. Finally, MPH-induced CPP was blocked by the dopamine D1 but not D2 antagonist, demonstrating the importance of the D1 receptor in the rewarding effects of MPH. These results demonstrate that dopamine mediates the rewarding effects of MPH in adolescence.
DEDICATION

This thesis is dedicated to my mentor Dr. Russell Brown and the Brown lab crew, specifically Ross Roeding, Blake Griffin, and Kate Burgess. Thank you all for all the endless hours you put into this study. Further, this thesis is dedicated to my husband Carlos and my son Jarrod. I give my deepest expression of love and appreciation for the encouragement that you gave and the sacrifices you made during the many challenges of graduate school and life. Thank you for the support and company during many late nights of work.

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

-Thomas A. Edison
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>ABSTRACT</strong></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>DEDICATION</strong></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>LIST OF FIGURES</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>Chapter</strong></td>
<td><strong>INTRODUCTION</strong></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Abuse Liability</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>The Dopamine System</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Dopamine Dysregulation and ADHD</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Mechanisms of MPH</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Effect of MPH on Reward</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Sex Differences and the Response to Psychostimulants</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Sex Differences and MPH in Animal Models</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Sex Differences and the Dopaminergic System</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>MPH Effect on DAT</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>The Adolescent Rat</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Conditioned Place Preference</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Research Questions Addressed in this Thesis</td>
<td>21</td>
</tr>
<tr>
<td><strong>Chapter</strong></td>
<td><strong>METHODS</strong></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Subjects</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Apparatus</td>
<td>24</td>
</tr>
</tbody>
</table>
3. RESULTS ........................................................................................................................................33
   Pre Conditioning Preference Test Experiment 1..........................................................33
   Post Conditioning Preference Test Experiment 1......................................................33
   Paired Context Distance Experiment 1........................................................................34
   Entries to Paired Context Experiment 1........................................................................35
   DAT Protein Experiment 1............................................................................................36
   Post Conditioning Preference Test Experiment 2......................................................37
   Entries to Paired Context Experiment 2........................................................................38
   Paired Context Distance Experiment 2........................................................................39
   Post Conditioning Preference Test D1 Experiment..................................................40
   Paired Context Distance D1 Experiment 3.................................................................41
   Entries to Paired Context D1 Experiment 3...............................................................42
   Post Conditioning Preference Test D2 Experiment 3................................................42
   Paired Context Distance D2 Experiment 3.................................................................43
   Entries to Paired Context Distance D2 Experiment 3................................................44
   Sex Differences in Response to D1/D2 Antagonists.................................................44

4. DISCUSSION .........................................................................................................................46
   DAT ........................................................................................................................................49
   Sex Differences ....................................................................................................................50
   Sex Difference and Response to Psychostimulants .................................................50
   Dopamine Receptor Density in Adolescence ..........................................................51
   Dopamine D1 Receptor .................................................................................................52
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Conditioned Place Preference Apparatus</td>
<td>25</td>
</tr>
<tr>
<td>2. Conditioned Place Preference Apparatus</td>
<td>26</td>
</tr>
<tr>
<td>3. Pre Conditioning Preference Experiment 1</td>
<td>33</td>
</tr>
<tr>
<td>4. Post Conditioning Preference Experiment 1</td>
<td>34</td>
</tr>
<tr>
<td>5. Paired Context Distance Experiment 1</td>
<td>35</td>
</tr>
<tr>
<td>6. Entries to Paired Context Experiment 1</td>
<td>36</td>
</tr>
<tr>
<td>7. DAT Protein Experiment 1</td>
<td>37</td>
</tr>
<tr>
<td>8. Post Conditioning Preference Ratio Experiment 2</td>
<td>38</td>
</tr>
<tr>
<td>9. Entries to Paired Context Experiment 2</td>
<td>38</td>
</tr>
<tr>
<td>10. Paired Context Distance Experiment 2</td>
<td>39</td>
</tr>
<tr>
<td>11. Post Conditioning Preference D1 Experiment 3</td>
<td>40</td>
</tr>
<tr>
<td>12. Paired Context Distance D1 Experiment 3</td>
<td>40</td>
</tr>
<tr>
<td>13. Entries to Paired Context D1 Experiment 3</td>
<td>41</td>
</tr>
<tr>
<td>14. Post Conditioning Preference Ratio D2 Experiment 3</td>
<td>41</td>
</tr>
<tr>
<td>15. Paired Context Distance D2 Experiment 3</td>
<td>42</td>
</tr>
<tr>
<td>16. Entries to Paired Context D2 Experiment 3</td>
<td>42</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Methylphenidate (MPH) is a psychostimulant that has been used in the clinical treatment of major depression, neurodegenerative disorders, cognitive enhancement in patients with brain tumors, HIV disease, fatigue and as a treatment for delirium and sedation associated with opioid use (Prommer, 2012). However, this drug is most frequently prescribed for the management of attention-hyperactivity disorder (ADHD) in children and adolescents (Goldman, Genel, Bezman, & Slanetz, 1998; Pliszka, 2007). In fact, MPH is the first choice of prescribed treatment for children and adolescents who have been diagnosed with this disorder (Dupont, Coleman, Bucher, & Wilford; 2008; Klein-Schwartz & Mcgrath, 2003; Prommer, 2012). Moreover, within the population of children, ADHD is one of the most common disorders, with approximately 3%-10% of school-aged children in the United States that are affected by this diagnosis (Buitelaar, 2002). Worldwide, the estimates for children and adolescents diagnosed with ADHD are approximately 5% (Polanczk, de Lima, Horta, Biederman, & Rohde, 2007).

Diagnosis is reliant on parent and teacher accounts as there is no current laboratory test able to confirm diagnosis (Rowland, Lesesne, & Abramowitz, 2002). Consequently, the incidence of ADHD can be an intricate process because of the subjective nature of parent and teachers account of a child’s behavior. Furthermore, this disorder has a high rate of comorbid diagnosis such as learning disability, conduct disorder and anxiety disorder (Rowland et al., 2002). The behavioral symptoms of these disorders often simulate the same behavioral symptoms of ADHD which can make diagnosis difficult and contribute to over diagnosis.

New diagnosed cases of ADHD have been increasing significantly and synonymously with the usage of prescription stimulants prescribed for treatment (Cox, Motheral, Henderson, &
Mager, 2003; Greenhill, Findling, & Swanson, 2002; Rowe, Robinson, & Gordon, 2005; Safer, Zito, & Fine, 1996; Olfson, Shaffer, Marcus, & Geenberg, 2003). It should be noted that a significant population of children who are diagnosed may not have ADHD (Mayes, Bagwell, & Erkulwater, 2008). Furthermore, prescription rates of MPH tripled during the early 1990s (Safer et al., 1996; Zito et al., 2000). The rise in prescription rate of MPH appeared to coincide with reports that varying dose amounts of MPH elicited tolerance, sensitization, and withdrawal (Dafny & Yang, 2006; Fone & Nutt, 2005). Interestingly, this suggests that MPH can elicit dependence and exhibit a potential for abuse.

Abuse Liability

The potential for abuse and dependence of MPH is alarming considering the rise in the amount of children misdiagnosed and subsequently prescribed a stimulant medication for treatment (Dupont et al., 2008; Klein-Schwartz & Mcgrath, 2003; Prommer, 2012). Evidence suggests that MPH abuse has noticeably increased over the past several years (Brookshire & Jones, 2012; Looby & Earleywine, 2011). In fact, of those persons age 12 and older, 4.2 million have used Ritalin or MPH recreationally at least once in their lifetime (Substance Abuse and Mental Health Services Administration, NSDUH, 2005). Additionally, in 2005 there were 3,212 MPH drug-related hospital emergencies and by 2010 that number has risen to 4,089 (Substance Abuse and Mental Health Services Administration, DAWN, 2010).

There are some factors that appear to contribute to the illicit use of MPH, and route of administration is a factor that appears to play an important role (Dupont et al.; Swanson and Volkow, 2008). Oral administration remains the most common method of administration and typically this has not been correlated with high rates of abuse (Mcgough, et al., 2006). However, Dupont et al. (2008) demonstrated that 86% of those college students who used MPH
recreationally did so by oral and intranasal routes of administration. Past studies have reported higher incidences of MPH abuse through inhalation, with 75% of abusers self-administering MPH through this route (Bright, 2008; Morton & Stockton, 2000). Further research has also suggested that oral administration of MPH may result in reinforcing effects and this effect appears to be completely dose-dependent (Jasinski, 2000; Rush & Baker, 2001), whereas other studies using self-report data have suggested that recreational use is common (Dupont et al., 2008, Teter, McCabe, LaGrange, Cranford, & Boyd, 2006). Finally, past studies have demonstrated that the use of extended-release MPH formulations have aided to eliminate the abuse liability (Kollins, Rush, Pazzaglia, & Ali, 1998; Parasrampuria et al., 2007; Spencer et al., 2006). However, given the route of administration (oral and intranasal) of typical recreational abuse, extended-release formulations of MPH may not negate the abuse liability of MPH.

The most common route of administration for recreational use of MPH is through intranasal administration (Bright, 2008) after several pills having been crushed. Intranasal administration avoids first past liver metabolism and is quickly absorbed into the bloodstream through the soft tissues in the mucous membrane. This allows for faster onset of bioavailability of the drug. Thus, MPH administered via this route negates differences in formulations, and rapid onset of the effects of the drug have been achieved. If the drug is taken through alternative routes of administration, it can reach the brain more rapidly and produce more prominent effects on the reward system (Dupont et al., 2008; Volkow & Swanson, 2003). Studies have shown that MPH bioavailability in the brain is increased in rats using the intraperitoneal (ip) route (Berridge et al., 2006). The ip route is similar in terms of pharmacokinetics to the intranasal route in humans, in that it avoids first pass metabolism. In the current study, rats were administered MPH
using the ip route. This is important, because the focus of the current study is on MPH abuse, and not MPH treatment of ADHD.

*The Dopamine System*

The mesolimbic dopaminergic system is formed by dopaminergic cell bodies within the ventral tegmental area (VTA). The VTA sends a major axonal projection to both the nucleus accumbens and frontal cortex, and this system forms the brain’s reward system. These brain areas also send reciprocal projections back to the VTA. This pathway is also known as the medial forebrain bundle. Dopamine (DA) has been shown to be vital in the rewarding properties of psychostimulants (George, Moal, & Koob, 2012). All addictive drugs have been shown to activate the mesolimbic DA pathway, including cocaine, amphetamine, and methamphetamine. Research has shown that all drugs of abuse increase DA release within this pathway of the brain’s reward system (Volkow et al., 1997).

Dopamine binds to two families of receptors: the D1 and D2. The D1 receptor has two receptor subtypes: the D1 and D5. The D2 receptor family has three receptor subtypes: the D2, D3, and D4. Both of these receptor families are metabotropic G-protein coupled dopamine receptors that negotiate the physiological functions of DA. Behaviorally, DA plays a major role in voluntary movement, reward, hormonal regulation as well as hypertension (Beaulieu & Gainetdinov, 2011). Thus, 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.

Research has demonstrated that dopamine D1 receptors are involved in the development of sensitization to the rewarding properties of psychostimulants (Meririnne, Kankaanpaa, &
Seppala, 2001). For example, it has been shown that the D1 antagonist SCH 23390 prevents self-administration of amphetamine prior to treatment (Pierre & Vezina, 1998) as well as cocaine induced conditioned place preference (Shippenberg, Heidbreder, & Lefevour, 1996). In contrast to D1-antagonism, the D2-antagonist raclopride (RAC) was ineffective in blocking conditioned place preference to cocaine. However, it should be noted that D2-antagonists have prevented development of sensitization to locomotor-stimulating effects of amphetamine and methamphetamine (Kuribara & Uchihashi, 1993; Meng, Feldpaush, & Merchant, 1998). Consequently, there is plausible evidence to suggest that D2 receptors may be involved in the rewarding properties of particular psychostimulants. One focus of the current study was to analyze the role of dopamine receptors on the associative rewarding effects of MPH.

**Dopamine Dysregulation and ADHD**

Research has demonstrated that DA dysregulation is implicated in those individuals diagnosed with ADHD (Swanson, & Volkow, 2008). DA is implicated in the brain as a mediator of reinforcement signals (Carmona et al., 2009) and if ADHD consists of alterations in reward processing, then altered dopamine functioning may trigger symptoms of ADHD (Tripp & Wickens, 2009). Tripp and Wickens (2009) have proposed a theory (dopamine transfer deficit) that suggests some symptoms of ADHD are a direct result of the failure of the transfer of the DA cell response to a cue that predicts reinforcement. Critically, previous imaging work has shown that children diagnosed with ADHD demonstrate a lower DA response in the ventral striatum to stimuli that involved anticipation of reward (Scherers et al., 2010). Therefore, it is hypothesized that there is reduced phasic DA neuronal response in those diagnosed with ADHD and MPH works to normalize this lack of response (Tripp & Wickens, 2008). Thus, it makes sense that the pharmacological properties of psychostimulants clinically prescribed to treat these disorders act
on the mesolimbic DA pathway. However, a variety of addictive drugs such as cocaine, amphetamine, and methamphetamine act directly on this pathway as well. Therefore, delayed reinforcement at the cellular level occurs by a reduction in phasic DA cell response to a cue that predicts reinforcement, thus rendering it ineffective. This would only occur after the positive reinforcer is delivered and would explain the unusual response to delay of reinforcement in children with ADHD versus children (Tripp & Wickens, 2009). Those children who do not have ADHD experience no delay in anticipatory dopamine signaling.

*Mechanism 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 diminishes synaptic clearance of these neurotransmitters, leaving behind high levels of monoamines in the synaptic cleft. This mechanism is similar to cocaine and should be of concern considering cocaine is a drug of abuse that has been shown to have reinforcing effects and is commonly abused (Swanson & Volkow, 2003). 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 (Volkow & Swanson, 2003). Studies have shown that 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). MPH induced increases of dopamine presumably underlies the reinforcing aspects of this drug, although its rewarding effects are dose-dependent (Volkow et al., 1997; Volkow et al., 1999).

*Effect of MPH on Reward*

Previous research has demonstrated that MPH resulted in producing conditioned place preference (Martin-Iverson, Ortmann, & Fibiger, 1985; Mithani, Martin-Iverson, Phillips, &
Fibiger, 1986). Most relevant to our work, Meririnne et al. (2001) demonstrated that doses of MPH ranging from 1.25 to 20 mg/kg produced CPP. Interestingly, a dose of 0.62 mg/kg showed only a trend to preference, and a dose of 0.31 mg/kg did not produce any preference. These two lower doses, when given IP, produce brain concentrations that are similar to therapeutic doses of MPH (Berridge et al., 2006; Devilbiss & Berridge, 2006). It has also been demonstrated that rats will self-administer MPH dose dependently on a fixed ratio (FR1) and progressive ratio (PR) schedules (Botly, Burton, Rizos, & Fletcher, 2008). Interestingly, the effects of the dopamine D1 receptor antagonist SCH 23390 and dopamine D2 receptor antagonist eticlopride at a dose of 0.01 and 0.03 mg/kg increased the number of MPH infusions on FR1 schedule and reduced breaking points on PR schedule. These results demonstrate the rewarding aspects of MPH and further demonstrate that this reinforcing efficacy of MPH is directly mediated by both D1 and D2 receptors.

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), and methamphetamine (Schindler, Bross, & Thorndike, 2002). 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 estrus 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. Frantz and Van Hartesveldt (1999) have shown that 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. Animals 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. A primary focus of the current study is to analyze sex differences in the associative effects of MPH during the adolescent period.

**Sex differences and MPH in Animal Models**

Although there has been relatively little research to analyze the effects of MPH in regards to sex differences in animal models, the few studies completely have generally shown that females demonstrate a more robust behavioral response to MPH in males. Brenhouse, Napierata, Kussmaul, Leussis, and Anderson (2009) reported that juvenile (P20) exposure to MPH (2 mg/kg) twice daily for 15 days resulted in aversions to cocaine-paired environments in young males, but this effect was opposite in females; in fact, juvenile exposure to MPH enhanced place preferences for cocaine-paired environments in female adolescent rats. Wooters, Dwoskin, and Bardo (2006) have shown that adult female rats demonstrated increased conditioned hyperactivity to a 3 or 10 mg/kg dose of MPH as compared to adult male rats. Consistent with this finding, Wagner and colleagues (2007) showed increases in activity in adult females affected by traumatic brain injury (TBI) in response to a 5 mg/kg dose of MPH, but TBI males demonstrated improved cognitive performance in response to MPH as compared to females.
Similarly, we have shown a 100% increase in locomotor activity in adolescent female rats as compared to adolescent male rats in response to a 5 mg/kg dose of MPH that was given every other day from postnatal day (P)33 to 49. In addition, we have also reported that adolescent females demonstrated sensitization to this same dose of MPH, whereas this was not shown in adolescent males (Brown, Hughes, Sheppard, Perna, & Ragsdale, 2012).

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. 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. 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 DA 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 the current study, we analyze the rewarding associative effects of MPH and the sex differences in this effect.

**MPH Effect on DAT**

The primary neurobiological mechanism for MPH is blockade of the DAT, and there have been several studies to analyze the effects of MPH on DAT protein. The DAT mediates the uptake of DA into neurons that is the primary mechanism through which DA is cleared from the synapse. In general, MPH has been shown to produce a significant decrease of DAT protein in both younger animals (Moll, Hause, Ruther, Rothenberger, & Huether, 2001) and in adults (Izenwasser et al., 1999) and reverse the increase in striatal DAT in an animal model of ADHD (Roessner et al., 2010). Further, in adult male rats, in vivo quantification of the DAT using small animal SPECT discovered a dose-dependent decrease of striatal DAT after iv administration of MPH (3 and 10 mg/kg) 2h post drug treatment, but nucleus accumbens was not analyzed (Nikolaus, Antke, Beu, & Muller, 2010). Finally, one study analyzed 0.75 and 1.5 mg/kg MPH given for 7 days reported no changes in the DAT of several brain areas, including the nucleus accumbens shell and core (Bello & Hajnal, 2006). Therefore, although in general it appears that MPH results in a significant decrease of DAT, most of these past studies have analyzed the striatum and there are no data on whether there may be sex differences in this response. The present study analyzed the effects of MPH on the DAT in adolescent rats, and both the dorsal striatum and nucleus accumbens were analyzed.

**The Adolescent Rat**

Before models of drug abuse can be discussed, the adolescent age in a rat must be defined. The age of adolescence in rodents has been debated in the literature and is a difficult period to precisely delineate. In the review by Smith (2003), it was stated that if one takes as boundaries
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, one reaches a conservative conclusion that for dosing to include adolescence, it should include the P28-60 range. In agreement with this age range, both 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. Although this is a fairly wide developmental period, it does include neurobiological changes as well as behaviors associated with adolescence and the transition from adolescence to adulthood. Neurobiologically, the approximate developmental period from P30 to P60 is characterized by steady increases in striatal dopamine and serotonergic transporter levels (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). Play behavior in rats peak between P30 and 40, and social play is more often displayed in adolescence as compared to adulthood (Varlinskaya, Spear, & Spear, 1999). Rats also display peak adrenocorticotropic hormone and corticosterone responses to stress during the adolescent period (Tarazi et al., 1998).

In contrast, Spear (2000) more narrowly defined adolescence in the rat as between the ages of P28 to P42, but this is the age of presexual maturation only and does not extend into postsexual maturation that occurs in adolescence. Both the common 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 (Kennedy, 1967), 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 effects of MPH during an approximate period between the ages of P30-60, 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.

Conditioned Place Preference

The present study is focused on the associative effects of MPH. 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; Tzschtentke, 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 paired 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 time in the drug-paired context as compared to saline controls (for review, see Bardo & Bevins, 2000).

To date there has only been one study that has analyzed the effects of MPH CPP in adolescent rats. Most recently de la Pena et al (2011) administered 1.25, 5 or 20 mg/kg of MPH to adolescent Wistar or Spontaneously Hyperactive rats (SHR) for 14 days from P21-35 before commencing conditioning from P39-45. This study demonstrated that Wistar rats, but not SHR animals, demonstrated CPP to all three doses of MPH.

Research Questions Addressed in this Thesis

The aim of this study was to examine the following:

1) Analyze MPH CPP in older rats using both a clinically relevant dose of MPH (1 mg/kg) and a dose of MPH (5 mg/kg) that is likely to be more relevant to abuse.

MPH CPP was analyzed in older adolescent rats using both a clinically relevant dose (1 mg/kg) and a dose of MPH (5 mg/kg) that is likely to be more relevant to abuse. The focus of this experiment was to compare the effects of a clinically relevant dose of MPH with a presumably abusive dose during a developmental period translatable to the diagnosis of ADHD. In addition, adolescence is a common developmental period when drug abuse initiates.
2) Analyze the effects of a relative high dose of MPH (5 mg/kg) on CPP in adolescent rats with a focus on sex differences and the dopamine system.

It was expected that female rats administered the 5 mg/kg dose of MPH would demonstrate enhanced CPP as compared to male rats given the same dose of MPH. This was based on previous research demonstrating that females show an enhanced behavioral response to psychostimulants as compared to males (Becker, 1999). Further, it was expected that female rats would demonstrate a higher level of activity and entries into the paired context as compared to male rats. This was based on previous research in our laboratory showing enhanced sensitization to MPH in females as compared to males (Brown et al., 2012). The roles of each of the dopamine receptors were analyzed in the effects of MPH on CPP, because the most dynamic changes in dopamine receptors during adolescence occur between the ages of P25 and P40 (see Andersen & Teicher, 2000).

3) Analyze the effect of a relatively high dose of MPH (5 mg/kg) for its effects on the DAT in the dorsal striatum and nucleus accumbens.

It was expected that MPH will produce a significant decrease of the DAT consistent with past literature (Moll et al., 2001), and that there will likely be sex differences in this response based on past data showing that adult female rats have significant decreases if the DAT compared to adult males (Harrod et al., 2004). To date, there have been no studies to analyze sex differences or the role of dopamine receptors in adolescent methylphenidate CPP.

4) The dopamine D1 and D2 receptors were investigated in the neurobehavioral underlying mechanisms of the effects of MPH.
It was expected that the Dopamine D1 receptor antagonist SCH 23390 would block the effects of MPH on CPP equally in both male and female rats, and would likely be more effective that the D2 antagonist eticlopride to block the effects of MPH. Eticlopride was predicted to block the effects of MPH on CPP more effectively in adolescent female rats as males have been reported having higher density of dopamine D2 receptors in striatum during adolescence as compared to females. (Anderson & Teicher, 2000). In order to gain a better understanding of the role of both D1 and D2 receptors, a D1 antagonist (SCH23390) and a D2 antagonist (eticlopride) were used to reduce the functionality of these receptors. Past research has demonstrated the likely probability that those areas in the brain that are dopamine-dense will have increased sensitivity to the addictive properties of MPH (Meredith & Steiner, 2006). Further, past work in our laboratory has shown that female adolescent rats sensitized to MPH, whereas male adolescent rats failed to produce sensitization (Brown et al., 2012). Therefore, in order to fully understand the effects of MPH with both D1 and D2 antagonism this study investigated sex differences with both antagonists.
CHAPTER 2

METHODS

Subjects

A total of 154 Sprague-Dawley rats (77 males, 77 females) were ordered from Harlan Inc. (Indianapolis, IN) and served as subjects in this experiment. All animals were 21 days of age upon arrival (P21), which corresponds to the age at which rats are weaned from the female dam. All animals were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited 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.

Apparatus

A three-chambered CPP box was employed for all experiments. 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 and white vertical or horizontal stripes. The middle compartment is painted solid gray. In addition, each CPP chamber also has different tactile surfaces along the floors of the boxes that help to make the 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.

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-Initial Preference Test](image1)

*Note.* The above picture shows the conditioned place preference boxes without the wooden dividers in.

*Figure 1. Conditioned Place Preference Apparatus-Initial Preference Test*

A photograph of the CPP apparatus without dividers is presented in Figure 2.
Note. The above picture shows dividers in the CPP apparatus for the 5 days of conditioning.

Figure 2. Conditioning Place Preference Apparatus-Conditioning

Procedure

In experiment 1, animals were postnatal day 43 (P43) when behavioral testing began. All animals were given a preference test on P43 to determine if there was an initial context or side preference of each subject. For the initial preference test, all animals were administered an intraperitoneal injection (ip) of saline 10 minutes prior to being placed into the CPP chamber with dividers removed and allowed to freely explore the apparatus. The purpose of this injection was to control for stress of the injection and to provide consistency in injection before each placement into the CPP apparatus. All animal behavior was recorded using Any Maze (Stoelting, Wood Dale, IL) software. An analysis of variance (ANOVA ) was used as the primary statistic with Fishers LSD as the post hoc test (P=.05).

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 MPH was randomized across subjects, and selection of the paired context was balanced across animals
administered saline. In animals given MPH, the paired context was the context that was paired in time with MPH. Controls were given saline in both contexts. In the morning session on conditioning days, all animals were given an ip injection of saline, and 10 minutes later placed into their assigned context for 10 minutes and behavior recorded. In the afternoon session (approximately 4 h later), animals in the MPH group were given an ip injection of MPH(1 m/kg or 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 the initial context preference across groups. Likewise, controls were given an ip injection of saline and placed into their assigned context. Conditioning occurred every consecutive day from P44-48.

The postconditioning test was conducted the day after conditioning was complete on P49. This test was identical to the initial preference test, with dividers removed all animals were ip administered saline to control for stress of injection, and allowed to freely explore the apparatus. For the postconditioning preference test, a preference ratio was used as the dependent measure to analyze preference. The preference ratio was calculated by dividing the time the animal spent in the paired context was by the overall time the animal spent in both the paired and unpaired contexts. This measure has been used in past work to determine preference in a three-chambered CPP apparatus (Gehrke, Harrod, Cass, & Bardo, 2003).

The day after post-conditioning (P50), all animals were sacrificed; however, only saline controls and animals conditioned to the 5 mg/kg dose of MPH were analyzed for DAT. The rationale for analyzing the DAT only in the group conditioned to 5 mg/kg of MPH was that these animals were the only group that demonstrated CPP. Further, the focus of the study was to better understand the rewarding aspects of MPH and not the effects of the clinically relevant dose (1
mg/kg) of MPH on the DAT. On P50, animals were rapidly decapitated, brain tissue removed, and then the dorsal striatum and nucleus accumbens were dissected away and flash frozen in cold (-20°C) isopentane. All tissue was stored in an ultra-low -80°C freezer (So Low Inc, Cincinnati, OH). A Western Blot analysis was used to determine DAT content in the dorsal striatum and nucleus accumbens. It is a widely accepted technique to detect specific proteins in tissue.

Western Blot Procedure

The following primary antibodies were used for the western blot: rabbit anti-DAT at 1:1000 (Sigma D6944), and mouse anti-GAPDH at 1:125,000 (Ambion AM4300). The secondary antibodies were: HRP donkey antirabbit (711-035-152, Jackson Immuno Labs) at 1:10,000 and HRP donkey antimouse (715-035-150, Jackson Immuno Labs) at 1:10,000.

Tissue was prepped in 2% SDS buffer solution with 1:100 phosphatase and protease inhibitor cocktails (Sigma—Aldrich P5726, P0044, and P8340), homogenized by sonication with ~7—10 pulses, allowed to settle, and sonicated again with ~7—10 pulses on ice. Once all samples were homogenized, samples were aliquoted and stored at −80 °C. Protein assays were conducted to determine equal loading with Pierce BCA Protein Assay Kit using a microplate procedure (ThermoScientific, 23255). Protein samples (30 ug protein per lane) were fractioned by SDS-PAGE and transferred to nitrocellulose membranes. Acrylamide gels were stained with Bio-Safe Commassie G-250 Stain (Bio-Rad 161-0786) for 30 minutes to confirm appropriate protein separation. Nitrocellulose membranes were stained with Ponceau S solution (Sigma P7170) to visualize transferred proteins and destained with 0.1 M NaOH.

The membranes were blocked for 1 hour at room temperature in Blotto (5% Carnation dried milk, 0.05% Tween 20 in TBS). Membranes were then incubated with primary antibody overnight (at concentrations listed above) at 4 °C. The next day, membranes were washed with
TBS repeatedly and incubated with the appropriate secondary in Blotto for 1 hour at room temperature. After additional TBS washes, chemiluminescence reagents (GE/Amersham ECL, Arlington Heights, IL) were applied. Membranes and gels were imaged with the Fujifilm LAS-4000 mini system. Western blots were stripped with Pierce Restore Western Blot Stripping Buffer (ThermoScientific 21059) for 30 minutes and reprobed with mouse anti-GAPDH for normalization. Semiquantitative analyses of immunoblots were performed by densiometric quantification with IMAGE J and expressed as DAT/GAPDH.

In Experiment 2, animals were postnatal day 32 (P32) when behavioral testing began. All animals were given their initial preference test on P32 to determine if there was an initial context preference for each subject. For 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 to freely explore the apparatus. All animal behavior was recorded using Any Maze software. Identical to Experiment 1, the ANOVA was used as the primary statistic with Fishers LSD as the post hoc test (P=.05). Although data are not shown, there was no initial context or side preference demonstrated by any group.

Conditioning was identical to Experiment 1, with a few exceptions. Conditioning began the day after the initial preference test on P33, and only one dose of MPH (5 mg/kg) was tested during this experiment. Animals were conditioned every second day and a postconditioning preference test was administered on P43. Thus, animals were tested from P33-43. Conditioning was identical to experiment 1 with the assignment of each paired context randomized across subjects. In animals given MPH, the unpaired context was the context which was not paired in time with MPH, and the paired context was the context that was paired in time with MPH. Controls were given saline in both contexts. On conditioning day, in the morning session all
animals were given an ip injection of saline and 10 minutes later placed into their assigned context for a 10 minute trial. In the afternoon session, animals in the MPH group were given an ip injection of MPH (5 mg/kg) and 10 minutes later, placed into the paired context for a 10 minute trial. The saline group was administered saline and placed into their “paired” context. The choice of the paired context was balanced across animals. Likewise, controls were given an IP injection of saline and placed into their assigned context. Conditioning occurred every second day from P33-41.

Post conditioning test was conducted 2 days after conditioning was complete on P43. A day of nondrug treatment was placed between the last day of conditioning and the postconditioning preference test to be consistent with conditioning. This test was identical to the initial preference test, with dividers removed and all animals were administered saline to control for stress of injection, and allowed to freely explore apparatus. All behaviors were measured by AnyMaze behavioral scanning software (Stoelting, Wood Dale, IL). For the post conditioning preference test, the preference ratio was used identically to Experiment 1.

In Experiment 3, animals were postnatal day 32 (P32) when behavioral testing began. All animals were given a preference test on P32 to determine if there was an initial context or side preference of each subject. For preference testing, all animals were administered an intraperitoneal injection (ip) of saline 15 minutes prior to being administered another ip injection of saline. Ten minutes after the last injection all animals were placed into the CPP chamber with dividers removed, and allowed to freely explore the apparatus for a period of 10 minutes. All animal behavior was recorded using Any Maze software. Identical to Expeirment 1 and 2, the ANOVA was used as the primary statistic with Fishers LSD as the post hoc test (P=.05).
Although data are not shown, there was no initial context or side preference demonstrated by any group.

Conditioning began the day after the initial preference test on P33, and removable dividers were placed into the apparatus. Conditioning was conducted similar to Experiments 1 and 2, but with a few exceptions. First, animals were given a dopamine D1 (SCH 23390) or D2 antagonist (Eticlopride HCl) before ip administration of saline or MPH. Animals were divided into eight separate experimental groups: a low dose eticlopride group (.01 mg/kg), a high dose eticlopride group (.03 mg/kg), MPH (5 mg/kg) group, saline control group, a low dose SCH 23390 (.01 mg/kg), a high dose SCH 23390 group (.03 mg/kg), MPH (5 mg/kg) group and saline control group. As in Experiments 1 and 2, the assignment of each context was randomized across subjects. In animals given eticlopride, SCH23390 and MPH, the unpaired context was the context in which animals were given saline, and the paired context was the context that was paired in time with the antagonist or MPH. Controls were given saline in both contexts. On conditioning day, in the morning session and all animals were given an ip injection of saline and 10 minutes later placed into their assigned context for a 10 minute trial. In the afternoon session, animals in the eticlopride, SCH23390 and MPH groups were given an ip injection of saline and 15 minutes later, placed into the paired context for a 10 minute trial. The choice of the paired context was balanced across animals. Likewise, controls were given an ip injection of saline followed 15 minutes later by another injection of saline and placed into their assigned context. Conditioning occurred every consecutive day from P33-37.

In the afternoon, the experimental groups were administered either eticlopride (.01 or.03 mg/kg), SCH23390 (0.1/ or 0.2 mg/kg) followed 15 minutes later by MPH (5 mg/kg), whereas the MPH (5 mg/kg) group was first given an IP injection dose of saline followed 15 minutes
later by MPH (5 mg/kg). The control groups were administered an ip injection of only saline for both injections. Approximately 10 minutes after the animal was administered either the MPH (5 mg/kg) injection (experimental group) or saline injection (controls), animals were placed into their respective conditioning contexts for a period of ten minutes. Doses of the antagonist compounds were based on past literature (Anderson & Teicher, 2000; Meririnne et al., 2001).

The postconditioning test was conducted the day after conditioning was conducted on P38. This test was identical to the initial preference test, with dividers removed all animals were administered saline followed 15 minutes later by another injection of saline and 10 minutes later placed into the CPP apparatus, and allowed to freely explore the apparatus. All behaviors were measured by AnyMaze behavioral scanning software (Stoelting, Wood Dale, IL). For the postconditioning preference test, the preference ratio was used identically to Experiment 1 & 2.
CHAPTER 3
RESULTS

Pre Conditioning Preference Test Experiment 1

Preference ratio is presented as a function of condition in Figure 3. A two-way ANOVA (sex, drug) revealed no significant main effects or interaction.

![Figure 3](image_url)

*Figure 3.* Time in paired context is presented as a function of group for initial preference test in Experiment 1. There were no significant main effects or interactions.

Post Conditioned Preference Test Experiment 1

Preference ratio is presented as a function of condition in Figure 4. A two-way ANOVA (sex, drug) of the preference ratio revealed a significant main effect of adolescent drug treatment $F(2,47)= 6.15, p <.005,$ but no significant main effect of sex nor an interaction of sex x adolescent drug treatment. Animals conditioned with 5 mg/kg dose of MPH showed a significantly higher preference ratio as compared to animals conditioned with 1 mg/kg dose of MPH and saline controls. Animals conditioned with 1 mg/kg dose of MPH did not show a significant difference from saline controls. The results demonstrate that a 5 mg/kg MPH was...
sufficient to increase preference in both adolescent male and female rats, but there were no sex differences revealed.

*Figure 4.* Preference ratio is presented as a function of a group for the post-conditioning preference test in Experiment 1. Groups that were conditioned with 5 mg/kg demonstrated an enhanced preference for the paired context compared to all other groups, and there were no sex differences. Asterisk (*) indicates group mean was significantly greater than groups conditioned with 1 mg/kg MPH and saline controls. All significant effects, p < .05.

*Paired Context Distance Test Experiment 1*

Paired context is presented as a function of condition in Figure 5. A two-way ANOVA revealed a significant main effect of sex F (1,47) = 4.44, p < .041, adolescent drug treatment F(2,47) = 21.82, p < .01, and significant interaction of sex x adolescent drug treatment F(2,47) = 6.71, p < .003. Females conditioned with 5 mg/kg of MPH demonstrated significantly higher levels of distance traveled in the paired contexts as compared to all other groups. Males conditioned to 5 mg/kg MPH showed a significant increase in distance traveled in the paired context as compared to female saline controls and males administered 1 mg/kg of MPH.
Interestingly, males conditioned to 1 mg/kg of MPH demonstrated less distance traveled in the paired context as compared to all other groups with the exception of the female saline group.

Figure 5. Distance in the paired context is presented as a function of group for the post conditioning preference test in experiment 1. Adolescent female rats conditioned with 5 mg/kg MPH traveled a longer distance than all other groups. Adolescent male rats conditioned with 5 mg/kg MPH traveled a longer distance than animals conditioned with 1 mg/kg or controls.

Entries to Paired Context Experiment 1

Entries to the paired context are presented as a function of condition in Figure 6. A two-way ANOVA revealed a significant main effect of adolescent drug treatment $F(1,47)=5.47$, $p<.008$. Animals conditioned to 5 mg/kg of MPH demonstrated an increased number of entries into the paired context as compared to all other groups. Animals conditioned to 1 mg/kg of MPH showed no significant difference as compared to saline controls and there were no sex differences.
Figure 6. Number of entries to the paired context is presented as a function of group for the post conditioning preference test in Experiment 1. Groups that were conditioned with 5 mg/kg MPH made more entries to the paired context compared to all other groups, and there were no sex differences. Double asterisk (**) indicates group demonstrated greater distance than all other groups; asterisk (*) indicates group mean was greater than groups conditioned with 1 mg/kg MPH and saline controls. All significant effects, p < .05.

DAT Protein Experiment 1

DAT protein is presented as a function of condition and brain area in Figure 7. A two-way ANOVA for the dorsal striatum revealed a significant main effect of adolescent drug treatment $F(1,34)=6.53$, $p<.016$. There were no significant main effects of sex or a significant main interaction of sex x adolescent drug treatment. There was a significant decrease (45%) in DAT in the dorsal striatum in both males and females. Specifically, the decrease in females was 46.4% and 44.3% in males. A two-way ANOVA for the nucleus accumbens revealed a significant main effect of adolescent drug treatment $F(1,34)=6.16$, $p < .019$. There were no significant main effects of sex or a significant main interaction of sex x adolescent drug treatment. There was a decrease (40.7%) in DAT in the nucleus accumbens in both males and females. Specifically, the decrease in females was 31.5% and 48.2% in males.
Figure 7. The dopamine transporter (DA) is presented as a ratio to total protein for both dorsal striatum and nucleus accumbens. In both brain areas, saline demonstrated a significant increase in DAT compared to groups treated with 5 mg/kg MPH. All significant effects, p < .05.

Post Conditioning Preference Test Experiment 2

Preference ratio is presented as a function of condition in Figure 8. A two-way ANOVA revealed a significant main effect of adolescent drug treatment F(1,31)=10.59, p<.003. There was no significant main effect of sex or sex x adolescent drug treatment. While MPH produced a significant increase in preference ratio in both male and females, no sex differences were found.
Figure 8. Preference ratio is presented as a function of group for the postconditioning preference test in Experiment 2. Groups that were conditioned with 5 mg/kg MPH demonstrated an enhanced preference for the paired context compared to all other groups, and there were no sex differences. Asterisk (*) indicates group mean was significantly greater than saline controls. All significant effects, p < .05.

Entries to Paired Context Experiment 2

Entries to the paired context are presented as a function of condition in Figure 9. A two-way ANOVA revealed no significant main effects or interactions, although the main effect of paired entries approached significance at p = .065.
Figure 9. Number of entries to the paired context is presented as a function of group for the post conditioning preference test. There were no significant main effects or interactions.

Paired Context Distance Experiment 2

Distance traveled in the paired context on the post conditioning test is presented a function of condition in Figure 10. There were no significant main effects or interactions. Interestingly, conditioning every second day appeared to change the associative strength of the context as compared to Experiment 1 according to the paired context distance entries to the paired context measures.
Figure 10. Distance in the paired context is presented as a function of group for the post conditioning preference test in Experiment 2. There were no significant main effects or interactions.

Post Conditioning Preference Test D1 Experiment 3

Post conditioning preference ratio of the D1 antagonist (SCH 23390) is presented as a function of condition in Figure 11. A two-way ANOVA of the preference ratio revealed a significant main effect of adolescent drug treatment $F(2,47=6.15, p<.005$, but no significant main effect of sex nor an interaction of sex x adolescent drug treatment.
Figure 11. A two-way ANOVA of the post-conditioning preference ratio revealed a significant main effect of adolescent drug treatment F(2,47)=6.15, p<.005, but no significant main effect of sex nor an interaction of sex x adolescent drug treatment.

Paired Context Distance D1 Experiment 3

Distance traveled in the paired context on the post-conditioning test is presented a function of condition in Figure 12. A two-way ANOVA revealed a significant effect of sex F(1,47)=4.44, p<.041, adolescent drug treatment F(2,47)=21.82, p<.01, and a significant interaction of sex x adolescent drug treatment F(2,47)=6.71, p<.003.
Figure 12. A two-way ANOVA revealed a significant main effect of sex $F(1,47)=4.44$, $p<.041$, adolescent drug treatment, $F (2,47)= 21.82$, $p < .01$, and a significant interaction of sex x adolescent drug treatment $F(2,47)= 6.71$, $p< .003$.

Entries to Paired Context D1 Experiment 3

Entries to the paired context are presented as a function of condition in Figure 13. A two-way ANOVA revealed a significant main effect of adolescent drug treatment $F(1,47)=5.47$, $p<.008$.

Figure 13. A two-way ANOVA revealed a significant main effect of adolescent drug treatment $F(1,47) =5.47$, $p<.008$ but no effect for sex or interaction of sex x adolescent drug treatment.
Post Conditioning Preference Test D2 Experiment 3

Post-Conditioning preference ratio of the D2 antagonist (eticlopride) is presented as a function of condition in Figure 14. A two-way ANOVA of the preference ratio revealed no significant main effect for adolescent drug treatment, sex or significant interaction of sex x adolescent drug treatment.

Figure 14. A two-way ANOVA revealed no significant main effect for adolescent drug treatment, sex or interaction of sex x adolescent drug treatment.

Paired Context Distance D2 Experiment 3

Distance traveled in the paired context on the post conditioning test is presented a function of condition in Figure 15. A two-way ANOVA revealed a significant main effect of adolescent drug treatment F(5,65)= 2.51, p<.041 but no significant main effect for sex or interaction of sex x adolescent drug treatment.
Figure 15. A two-way ANOVA revealed a significant main effect of adolescent drug treatment \( F(5,65)= 2.51, p<.041 \), but no significant main effect for sex or interaction of sex x adolescent drug treatment.

Entries to Paired Context D2 Experiment 3

Entries to the paired context are presented as a function of condition in Figure 16. A two-way ANOVA revealed a significant main effect of adolescent drug treatment \( F(5,65)= 2.51, p<.041 \), but no significant main effect for sex or interaction of sex x adolescent drug treatment.

Figure 16. A two-way ANOVA revealed a significant main effect of adolescent drug treatment \( F(5,65)= 2.51, p<.041 \), but no significant main effect for sex or interaction of sex x adolescent drug treatment.

Sex Differences in in Response to Dopamine Antagonist D1 & D2
A two-way ANOVA revealed a significant main effect of adolescent drug treatment F(5,73)=9.403, p<.000. There was no significant main effect of sex or sex x adolescent drug treatment. Analysis of the significant effect revealed that MPH-induced CPP was blocked in both males and females by the dopamine D1 antagonist SCH 23390 but not the D2 antagonist eticlopride. Post hoc analysis with Fishers LSD revealed that high dose SCH 23390 blocked CPP in females administered the high dose of SCH 23390 (p < .002) and females administered the low dose of SCH 23390 (P < .004). Further, CPP was also blocked in males administered the high dose of SCH 23390 (p < .000) and low dose males (p < .001). The D2 antagonist eticlopride failed to block CPP in high dose females (p > .05); however, CPP was blocked in low dose females (p < .002). The high dose males (p > .05) and low dose males (p > .05) administered the D2 antagonist eticlopride failed to block CPP. This demonstrates a sensitivity of dopamine receptors in relation to sex differences and highlights the existence of sex differences in dopamine density.
CHAPTER 4
DISCUSSION

The results of the present study report several important findings relative to the rewarding associative effects of MPH and are consistent with past work on the mechanisms of MPH and other psychostimulants. First, all three experiments consistently show that MPH produced a conditioned place preference in adolescent rats. Second, this study demonstrated that the dopamine D1 antagonist (SCH23390) but not the D2 antagonist (eticlopride) blocked MPH-induced CPP, demonstrating the importance of the D1 receptor in the rewarding effects of MPH. Further, these results demonstrate that dopamine mediates the rewarding effects of MPH in adolescence.

The behavioral effects of MPH appear to be dose-dependent. The 1 mg/kg dose of MPH is consistent with the brain concentration of a clinically therapeutic dose (Berridge et al., 2006; Devilbiss & Andrzejewski, 2006), whereas the 5 mg/kg dose of MPH may be more relevant to an abused dose. In experiment 1, both males and females conditioned with the 5 mg/kg dose of MPH produced CPP, whereas animals conditioned to 1 mg/kg dose of MPH did not produce CPP and, thus, did not significantly differ from saline controls. Furthermore, males who were administered the 5 mg/kg dose of MPH demonstrated significant increases in distance traveled in the paired context as compared to males administered 1 mg/kg of MPH and female saline controls. Thus, it appears that a 5 mg/kg dose of MPH is rewarding whereas a 1 mg/kg dose of MPH is not rewarding according to the behavioral data shown here.

In the present study, animals were administered MPH using the ip route of administration. Studies have shown that MPH bioavailability in the brain is increased in rats using the intraperitoneal (ip) route (Berridge et al., 2006). The use of this route of administration, which is
inconsistent with the route typically administered clinically, is more consistent with recreational use of MPH. When taken recreationally, MPH is typically administered via the intranasal route (Teter et al., 2006). This route of administration avoids the body’s first pass metabolism by the liver, thereby delivering higher concentration levels of the drug to the brain when compared to taking the drug orally. The ip route of administration is consistent with MPH bioavailability via the intranasal route. Further, this route provides intense drug effect with a faster onset. It is not known what long-term effect on the brain this drug may have for those recreational users who administer this drug via snorting, and this should be of concern regarding MPH abuse (Carlezon & Konradi, 2004). Consequently, the faster onset and intense drug effect could alter the behavioral response to the drug. This is important because the focus of the present study is on MPH abuse, and dosage appears to play a role in abuse liability.

Consistent with this finding, Experiment 1 demonstrated that the higher dose (5 mg/kg) of MPH produced a significant CPP, whereas those animals conditioned with the lower dose (1 mg/kg) of MPH did not significantly differ from saline controls. Further, evidence of dose dependency was demonstrated in Experiment 2 when animals administered the higher dose (5 mg/kg) of MPH produced a significant CPP as compared to all other groups even though the drug schedule had been changed to an every second day paradigm. Also, the animals used in this experiment were a different age than those used in Experiment 1. Previous research has demonstrated that males have a higher density of D1 receptors in the nucleus accumbens during adolescence as compared to females (Anderson & Teichner, 2000). Also, it is known that sex differences exist during adolescence within the development of D2 receptors. This is important because the dopamine system is changing very quickly during this developmental period and previous research has demonstrated that MPH administered at varying developmental periods
has been shown to change behavioral sensitivity to numerous psychostimulants (Achat-Mendes & Anderson, 2003; Burton, Nobrega & Fletcher, 2010).

In contrast to past work by Merinne et al. (2001) demonstrating that a similar dose of MPH (1.25 mg/kg) produced CPP in adult male rats, results from Experiment 1 demonstrated that the 1 mg/kg dose of MPH did not result in CPP. In addition, this dose resulted in hypoactivity on distance traveled within the paired context. While these results are dissimilar, it is important to keep in mind that there were several methodological differences between the two studies. First, Merinne et al. (2001) used adult male rats as subjects, whereas the current study used both male and female adolescent rats. These disparate results suggest there is an age difference in regards to the reward sensitivity to MPH. This may be due to the a developing dopamine system in adolescence as compared to a mature dopamine system in adults. The developmental age of the animal is important because previous research with psychostimulants has demonstrated that changes within the brain’s reward system from adolescence to adulthood could contribute to the age differences that may exist in the rewarding associative effects of MPH (Bolanos, Glatt, & Jackson, 1998; Caster, Walker & Kuhn, 2005; Faraday, Elliot, et al., 2001) Secondly, the present study used a 10 minute conditioning trials for 5 consecutive days whereas Merrine et al. (2001) conditioned animals for 40 minute trials over the course of 3 consecutive days. Finally, and possibly most importantly, Merinne et al. (2001) used a two chamber biased CPP paradigm, in that animals were conditioned against their natural preference from the initial preference test. The current study used an unbiased CPP paradigm in which the animal was randomly assigned a context with no initial preference shown.
DAT

Results from Experiment 1 demonstrated a significant decrease of striatal and accumbal (DAT) density in both adolescent male and female rats that were administered the 5 mg/kg dose of MPH. Although surprisingly not much work has been done analyzing the effects of MPH on DAT protein, a previous study by Moll et al. (2001) has shown that a 2 mg/kg dose of MPH administered to older adolescent and adult rats produced a significant decrease in (DAT) density in the striatum and that this decrease was approximately 45% as compared to controls (Moll et al., 2001). This is consistent with the present study, because results demonstrated a 45% decrease in the striatum and 40.7% decrease in the nucleus accumbens. There were some methodology differences that should be noted, in that Moll et al. (2001) administered a lower dose (2 mg/kg) of MPH than the current study, albeit for a longer period, and administered MPH orally through the animals’ drinking water. Oral administration would result in a lower bioavailability of MPH in the brain as compared to ip administration. In addition, the DAT analysis used in the Moll et al. (2001) study was analyzed via ligand binding immunohistochemical assays, whereas the present study analysis was performed by Western blot assay. Thus, the assay used by Moll et al., (2001) may actually have higher sensitivity than the Western blot used in the present investigation.

ADHD medications are suggested to be effective because they increase dopamine levels in an abnormal hypofunctioning system of the dopaminergic system (Seeman & Madras, 2002). This overall “dopamine transfer deficit” exists in the disorder and this could be related to the therapeutic mechanisms of MPH, as it produces a decrease in DAT and increased availability of DA in the synapse (Tripp & Wickens, 2009). However, the focus of the current study is the abuse liability of MPH and, thus, this study used a non-ADHD rodent model. Therefore, the
reduction of availability of DAT in those brain areas that are associated with drug reward may contribute to the behavioral effects observed in the current study. This increased availability in synaptic dopamine, resulting in an heightened dopaminergic response in both the nucleus accumbens and dorsal striatum, appears to be the underlying mechanism of the rewarding aspects of MPH, as dopamine D1 antagonism blocked this effect. Both brain areas are indicated in the drug reward pathway and a decrease in DAT density could have deleterious effects on the brain’s reward system. Past work has demonstrated that MPH administered during critical developmental periods has altered behavioral sensitivity to other psychostimulants (Achat-Mendes et al., 2003; Burton et al., 2010).

Sex Differences

Sex Differences and the response to Psychostimulants

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). 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), and methamphetamine (Schindler et al., 2002). In contrast to past work, the current study revealed no sex differences in the conditioned effects of MPH in older adolescents, but there was a sex difference in younger adolescents. However, in Experiment 1, females administered the 5 mg/kg dose of MPH demonstrated an increase in distance traveled in the paired context as compared to all other groups. However, it should be noted that sensitization and CPP are two distinct behavioral tasks, and the motor activating effects of MPH may likely play a larger role in sex differences and behavioral response to MPH.
Most relevant to the present work, previous research has reported that adult female rats demonstrated increased conditioned hyperactivity to a 3 or 10 mg/kg dose of MPH as compared to adult male rats (Wooters et al., 2006). In the present study in Experiment 1, a sex difference was observed on distance traveled in the paired context, in that adolescent female rats administered the 5 mg/kg dose of MPH demonstrated an increase in distance traveled as compared to all other groups using an every consecutive day methodology. Interestingly, in Experiment 2, when the conditioning methodology was changed to every second day, there was no sex difference observed. Thus, it appears that conditioning animals using an every second day paradigm changes the associative strength of MPH.

*Dopamine Receptor Density in Adolescence*

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. As mentioned, there are sex differences in dopamine receptor density in adolescence (Anderson & Teicher, 2000). The results from the current study demonstrated that MPH-induced CPP was blocked by the dopamine D1 antagonist SCH 233990. These results are consistent with past work on D1 receptors. For example, it has been shown that the D1 antagonist SCH 23390 prevents self-administration of amphetamine prior to treatment (Pierre & Vezina, 1998), as well as cocaine induced conditioned place preference (Shippenberg et al., 1996). Interestingly, MPH-induced CPP was not blocked by pretreatment with the dopamine D2 antagonist eticlopride. These results are consistent with past work on the role of dopamine D2 receptors in MPH conditioned place preference (Meririnne et al., 2001). For example, in contrast to D1-antagonism, the D2-antagonist raclopride (RAC) was ineffective in blocking conditioned place preference to cocaine. However, it should be noted that
D2-antagonists have prevented development of sensitization to locomotor-stimulating effects of amphetamine and methamphetamine (Kuribara & Uchihashi, 1993; Meng et al., 1998).

**Dopamine D1 Receptor**

Results of this study showed that MPH-induced CPP was blocked by the dopamine D1 but not the D2 antagonist, which demonstrates the importance of the D1 receptor in the rewarding effects of MPH. It is known that there are sex differences in dopamine receptor density in adolescence (Anderson & Teicher, 2000). These results are consistent with past work on D1 receptors. For example, it has been shown that the D1 antagonist SCH 23390 prevents self-administration of amphetamine prior to treatment (Pierre & Vezina, 1998) as well as cocaine induced conditioned place preference (Shippenberg et al., 1996).

**Dopamine D2 Receptor**

The dopamine D2-like antagonist eticlopride failed to block CPP; however, this is consistent with past literature (Meririnne et al., 2001). It is important to understand that eticlopride is ubiquitous, in that it will block all types of D2 receptors in the synaptic cleft. Dopamine D2 receptors are found both pre- and postsynaptically and exist as autoreceptors on the presynaptic terminal. Autoreceptors are found on the presynaptic side, on the presynaptic terminal where neurotransmitters are released into the synaptic cleft. The presynaptic neuron is inhibited when dopamine hits the presynaptic D2 receptors and this feedback loop” helps modulate the reuptake of excess dopamine in the synaptic cleft (Dreyer & Hounsgaard, 2013). Therefore, when the antagonist eticlopride blocks the inhibitory autoreceptors, dopamine continues to be released into the synaptic cleft, allowing it to bind both dopamine D1 and D2 receptors. When only the postsynaptic D2 receptor is blocked, it would be expected to see similar results as to the D1
antagonist. However, eticlopride blocks the D2 receptor both pre and postsynaptically and therefore failed to block MPH CPP, whereas the D1 antagonist SCH 23390 produced blocked MPH CPP. Consequently, there is plausible evidence to suggest that D2 receptors may be involved in the rewarding properties of particular psychostimulants.

*Translational Relevance*

The findings of the current study demonstrate that MPH, when administered in a relatively high dose (5 mg/kg) produces changes in the dopamine system during a critical developmental period (adolescence) that could have deleterious effects on the dopamine system into adulthood. It is known that MPH has potential abuse liability because like cocaine, MPH inhibits dopamine reuptake which results in increased dopamine levels in the nucleus accumbens and striatum. There is a continued rise in the number of children misdiagnosed and subsequently prescribed a stimulant medication for treatment (Dupont et al., 2008; Klein-Schwartz & McGrath, 2003; Prommer, 2012). Evidence suggests that MPH abuse has noticeably increased over the past several years (Brookshire & Jones, 2012; Looby & Earleywine, 2011). This is especially important because a decrease in DAT may have long term consequences on the brain’s reward system. For example, when DAT is reduced in the brain’s reward pathway this may result in an increase of synaptic dopamine availability and thus an enhanced dopaminergic response in the nucleus accumbens and striatum. Further, previous research demonstrated that MPH exposure during early prenatal period increased sensitivity to appetitive stimuli in adulthood (Crawford et al., 2007). Moreover, MPH has been shown to alter behavioral sensitivity to other psychostimulants if administered during critical developmental periods, including adolescence (Achat-Mendes et al., 2003; Burton et al., 2010). The current study adds critical information to the profile of MPH and is one of the first studies to thoroughly analyze sex differences in the
behavioral response to MPH in adolescence, and increases the evidence for similarities between MPH and other psychostimulants.

Conclusions

The aim of this study was to determine the effects of a clinically relevant and relatively high dose (5 mg/kg) of MPH using a CPP paradigm in younger and older adolescent rats (P33-39, P44-49), analyze the effects of MPH on DAT in older adolescent rats, and analyze the roles of dopamine D1 and D2 receptor in this effect. Results generally found that animals administered 5 mg/kg but not 1 mg/kg MPH, resulted in a significant preference that was more robust in younger adolescent males. The 5 mg/kg dose of MPH also resulted in a significant decrease of the dopamine transporter in both the nucleus accumbens and striatum, revealing dopamine clearance is decreased by MPH in brain areas that mediate reward. Finally, MPH-induced CPP was blocked by the dopamine D1 antagonist SCH 23390 but not the dopamine D2 antagonist eticlorpide. This is important because these results demonstrate that dopamine mediates the rewarding effects of MPH in adolescence.

Previous research on sex differences in the conditioned effects of MPH have demonstrated that adult female rats significantly increased conditioned hyperactivity to a 3 and 10 mg/kg dose of MPH as compared to adult male rats (Wooters et al., 2006). The results from experiment 1 demonstrate that adolescent females administered 5 mg/kg of MPH significantly increased distant traveled in the paired context as compared to adolescent males administered the same dose of MPH as well as controls. However, results from experiment 2 did not yield the same effect and thus it appears that MPH administered every second day changes the associative strength of the drug to the environment. Further, research has demonstrated that juvenile (P20) animals administered 2 mg/kg of MPH twice daily for a period of 15 days resulted in conditioned
place aversion in the cocaine-paired context in males as compared to females (Brenhouse et al., 2009). Although there have been very few studies on the conditioning effects of MPH and sex differences, it appears that sex differences in response to MPH should be further investigated.

Past work in our laboratory has demonstrated that female adolescent rats sensitized to 5 mg/kg MPH but failed to sensitized to the lower dose (1 and 3 mg/kg) of MPH (Brown et al., 2012). However, in past work, animals were sensitized to MPH using an every second day administration paradigm. Based on these past data, Experiment 2 was designed to analyze the effects of an every second day conditioning paradigm on MPH CPP. Consistent with this methodology, past research has shown that those individuals who begin to use drugs recreationally typically have a time period between drug administrations (Gawin, 1991; Griffiths, 1993). Also, the past work in our laboratory on sensitization demonstrated that MPH sensitization peaked at P41, which was the fifth day of administration. Based on findings that drug sensitization typically increases dopaminergic activity, it was surmised that this peak was likely due to peak dopaminergic response in females at this time point. The present experiment analyzed MPH CPP on a different adolescent developmental period by testing a different age within adolescence.

Another focus of the current study was to analyze the role of dopamine receptors on the associative rewarding effects of MPH. The brain’s dopaminergic system is known to play a critical role in mediating the rewarding properties of psychostimulants. Previous research has demonstrated that both dopamine D1 and D2 receptors are involved in the rewarding properties of particular psychostimulants including MPH (Pierre & Vezina, 1998; Shippenberg et al., 1996). In order to investigate the role of D1 and D2 receptors in mediating the rewarding aspects of MPH, the D1 antagonist SCH 23390 and D2 antagonist D2 eticlopride were used in this study to
block the effects of MPH. SCH 23390 has been prevalently used in studies analyzing the role of the dopamine D1 receptor in drug sensitization and its effects on reward (eg, Meririnne et al., 2001). SCH 23390 is also extremely selective to the D1 receptor as compared to the D2 receptor. However, across several studies, several different D2 receptor antagonists have been used. We chose to use eticlopride HCl, because this antagonist blocks D2-like receptors, meaning it blocks D2, D3, and D4 receptors with relatively equal affinity, all of which are D2 receptor subtypes.

The current study demonstrated that a 5 mg/kg dose of MPH produced CPP in both adolescent male and female rats. The dosage amount administered is approximately 5 times the amount of a clinically relevant dose. The results of the current study demonstrated that regardless of the differing age in adolescence, a 5 mg/kg dose of MPH produced CPP in the 5 consecutive days and every other day CPP paradigm. Further, a dose 5 times the clinically relevant dose of MPH produced significant decreases of the DAT in the striatum and nucleus accumbens. Significant increases of DAT in these specific brain regions have implications in the brain’s reward system and in dopaminergic plasticity. Therefore, it is important to point out that the focus of the current study is the potential abuse liability of MPH during a critical time in adolescence when children are more likely to experiment with recreational drugs. The results of the current study necessitate the need for future research on how the underlying mechanisms of the effects of MPH affect behavior and plasticity of the dopamine system.
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