METHYLPHENIDATE TREATMENT AND REARING ENVIRONMENT:
EFFECTS ON THE DOPAMINE SYSTEM AND VULNERABILITY TO SUBSTANCE
ABUSE IN ANIMAL MODELS

BY

KATHRYN ESTELLE GILL

A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES
in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
Physiology and Pharmacology
December 2013
Winston-Salem, North Carolina

Approved by:
Linda J. Porrino, Ph.D., Advisor
Beth A. Reboussin, Ph.D., Chair
Thomas J.R. Beveridge, Ph.D.
Michael A. Nader, Ph.D.
Jeffrey L. Weiner, Ph.D.
ACKNOWLEDGEMENTS

This fall marks nine years since I first entered the Porrino lab as a technician, fresh out of college, wide-eyed, with a huge desire to learn and do, but without any solid direction. Linda gave me a direction with her brilliant proposal to look at the effects of methylphenidate in monkeys. Although I bounced in and out of the lab for several years before committing to graduate school in 2008, I was always drawn to the methylphenidate study. The project captured me from day one, and this final product is the result of Dr. Porrino’s commitment, her guidance and expertise, mentorship, and friendship for the better part of a decade. Thank you Linda, for supporting me, pushing me, knowing when not to push, and never, ever giving up on me. I truly value all the years I’ve spent as your student.

Tom Beveridge has spent more hours in the lab with me and discussing my study designs, data analysis, and interpretations than anyone else. He has been involved in every aspect of the rodent studies from start to finish, from questioning my written rationale to helping me harvest brains. I do not know how I would have done this without his mentorship. Thank you for being a fantastic teacher and for your friendship throughout this process.

Thank you to Jeff Weiner for opening up his lab to me and making me part of the alcohol group during the past several years. His guidance was absolutely critical in the design and completion of this work. Thank you, Jeff, for always being able to get me excited about my data, even when it was exactly the opposite of what we expected/hoped it would look like. You always made me feel encouraged about this project.
A large part of my knowledge about the field of behavioral pharmacology can be attributed to the expertise of Mike Nader. I appreciate so much all the times over the years that you have reached out to get me involved in lab exchanges, summer behavioral pharmacology classes, and conferences. I have felt like an honorary member of the Nader lab on many occasions and have been grateful for your insight throughout my time at Wake. Thank you.

Beth Reboussin has helped me with every statistical problem that I have run across in graduate school. I really appreciate the fact that she refused to answer when I asked her, “How many subjects do I need?” Instead, she gave me exactly the correct amount of information I needed to be able to figure it out and now I pride myself on the fact that I can do my own power calculations. My (still limited, but sufficient) statistical know-how is a credit to her teaching. Thank you so much for the guidance you’ve given me on numerous projects throughout my time as a graduate student.

I am overwhelmed with gratitude for all of my committee members named above. I did not take the easiest road through graduate school. There were many opportunities when quitting and finishing with a master’s degree would have been the easiest option. And yet, that option never crossed the lips of any one of my committee members. When I said I was pregnant (twice!), all I heard was “Congratulations!” When I said I was moving to Kentucky, all I heard was “How can we help?” This extension of encouragement and support was, and still is, mind boggling. I am truly fortunate to have had such an incredible group of mentors along this journey. Thank you all so much.

I am thankful for the help of all of the Porrino Lab members past and present, but especially to Hilary Smith. Hilary taught me everything I know about working in the lab, she rescued me countless times from broken cryostats and centrifuges, taught me how
to run experiments, keep impeccable records, identify brain regions, do lunges in the darkroom to pass the time, and so on. Hilary was the first person I went to with any problem, and usually the last person I had to go to because she always knew how to solve it. Even problems that weren’t technical in nature could be solved by Hilary with a glass of wine and a long conversation. Thank you for being a great teacher, but more importantly, a truly incredible friend.

Ann Chappell was an integral part of the second phase of this project. She gave up several weekends to be in the lab with me running animals on behavioral tasks, when she had absolutely no responsibility to do so. She helped me with every single rodent procedure described here, and I’m pretty sure my rats preferred her to me. Also, thanks to Ann’s expertise, I have won at least $50 at the Keeneland horse racing track, which is an added benefit. In all seriousness, I would not have been able to complete the alcohol portions of this project without Ann’s help and I am extremely grateful for it.

Thank you also to Katy Lack and Erin Shannon Hatzis, who were both post-docs in the lab during my early years of graduate school. They showed me that it was possible to get a Ph.D., while married to a medical resident, with kids at home. They were both ready with words of encouragement any time I was struggling with school or life. Both of their friendships have been invaluable.

Thank you also to Mack Miller, who taught me a great deal about imaging, and from whom I learned that there are real-life ninjas who are capable of being absolutely, completely silent. I owe thanks to many others who have been in the Porrino lab during my tenure in graduate school for their support, guidance, feedback on papers and talks, brainstorming sessions during lab meeting and lunch, and friendship. Of these
individuals, thank you especially to Ashley Morgan, Colleen Hanlon, and Michael Wesley.

In addition to these individuals, I want to thank the remaining members of the Department of Physiology and Pharmacology for their support through this process. Notably, Brian McCool, who made himself available to me on many occasions to give advice on alcohol studies, answer behavioral questions, and offered to help contact people when I moved to Kentucky. Also, special thanks to Allyn Howlett, our program director, who has been extremely flexible with my situation and has enabled me to complete this process from afar. Many other faculty members, including Sara Jones, Anthony Ligouri, and Paul Czoty have given me advice and feedback on this project and I am grateful to all of them for imparting their wisdom over the years.

I can honestly say that this could not have been done without the support of my family. My incredible parents, John Gill and Mary Klein-Gill, and my wonderful in-laws, Susan and Charlie Bounds, helped move me back and forth from Kentucky three times in order for this work to be completed. Mom and Susan gave up months of their own lives to live with me in North Carolina and provide childcare while I spent all my available time in the lab for three and half months. And during the final several months of writing and editing, either Mom and Dad or Susan and Charlie have been with us in Kentucky almost the entire time and have kept us fed and in clean clothes, and kept the kids happy while I locked myself in my basement office and Mike worked 100 hours/week. I am an extremely lucky person to have such an incredible support network.

To Mom and Dad, especially, thank you for believing in me and encouraging me. Mom, my greatest aspiration is to be as wonderful a mother to my children as you have been to me. Dad, you have been an incredible role model not only in your perseverance
and work ethic, but in your unique ability to focus on things that are truly important and let the rest roll of your back. I love you both.

The greatest thanks of all go to my husband, Mike. There are no words that are adequate for expressing my gratitude for his support through this process. He continues to amaze me with his unwavering encouragement, regardless of the sacrifices we both have made get to this point. Thank you for always putting me, Elodie, and Jefferson ahead of yourself. Thank you for being an incredible father and husband and for being by my side every step of the way. You are the smartest and the hardest working person I have ever known and a daily inspiration to me. I love you.

To my amazing, beautiful, children, Elodie and Jefferson. Thank you for making my world so much brighter. May you always follow your dreams.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>FIGURES AND TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTERS</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1. Emergence of ADHD and Methylphenidate Treatment</td>
<td>1</td>
</tr>
<tr>
<td>2. The Dopamine System: Overview and Ontogeny</td>
<td>8</td>
</tr>
<tr>
<td>3. Methylphenidate’s Mechanism of Action</td>
<td>17</td>
</tr>
<tr>
<td>4. Acute Methylphenidate Treatment in Animals</td>
<td>22</td>
</tr>
<tr>
<td>5. Chronic Methylphenidate Treatment in Animals</td>
<td>25</td>
</tr>
<tr>
<td>6. Factors that Influence Vulnerability to Substance Abuse</td>
<td>28</td>
</tr>
<tr>
<td>7. Influence of Environment on Behavior, Dopamine, and Vulnerability to Substance Abuse</td>
<td>35</td>
</tr>
<tr>
<td>8. Purpose of these Studies</td>
<td>49</td>
</tr>
<tr>
<td>2. Chronic treatment with extended release methylphenidate</td>
<td></td>
</tr>
<tr>
<td>does not alter dopamine systems or increase vulnerability for cocaine self-administration: A study in nonhuman primates</td>
<td>109</td>
</tr>
<tr>
<td>Published in Neuropsychopharmacology, November, 2012</td>
<td></td>
</tr>
<tr>
<td>3. The effects of rearing environment and chronic methylphenidate administration on behavior and dopamine receptors in juvenile rats</td>
<td>150</td>
</tr>
<tr>
<td>Published in Brain Research, August, 2013</td>
<td></td>
</tr>
<tr>
<td>4. Chronic methylphenidate treatment during early life is associated with greater ethanol intake in socially isolated rats</td>
<td>197</td>
</tr>
<tr>
<td>In preparation for submission to Alcoholism: Clinical and Experimental Research</td>
<td></td>
</tr>
<tr>
<td>Chapter 4 Supplementary Material: Performance on the elevated plus maze is associated with drinking behavior</td>
<td>230</td>
</tr>
</tbody>
</table>
5. Discussion.........................................................................................................................236
   1. Main Effects of Methylphenidate.................................................................239
   2. Main Effects of Environment.................................................................248
   3. Interaction between Environment and Methylphenidate.............250
   4. Final thoughts..........................................................................................252

CURRICULUM VITAE........................................................................................................270
# FIGURES AND TABLES

## CHAPTER ONE

| Figure 1 | Rodent post-natal days and monkey ages compared to human developmental stages. | 10 |
| Table I | Formulations of methylphenidate available to treat ADHD as of 2013 | 3 |
| Table II | Effects of enriched environment on behavior | 38 |
| Table III | Effects of isolated environment on behavior | 39 |
| Table IV | Effects of enriched environment on the dopamine system | 43 |
| Table V | Effects of isolated environment on the dopamine system | 44 |

## CHAPTER TWO

| Figure 1 | Standard measures of growth, weight, crown to rump length and Body Mass Index (BMI), were measured at baseline, after 12 months of treatment, and after 3-5 months of drug washout. | 123 |
| Figure 2 | Dopamine D2/D3 receptor availability as measured by $[^{18}\text{F}]$FCP in MPH-treated and control monkeys in the caudate nucleus, putamen and ventral striatal regions. | 125 |
| Figure 3 | Binding of $[^{18}\text{F}]$ FCP to dopamine D2/D3 receptors in the striatum decreases over a 12-month period in control and MPH treated animals. | 127 |
| Figure 4 | Acquisition of cocaine reinforcement in MPH-treated and control monkeys | 132 |
| Table 1 | Effects of methylphenidate treatment on $[^{18}\text{F}]$FCT distribution volume ratios | 129 |
| Table 2 | Baseline food-maintained responding and number of sessions for response extinction | 131 |
CHAPTER THREE

Figure 1  Behavior in the locomotor chamber.....................158
Figure 2  Time spent in the open arms of the elevated plus maze...........161
Table 1  The density of D1-like receptors in the striatum..................163
Table 2  The density of D2-like receptors in the striatum..................165

CHAPTER FOUR

Figure 1  Daily g/kg EtOH intakes.........................................208
Figure 2  Ethanol Preference in Isolated Animals..........................210
Figure 3  Elevated Plus Maze Behavior.....................................212
Figure 4  Blood Ethanol Concentrations....................................214
Figure 5  Sucrose drinking.....................................................216

CHAPTER FOUR (supplement)

Figure 1  Drinkers versus Nondrinkers on the Elevated Plus Maze..........233

CHAPTER FIVE

Figure 1  Percentage of boys and girls ages 4-17 who were diagnosed with ADHD and taking medication to treat ADHD as of 2007-2008.................................237
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>ADX</td>
<td>Adrenalectomized</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BEC</td>
<td>Blood Ethanol Concentration</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned Place Preference</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate-Putamen</td>
</tr>
<tr>
<td>D&lt;sub&gt;2L&lt;/sub&gt;</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;-long</td>
</tr>
<tr>
<td>D&lt;sub&gt;2S&lt;/sub&gt;</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;-short</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine Transporter</td>
</tr>
<tr>
<td>dIPFC</td>
<td>dorsolateral Prefrontal Cortex</td>
</tr>
<tr>
<td>DSM II</td>
<td>Diagnostic and Statistic Manual- II</td>
</tr>
<tr>
<td>DSM IV-tr</td>
<td>Diagnostic and Statistic Manual- IV-text revised</td>
</tr>
<tr>
<td>DVR</td>
<td>Distribution Volume Ratio</td>
</tr>
<tr>
<td>EE</td>
<td>Environmentally Enriched</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-maximal Effective Concentration</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FCP</td>
<td>Fluoroclebopride</td>
</tr>
<tr>
<td>FCT</td>
<td>-(+)-(4-fluorobenzyl)-2β-propanoyl-3β-(4-chlorophenyl)tropane</td>
</tr>
<tr>
<td>FR</td>
<td>Fixed Ratio</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>mPFC</td>
<td>medial Prefrontal Cortex</td>
</tr>
<tr>
<td>MPH</td>
<td>Methylphenidate</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTA</td>
<td>Multimodal Treatment of ADHD</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NET</td>
<td>Norepinephrine Transporter</td>
</tr>
<tr>
<td>OROS-MPH</td>
<td>Osmotic Release Oral System- Methylphenidate</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PH</td>
<td>Pair-housed</td>
</tr>
<tr>
<td>PND</td>
<td>Post Natal Day</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin Transporter</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously Hypertensive Rat</td>
</tr>
<tr>
<td>SI</td>
<td>Socially Isolated</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
</tbody>
</table>
ABSTRACT

METHYLPHENIDATE TREATMENT AND REARING ENVIRONMENT: EFFECTS ON THE DOPAMINE SYSTEM AND VULNERABILITY TO SUBSTANCE ABUSE IN ANIMAL MODELS

Dissertation under the direction of Linda J. Porrino, Ph.D.,
Professor and Chair of Physiology and Pharmacology

The purpose of these studies was to investigate the effects of chronic methylphenidate treatment, at doses that have been associated with clinical efficacy in children, on the dopamine system and vulnerability to substance abuse in animal models. Methylphenidate is the most commonly prescribed stimulant to treat Attention Deficit Hyperactivity Disorder. As a dopaminergic agent, it has potential to cause long-term alterations of a neural system that has been associated with numerous psychopathologies, including substance abuse. Yet, few studies have examined its long term effects on the brain. Another factor that can influence vulnerability to substance abuse is early rearing environment, as children raised in negative environments are more likely to develop substance abuse disorders. These studies examined the interaction of environment and methylphenidate on the dopamine system and vulnerability to cocaine and alcohol abuse.

Both nonhuman primate and rodent models were used in this series of studies. Nonhuman primates were treated with an extended release formulation of methylphenidate and rodents were treated with a consistent subcutaneous infusion of methylphenidate. Rodents were also exposed to different environmental conditions during the treatment phase. In both species, the age of treatment, duration of treatment, and doses used were matched as closely as possible to the human condition. Positron
Emission Tomography (in nonhuman primates) and in vitro autoradiography (in rodents) were employed to evaluate the availability and density of dopamine receptors and transporters. Nonhuman primates and rodents were then exposed to cocaine and alcohol self administration, respectively.

Methylphenidate treatment had no effect on the dopamine systems of nonhuman primates or rodents, though treatment plus drug washout altered the trajectory of D2-like receptor development in treated nonhuman primates. Social isolation was associated with greater levels of D1-like receptors in rats. Methylphenidate did not alter vulnerability to cocaine self-administration, but it did increase ethanol intakes and preferences in socially isolated rodents. In conclusion, methylphenidate may not have long-term effects on the dopamine system in treated children, but it may increase vulnerability to substance abuse in a subset of individuals that have a negative environmental history.
CHAPTER ONE
INTRODUCTION

1. Emergence of ADHD and MPH treatment

Methylphenidate (MPH) is a commonly prescribed stimulant drug to treat Attention Deficit Hyperactivity Disorder in children and adults. Recent estimates suggest that close to 5% of children in the United States are currently treated with stimulant drugs, either MPH or amphetamines (CDC, 2005; Visser et al., 2007; Visser et al., 2013). The use of stimulant drugs in children dates to 1937, when the first published report emphasized the dramatic improvements in school performance of “behavior problem” children that were administered Benzedrine, an amphetamine (Bakwin, 1948; Bradley, 1937; Bradley and Bowen, 1940; Bradley and Green, 1940; Bradley and Bowen, 1941; Bradley, 1950). “Hyperkinetic reaction of childhood” was first recognized as a psychiatric illness and added to the Diagnostic and Statistical Manual II (DSM II) in 1968 (Baumeister et al., 2012). This disorder became known as “Attention Deficit Disorder” in 1980 and more recently, Attention Deficit Hyperactivity Disorder (ADHD) (Mayes et al., 2008; Michels, 1993). Currently, ADHD is the most commonly diagnosed psychiatric disorder in minors (Williams et al., 2004). In the Diagnostic and Statistical Manual-IV-text revised, (DSM-IV-tr), ADHD is defined as a disorder that is characterized by symptoms of inattention (e.g. has trouble keeping attention to tasks or play activities, does not give close attention to details or makes careless mistakes, is often easily distracted, etc), hyperactivity (e.g. often fidgets with hands or feet when sitting still is expected, often has trouble playing or doing leisure activities quietly, etc), and impulsivity (e.g. often blurts out answers before questions have been finished, often has trouble waiting one’s turn, etc) that are disruptive and inappropriate for the developmental level (American Psychiatric Association, 2000). For diagnosis, these symptoms must have
been present for at least 6 months and there must be clear evidence of significant impairment in school, social, or work functioning.

Methylphenidate was first synthesized in 1944 (Pannizon, 1944), was patented ten years later and was originally put on the market by CIBA-Geigy pharmaceutical company in the immediate release formulation of Ritalin® for indications such as lethargy, chronic fatigue, and depressed states (Meier et al., 1954; Physician’s Desk Reference, 1956). The first reports of the effectiveness of MPH for behavior disorders in children were published in the late 1950s (Barrett et al., 1956; Blair et al., 1959; Percheson et al., 1959; Zimmerman and Burgemeister, 1958), with multiple studies documenting that MPH administration to children afflicted by “hyperkinetic reaction” acutely improved school performance, attention, and task performance (Conners et al., 1964; Conners, 1966). Since these early studies, for the past 60 years, MPH has been shown to be extremely effective in reducing the core symptoms of ADHD (see section 1.1). Currently, MPH usage is approved by the Food and Drug Administration for the treatment of ADHD and narcolepsy. The original formulation of immediate-release MPH is now trademarked as Ritalin® by Novartis Pharmaceuticals, but there are multiple generic formulations and extended-release formulations also available (Table 1). MPH and particularly its various extended-release formulations are now the most commonly prescribed drugs for the treatment of ADHD (Brown et al., 2005), over amphetamines and non-stimulant alternatives.
Table I. Formulations of methylphenidate available to treat ADHD as of 2013.

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritalin®</td>
<td>Immediate Release</td>
</tr>
<tr>
<td>Metadate CD™</td>
<td>Extended Release (Controlled Delivery)</td>
</tr>
<tr>
<td>Methylin ER®</td>
<td>Extended Release</td>
</tr>
<tr>
<td>Ritalin LA®</td>
<td>Extended Release (Long Acting)</td>
</tr>
<tr>
<td>Ritalin SR®</td>
<td>Extended Release (Sustained Release)</td>
</tr>
<tr>
<td>Concerta®</td>
<td>Osmotic-controlled Release Oral delivery System (OROS)</td>
</tr>
<tr>
<td>Daytrana™</td>
<td>Transdermal System</td>
</tr>
</tbody>
</table>

1.1 Effectiveness of MPH as a treatment for ADHD

Children with ADHD respond to MPH or other stimulant therapies in approximately 80% of cases (Adesman and Morgan, 1999; Bennett et al., 1999; Goldman et al., 1998), making these agents an extremely effective first line treatment (for review see Findling, 2008), and far more effective than behavioral therapies alone (MTA Cooperative Group, 1999). To examine the effects of MPH in natural settings, many studies have relied on teacher rating scales as outcome measures, such as the Conners ADHD/DSM-IV scales for teachers, the Conners Global Index teacher scale, and the Inattention/Overactivity with Aggression teacher rating. Using these scales, studies consistently show the significant benefits of MPH over placebo in improving attention and quelling hyperactivity in the classroom (Biederman, 2003; Greenhill et al., 2002; Greenhill et al., 2006; Wolraich et al., 2001). In addition, children with ADHD who are treated with stimulant drugs consistently exhibit improved social skills and academic achievement (Greenfield et al., 1988; Hechtman et al., 1984).

In laboratory settings, it is clear that MPH improves the performance of children and adults with ADHD on a multitude of tasks. With MPH treatment, ADHD patients perform better on cognitive performance tasks that require attention (Ackerman et al.,
1982; Reid and Borkowski, 1984), they have improved language processing (Ballinger et al., 1984), and they have faster reaction times and decreased impulsivity on measures of inhibitory processing (Aron et al., 2003; DeVito et al., 2008; DeVito et al., 2009; Klorman et al., 1991; Rapport et al., 1985; Scheres et al., 2003; Tannock et al., 1989; Trommer et al., 1991; Turner et al., 2005). In fact, when compared to normal, healthy children, children with ADHD that have been treated with MPH have been shown to perform just as well on a task that measures impulsivity (Malone and Swanson, 1993).

More recent work has refined earlier studies, testing broader populations, using more stringently matched control subjects, and examining new formulations of medication, for example. Overwhelmingly, the literature continues to document MPH’s effectiveness in treating classic ADHD symptoms of inattention (Epstein et al., 2006; Konrad et al., 2004; Konrad et al., 2005; Riccio et al., 2001; Tucha et al., 2006a; Tucha et al., 2006b; Zeiner et al., 1999) and impulsivity (Konrad et al., 2004; Konrad et al., 2005; Vaidya et al., 1998). In addition, some studies have concluded that MPH improves executive functions such as working memory (Strand et al., 2012; Zeiner et al., 1999), processing speed (Bedard et al., 2002; Epstein et al., 2006), reaction time, (Spencer et al., 2009) and set shifting (Konrad et al., 2004; Konrad et al., 2005; Mehta et al., 2004; Pietrzak et al., 2006). MPH has also been shown to increase motivation in children with ADHD. For example, MPH treatment of children with ADHD had an additive effect to motivational incentives given to maintain attention to a task, enhancing stimulus salience and the allocation of intentional resources to the task (Groom et al., 2010). However, it must be noted that some other studies have failed to show dramatic performance-enhancing effects of MPH on various domains, indicating that the effects of MPH may be task-specific. For example, Blum and colleagues (2011) found that MPH improved performance on tasks measuring response inhibition, but failed to affect
performance on working memory tasks. Nonetheless, the large majority of evidence suggests that acute MPH treatment is highly effective in reducing core symptoms of ADHD.

1.2 Side effects

Despite the overwhelming success of these drugs in clinical populations, there is concern over the potential for MPH treatment to have a negative side effect related to physical growth in children. Several studies have reported reduced height gains in children currently treated with stimulant medication (Faraone et al., 2006; Safer et al., 1972; Zhang et al., 2010). The Multimodal Treatment of ADHD (MTA) study, which was a very controlled examination of ADHD medications in a large sample of children, reported that two years of stimulant treatment was associated with a reduction in height and weight gain in treated children when compared to age-matched controls (Swanson et al., 2007). Another study reported reduced height and weight gains in treated children, but only after four years of continuous high dose treatment (Charach et al., 2006). In contrast, there are also studies that have reported no effects on growth in children treated with stimulants (Biederman et al., 2010; Pliszka et al., 2006; Spencer et al., 2006b). Other reported side effects are relatively few and mild and include complaints such as insomnia, decreased appetite, stomach pain, headache, and irritability (Barkley, 2002; Smucker and Hedayat, 2001). While the potential effect of chronic MPH treatment on growth is an area that warrants further investigation (see Chapter Two), there is still general consensus that MPH is safe and effective when used properly (Mayes et al., 2008), and it remains a first-line treatment for ADHD.

1.3 Prevalence of ADHD and MPH use
As both clinical and preclinical literature have continued to support the use of MPH as an effective therapy for ADHD, its use has skyrocketed (Zito et al., 2003; Zuvekas et al., 2006), coincident with the increasing prevalence of ADHD diagnoses. ADHD is now estimated to affect 6-9% of school-age children (Visser et al., 2007), making it the most prevalent psychiatric disorder of childhood (Williams et al., 2004). The 1990s saw a four-fold increase in rates of stimulant drug therapy for ADHD (Olfson et al., 2002), though recent data suggests an attenuation of that trajectory in the early 2000s (Zuvekas et al., 2006). Currently, approximately 5% of children in the United States are treated with prescription stimulant drugs (CDC, 2005), a number that is much greater than the rest of the world’s children combined (Buitelaar et al., 2004).

The expansion of MPH prescriptions has included a substantial increase in the number of adult prescriptions. There was a 90% increase in adult stimulant prescriptions to treat ADHD between 2002 and 2005, and adult prescriptions are estimated to now make up almost one-third of total prescriptions in the United States (Okie, 2006). Recent studies suggest that MPH and other stimulants are equally efficacious in adults as they are in children (Aron et al., 2003; Faraone et al., 2004). Additionally it is now becoming clearer that 50-80% of children with an ADHD diagnosis will continue to display symptoms as adults (Barkley, 2002; Biederman et al., 1996; Gittelman et al., 1985; Kessler et al., 2005; Mannuzza et al., 2003a; Mayes et al., 2008; Molina and Pelham, 2003), thus the trend of treating adults is likely to continue.

In addition to the treatment of adults, there has also been a movement to treat younger and younger children, starting in the pre-school years (DeBar et al., 2003; Kratochvil et al., 2004; Kratochvil et al., 2006; Rappley et al., 1999; Rappley et al., 2002; Zito et al., 2000). While there is evidence that MPH will attenuate symptoms of ADHD in children as young as two or three years old, there is considerable controversy over the
validity and accuracy of diagnosing ADHD in children this young (Coyle, 2000; Zito et al., 2000). Controversy surrounding the prescription of stimulant drugs is not limited to young children, however. The rapid rise of ADHD diagnosis that has led to the boom in prescriptions written for stimulants has generated intense public debate for decades (Cowart, 1988; Eberstadt, 1999; Huessy, 1988; Wolraich et al., 2007).

One point of critique is wide demographic variation in rates of ADHD diagnosis and treatment (Barbaresi et al., 2002; Bokhari et al., 2005; Cox et al., 2003; Olfson et al., 2002; Zito et al., 2003). Children in the southeast and midwest regions of the United States are significantly more likely to be diagnosed with ADHD than their northeastern or western counterparts (Cox et al., 2003). Per-capita rates of ADHD drug prescription are increased in counties that have greater populations, higher income, lower unemployment, more physicians, and higher student-to-teacher ratios (Bokhari et al., 2005). Additionally, several policy changes in the early 1990’s coincided with the expansion of stimulant prescriptions written to children, including the addition of ADHD as a disability category in the Individuals with Disabilities Education Act (Reid et al., 1994). The demographic variability in diagnosis and the policy changes that coincided with the increase in MPH prescriptions have led many to question whether or not children are being appropriately diagnosed and treated. In addition, there is now evidence that suggests that physicians are intentionally prescribing MPH and other stimulants to children solely for the purposes of “neuroenhancement” without a diagnosis of ADHD (Graf et al., 2013). Likewise, college-students without a history of ADHD diagnosis are seeking stimulant treatment for the purpose of having a competitive edge, academically (McCabe et al., 2009; Rabiner et al., 2009). There is, additionally, an argument that stimulant medications should be available over-the-counter to the general public as “cognitive enhancers” (Diller, 1998; Hyman et al., 2006). This argument is
based on the idea that stimulant drugs, such as MPH, are completely safe, have limited abuse potential, and can benefit healthy individuals in addition to ADHD patients. In reality, however, little is known about the long-term consequences of MPH on the brain or behavior, its potential to influence substance abuse, or even its affect on physical growth.

The studies presented here were undertaken to answer some of the outstanding questions about the effects of MPH on these outcomes using animal models, which are not constrained by many of the confounds of human studies (see section 8.1 for further explanation). MPH was chosen over other stimulants, such as amphetamine, because it is the most commonly prescribed stimulant medication for ADHD (Brown et al., 2005). We examined its impact on physical growth (see Chapter Two) and the development of the dopamine system (Chapters Two and Three), as well as the potential for MPH to affect vulnerability to substance abuse (Chapters Two and Four). In addition, we investigated the interaction of MPH with environmental influences that are known to affect many of the same systems as stimulant treatment (Chapters Three and Four). These studies attempted to model current clinical practice in the use of extended release formulations of medication, at doses that produce blood plasma levels of drug associated with clinical efficacy, and at developmentally appropriate age ranges for treatment. The results of these investigations are important additions to the extant literature on the long-term effects of MPH treatment.

2. The Dopamine System: Overview and Ontogeny

The dopamine system is one of the primary sites of action for MPH. Dopamine itself is a monoamine neurotransmitter that belongs to the catecholamine family, which
also includes epinephrine and norepinephrine. As a neurotransmitter, dopamine is a signaling molecule within the central nervous system that is released by neurons to influence brain activity through its interactions with 5 subtypes of dopamine receptors and its transporter. Dopamine signaling has been implicated in reward learning, motor activity, motivation, and executive functioning. Disturbances of the dopamine system have been associated with multiple diseases such as Parkinson’s disease, schizophrenia, ADHD, and substance abuse. The mechanism of action of MPH on the dopamine system will be further discussed in section 3. This section reviews the basic anatomy of the dopamine system, including its major pathways and receptor families.

Additionally, this section reviews some of the major developmental changes within the dopamine system that occur during childhood and adolescence in humans, monkeys, and rats. It is important to note that MPH is most likely to be prescribed during the juvenile and adolescent period in children. During these stages, the brain is undergoing substantial development, including prefrontal cortical development, pruning of synapses in cortical and subcortical regions, and continued myelination of neurons to increase efficiency (Andersen, 2005). These processes occur in all mammalian species, including humans, monkeys, and rats, with similar external developmental milestones (Andersen, 2003; Figure 1) and MPH treatment during development has potential to disrupt normal maturational processes. Thus, it is important to study the effects of MPH on the dopamine system within the framework of normal dopaminergic development, as was one goal of the studies reported in Chapter Two.
Figure 1. Rodent post-natal days and monkey ages compared to human developmental stages. Data are adapted from Andersen et al., 2003; Malkova et al. 2006; Saxton & Lotz, 1980.
2.1 Pathways

The primary action of MPH in the brain is within the dopamine system and therefore encompasses the mesolimbic, mesocortical, and nigrostriatal dopamine pathways, affecting a large array of brain structures and processes. The mesolimbic pathway begins with dopaminergic cell bodies in the ventral tegmental area (VTA) of the midbrain, which send dopaminergic projections to the nucleus accumbens (NAcc) region of the ventral striatum, an area critical in mediating the reinforcing effects of drugs (for review see Sesack and Grace, 2010). The mesolimbic pathway also includes dopaminergic projections to the ventral pallidum, hippocampus, amygdala, and medial prefrontal cortex (mPFC) (Moore and Bloom, 1978; Oades and Halliday, 1987; Simon et al., 1980). This system has been associated with numerous behaviors, most notably motivation, incentive salience, and drug reinforcement. The mesocortical dopamine pathway comprises neurons that also arise in the VTA, but send their projections directly to the prefrontal cortex, particularly the dorsolateral prefrontal cortex (dLPFC), which is known for its role in cognition and executive function (Goldman-Rakic, 1987; Goldman-Rakic, 1988). Finally, the nigrostriatal pathway begins in the dopaminergic cell bodies of the substantia nigra which send axonal projections to the dorsal striatum, including the caudate and putamen, which are involved in motor function, such as initiation of movement and stereotypy (Moore and Bloom, 1978). Thus, it is clear that disruptions of any of these pathways may result in ADHD-like symptoms, including the loss of motivation and focus, executive control, and increases in motor activity.

2.2 Development of dopaminergic innervation

At birth, the dopamine system pathways are relatively well established in rats (Berger et al., 1985; Hu et al., 2004) nonhuman primates (Lidow et al., 1991), and
humans (Meng et al., 1999; Verney et al., 1991). Cell birth, neuronal differentiation, and migration of neurons along the mesolimbic, mesocortical, and nigrostriatal pathways to target areas are almost complete very early in life (Andersen, 2005). Since dopamine containing neurons have reached the cortex, where D1-like and D2-like receptors are present, dopaminergic neurotransmission is possible at birth (Wahlstrom et al., 2010). However, there is still considerable development of this system throughout the juvenile and adolescent years. In the nonhuman primate brain, there is evidence that dopamine neuron innervation to the prefrontal cortex increases during childhood and peaks in adolescence (Rosenberg and Lewis, 1994; Rosenberg and Lewis, 1995). Likewise, some studies suggest that dopamine concentration in the PFC is at its peak during adolescence in the nonhuman primate brain (Goldman-Rakic and Brown, 1982), yet human findings are inconclusive, with some showing age-related decreases in dopamine concentration and some showing no changes during development (Haycock et al., 2003; Kalaria et al., 1993). In rats, dopamine innervation of the cortex and dopamine concentration increases through adolescence and continues into early adulthood (Berger et al., 1985; Giorgi et al., 1987; Kalsbeek et al., 1988), differing from the primate models of development in which those measures peak during adolescence.

2.3 Dopamine receptors

Dopamine receptors are all members of the seven transmembrane guanine nucleotide regulatory protein (G-protein) –coupled receptor family (GPCRs) and thus share most of their structural characteristics (Probst et al., 1992). There are two classes of dopamine receptors that have been identified thus far: The D1-like receptor family which includes D1 and D5 receptor subtypes and the D2-like receptor family which includes D2, D3, and D4 receptor subtypes. The current classification system is based upon the functional properties of the receptors, namely which G-proteins they are
coupled to and how their stimulation affects cell signal transduction (Glickstein and Schmauss, 2001). Within the classes, the receptor subtypes share high homology in their transmembrane domains and their affinities for classic D1-like and D2-like receptor ligands (Civelli et al., 1993; Gingrich and Caron, 1993; Jackson and Westlind-Danielsson, 1994; Levant, 1997; Missale et al., 1998; Seeman and Van Tol, 1994; Sokoloff and Schwartz, 1995). The vast majority of anatomical localization studies for receptor protein and messenger Ribonucleic Acid (mRNA) have been done in non-human primate and rodent tissue, and thus may not be perfectly comparable to the human brain. However, the DA system is highly conserved among species which suggests that there is translational value (Richfield et al., 1987).

2.3.1 D1-like receptors

D1-like receptors are widely expressed throughout the brain. The D₁ subtype in particular is expressed at higher levels than any other dopamine receptor (Deary et al., 1990). D₁ receptor mRNA can be found in the striatum, NAcc, and olfactory tubercle, and D₁ receptor protein has been identified in the limbic system, basal ganglia, thalamus, and hypothalamus (Fremeau et al., 1991; Weiner et al., 1991). By comparison the D₅ receptor is poorly expressed (Missale et al., 1998). Reports have localized the protein to the hippocampus, lateral mamillary nucleus, and parafascicular nucleus (Meador-Woodruff et al., 1992; Tiberi et al., 1991), and receptor mRNA has been found in several regions including the lateral thalamus and striatum (Choi et al., 1995; Huntley et al., 1992; Rappaport et al., 1993). D₁ and D₅ receptors are co-expressed in the pyramidal neurons of the prefrontal, premotor, cingulate, and entorhinal cortices as well as the hippocampus and dentate gyrus, and are most frequently found postsynaptically (Bergson et al., 1995a; Bergson et al., 1995b; Huang et al., 1992; Smiley et al., 1994).
2.3.2 D2-like receptors

The D₂ receptor subtype is found mainly in the striatum, the olfactory tubercle, and the core and shell of the NAcc (Bouthenet et al., 1991; Diaz et al., 1994; Diaz et al., 1995; Jackson and Westlund-Danielsson, 1994; Le Moine et al., 1990; Le Moine and Bloch, 1995). Its mRNA is expressed in prefrontal, cingulate, entorhinal, and temporal cortices, as well as in the amygdala, hippocampus, substantia nigra, and VTA (Bouthenet et al., 1991; Meador-Woodruff et al., 1989). Two splice variants of the D₂ receptor have been identified and are called D₂-short (D₂S) and D₂-long (D₂L) (Tan et al., 2003). The splice variants differ slightly in their regional distribution, with D₂S predominating in the cell bodies and axons of the mesencephalon and hypothalamus, and the D₂L predominating in the striatum, NAcc, and other structures that are targeted by dopamine neurons (Khan et al., 1998a; Khan et al., 1998b). While it appears that both isoforms are located pre- and post-synaptically, there is evidence to suggest that postsynaptic effects of D₂ receptors are primarily mediated by the D₂L, and presynaptic effects are primarily mediated by the D₂S (Khan et al., 1998a; Khan et al., 1998b; Lindgren et al., 2003). Presynaptically, D₂ receptors function as autoreceptors, providing feedback inhibition by limiting cell firing rate, limiting dopamine synthesis within the cell, or limiting DA release (Cragg and Greenfield, 1997).

The D₃ subtype is highly expressed in limbic regions such as the NAcc (Landwehrmeyer et al., 1993a; Landwehrmeyer et al., 1993b), but is poorly expressed in the dorsal striatum, particularly when compared to the D₂ subtype (Bouthenet et al., 1991; Levesque et al., 1992; Sokoloff et al., 1990). D₃ receptor mRNA is also found in the substantia nigra and VTA, but in a minority of dopamine neurons (Diaz et al., 1994; Diaz et al., 1995). Unlike the D₂ and D₃ receptor subtypes, the D₄ receptor is expressed primarily in the frontal cortex (Mrzljak et al., 1996; O'Malley et al., 1992), with only low
levels of expression in the basal ganglia (Mrzljak et al., 1996; Patel et al., 1997; Rappaport et al., 1993; Schlachter et al., 1997).

2.3.3 Dopamine receptor development

Dopamine receptor densities undergo substantial changes during the juvenile and adolescent period. In nonhuman primates, dopamine receptor densities in the premotor, motor, somatosensory and occipital cortices peak early in life, at about 2 to 4 postnatal months. They then begin a slow and gradual decline until reaching adult levels at about 5 years of age (Lidow et al., 1991; Lidow and Rakic, 1992). A similar decline has also been documented in humans (Meng et al., 1999; Seeman et al., 1987). In a large post mortem human study, Seeman and colleagues (1987) reported peak levels of D2-like receptor density at 5 years of age, followed by a sharp decline into early adolescence (14 years old), and a gradual decrease throughout life. Indeed, synaptic density in humans in the frontal cortex has been reported to decline by 40% between the ages 5 and 15 (Huttenlocher, 1979). More recent studies have reported that gray matter volume in the cortex peaks slightly later in life, around 9-10 years old in girls and 10-11 years old in boys (Giedd, 2004; Giedd et al., 2008), before it declines, an effect attributed to synaptic overproduction and pruning. Generally, it appears that subcortical areas, such as the striatum, overproduce and prune slightly earlier than cortical regions (Thompson et al., 2000), though the overall pattern of early overproduction and then gradual declines is consistent throughout the brain.

There is evidence of similar overproduction and pruning of synapses in rodent models, especially among dopamine receptors in the striatum. Most studies suggest that D2-like receptors in the rodent caudate putamen increase from birth until post natal day (PND) 35-40, which corresponds to adolescence (Andersen et al., 1997; Gelbard et
Similarly, D1-like receptors in the NAcc have been reported to increase until adolescence (Leslie et al., 1991), and then decrease into adulthood, though there are also suggestions of a second peak around PND 80 (Andersen et al., 1997; Teicher et al., 1995). The adult increase in receptors in the NAcc was limited to the D1-like population; D2-like receptors did not show similar increases (Andersen et al., 1997; Teicher et al., 1995).

2.4 Dopamine transporter function and development

The dopamine transporter (DAT) is primarily located on the presynaptic terminals of the dopamine synapse. It regulates dopamine transmission in the brain by actively transporting extracellular dopamine back into the presynaptic neuron, thereby attenuating dopamine signaling. The DAT is widely distributed throughout the brain on dopaminergic neuron terminals in the mesolimbic, mesocortical, and nigrostriatal pathways (Marshall et al., 1990; Richfield, 1991) and plays a primary role in the effects of MPH, as well as the regulation of stimulant drugs of abuse, as will be discussed in greater detail below.

Like the dopamine receptor families, numerous studies have documented declines in DAT expression with age in primates and rodents (Hebert et al., 1999; Ma et al., 1999; Volkow et al., 1996). However, the decline in DAT seems to begin later in life than the decline in dopamine receptors (Volkow et al., 1996). In human post-mortem tissue, DAT expression in the basal ganglia peaked during adolescence (14-15 years old), then slowly declined (Meng et al., 1999). In rodents, in the striatum, DAT density has been reported to increase until puberty, then decline into adulthood (Moll et al., 2000), a timeline that is remarkably similar to humans and nonhuman primates.
The developmental changes within the dopamine system in early life and during adolescence may be affected by chronic drug treatment. It is important to consider the effects of MPH treatment within the context of the normal development of the dopamine system. Thus, investigating the normal development of the dopamine system, specifically D2-like receptors and DATs, was an additional goal of the studies presented in Chapter Two. An overview of how MPH exerts its effects on the dopamine system is presented in the following section.

3. Methylphenidate’s Mechanism of Action

3.1 Binding to the DAT, elevating extracellular dopamine

Early mechanistic in vitro studies localized the binding site of MPH to dopaminergic nerve terminals (Janowsky et al., 1985) with the highest binding in the caudate putamen, olfactory tubercle, nucleus accumbens, bed nucleus, and median eminence. Additionally, the binding of [3H]MPH was disrupted by a 1000-fold excess of nomifensine, a potent DAT inhibitor (Unis et al., 1985). Later studies confirmed that, like other stimulant drugs such as cocaine, MPH acts by blocking the DAT and inhibiting dopamine uptake from the synaptic cleft (Patrick et al., 1987) resulting in increased extracellular dopamine in the striatum and other regions (Butcher et al., 1991; Hurd and Ungerstedt, 1989; Kuczenski and Segal, 1997; Woods and Meyer, 1991). In humans, a typical, oral therapeutic dose of MPH will block about 60% of DATs in the striatum, where binding is highest (Volkow et al., 1995), with peak uptake in the brain at around 60 minutes (Volkow et al., 1998). Using Positron Emission Tomography (PET), Volkow and colleagues (2001b) showed that acute doses of MPH resulted in a decrease in [11C]raclopride binding to D2-like receptors due to competition with increased
endogenous dopamine in healthy volunteers. Interestingly the displacement of raclopride was greatest when the subjects were engaged in a cognitive task, suggesting that MPH is more effective with increasing cognitive load (Rosa-Neto et al., 2005; Volkow et al., 2004).

3.2 Pharmacokinetic Profile of MPH

3.2.1 Immediate release formulations

In humans, MPH, in the immediate release formulation commonly known as Ritalin®, is readily absorbed after oral administration. It reaches peak plasma concentrations within 1 to 3 hours, with a high degree of individual variability, and has a half life of 2.5-3 hours, and then is rapidly metabolized by de-esterfication to ritalinic acid and is excreted in urine (Aoyama et al., 1994; Leonard et al., 2004; Patrick et al., 1981; Wargin et al., 1983). As MPH taken orally is subject to substantial first pass metabolism, the average bioavailability of a typical therapeutic dose (~0.25 mg/kg) in children is only 31%, and there is substantial inter-subject variability, with values ranging from 10%-50% (Chan et al., 1983). The individual variability in absorbance rates and bioavailability is likely one reason why different children respond optimally to a varying range of doses.

The pharmacokinetics of MPH also varies substantially by species. In rats and monkeys, absolute bioavailability of MPH after oral administration is even lower than in humans, at only 20% (Wargin et al., 1983). Furthermore, MPH is far less potent at occupying the DAT in nonhuman primates than in humans. The half-maximal effective concentration (EC50) for DAT occupancy after oral MPH has been shown to be 21.5 mg/kg in nonhuman primates (Wilcox et al., 2008), while in humans it is 0.25 mg/kg (Volkow et al., 1998). In rats, absorption and clearance of MPH are much more rapid than in humans. The rate of absorption after an oral dose of 1 mg/kg MPH in a rat has
been calculated to be only 10 minutes until peak serum concentration (Patrick et al., 1984) and the half life has been reported to be less than 30 minutes (Gal et al., 1977). Due to the rapidity of absorbance and clearance in rodents, most studies have used higher doses (1-3 mg/kg, 2-3 times daily, normally) to replicate human dosing patterns. Another strategy has been to measure blood serum levels of MPH after dosing and to titrate doses to blood plasma levels of 10-15 ng/mL that have been associated with clinical efficacy in children (see Chapter Two; Kuczynski and Segal, 2005; Swanson and Volkow, 2003). Strong correlations between MPH plasma concentration and DAT occupancy have been established, suggesting that plasma measurements are good indicators for monitoring delivery to the brain (Leonard et al., 2004).

3.2.2 Extended release formulations

In recent years, extended-release formulations of MPH such as Concerta® or Metadate CD™ have been developed to overcome problems associated with immediate-release formulations such as multiple daily doses, and acute tolerance to drug effects. These drugs are similar to the immediate release formulations in terms of the extent of absorption, but have been shown to have a much longer uptake and duration in the plasma, as expected (Markowitz et al., 2003; Patrick et al., 1989). Spencer and colleagues (2006a) have directly compared the pharmacokinetic profiles and DAT occupancies of equivalent doses of immediate-release MPH versus a long acting, osmotic release oral system (OROS-MPH, Concerta®) formulation. They found that MPH plasma levels were higher from hours 1-3 post administration of the immediate release formulation, the levels for the two formulations converged at hour 4, and the levels were lower for immediate release MPH than for OROS-MPH at hours 5 to 10. While the maximum concentrations of the two drugs in plasma were not significantly different, the time to max concentration was significantly longer (7.5 ± 1.2 hours) after
the extended release formulation than the immediate release formulation (2.2 ± 0.8 hours) (Spencer et al., 2006a). Importantly, DAT occupancies measured by PET mirrored the blood plasma levels such that, after 1 hour, DAT occupancy with immediate-release MPH was greater than osmotic-release MPH, the values converged at hours 3 and 5, and then DAT occupancy was greater following OROS-MPH at hour 7. Also in accordance with blood plasma levels, the average maximum occupancy was similar for the two formulations (immediate-release 67.6 ± 5.5%; osmotic release 71.5 ± 7.7%) and the time to maximum occupancy was 3 times greater after OROS-MPH (5 ± 2.5 hours) than immediate-release MPH (1.7 ± 1.0 hours) (Spencer et al., 2006a).

Extended-release formulations are relatively new, and are reliant upon oral administration of a pill or capsule in almost all cases, and consequently they are difficult to administer to nonhuman primates and rodents. Thus there are few published studies of extended-release MPH in animals, and, to the best of our knowledge, no published pharmacokinetic studies, despite the fact that these formulations are now the most commonly prescribed. We were able to successfully administer oral doses of extended-release MPH (Metadate CD™) to juvenile nonhuman primates in the experiment described in Chapter Two, which is of great translational value to the human condition.

3.3 Effects at other Monoamine Transporters

In addition to blocking the DAT, MPH has been shown to act at the norepinephrine transporter (NET), and numerous studies in vitro and in vivo have shown increases in brain concentrations of both dopamine and norepinephrine after MPH administration (Ferris et al., 1972; Gatley et al., 1996; Patrick et al., 1987; Ritz et al., 1987; Ross and Kelder, 1979). Several studies in rodents have reported greater increases in hippocampal or prefrontal norepinephrine after low doses of oral MPH than
in striatal dopamine, and have thus suggested that “clinically-relevant” doses preferentially affect the norepinephrine system (Berridge et al., 2006; Kuczenski and Segal, 2002). Furthermore, in vitro studies have suggested that MPH has an even higher affinity for the NET than for the DAT (K_i = 38 nmol/L vs. 193 nmol/L) (Eshleman et al., 1999; Ferris et al., 1972), a finding that was recently supported in vivo in humans (Hannestad et al., 2010). Hannestad and colleagues (2010) used PET to measure NET occupancy after acute doses of MPH and reported that a therapeutic dose of MPH that occupied 60-70% also occupied 70-80% of NETs, suggesting stronger NET involvement. Other stimulants such as amphetamine and cocaine are known to act on both the DAT and the NET, and have action at the serotonin transporter (SERT) as well. However, there is little evidence to suggest that MPH has any affect on the SERT (Gatley et al., 1996; Kuczenski and Segal, 1997; Leonard et al., 2004).

While the norepinephrine system is clearly affected by MPH, many of the behaviors that are known to be influenced by MPH, as well as involved in ADHD, are associated with dopamine function. Additionally, the dopamine system plays a significant role in drug reward and vulnerability to substance abuse, which will be reviewed in section 6.1. As the question of whether or not early MPH treatment influences future substance abuse was of great importance in the present investigations, the emphasis of this present set of studies was therefore on the dopamine system. These questions were addressed in animal models (see section 8.1 for rationale) and thus the existing literature on the effects of acute and chronic MPH treatment on behavior and the dopamine system in animals is discussed below.
4. Acute Methylphenidate treatment in animals

MPH treatment in animals has been shown to affect many behaviors that are mediated by dopamine systems, including locomotor behavior, impulsivity, and attention (reviews: Albin et al., 1995; Berridge, 2007; Salamone, 2002; Salamone and Correa, 2002). Additionally, MPH has been shown to affect measures of cognitive performance, anxiety-like behavior, exploratory behavior, and spatial learning. However, it is clear that the effects of MPH on animal behavior vary depending on several critical factors such as MPH dose, the route of administration, and the age of the animal at testing. Additionally, there are substantial differences between the effects of acute treatment and chronic treatment, which will be covered below.

4.1 Behavioral effects of acute MPH treatment: critical factors

4.1.1 Dose-dependency

Importantly, similar to other stimulant drugs, the effects of MPH on behavior in animals have been shown to be largely dose-dependent. For example, locomotor activity has been shown to be unaffected by low (2 mg/kg), oral doses of MPH in rodents (Kuczenski and Segal, 2002), yet increased by doses of 10- (Torres-Reveron and Dow-Edwards, 2005), or 20 mg/kg (McNamara et al., 1993). Torres-Reveron and Dow-Edwards (2005) showed that a 20 mg/kg dose of MPH was associated with lower levels of locomotor activity, as measured by total distance travelled, compared to 10 mg/kg, yet the rats did engage in greater levels of high intensity stereotypical behavior after 20 mg/kg MPH (Torres-Reveron and Dow-Edwards, 2005). Dose-dependency has also been observed on other tasks, such as delayed alternation, a cognitive task whereby rats were required to alternate arms of T-maze for a reward. The acute doses of MPH tested were 0.5, 1.0, 1.5, 2.0, and 3 mg/kg, delivered orally. Performance increased with
dose until it peaked at 1.5 mg/kg, but was disrupted by higher doses (Arnsten and Dudley, 2005).

There are also dose dependent effects of stimulants on measures of impulsivity. At low doses, MPH has been shown to improve performance, decreasing impulsive-like behavior (Pattij et al., 2007; Perry et al., 2008; Puumala et al., 1996; van den Bergh et al., 2006; van Gaalen et al., 2006; Wooters and Bardo, 2011). At higher doses, though, MPH acts to increase impulsivity (Kuczenski and Segal, 1997). Effects also vary depending on the baseline performance of the animals. Stimulant drugs usually increase impulsivity on the 5-choice serial response time task (Adriani et al., 2006; Floresco et al., 2008; Isles et al., 2003; Richards et al., 1999; van Gaalen et al., 2006; Wade et al., 2000), unless rats have an unusually high rate of premature responses to begin with, in which case stimulant administration improves performance (Puumala et al., 1996). Thus, at the low doses normally used therapeutically, MPH is likely to improve performance on tasks that measure impulsive-like behavior.

4.1.2 Route of administration

The route of administration of the drug has also been shown to be a critical factor that influences behavior outcomes as well as brain dopamine measures. For example, previous studies have shown that 2 mg/kg MPH will enhance locomotor activity when administered by intraperitoneal injection, but the same dose has no effects when given intragastrically (Gerasimov et al., 2000) or orally (Kuczenski and Segal, 2002). Similarly, Griggs and colleagues (2010) reported that intraperitoneal injection of MPH resulted in hyperactivity in the open field, while continuous administration resulted in significantly lower levels of activity, even when dosages were normalized between groups.
4.1.3 Age-dependency

There is also evidence that stimulant effects in rodents are age-dependent. For example, late-adolescent animals (PND 45) have been shown to have lower levels of stereotypical behavior in response to high doses (20 mg/kg) of MPH than juveniles (PND 21), or adults (PND 60) (Torres-Reveron and Dow-Edwards, 2005). Other studies have reported that low doses of MPH stimulate levels of locomotor behavior to a greater degree in juvenile rats than in adult rats (Heyser et al., 2004; Wooters et al., 2006).

Among all of these variables, one consistent treatment pattern emerges. That is, orally administered, low doses of MPH generally do not have adverse behavioral consequences in juvenile and adolescent rats. In fact, this type of dosing regimen has been associated with beneficial effects on several behaviors. For example, 2.5-3 mg/kg oral MPH has been associated with lower levels of anxiety-like behavior and better performance on attentional tasks (Koike et al., 2009; Zhu et al., 2010). In addition, the same dose has been shown to lead to greater exploratory behavior and performance on a radial arm maze (Dow-Edwards et al., 2008). Contrarily, higher doses have often been associated with negative effects. An acute dose of 10 mg/kg has been reported to lead to impaired recognition memory (Chuhan and Taukulis, 2006), in addition to the locomotor stimulating effects of this dose (Torres-Reveron and Dow-Edwards, 2005). Thus, at low doses that might be considered “clinically-relevant”, based on the blood levels of drug that they produce (Kuczynski and Segal, 2005), acute MPH does not appear to have adverse behavioral consequences in animals.

4.2 Dopaminergic effects of acute MPH treatment in animals

As in human studies, acute administration of MPH in animals has been consistently shown to increase extracellular dopamine levels (Berridge et al., 2006; Huff
and Davies, 2002; Jezierski et al., 2007; Koda et al., 2010; Sproson et al., 2001; Walker et al., 2010; Weikop et al., 2007). As the dopamine system is undergoing substantial development during the juvenile and adolescent period, the age of the animal during MPH testing has been shown to influence the effects of acute MPH treatment on brain dopamine levels. For example, Walker and colleagues (2010) showed that 10 mg/kg MPH had a much greater effect of increasing extracellular dopamine in adolescent rats (PND 28) when compared to adult rats (PND 65). This finding is consistent with behavioral studies that report greater MPH-stimulated locomotor activity in adolescent rats when compared to adult rats (Wooters et al., 2006).

5. Chronic MPH treatment in animals

While studies of acute treatment in animals have been valuable for delineating the drug mechanisms of action and the subsequent behavioral outcomes, most children and adults take MPH daily for many years. Thus studies of chronic treatment in animals are necessary to reveal long-term treatment effects. Results have shown that behavioral effects of chronic MPH treatment can vary greatly from acute treatment effects.

5.1 Behavioral effects of chronic treatment

Similar to acute treatment, the effects of chronic treatment are influenced by MPH dose, age of animal and route of administration. Doses of 2-10 mg/kg/day for several weeks have been shown to lead to lower levels of locomotor activity (Bethancourt et al., 2011; Bolanos et al., 2003; Britton and Bethancourt, 2009), yet doses of 44 mg/kg/day for seven days have been associated with much greater levels of locomotor activity (Izenwasser et al., 1999). Bolanos and colleagues (2003) documented higher levels of anxiety-like behavior after 2 weeks of 4 mg/kg/day MPH.
treatment by injection, yet Britton and Bethancourt (2009) showed that 7 weeks of 4, 6, or 10 mg/kg/day MPH, administered orally, had no effect on anxiety-like behavior.

5.2 Dopaminergic effects of chronic treatment

Within the dopamine system, chronic MPH treatment has been associated with a number of alterations. Several studies have documented reduced DAT density after chronic MPH treatment at various doses (Izenwasser et al., 1999; Moll et al., 2001; Simchon et al., 2010), though it appears these effects may be dependent on route of administration. DAT density was lower in animals that received intraperitoneal injections of MPH, but not in animals that had a continuous infusion of MPH for 7 days, when compared to control animals (Izenwasser et al., 1999). MPH has also been associated with significantly downregulated basal dopamine levels in the nucleus accumbens and mPFC after 24 days of 10 mg/kg day injections (Jezierski et al., 2007), as well as an overall reduction of the number of dopamine neurons in the substantia nigra (Sadasivan et al., 2012). In mice, chronic treatment with a high dose of MPH (15 mg/kg/day) has been found to increase the density of dendritic spines on D1-like and D2-like receptor expressing medium spiny neurons in the nucleus accumbens (Kim et al., 2009). Additionally, Thanos and colleagues (2007) reported that 8 months of daily, oral MPH treatment at 2 mg/kg/day increased D2-like receptor availability in the striatum.

5.3 Duration of treatment

In addition to the influence of dose level, route of administration, and age of animals, the above studies highlight another critical factor in studies of chronic treatment, which is the duration of treatment. In the literature reviewed above, “chronic” treatment in rodents ranges from 5 days to 8 months of daily MPH administration. Studies that have directly compared duration of treatment have documented differences
in locomotor and anxiety-like behavior after 4 or 7 weeks of treatment, holding all other variables constant (Britton and Bethancourt, 2009). Similarly, while 8 months of oral 2 mg/kg MPH treatment was associated with increased D2-like receptor availability, 2 months of treatment at the same dose and through the same route of administration was associated with decreased D2-like receptor availability (Thanos et al., 2007). Thus, both behavior and dopaminergic measures can be significantly affected by duration of treatment, holding all other variables constant.

The wide variability in duration of treatment in previous literature has made comparing results between studies extremely difficult. In the current series of studies, we attempted to address this issue by administering MPH during developmentally-relevant stages in nonhuman primates and rodents. Hence, monkeys were treated for one year between the ages of 3.5 years and 4.5 years (Chapter Two), and rats were treated for three weeks from PND 28 to 49 (Chapters Three and Four). These ages correspond to late childhood and adolescence (Figure 1), well within the range at which treatment is often initiated in children.

In addition, as the dopamine system is known to mediate the rewarding effects of drugs (see section 6.1) an important question that derives from this is how changes in the dopamine system influence vulnerability to the reinforcing effects of drugs. Specifically, does MPH interact with the developing dopamine system to influence vulnerability to substance abuse? In Chapters Two and Four of the present report, the effects of chronic MPH treatment on future vulnerability to cocaine abuse (Chapter Two) and alcohol abuse (Chapter Four) were investigated.
6. Factors that Influence Vulnerability to Substance Abuse/Addiction

6.1 Dopamine’s involvement with drug reward

As mentioned above, midbrain dopamine signaling is central to the rewarding effects of drugs of abuse, and is intricately involved in the development of substance abuse disorders (Hyman et al., 2006; Robinson and Berridge, 1993). It is generally accepted that the rewarding effects of drugs are due to their ability to increase dopamine in the mesolimbic pathway, and particularly in the nucleus accumbens (Koob, 1998). However, there are important roles for the mesocortical and nigrostriatal dopamine pathways as well (Wise, 2009), and it is clear that stimulation of either the VTA or the substantia nigra is rewarding (Prado-Alcala et al., 1984; Prado-Alcala and Wise, 1984). Stimulants (Drevets et al., 2001; Volkow et al., 2001b), alcohol (Boileau et al., 2003; Ramchandani et al., 2011), and marijuana (Bossong et al., 2009) all increase dopamine in the ventral striatum by various means in human subjects as well as in animal models (Di Chiara and Imperato, 1988). Dopaminergic antagonist administration and lesions of the ventral striatum will, on the other hand, attenuate the reinforcing effects of drugs (Maldonado et al., 1993; Roberts et al., 1980). However, it must be noted that stimuli with aversive properties will also increase dopamine in the ventral striatum (Imperato et al., 1992; Kalivas and Duffy, 1995; McCullough and Salamone, 1992). Thus, the effects of dopamine in this region are not necessarily associated with the hedonic or pleasure inducing effects of drugs, so much as the salience, or the incentive value of the stimuli (Ikemoto, 2010).

Despite the fact that drugs of abuse share the characteristic of increasing dopamine transmission in the ventral striatum, simply increasing dopamine transmission by taking drugs is not sufficient for an individual to develop a substance abuse disorder.
or addiction. Indeed, only approximately 10-20% of individuals who use illicit drugs will end up fitting diagnostic criteria for drug dependence (Volkow et al., 2005; Volkow et al., 2010). Determining the factors that make individuals vulnerable to drug dependence has therefore been a major focus of addiction science. Research has shown that both dopaminergic and environmental influences play a role in the development of substance abuse patterns, that adolescence is a critical period, and that the dopamine D2-like receptor family is involved.

6.2 The influence of D2-like receptors on vulnerability to substance abuse

Of all the dopamine system components, the one that has been most highly implicated in vulnerability to addiction is the D2-like receptor protein. For example, one important PET study of non-drug using individuals quantified the availability of D2-like receptors using $[^{11}\text{C}]$raclopride before and after MPH treatment (Volkow et al., 1999). After the initial, pre-drug PET scan, each individual was given a dose of MPH and a survey that measured the subjective effects of the drug. Approximately half of the subjects found the MPH dose pleasurable, while the remaining half were indifferent or found the MPH aversive. The self-reported subjective experience of the drug effects were negatively correlated with the D2-like receptor availability of the subjects. Thus, individuals with greater levels of D2-like receptors tended to find the MPH unpleasant and those with lower levels found it pleasant (Volkow et al., 1999). The conclusion drawn from this study was that there is an inverse relationship between D2-like receptor density and the reinforcing properties of stimulant drugs like MPH. These findings have been extended into animals models. Morgan and colleagues (2002) reported that environmental conditions, such as social rank within a housing group, could affect D2-like receptor density in male cynomolgus monkeys. Specifically, monkeys that became dominant, when socially housed, had a 20% increase in D2-like receptor density.
Importantly, these monkeys self administered cocaine at lower rates and had lower intakes when compared with monkeys that were subordinate within the social group (Morgan et al., 2002).

In addition to the studies described above, there is evidence that individuals dependent on cocaine (Volkow and Fowler, 2000), methamphetamine (Volkow et al., 2001a), alcohol (Volkow et al., 2007), and nicotine (Fehr et al., 2008) have lower levels of D2-like receptor availability than non-drug using individuals. These low levels have been associated with lower activation in areas of the brain that are involved in compulsive behavior, including the orbital frontal cortex and anterior cingulate cortex (Baxter et al., 1987; Brown et al., 2006; Volkow et al., 1992). In addition, higher than normal levels of D2-like receptor availability are seen in non-alcohol using 1st degree family members of alcoholics. As alcoholism is highly heritable, this finding suggests that having high D2-like receptor availability confers some kind of protection against developing alcoholism (Volkow et al., 2006). Taken together, the D2-like receptor family is clearly an important target to examine when investigating changes in the dopamine system that may confer vulnerability to substance abuse disorders. As discussed in section 2.3.3, dopamine receptors, including D2-like receptors, undergo substantial changes during the adolescent period. These changes may relate to notable behavioral changes during adolescence, a critical period for the initiation of substance abuse.

6.3 The adolescent period

Adolescence, defined as the ages from 10 years to 19 years old in humans (World Health Organization, 2010) is a transitional period marked by behavioral, hormonal, and neurochemical changes that prepare an individual for independent survival (Casey et al., 2008; Doremus-Fitzwater and Spear, 2007; Spear, 2002;
Wahlstrom et al., 2010). There is a plentiful literature covering the myriad of physical and social changes that take place during this developmental stage. Here, only the aspects pertinent to the present investigations are briefly reviewed.

The adolescent period is critical for the development of addiction behaviors. The continued maturation of the PFC during this stage is associated with performance on a wide variety of tasks including those measuring planning (Asato et al., 2006; Luciana et al., 2009), concept formation (Chelune and Baer, 1986), working memory (Conklin et al., 2007; De Luca et al., 2003; Luciana et al., 2005), impulsivity (Olson et al., 2007; Steinberg et al., 2009), and motivated decision making (Cauffman et al., 2010; Crone and van der Molen, 2004; Hooper et al., 2004; Overman et al., 2004). It is thought that the immature PFC may be the reason why adolescents are typically more impulsive than adults, are novelty-seekers, and tend to take greater risks without anticipation of adverse consequences (Laviola et al., 2003; Steinberg, 2008). In addition, the effects of social dynamics are at an all time high, as adolescents often leave the “family nest” and are heavily influenced by their peers (Forbes and Dahl, 2005; Steinberg, 2010a; Steinberg, 2010b).

These behavioral outcomes are associated with underlying changes in the adolescent dopamine system. One theory of brain dopamine development during adolescence suggests that increases in tonic dopamine activity at this age mediates incentive-motivated approach behavior (Depue and Collins, 1999) and promotes reward-seeking through activity in limbic, striatal, and prefrontal networks (Wahlstrom et al., 2010). Heightened tonic dopamine activity within this system may result in an over-activation of incentive motivation in the absence of reliable behavioral control, which is modulated by an as-yet immature, PFC (Wahlstrom et al., 2010). It is clear that there are age related differences in orbital frontal cortex and ventral striatum activity when
individuals make responses to rewards or reward-relevant decisions. Adolescents have been shown to have heightened activity to rewarding stimuli in these regions when compared to adults (Ernst et al., 2005; Galvan et al., 2006; Van Leijenhorst et al., 2010). Regardless of the exact neural mechanisms by which adolescents are more vulnerable to the rewarding effects of drugs, it is clear that this developmental stage is critical for the initiation of substance abuse. At this age, adolescents often experiment with or use cigarettes and marijuana, and occasionally “harder” drugs such as cocaine and heroin. However, the most commonly abused substance during adolescence is alcohol.

6.4 Adolescence and alcohol drinking

Compared to adults, adolescents appear to be less sensitive to the negative effects of alcohol that regulate consumption, yet more sensitive to the positive, reinforcing effects, serving to increase intakes (Spear and Varlinskaya, 2005). Epidemiological studies have reported that, by 12th grade, 7 of 10 high school students have experimented with alcohol, 54% reported having been drunk at least once in their life, and 24% reported at least one episode of binge drinking, defined as greater than 5 drinks for a male and 4 drinks for a female, in the last two weeks (Johnston et al., 2013). These rates are far higher than any other abused substance. For example, the second most commonly abused drug in this age group, marijuana, had been used by only 45% of 12th graders, compared to the 70% who reported alcohol use (Johnston et al., 2013). Additionally, this behavior has been shown to increase during college years. Thirty-seven percent of college students reported one episode of binge drinking in the past two weeks and almost 50% reported having been drunk at least once in the past 30 days (Johnston et al., 2013). Alcohol abuse in this age group has been associated with numerous immediate negative consequences, including personal injury, risky sexual behavior, and injury to others, as well as long term consequences such as poorer
academic performance and increased likelihood of substance dependence in adulthood. Studies are clear that individuals who begin using drugs earlier than their peers are more likely to develop a substance abuse disorder in adulthood than individuals who begin using drugs later in life (Anthony and Petronis, 1995; Clark et al., 2005; Prescott and Kendler, 1999).

Age-related differences in the rewarding properties of alcohol that lead to greater alcohol intakes during adolescence may be related to the developmental stage of the dopamine system. In rats, acute alcohol exposure during adolescence has been shown to result in a greater increase in dopamine release in the NAcc than during adulthood (Pascual et al., 2009; Philpot et al., 2009; Soderpalm et al., 2009), which may explain why alcohol appears to be more rewarding to adolescents (Doremus-Fitzwater et al., 2010; Pautassi et al., 2008; Ristuccia and Spear, 2008). Despite the fact that 5-6% of children will have a history of MPH prescription upon reaching college, and that MPH is known to affect the dopamine system, few studies have attempted to determine whether chronic MPH in childhood affects future alcohol or drug use.

6.5 Methylphenidate treatment and vulnerability to substance abuse

Human studies that have attempted to answer this question are naturally confounded by the presence of ADHD in the tested population. The disorder itself is associated with a two-fold greater risk for the development of a substance abuse disorder (Biederman et al., 1995; Biederman et al., 1997; Gittelman et al., 1985; Milberger et al., 1997a; Milberger et al., 1997b). Adolescents with ADHD have a higher rate of conduct disorder and substance abuse than healthy adolescents (Barkley et al., 1990; Biederman et al., 1996; Molina and Pelham, 2003). However, most recent clinical literature suggests that early stimulant medication for ADHD may actually have a
protective effect against the development of substance abuse disorders in adulthood (Biederman, 2003; Fischer and Barkley, 2003; Katusic et al., 2005; Mannuzza et al., 2003b; Volkow and Swanson, 2008; Wilens et al., 2003). Medicated ADHD adolescents have been reported to have an 85% reduction in risk for substance abuse disorder when compared to unmedicated ADHD subjects (Biederman et al., 2009). In one study that reported that MPH treatment was associated with an increase in the likelihood of trying cocaine, that result seemed to be explained by the presence of comorbid conduct disorder within the test population (Barkley et al., 2003). Despite these results, it must be noted that studies of stimulant medication in human populations are limited by the difficulty of finding non-medicated children with ADHD and there is likely to be considerable selection bias (Volkow and Insel, 2003). Thus, animal models are important to add to the current knowledge of the effects of stimulant medication.

However, results in animal models have been equivocal. In rodents, Thanos and colleagues (2007) showed that 8 months of oral chronic MPH treatment at low doses increased D2-like receptor availability in the striatum and decreased cocaine self-administration. Likewise, several studies have reported that two weeks of MPH administration in young rodents was associated with a greater response to the aversive effects of cocaine as measured by conditioned place preference (CPP) (Andersen et al., 2002; Carlezon et al., 2003). In contrast to the CPP results, several other studies have shown that young rats that have been exposed to chronic MPH treatment prior to testing for cocaine self-administration tended to exhibit enhanced vulnerability to the reinforcing effects of cocaine (Brandon et al., 2001; Crawford et al., 2011; Harvey et al., 2011). Again, there is evidence that this effect may be dependent on route of administration, as daily injections of MPH increased the response to cocaine, but administration by osmotic minipump had no effect, in at least one study (Griggs et al., 2010). Similarly, the dose
of MPH has been shown to affect cocaine reinforcement, as pretreatment for 9 days with intraperitoneal injections of 5 mg/kg of MPH had no effect, but a single MPH dose of 20 mg/kg significantly reduced latency to acquire cocaine self-administration (Schenk and Izenwasser, 2002).

Interestingly, despite the fact that alcohol is the most commonly abused substance in adolescents and young adults, very few studies have examined the impact of prior chronic MPH treatment on future alcohol abuse. To the best of our knowledge, only two studies have been completed and they reported conflicting results, with one suggesting that MPH treatment enhanced future ethanol (EtOH) intake (Vendruscolo et al., 2008), and the other reporting no association between MPH treatment and EtOH intake (Soeters et al., 2008). Here we examined vulnerability to both cocaine and alcohol in two different animal models. In Chapter Two, we examined the impact of chronic MPH treatment during adolescence on future vulnerability to cocaine abuse in nonhuman primates. In Chapter Four, juvenile rats were treated with MPH for three weeks during adolescence and then EtOH intakes and preferences were measured. Additionally, we examined the interaction of MPH treatment with early rearing environment on behavior and vulnerability to substance abuse, a topic that is reviewed in greater detail below.

7. Influence of environment on behavior, dopamine, and vulnerability to substance abuse

In rodent models, the housing environment has been shown to have powerful and enduring effects on behavioral phenotypes and dopamine physiology, particularly during the juvenile and adolescent stages. Typically, two conditions are examined:
enriched environment and isolated/impoverished environment, often with a standard housing condition (e.g. two rats to a standard sized cage without additional toys) serving as a control between the two extremes. Enriched conditions are variable among studies, but normally include social housing with at least 2, but usually more than 2 rats per cage as well as some access to toys or climbing structures that are changed frequently. Contrarily, isolated rats are housed singly without access to toys or other rats. Both of these conditions have been found to alter behavior, dopamine neurochemistry, and vulnerability to substance abuse when compared to standard pair-housing.

7.1 Effects of environment on behavior

Many studies have looked at the effects of rearing conditions on behavior and the results are largely summarized in Tables II and III. Most studies that specifically examine social and environmental enrichment in the form of multiple rats per cage, large cages, climbing structures, and toys, compare enriched animals to socially isolated animals as a control group. However, studies that are specifically investigating the effects of isolation compare isolated animals to group-housed animals without additional environmental enrichment. This is one reason that comparing results between studies of enrichment and isolation is difficult. Additionally, there are wide variations in the duration of exposure to the environment, the complexity of the environment, and the age of the animals during exposure. Most studies focus on the immediate postweaning period (starting at PND 21) as this stage has been shown to be critical in the formation of enduring behavioral phenotypes.

One of the most consistent findings is that enriched rats have reduced spontaneous locomotor activity (Bardo et al., 1995; Bowling et al., 1993; Brenes et al., 2008; Del Arco et al., 2007a; Segovia et al., 2008), which results in larger increases in
locomotor activity after challenge with a stimulant drug, such as amphetamine (Bardo et al., 1995). However, enriched rats are less susceptible to locomotor sensitization than isolated rats following repeated amphetamine injections (Bardo et al., 1995), which is consistent with their reported resistance to the rewarding effects of drugs (Stairs and Bardo, 2009). Contrarily, socially isolated animals have consistently been shown to have greater levels of spontaneous locomotor activity in the open field than group-housed animals (Fabricius et al., 2011a; King et al., 2009; Shao et al., 2009; Varty et al., 2000). Other consistent findings include greater levels of anxiety-like behavior (Chappell et al., 2013; Lodge and Lawrence, 2003; Lukkes et al., 2009; McCool and Chappell, 2009; Yorgason et al., 2013), and decreases in sensory motor gating (Fabricius et al., 2011b; Varty et al., 2000; Wang et al., 2012) among isolated animals when compared to group-housed controls.
Table II. Effects of enriched environment on behavior

<table>
<thead>
<tr>
<th>Reference</th>
<th>Conditions</th>
<th>Age of animals during exposure to environment</th>
<th>Effect</th>
<th>Comparison Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Locomotor Activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowling et al., 1993</td>
<td>12-13 rats, large cages, toys</td>
<td>PND 21-60</td>
<td>↓ baseline locomotor activity; ↑ amphetamine stimulated locomotor activity</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Del Arco et al., 2007a</td>
<td>10-12 rats, large cages, toys</td>
<td>Adults: 3 months - 15 months</td>
<td>↓ locomotor activity in the open field</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Del Arco et al., 2007b</td>
<td>10-12 rats, large cages, toys</td>
<td>Adults: 3 months - 6 months</td>
<td>↓ spontaneous locomotor activity</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Segovia et al., 2008</td>
<td>10-12 rats, large cages, toys</td>
<td>Adults: 3 months-6 months, 12 months, 24 months</td>
<td>↓ spontaneous locomotor activity in 6 month old and 12 month old rats, no difference in older rats</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Brenes et al., 2008</td>
<td>15 rats, large cage, toys</td>
<td>PND 30-114</td>
<td>↓ locomotor activity in the open field than other two groups</td>
<td>Isolated animals, and groups of 3</td>
</tr>
<tr>
<td>Bardo et al., 1995</td>
<td>12-13 rats, large cages, toys</td>
<td>PND 21-50</td>
<td>↑ amphetamine stimulated locomotor activity; ↓ locomotor sensitization to repeated amphetamine injection</td>
<td>Isolated animals</td>
</tr>
<tr>
<td><strong>Other behaviors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brenes and Fornaguera, 2008</td>
<td>15 rats, large cage, toys</td>
<td>PND 30-114</td>
<td>↑ levels of swimming and climbing, ↓ immobility in the forced swim test than other groups</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Brenes and Fornaguera, 2009</td>
<td>12 rats, large cage, toys</td>
<td>PND 28-60</td>
<td>No difference in elevated plus maze activity, ↑ levels of swimming and climbing, ↓ immobility in the forced swim test than other two groups</td>
<td>Isolated animals, and groups of 3</td>
</tr>
<tr>
<td>Reference</td>
<td>Conditions</td>
<td>Age of animals during exposure to environment</td>
<td>Effect</td>
<td>Comparison Group</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>---------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td><strong>Locomotor Activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shao et al., 2009</em></td>
<td>Single housing</td>
<td>PND 38-51</td>
<td>↑ locomotor activity in the open field</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td><em>Varty et al., 1999</em></td>
<td>Single housing</td>
<td>PND 21-77</td>
<td>↑ locomotor activity in the open field</td>
<td>Groups of 3 animals</td>
</tr>
<tr>
<td><em>King et al., 2009</em></td>
<td>Single housing</td>
<td>PND 21-36</td>
<td>↑ locomotor activity in the open field</td>
<td>Groups of 3 animals</td>
</tr>
<tr>
<td><em>Wang et al., 2012</em></td>
<td>Single housing</td>
<td>PND 28-91</td>
<td>↑ locomotor activity in the open field</td>
<td>Groups of 3-4 animals</td>
</tr>
<tr>
<td><em>Fabricius et al., 2011</em></td>
<td>Single housing</td>
<td>PND 25-81</td>
<td>↑ locomotor activity in the open field</td>
<td>Groups of 5 animals</td>
</tr>
<tr>
<td><em>Chappell et al., 2013</em></td>
<td>Single housing</td>
<td>PND 28-70</td>
<td>↑ locomotor response to a novel environment</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td><em>Hall et al., 1998b</em></td>
<td>Single housing</td>
<td>PND 21-85</td>
<td>↑ levels of locomotor activity in the open field that dissipated with repeated testing and handling</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td><strong>Anxiety-like behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chappell et al., 2012</em></td>
<td>Single housing</td>
<td>PND 28-70</td>
<td>↑ levels of anxiety-like behavior</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td><em>McCool and Chappell, 2009</em></td>
<td>Single housing</td>
<td>PND 28-70</td>
<td>↑ anxiety-like behavior on the elevated plus maze</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td><em>Lodge et al., 2003</em></td>
<td>Single housing</td>
<td>PND 21-83</td>
<td>↑ anxiety-like behavior on the elevated plus maze</td>
<td>Groups of 3-4 animals</td>
</tr>
<tr>
<td><em>Yorgason et al., 2013</em></td>
<td>Single housing</td>
<td>PND 28-77</td>
<td>↑ in anxiety-like behavior, enduring 4 months past original testing</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PND 28-77, then all animals singly housed</td>
</tr>
</tbody>
</table>
### Other behaviors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Housing Condition</th>
<th>PND</th>
<th>Observations</th>
<th>Group Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baarendse <em>et al.</em>, 2013</td>
<td>Single housing, then resocialize at PND 42</td>
<td>PND 21-42</td>
<td>At PND 82, impairments in decision making (gambling task), ↓ performance on the 5-CSRTT (impulsivity task)</td>
<td>Pair-housed animals</td>
</tr>
<tr>
<td>Hall <em>et al.</em>, 1998a</td>
<td>Single housing</td>
<td>PND 21-105</td>
<td>No differences in immobility, swimming, or climbing on the forced swim test</td>
<td>Pair housing</td>
</tr>
<tr>
<td>Fitzgerald <em>et al.</em>, 2013</td>
<td>Single housing</td>
<td>PND 23-79</td>
<td>↓ of pre-pulse inhibition to acoustic startle</td>
<td>Groups of 3 animals</td>
</tr>
<tr>
<td>Wang <em>et al.</em>, 2012</td>
<td>Single housing</td>
<td>PND 28-91</td>
<td>↓ prepulse inhibition to acoustic startle</td>
<td>Groups of 3-4 animals</td>
</tr>
<tr>
<td>King <em>et al.</em>, 2009</td>
<td>Single housing</td>
<td>PND 21-36</td>
<td>↓ ability to discriminate between familiar and novel objects</td>
<td>Groups of 3 animals</td>
</tr>
</tbody>
</table>
7.2 Effects of environment on the dopamine system

In addition to influencing behavioral phenotypes, environmental conditions have been shown to have a significant impact on multiple aspects of dopamine system function (see Tables IV and V). While some of the behavioral effects of enrichment and isolation (e.g. locomotor activity and anxiety-like behavior) are largely agreed upon, there is little consensus surrounding the effects of environment on the dopamine system. Again, the results of these studies are influenced by the specific housing condition as well as the duration of exposure, but also variables such as rodent strain and age of subjects. As discussed previously (section 2), the dopamine system is undergoing substantial reorganization in rodents during the postweaning period, and thus duration of exposure and the age of the animal at testing is likely to have a significant impact on dopaminergic measures. For example, basal dopamine level has been shown to be higher in the mPFC of Sprague Dawley rats that were socially isolated from PND 21-85 (Han et al., 2011), but not changed in Lister-hooded rats that were isolated from PND 28-56 (Dalley et al., 2002), and lower in Lister-hooded rats isolated from PND 25-109 (Fabricius et al., 2011a), when compared to group housed animals.

Rearing condition has been shown to lead to alterations in the densities of D1-like and D2-like receptors in previous literature, though there is no consensus on the direction of the changes. Specifically, Bardo and Hammer (1991) documented no differences in D1-like or D2-like receptor density associated with rearing in an isolated, group-housed, or enriched environment in Sprague Dawley rats exposed to environmental conditions from PND 30-60. D1-like receptor concentration and sensitivity has been reported to be greater in socially-isolated mice (Gariepy et al., 1995) and adult rats (Del Arco et al., 2007a) than in group-housed animals, though other studies have also reported no changes in D1-like receptors associated with social
isolation in younger animals (Djouma et al., 2006). The effects of environmental conditions on D2-like receptors have been of particular interest because of their association with vulnerability to substance abuse, which is known to be altered by rearing condition (see section 7.3). However, the effects of environment on the D2-like receptor are also unclear as there are reports of increases (Djouma et al., 2006; King et al., 2009), decreases (Hall et al., 1998c), and no changes (Bardo and Hammer, 1991; Del Arco et al., 2004; Malone et al., 2008) in D2-like receptor concentration after social isolation, compared to group housing. Thus, questions remain as to the effects of environmental enrichment and social isolation on D1-like and D2-like receptors. To address this, one goal of the studies presented in chapter 3 was to investigate the effects of rearing condition on the concentrations of both families of dopamine receptors in the striatum during a critical period of neurodevelopment, the postweaning period until early adulthood.
### Table IV: Effects of enriched environment on the dopamine system

<table>
<thead>
<tr>
<th>Reference</th>
<th>Conditions</th>
<th>Age of animals during exposure to environment</th>
<th>Effect</th>
<th>Comparison Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lack et al., 2010</td>
<td>Pair-housed with toys</td>
<td>PND 28-58</td>
<td>↑ glucose utilization in the nucleus accumbens core and shell</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Bowling et al., 1993</td>
<td>12-13 rats, large cages, toys</td>
<td>PND 21-60</td>
<td>↑ dopamine synthesis in the striatum in response to amphetamine <em>in vivo</em></td>
<td>Isolated animals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ dopamine metabolism in the nucleus accumbens in response to amphetamine <em>in vivo</em>;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ baseline tissue concentration of dopamine in the striatum <em>in vitro</em></td>
<td></td>
</tr>
<tr>
<td>Zhu et al., 2004</td>
<td>8-12 rats, large cages, toys</td>
<td>PND 21-53</td>
<td>↓ maximum velocity of dopamine uptake in mPFC</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Zhu et al., 2005</td>
<td>8-12 rats, large cages, toys</td>
<td>PND 21-53</td>
<td>↓ cell surface expression of DAT in mPFC</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Del Arco et al., 2007</td>
<td>10-12 rats, large cages, toys</td>
<td>Adults: 3 months - 6 months</td>
<td>↓ sensitivity of D1-like receptors in the PFC</td>
<td>Isolated animals</td>
</tr>
<tr>
<td>Reference</td>
<td>Conditions</td>
<td>Age of animals during exposure to environment</td>
<td>Effect</td>
<td>Comparison Group</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>----------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Dopamine Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitzgerald et al., 2013</td>
<td>Single housing</td>
<td>PND 23-79</td>
<td>↓ D2-like receptor density in prelimbic area of prefrontal cortex</td>
<td>Groups of 3 animals</td>
</tr>
<tr>
<td>Hall et al., 1998</td>
<td>Single housing</td>
<td>PND 21-106</td>
<td>↓ D2-like receptors in the nucleus accumbens</td>
<td>Groups of 4 animals</td>
</tr>
<tr>
<td>King et al., 2009</td>
<td>Single housing</td>
<td>PND 21-36</td>
<td>↑ in striatal D2-like receptors</td>
<td>Groups of 3 animals</td>
</tr>
</tbody>
</table>
| Djouma et al., 2006 | Single housing | PND 21-84                                    | ↑ D2-like receptor binding in nucleus accumbens, basolateral amygdala, central amygdala, substantia nigra
No change in D1-like receptor binding | Groups of 3-4 animals                                  |
<p>| Bardo and Hammer, 1991 | Single housing | PND 30-60                                    | No change in D1-like or D2-like receptor binding                       | Groups of 3 animals, or group of 11 animals with toys (enriched) |
| Del Arco et al., 2004 | Single housing | PND 21-78                                    | No change in the number, affinity, or functional efficacy of D2-like receptors | Groups of 4 animals                                  |
| Malone et al., 2008 | Single housing | PND 21-78                                    | No changes in D2-like receptor expression in caudate putamen or nucleus accumbens | Groups of 6 animals                                  |
| <strong>Other Measures</strong>   |                |                                              |                                                                        |                                                      |
| Shao et al., 2009   | Single housing | PND 38-51                                    | ↑ dopamine concentration in the nucleus accumbens                      | Groups of 4 animals                                  |
| Yorgason et al., 2013 | Single housing | PND 28-77                                    | ↑ stimulated dopamine release and reuptake in the nucleus accumbens    | Groups of 4 animals                                  |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Housing</th>
<th>Age (PND)</th>
<th>Findings</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabricius et al., 2011</td>
<td>Single housing</td>
<td>25-109</td>
<td>↑ dopamine release in mPFC and Nacc in response to amphetamine challenge</td>
<td>Groups of 5 animals</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ basal DA release in the mPFC</td>
<td></td>
</tr>
<tr>
<td>Fabricius et al., 2010</td>
<td>Single housing</td>
<td>25-109</td>
<td>↑ number of spontaneously active DA neurons in the VTA</td>
<td>Groups of 5 animals</td>
</tr>
<tr>
<td>Miura et al., 2002</td>
<td>Single housing</td>
<td>77-105</td>
<td>↓ dopamine turnover in the mPFC and nucleus accumbens</td>
<td>Groups of 3 animals</td>
</tr>
<tr>
<td>Wang et al., 2012</td>
<td>Single housing</td>
<td>28-91</td>
<td>↓ dendritic complexity and spine density in the mPFC</td>
<td>Groups of 3-4 animals</td>
</tr>
</tbody>
</table>
7.3 Effects of environment on vulnerability to substance abuse

While there is discrepancy surrounding the exact effects of social isolation and environmental enrichment on dopaminergic parameters, the effects of early environmental conditions on vulnerability to substance abuse are mostly consistent. For the purposes of this review, increased “vulnerability” to the rewarding effects of drug can be reflected by greater intake of drugs, more rapid acquisition or escalation of drug taking, or increased potency of drugs on various behaviors (e.g. responding for lower doses of drug). Generally, environmental enrichment has been shown to confer protection against the rewarding effects of drugs (for review see Stairs and Bardo, 2009), while social isolation has been regularly associated with increased sensitivity to multiple drugs of abuse including stimulants and EtOH.

Environmentally enriched rodents have been shown to have lower levels of amphetamine intake than socially isolated animals (Bardo et al., 2001; Green et al., 2002). Importantly, this was shown at low unit doses of amphetamine (0.03 mg/kg/infusion), but not at high doses (0.1 mg/kg/infusion), indicating that enriched animals were less sensitive to lower doses of drug than isolated animals, but not higher doses (Bardo et al., 2001). Similarly, establishing a dose-response curve using a fixed ratio schedule of reinforcement, Green and colleagues (2002) reported that enriched rats had lower amphetamine intakes than isolated rats at low doses of drug. In addition, using a progressive ratio schedule, enriched rats maintained lower breakpoints for amphetamine responding than isolated rats. Enriched rats have been shown to extinguish responding for amphetamine more quickly than isolated rats and require higher doses of amphetamine as a pretreatment for reinstatement (Stairs et al., 2006). As with amphetamine, enriched animals have been shown to have lower intakes of cocaine (Gipson et al., 2011; Puhl et al., 2012) and even MPH (Alvers et al., 2012),

46
when tested in self-administration paradigms. These effects are not limited to stimulants, as enriched rats have been shown to be less sensitive to the effects of heroin when tested using CPP (El Rawas et al., 2009), and nicotine (Adams et al., 2013). Enriched rats are also less vulnerable to alcohol drinking, as they have been shown to have lower EtOH intakes and preferences, and lower motivation to obtain EtOH than rats raised in isolated conditions (Deehan et al., 2011).

Social isolation of rodents, on the other hand, has been shown to increase vulnerability to a number of drugs of abuse. For example, isolated rats have been shown to acquire cocaine self-administration at a much greater rate than rats housed or environmentally enriched. They also escalated their intake of cocaine faster than enriched rats when given long-access to self-administer (Gipson et al., 2012). Additionally, as mentioned above, isolated rats have been shown to have greater intakes of amphetamine than enriched rats (Bardo et al., 2001; Green et al., 2002). Short term neontatal isolation (pre-weaning) has been shown to increase responding for cocaine on both fixed ratio and progressive ratio schedules (Zhang et al., 2005), and enhance cocaine seeking during extinction and cue-induced reinstatement (Lynch et al., 2005). Rats isolated for three weeks during early life have been shown to drink greater amounts of a morphine solution in adulthood than group-reared animals (Raz et al., 2010).

Environmental conditions have also been shown to have a significant impact on future EtOH abuse in both rodents and monkeys. In rodents, social isolation early in life has been shown consistently to increase EtOH drinking in adulthood, using multiple EtOH drinking paradigms. Several studies have shown that isolated rats and mice have greater EtOH intakes and preferences than group-reared rodents when exposed to a home-cage drinking, two-bottle choice procedure, where one bottle is filled with an EtOH solution and the other choice is water (Advani et al., 2007; Chappell et al., 2013; Ehlers
et al., 2007; Hall et al., 1998d; Juarez and Vazquez-Cortes, 2003; Lodge and Lawrence, 2003; McCool and Chappell, 2009; Wolffgramm, 1990). Early life isolation has also been associated with greater responding for EtOH in an operant procedure (Deehan et al., 2007; McCool and Chappell, 2009). These effects are highly consistent between studies even at a variety of EtOH concentrations whether 5% (Advani et al., 2007; Lodge and Lawrence, 2003), 8% (Juarez and Vazquez-Cortes, 2003), or 10% (Chappell et al., 2013; Deehan et al., 2007).

In nonhuman primates, nursery-rearing versus mother-rearing is a procedure that has been well-validated as a model of early life stress (Harlow and Harlow, 1965). In the nursery-reared condition, monkeys are separated from their mothers and raised independently in a peer group. Studies using this model have shown that nursery-reared animals, both males and females, have greater EtOH intakes than their mother-reared counterparts (Barr et al., 2004; Fahlke et al., 2000; Higley et al., 1991). Thus, early life stress, either in the form of social isolation in a rodent or nursery-rearing in a monkey, clearly enhances vulnerability to alcohol abuse in animal models.

Clearly, early rearing environment has a powerful influence on dopamine neurochemistry and substance abuse vulnerability. Chronic MPH administration has further potential to modulate this system and alter behaviors including locomotor behavior, anxiety-like behavior, and vulnerability to drug abuse. Despite the fact that there are numerous studies examining both variables separately, the interaction between environment and chronic MPH has never been explicitly explored, and thus was a major goal of this work. The influence of environment on behavior and its interaction with MPH is critical, as children who have experienced early life adversity are more likely to be diagnosed with ADHD (Bornovalova et al., 2010; Bornovalova et al., 2013; Caspi et al., 2004; Stanger et al., 2004; Stormshak et al., 2000) and have
substance abuse problems as teenagers and adults (Dube et al., 2003; Gerra et al., 2009; Gerra et al., 2010; Schafer et al., 2010). Chapter Three presents the interaction of environment and chronic MPH treatment on locomotor behavior, anxiety-like behavior, and dopamine receptors in juvenile rats. In Chapter Four, this study was extended to examine the interaction of these variables on adult EtOH intake and preference.

8. Purpose of these studies

These studies were conducted to examine the effects of early chronic methylphenidate treatment, alone and in combination with environmental manipulations, on dopaminergic receptors and vulnerability to substance abuse. The dopaminergic targets that were examined, D1-like receptors (rats), D2-like receptors (rats and monkeys) and DATs (monkeys), were selected for their known involvement in the pharmacodynamics of MPH as well as substance abuse pathology. As cocaine is a psychostimulant with very similar actions in the brain as MPH, vulnerability to cocaine abuse after MPH treatment was studied in nonhuman primates. Alcohol was chosen as a second target for the rodent studies because of the prevalence of alcohol abuse in the young adult population. Finally, the interaction of MPH and environmental conditions was investigated in the rodent model as environmental enrichment and social isolation are known to affect both the dopamine system and drug and alcohol abuse. The results of these studies provide a broad, multi-species overview of the effects of MPH on different dopamine receptors and vulnerability to cocaine and alcohol abuse.

8.1 Rationale for the use of animal models

We chose to address these questions using animal models. As mentioned previously, human studies are subject to numerous confounds, such as selection biases,
the presence of comorbid psychopathologies, and problems of attrition in longitudinal studies. In studies of children with ADHD, particular attention must be paid to drug histories as many children have been prescribed to multiple drugs, at widely varying doses, and for varying durations. In addition, there are numerous dosing regimens that can be followed, including once-daily dosing with long-acting or extended release formulations, 2-3 times daily dosing with short-acting formulations, dosing only during school days (Monday-Friday) versus seven days per week, and the inclusion or exclusion of “drug holidays” such as Christmas break and/or summer vacation. In addition, factors such as parental involvement and support, socioeconomic status, peer relationships, academic support and IQ all affect outcome measures in epidemiological studies. Finally, studies of the effects of MPH treatment in children with ADHD often rely on parental, teacher, and self reporting for the outcome measures, rather than objective assessments.

Some human studies have attempted to control for these various factors to isolate the effects of specific drug treatments. For example, an excellent epidemiological investigation, the Multimodal Treatment of ADHD (MTA) study, attempted to carefully control all of these factors and extensively screened their subjects. In that screening process, 87% of subjects who were originally screened (n=4541) were eliminated, for a final sample of 579 children diagnosed with ADHD. Subjects were eliminated for various criteria including but not limited to, age and grade, IQ, parent refusal, teacher refusal, and incomplete questionnaires. Still, despite this rigorous selection process, much of the final sample of children with ADHD had comorbid diagnoses of anxiety disorder (30%), conduct disorder (15%), and oppositional-defiant disorder (40%), among others. Additionally, at least 30% of the sample had a prior history of ADHD medication. There were also significant differences in many of the demographic variables between test
locations, highlighting the potential impact of environmental differences on the outcome measures (MTA Cooperative Group, 1999). While information gleaned from the MTA and similar studies is immensely important, animal models provide an even more refined examination of the variables of interest, methylphenidate and environment, by affording the careful control of external variables.

In addition, within large scale clinical studies, it is difficult to examine the actions of MPH on the dopamine system. Patients are highly variable in drug and alcohol use, medication use, genetics, and/or dietary components that may influence the baseline state of the dopamine system and drug pharmacokinetics. In addition, procedures such as PET that can be used to quantify dopaminergic measures involve injections of radioactive ligands and occasionally sedatives in young children in order to complete the procedure. Thus, there are significant logistical difficulties to be overcome when examining the effects of MPH on the dopamine system in children. By using an animal model, the effects of prior drug and behavioral history, genetics, diet, and environment can be regulated and their impact on the dopamine system can be eliminated as a potential confound. Additionally, it is important to note that the dopamine system is highly conserved between species, thereby increasing the translational value of studies in animal models.

8.1.1 Nonhuman primates

In Chapter Two, a nonhuman primate model of development was employed to examine the long-term effects of MPH treatment on the developing dopamine system. Having an approximately 95% gene homology with humans, and complex social and behavioral repertoires, rhesus macaques are excellent models of human behavior (Hacia et al., 1998). Rhesus monkeys also have relatively long developmental periods...
of childhood and adolescence that are marked by hormonal and physiological maturation that is very similar to humans (Bennett and Pierre, 2010) which make them very valuable for longitudinal, developmental studies. They also have close homology to humans in terms of neuroanatomy and neurochemistry of the dopamine system (Weerts et al., 2007). Dopamine transporter homology is 98.9% between humans and monkeys thus it can be expected that the pharmacodynamic properties of MPH at the DAT will be similar between species (Miller et al., 2001). Additionally, comparative studies have concluded that the organization of dopamine afferents and dopamine receptors in the prefrontal cortex (Goldman-Rakic et al., 1992) and receptors in the basal ganglia (Richfield et al., 1987) are similar between humans and monkeys. Finally, monkeys have proven to be excellent models of human drug abuse, readily self-administering drugs such as cocaine, methamphetamine, alcohol, and nicotine (Griffiths, 1980; Spealman and Goldberg, 1978; Weerts et al., 2007).

8.1.2 Rats

Rodents have also been shown to be valuable models for dopamine neurophysiology and drug self-administration. In Chapters Three and Four, a rodent model was used to examine the interaction of environment and chronic MPH treatment on the dopamine system and future alcohol drinking. As with nonhuman primates, it has been shown that the distributions of D1-like and D2-like receptors in the basal ganglia are remarkably similar between rats and humans, (Richfield et al., 1987; Weerts et al., 2007). The dopaminergic regions of interest in the present investigation were all located within the basal ganglia, which strengthens the use of a rodent model for this purpose.

In rats, social isolation and environmental enrichment are well established procedures that have notable effects on behavior and dopaminergic transmission, as
seen above (section 7). While developmental periods in a rodent are much shorter than monkeys or humans, there is ample evidence that environmental manipulations during the 3-4 week postweaning period of early adolescence to young adulthood is sufficient to induce long-lasting behavioral phenotypes. Finally, like nonhuman primates, rodents are good models for studies of drug self-administration and have been shown to self-administer many drugs, including alcohol.

8.2 Extended release formulation of MPH

The vast majority of studies of chronic MPH treatment in nonhuman primate and rodent models have administered drug via intraperitoneal injection (for example Andersen et al., 2002; Carlezon et al., 2003) or orally (Soto et al., 2012; Thanos et al., 2007), often twice a day to model the immediate release formulations of MPH, such as Ritalin®. However, extended release formulations, such as Concerta®, and Metadate CD™ are the most commonly prescribed medications. As reviewed above (section 3.2), these formulations, taken orally, are designed to release an immediate bolus of drug and then slowly continue to release drug over a 12 hour period, keeping blood plasma levels within the therapeutic range of 10-15 ng/ml (Volkow and Swanson, 2003). The drugs were originally designed to overcome problems of acute tolerance seen with immediate release formulations (Stein et al., 1996) and to eliminate the need for multiple drug doses per day. Advantages of these formulations include increased compliance, elimination of the need for dosing at school or work which can cause embarrassment and stigmatization, and alleviation of issues of medical privacy (Connor and Steingard, 2004; Swanson et al., 2004; Weiss and Weiss, 2004). Like the immediate release formulations, extended release MPH has shown great efficacy reducing the symptoms of ADHD in humans. Here, in the nonhuman primate model, we used an extended release formulation of MPH delivered orally in a pudding vehicle and adjusted doses monthly to
attain blood plasma levels that have been associated with clinical efficacy in children. In the rodent model, we implanted osmotic minipumps (Alzet©; Durect Corporation, Cupertino, CA) to deliver, subcutaneously, a steady dose of MPH over a three week period to attempt to better model current clinical practice. Several doses of MPH were tested and blood levels of drug were determined at the end of the procedure. The use of these dosing regimens is innovative and is one factor that separates this series of studies from other investigations of chronic MPH treatment.

8.3 Overall goal, AIMS, and hypotheses

The overall goal of this series of studies was to determine the effects of MPH on the dopamine system of juvenile animals and future vulnerability to substance abuse in adulthood. A second goal was to determine, in a rodent model, the interaction of MPH with the environment, as environmental conditions early in life are known to influence dopaminergic development and substance abuse. To these ends, three AIMS were developed:

1) To determine the effects of MPH treatment on the development of D2-like receptors and DATs, physical growth, and cocaine abuse vulnerability in a nonhuman primate.

2) To determine the interaction of MPH treatment and environmental condition, enrichment versus isolation, on behavior and D1- and D2-like receptor density in juvenile rats.

3) To determine the interaction of MPH treatment and environmental condition, enrichment versus isolation, on future vulnerability to alcohol abuse in rats.
In AIM 1 (Chapter Two), we measured the development of dopamine receptors and DATs using PET, a non-invasive imaging technique for quantifying the availability of receptors using radioactive ligands. The use of PET and nonhuman primates, because of their relatively long developmental stages when compared to rodents, allowed the ability to take longitudinal measurements of normal dopamine system development and MPH-treated dopamine system development. Additionally, to address concerns about the effects of MPH treatment on physical growth in humans, height and weight were measured over an 18 month period. Finally, as young adults, these animals were tested in a cocaine self-administration paradigm to determine whether MPH altered vulnerability to cocaine abuse.

In nonhuman primates, we hypothesized that chronic MPH treatment would have a profound impact on the development of the dopamine system, when compared to untreated animals. Previously, Thanos et al., (2007) reported that long-term MPH treatment, administered orally at low doses, resulted in greater D2-like receptor availability in rodents, when compared to untreated animals. Higher levels D2-like receptor availability are associated with lower vulnerability to substance abuse (Morgan et al., 2002; Volkow et al., 1999; Volkow et al., 2006) and most clinical studies suggest that early stimulant treatment may be protective against substance abuse (Biederman et al., 2003; Biederman et al., 2008, Wilens et al., 2008). Thus, our primary hypothesis was that MPH would be associated with higher levels of dopamine D2-like receptor availability and that this would be reflected by lower rates of cocaine self-administration in the treated group.

In AIM 2 and 3 (Chapters Three and Four, respectively), a rodent model was employed to investigate the interaction between MPH treatment and the effects of early rearing environment on the dopamine system, locomotor and anxiety-like behavior, and
future vulnerability to alcohol abuse. The measures of locomotor activity and anxiety-like behavior were chosen as they are both known to be affected by environmental conditions, and thus, they served as important validation for the efficacy of our housing procedure. Additionally, locomotor behavior is known to be affected by stimulant drugs, such as MPH, and anxiety-like behavior is associated with EtOH intake in rodents (McCool and Chappell, 2009). Therefore, these were appropriate behaviors to examine as part of an investigation of the effects of environment and chronic MPH treatment on future alcohol drinking. In AIM 2, at the conclusion of a 4-week housing period with 3 weeks of drug administration, rats were sacrificed and brains were harvested and prepared for *in vitro* autoradiography to quantify the density of D1-like and D2-like receptors in striatal brain regions. In AIM 3, after the same duration of drug and housing exposure, rats were exposed to a two-bottle choice, limited access, EtOH drinking procedure.

As described in the previous section, social isolation is known to enhance vulnerability to the rewarding effects of psychostimulants, alcohol and other drugs. Thus, social isolation of young rodents was expected to be associated with a lower density of D2-like receptors in the striatum in isolated rats, when compared to rats reared in environmentally enriched conditions. Acute MPH treatment has been shown to improve impulsivity in socially isolated rats while having no effect on environmentally enriched rats (Perry et al., 2008), potentially due to underlying differences in the dopamine system associated with rearing environment. Accordingly, we hypothesized that chronic MPH treatment would have effects on the dopamine systems and EtOH intakes of socially isolated animals, while not affecting enriched animals. Specifically, we anticipated that chronic MPH treatment would be associated with greater D2-like receptor densities in treated socially isolated animals, when compared to untreated
isolated animals. In turn, we expected that socially isolated animals would have a
greater vulnerability to EtOH self-administration when compared to enriched animals,
and that MPH treated isolated animals have lower EtOH intakes than untreated isolated
animals.

This series of studies was innovative in the use of extended release formulations
of MPH, the use of nonhuman primates to study normal dopaminergic development and
physical growth in tandem with the effects of MPH on these measures, and in its direct
examination of the interaction between rearing environment and chronic MPH treatment.
The results presented here add important information to the existing knowledge of the
effects of MPH on dopamine system development and future vulnerability to substance
abuse, and highlight the importance of considering environmental conditions when
prescribing MPH to children.
References


terminal fields in the motor, visual (area 18b) and retrosplenial cortex in the
young and adult rat. Immunocytochemical and catecholamine histochemical
analyses. Neuroscience. 15, 983-98.

Characterization of subtype-specific antibodies to the human D5 dopamine
receptor: studies in primate brain and transfected mammalian cells. Proc Natl
Acad Sci U S A. 92, 3468-72.

Bergson, C., Mrzljak, L., Smiley, J.F., Pappy, M., Levenson, R., Goldman-Rakic, P.S.,
1995b. Regional, cellular, and subcellular variations in the distribution of D1 and

Berridge, C.W., Devilbiss, D.M., Andrzejewski, M.E., Arnsten, A.F., Kelley, A.E.,
Schmeichel, B., Hamilton, C., Spencer, R.C., 2006. Methylphenidate
preferentially increases catecholamine neurotransmission within the prefrontal
cortex at low doses that enhance cognitive function. Biol Psychiatry. 60, 1111-20.

Berridge, K.C., 2007. The debate over dopamine's role in reward: the case for incentive

term oral methylphenidate treatment on spontaneous and learned fear behaviors.
Neurosci Lett. 496, 30-4.

Psychoactive substance use disorders in adults with attention deficit hyperactivity
152, 1652-8.

Biederman, J., Faraone, S., Milberger, S., Curtis, S., Chen, L., Marrs, A., Ouellette, C.,
Moore, P., Spencer, T., 1996. Predictors of persistence and remission of ADHD


Fehr, C., Yakushev, I., Hohmann, N., Buchholz, H.G., Landvogt, C., Deckers, H.,
Eberhardt, A., Klager, M., Smolka, M.N., Scheurich, A., Dielentheis, T., Schmidt,
Association of low striatal dopamine d2 receptor availability with nicotine
dependence similar to that seen with other drugs of abuse. Am J Psychiatry. 165,
507-14.

of amphetamine, deoxypipradrol and methylphenidate to inhibit the uptake of
tritiated catecholamines into rat cerebral cortex slices, synaptosomal
preparations of rat cerebral cortex, hypothalamus and striatum and into


Fitzgerald, M.L., Mackie, K., Pickel, V.M., 2013. The impact of adolescent social isolation
on dopamine D2 and cannabinoid CB1 receptors in the adult rat prefrontal

Floresco, S.B., Tse, M.T., Ghods-Sharifi, S., 2008. Dopaminergic and glutamatergic
regulation of effort- and delay-based decision making.
Neuropsychopharmacology. 33, 1966-79.

understanding child and adolescent depression? Dev Psychopathol. 17, 827-50.

Fremeau, R.T., Jr., Duncan, G.E., Fornaretto, M.G., Dearry, A., Gingrich, J.A., Breese,
supports a role in cognitive, affective, and neuroendocrine aspects of

Gal, J., Hodshon, B.J., Pintauro, C., Flamm, B.L., Cho, A.K., 1977. Pharmacokinetics of
methylphenidate in the rat using single-ion monitoring GLC-mass spectrometry. J

Earlier development of the accumbens relative to orbitofrontal cortex might

conditions alter social reactivity and D1 dopamine receptors in high- and low-
aggressive mice. Pharmacol Biochem Behav. 51, 767-73.

Gatley, S.J., Pan, D., Chen, R., Chaturvedi, G., Ding, Y.S., 1996. Affinities of
methylphenidate derivatives for dopamine, norepinephrine and serotonin

development of dopamine D1 and D2 receptor sites in rat striatum. Brain Res
Dev Brain Res. 49, 123-30.

Gerasimov, M.R., Franceschi, M., Volkow, N.D., Gifford, A., Gatley, S.J., Marsteller, D.,
methylphenidate administration: A microdialysis and locomotor activity study. J
Pharmacol Exp Ther. 295, 51-7.

Gerra, G., Leonardi, C., Cortese, E., Zaimovic, A., Dell'agnello, G., Manfredini, M.,
neglect and parental care perception in cocaine addicts: relation with psychiatric


Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., Caron, M.G., 1998. Dopamine receptors: from structure to function. Physiol Rev. 78, 189-225.


CHAPTER TWO

CHRONIC TREATMENT WITH EXTENDED RELEASE METHYLPHENIDATE DOES NOT ALTER DOPAMINE SYSTEMS OR INCREASE VULNERABILITY FOR COCAINE SELF-ADMINISTRATION:

A STUDY IN NONHUMAN PRIMATES

Kathryn E. Gill¹
Peter J. Pierre¹
James Daunais¹
Allyson J. Bennett¹
Susan Martelle¹
H. Donald Gage²
James M. Swanson³
Michael A. Nader¹,²
Linda J. Porrino¹,²

¹Department of Physiology and Pharmacology
²Department of Radiological Sciences
Wake Forest School of Medicine
Winston Salem, NC 27157

³Child Development Center
University of California, Irvine
Irvine, CA 92612

The following manuscript was published in Neuropsychopharmacology in November 2012. Stylistic variations are due to the requirements of the journal. Kathryn E Gill contributed to study design, collected and analyzed data, and prepared the manuscript.
Abstract

Despite the widespread use of stimulant medications for the treatment of Attention Deficit Hyperactivity Disorder, few studies have addressed their long-term effects on the developing brain or susceptibility to drug use in adolescence. Here, we determined the effects of chronic methylphenidate treatment on brain dopamine systems, developmental milestones and later vulnerability to substance abuse in juvenile nonhuman primates.

Male rhesus monkeys (approximately 30 months old) were treated daily with either a sustained release formulation of methylphenidate or placebo (N=8/group). Doses were titrated to achieve initial drug blood serum levels within the therapeutic range in children and adjusted throughout the study to maintain target levels. Growth, including measures of crown-rump length and weight, was assessed before and after one year of treatment and after 3-5 months washout. Additionally, Positron Emission Tomography scans were performed to quantify binding availability of D2/D3 receptors and dopamine transporters. Distribution volume ratios were calculated to quantify binding of \[^{18}F\]fluoroclobopride (dopamine D2/D3) and \[^{18}F\]-(+)-N-(4-fluorobenzyl)-2β-propanoyl-3β-(4-chlorophenyl)tropane (dopamine transporter; DAT). Chronic methylphenidate did not differentially alter the course of weight gain or other measures of growth, nor did it influence DAT or D2/D3 receptor availability after one year of treatment. However, after washout, the D2/D3 receptor availability of MPH-treated animals did not continue to decline at the same rate as control animals.

Acquisition of intravenous cocaine self-administration was examined by first substituting saline for food reinforcement and then cocaine doses (0.001-0.1 mg/kg/injection) in ascending order. Each dose was available for at least 5 consecutive
sessions. The lowest dose of cocaine that maintained response rates significantly higher than saline-contingent rates was operationally defined as acquisition of cocaine reinforcement. There were no differences in rates of acquisition, overall response rates, or cocaine intake as a function of cocaine dose between groups.

In an animal model that closely mimics human development, chronic treatment with therapeutic doses of sustained release methylphenidate did not have a significant influence on the regulation of dopamine transporters or D2/D3 receptors, or on standard measures of growth. Furthermore, this treatment regimen and subsequent drug washout did not have an impact on vulnerability to cocaine abuse.

Keywords: Dopamine, Addiction & Substance Abuse, Psychostimulants, Development / Developmental Disorders, Monkeys, methylphenidate, Attention Deficit Hyperactivity Disorder, PET
Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most commonly diagnosed psychiatric disorder of childhood and adolescence, affecting an estimated 7.8% of children ages 4-17 in the United States (Visser et al, 2007). Stimulant medications including methylphenidate (MPH) and amphetamine have been the most frequently prescribed treatments for ADHD since their introduction in the 1960’s, and their use has steadily increased over time (Swanson and Volkow, 2009).

MPH acts at the dopamine transporter (DAT) to increase concentrations of extracellular dopamine (DA) in the mesocorticolimbic and mesostriatal systems by blocking reuptake (Schweri et al, 1985; Volkow et al, 1998). Like cocaine and methamphetamine, there is evidence that MPH causes significant neuroadaptations within the DA system (Self, 2004; Wolf et al, 2004). However, few studies have examined long-term consequences following chronic use of MPH in children and adolescents. Thus, one goal of the present study was to characterize the changes in DAT and DA receptor function following one year of MPH treatment in juvenile nonhuman primates.

While there is considerable evidence of the efficacy of stimulant medications in reducing the symptoms of ADHD, there are questions about the potential for adverse consequences accompanying chronic treatment. There is evidence for modest growth suppression (Safer et al, 1972; Zhang et al, 2010), although this is far from consistent. Some studies of MPH-treated children have reported reduced growth curves for both height and weight compared to age-matched controls (Swanson et al, 2007), whereas others reported no effects on growth (Biederman et al, 2010). Thus, a second goal of
the present study was to characterize growth measures in juvenile monkeys treated with MPH and control animals.

One of the greatest concerns surrounding the use of stimulant medications in childhood is an increased risk of substance abuse in adolescence. Several studies of children with ADHD have suggested that treatment does not increase (Biederman \textit{et al}, 2008; Mannuzza \textit{et al}, 2008; Molina \textit{et al}, 2009), or may even have a protective effect against the development of substance abuse disorder (Barkley \textit{et al}, 2003; Biederman \textit{et al}, 1999). However, at least one study found some evidence for an increased risk of tobacco and cocaine use in adults treated as children (Lambert and Hartsough, 1998).

Because of the complex interactions among dosing regimens, diagnoses, duration of treatment, co-morbidity, etc. in studies of children and adolescents with ADHD, there have been attempts to answer this question using animal models, almost exclusively in rodents. While some studies have reported that MPH-treated animals are more susceptible to the rewarding effects of cocaine (Brandon \textit{et al}, 2001), other studies have documented reduced rates of cocaine self-administration (Thanos \textit{et al}, 2007), or less sensitivity to cocaine (Andersen \textit{et al}, 2002; Carlezon \textit{et al}, 2003) post-MPH treatment. The equivocal results may be due to differences in routes of administration, drug doses, drug formulations, and durations of treatment (Volkow and Insel, 2003). The discrepancies underscore a major problem with developmental studies in rodents, which is the relatively short window of pre- and peri-adolescence/adolescence in this species.

Given the limitations of studies in rodents, the current study employed juvenile nonhuman primates to measure the effects of the chronic MPH administration. Nonhuman primates provide exceptional models for developmental research because
they undergo a relatively long childhood and adolescent periods, marked by hormonal and physiological maturation similar to humans (Bennett and Pierre, 2010). Additionally, nonhuman primates have close homology to humans in terms of neuroanatomy and neurochemistry of the DA system, as well as complex social and cognitive behavioral repertoires (Weerts et al, 2007).

Recently, controlled-release formulations of MPH such as Concerta® and Metadate CD® have replaced immediate release, short-acting formulations such as Ritalin® as the most commonly prescribed form of medication. In children, these formulations of MPH have been reported to be clinically effective for as long as 12 hours after administration and obviate the need for repeated dosing throughout the day (Pelham et al., 2001; Swanson et al., 2004). They make use of first-order drug delivery profiles to overcome the acute tolerance that occurs with immediate-release dosing and are designed to release an initial bolus followed by continuous dosing for approximately 6 hours after administration (Swanson et al, 1999). In order to best model current clinical practice, nonhuman primates in the present study were treated with a controlled release formulation of MPH at doses targeted to be within a clinically relevant range (Swanson and Volkow, 2003).

Thus, the goals of this study were to evaluate the effects of the chronic administration of a commonly used formulation of MPH on measures of DA function, growth, and vulnerability to the reinforcing effects of cocaine in juvenile nonhuman primates.
Methods

Subjects. Sixteen socially housed young male rhesus monkeys were studied in two cohorts (cohort=8; mean age 40.5 and 38.0 mo. at time of initiation of drug treatment). Each cohort was divided into two groups (N=4, housed as a unit): Drug-treated and Vehicle-treated. Groups were initially matched as closely as possible on baseline age and body weight. Monkeys were fed individually and maintained on 200 kcal diet (Purina 5038) as prescribed for young animals at our primate center with ad libitum water. Diet amounts were calculated for each individual, remaining chow was weighed after each meal and chow amounts were adjusted as body weight increased. All animals were considered in the late juvenile/early adolescent stage of development at the start of the study based on a constellation of measurements including weight, crown-rump length, testis size, canine length, femur length, and abdominal and chest circumference (Bennett et al., 2010). Animals were trained to present their leg for conscious femoral venipuncture. Animal housing, handling, and experimental procedures were performed in accordance with the 2003 National Research Council Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. Experimental protocols and environmental enrichment plans were approved by the Animal Care and Use Committee of Wake Forest University.

Phase 1: Drug Treatment, Developmental and Dopamine System Markers

Drugs and Dosing Procedures: The contents of individual Metadate CD® (generously provided by UCB, Inc., Smyrna, GA) capsules (20 mg) were suspended in 10 ml of pudding (Hunts Snack Pack™, Con Agra Foods, NE). Control subjects received 10 ml of pudding only. Doses were chosen to approximate clinically relevant levels in children.
of 10-15 ng/ml blood serum (Swanson et al., 2003). All animals received one capsule (20 mg) initially, then each monkey’s dose was adjusted to target serum levels. Serum levels were re-determined monthly and doses adjusted to account for weight gain and any changes in MPH levels. Blood was collected 1.5 hr following dose consumption to target peak drug levels, centrifuged, serum fraction separated and frozen at -70°C. Analysis was performed (Medtox Laboratories, MN) using gas chromatography. Preliminary studies showed that blood serum levels after the immediate release phase were maintained during the 2-6 hour extended release phase post-administration. For one hour each morning monkeys were separated into individual quadrants of the home cage for feeding and dosing and then returned to their social groups. Monkeys voluntarily consumed drug or vehicle through a 60 ml syringe. After one hour, each animal’s chow was removed and weighed and then adjusted to accommodate a positive growth trajectory (NRC/NAS, 2003). The amount of chow consumed did not differ between groups over the course of the study. This procedure was repeated daily for 12 months.

*Morphometric measurements:* Morphometric measurements were taken at baseline (two weeks prior to initiation of drug administration), treatment (after 12 months of drug administration), and washout (3-5 months after cessation of treatment). Measurements were collected following anesthesia with ketamine hydrochloride (15 mg/kg, IM). Crown-rump length (cm) was measured using a 60 cm sliding caliper (Anthropometer Model 01290; Lafayette Instrument Company, Lafayette, IN) by measuring from the most distal part of the left ischial callosite to the top of the skull. Body mass index (BMI) was calculated as body weight/crown-to-rump length.
Positron Emission Tomography (PET) imaging of dopamine D2/D3 receptors and dopamine transporters: PET imaging studies to measure DA D2/D3 receptor and DAT availability were carried out at similar time points as the morphometric measures: baseline, treatment, and washout. Treatment measurements were made within one week following the cessation of treatment, and at least 24 hours after final dosing to allow for drug clearance. Washout scans were performed 3-5 months later. At each time point, T1 weighted magnetic imaging resonance imaging (MRI) scans were acquired on a 3.0T GE Scanner for co-registration and definition of regions of interest (ROIs).

Details of PET data acquisition procedures for both D2/D3 receptors and DATs have been described previously (Czoty et al., 2007). Briefly, monkeys were anesthetized with 10 mg/kg ketamine, intubated, and anesthesia maintained by 1.5% isoflurane. Once in the scanner, a venous catheter was inserted percutaneously for tracer injection. To quantify D2/D3 receptor availability, approximately 5 mCi of $[^{18}\text{F}]$fluoroclebopride (FCP), a high affinity D2/D3 ligand, was injected followed by 3 ml heparinized saline. On a second scanning day (separated by at least one week), approximately 5 mCi of $[^{18}\text{F}]$-(+)-N-(4-fluorobenzyl)-2β-propanoyl-3β-(4-chlorophenyl)tropane (FCT) was injected to quantify DAT.

To eliminate movement during the scan, a paralytic (0.07 mg/kg vecuronium Br, i.v.) was administered 15 minutes prior to the start of image acquisition. Respiration was maintained by a ventilator with supplemental doses administered as needed. Body temperature was maintained by a heating pad, fluids maintained by intravenous normal saline, and vital signs monitored throughout. At scan conclusion, animals received an
injection of neostigmine (0.07 mg/kg) and glycopyrrolate (0.02 mg/kg) to reverse the paralytic effects. Once recovered, monkeys were returned to their home environments and monitored until normally behaving.

Image acquisition occurred on a GE Advance NXi PET Scanner (General Electric Systems, Milwaukee, WI) with an effective resolution of approximately 4 mm. Data were analyzed using PMOD software (PMOD; Zurich, Switzerland). ROIs were drawn on coronal sections in the ventral striatum, caudate nucleus, and putamen of each monkey’s MRI at each time point. PET scans were manually co-registered to the corresponding MRIs and ROIs transferred to co-registered PET images. The dependent measure was the distribution volume ratio (DVR), defined as the distribution volume of the radiotracer in ROI relative to the distribution volume of the radiotracer in a receptor-less reference region (cerebellum). DVRs were determined by using the “Logan method” of analysis as implemented in PMOD (Logan et al, 1996). DVRs were calculated for bilateral caudate nucleus, putamen, and ventral striatum for each PET scan.

Statistical Analysis: Morphometric data (body weight, crown-rump length and BMI) and PET data (D2-like receptor and DAT DVRs) were analyzed by means of a two-way analysis of variance (ANOVA) (treatment group X time point; time as a repeated measure), followed by post-hoc comparisons (Tukey tests) where appropriate. In all cases, p<0.05 was considered statistically significant.
Phase 2: Cocaine Self-administration

After completion of Phase 1 testing, monkeys were individually housed with visual and auditory contact with each other, fitted with aluminum collars (Primate Products, Redwood City, CA) and trained to sit calmly in a primate chair (Primate Products). Monkeys were weighed weekly and fed enough food daily (Purina Monkey Chow and fresh fruit and vegetables) to accommodate normative growth (NRC/NAS, 2003) while maintaining food-based responding. Water was available ad libitum.

Surgery. Under sterile conditions, each monkey was prepared with a chronic indwelling venous catheter, implanted in the femoral or jugular vein. The distal end of the catheter was attached to a subcutaneous vascular port (Access Technologies, Skokie, IL), as described previously (Czoty et al, 2005). Each port and catheter was filled with heparinized saline solution (100 units/ml) after every experimental session to prolong catheter patency. Prior to each self-administration session, the animal's back was cleaned with chlorhexidine acetate solution and 95% EtOH and the port was connected to the infusion pump via a 22-gauge Huber Point Needle (Access Technologies). Before starting the session, the pump was operated for approximately 3 seconds to fill the port and catheter line with the dose of cocaine available for that session.

Apparatus. The apparatus was a ventilated, sound-attenuating chamber (1.5 x 0.74 x 0.76 m; Med Associates, East Fairfield, VT) consisting of two photo-optic switches (Model 117-1007; Stewart Ergonomics, Inc., Furlong, PA) on one side with a horizontal row of three stimulus lights positioned above each switch. A food receptacle between the switches was connected to a pellet dispenser (Med Associates) for delivery of 1-g
banana-flavored food pellets (Bio-Serv., Frenchtown, NJ). An infusion pump (Cole-Palmer, Inc., Chicago, IL), which delivered solutions at a rate of approximately 1.5 ml/10 sec, was located on the top of the chamber.

Procedure. Monkeys were trained to respond on the left or right response switch, under a fixed-ratio (FR) 30 schedule of reinforcement in which a food pellet was delivered after the 30th response, followed by a 10-s timeout. Sessions ended after 15 or 30 reinforcers (depending on the monkey) or 60 min, whichever occurred first. After catheter implantation, food-maintained responding was re-established and, when stable (< 20% variability over 3 consecutive sessions with no trends in responding), saline was substituted for food pellets for at least 5 consecutive sessions and until responding declined by at least 80% of food-reinforced responding for 3 consecutive sessions with no trends in responding. The total number of sessions needed to meet this criterion is presented (Table 2). Next, food-maintained responding was re-established and then different doses of cocaine HCl (National Institute on Drug Abuse, Bethesda, MD, dissolved in sterile 0.9% saline) were substituted for the food pellets in ascending order from 0.001 mg/kg/injection, increasing in half log units to 0.1 mg/kg/injection. For cocaine substitution studies, sessions ended after 30 injections or 60 min, whichever occurred first. Each dose was available for at least 5 consecutive sessions until responding was deemed stable (response rate mean ±20% with no trends for 3 consecutive sessions). There was a return to food-reinforced responding for at least 3 sessions before the next cocaine dose was tested, until the cocaine dose-response curve had been generated.
Statistical analysis. To assess acquisition of cocaine reinforcement, a cocaine dose was operationally defined as reinforcing by using two-tailed t-tests comparing 3-day mean response rates for a given dose of cocaine to mean response rates when saline was available. Survival curves were generated and the number of monkeys that acquired at each cocaine dose was compared for both groups of monkeys. Mean response rates (responses/second) and total cocaine intake (mg/kg/session) were calculated and dose-response curves were compared between groups using repeated measures ANOVA, followed by post-hoc analysis (Tukey test). In all cases, p<0.05 was considered statistically significant.

Results

Serum Concentrations. Initial MPH doses were 20 – 40 mg (average = 5.9 ± 1.2 mg/kg (mean ± SD)) with serum levels averaging 10.73 ± 1.10 ng/ml (mean ± SD). Over the course of the 12-month treatment period, dose increases resulted in final doses of 60 mg in 6 animals and 20 and 120 in the other two (average = 6.8 ± 1.7 mg/kg (mean ± SD)) and final serum levels of 13.623 ± 1.15 ng/ml (mean ± SD). There was no relationship between serum levels or doses and changes in growth or dopamine markers.

Morphometrics. Two-way repeated measures (time x drug condition) ANOVAs were used to analyze growth data. There were significant main effects of time in all three morphometric measures (Weight: F(2,28) = 113.04, p < 0.001; Crown to Rump: F(2,28) = 116.68, p < 0.001; BMI: F(2,28) = 40.49, p < 0.001), indicating that the monkeys were growing over the study period (Fig. 1). There were no significant effects of drug treatment on any measure (Weight: F(1,14) = 0.027; Crown to Rump: F(1,14) = 0.035; BMI:
$F_{1,14} = 0.09$), indicating that control and MPH-treated animals were growing at similar rates. Finally, the interaction of treatment with time was not significant for any measure.

**PET Measurements: D2-like receptor availability.** Two-way repeated measures ANOVAs confirmed significant main effects of time in the caudate nucleus, putamen, and ventral striatum (Caud: $F_{2,28} = 14.70$, $p < 0.001$; Put: $F_{2,28} = 27.59$, $p < 0.001$; VS: $F_{2,28} = 12.69$; $p < 0.001$), indicating that D2/D3 receptor availability decreased from baseline through treatment and washout (Figs 2; 3). There were no significant main effects of drug treatment in any of the brain regions (Caud: $F_{1,14} = 0.032$; Put: $F_{1,14} = 0.024$; VS: $F_{1,14} = 0.042$). This is illustrated in Figure 3 which depicts a representative animal from both the control- and MPH- treated groups at baseline and treatment time points. Both animals had reduced D2/D3 receptor availability after treatment, but did not differ from each other.

There was a significant interaction between drug and time in the putamen ($F_{2,28} = 4.32$, $p < 0.05$; Fig 2B). Post-hoc t-tests on the absolute value of the DVRs between treatment and control groups were not significant. However, there was a significant difference between control- and MPH- treated animals in the magnitude of the change in DVR (calculated as washout DVR- treatment DVR) between the treatment and washout time points in the putamen ($t_{14} = 3.11$; $p < 0.01$). This suggests that, over the course of washout, D2/D3 receptor availability in the putamen of MPH treated monkeys did not continue to decrease at the same rate as in control monkeys.
Figure 1.
**Figure 1.** Standard measures of growth, weight, crown to rump length and Body Mass Index (BMI), were measured at baseline, after 12 months of treatment, and after 3-5 months of drug washout. Animals in both control- and MPH-treatment groups showed normal gains in weight and length over the study period. No differences were observed between groups. Insets depict the absolute change in weight, crown-rump length and BMI between baseline and treatment and treatment and washout timepoints.
Figure 2.
Figure 2. Dopamine D2/D3 receptor availability as measured by $^{18}$F]FCP in MPH-treated and control monkeys in the caudate nucleus, putamen and ventral striatal regions. Relative distribution volume ratios (mean +/- SEM) show no differences in D2/D3 receptor availability between control and MPH-treated animals. However, a significant decrease in D2/D3 receptor availability from baseline to the end of the treatment period was found in all three regions ($p < 0.05$). Insets depict the absolute change in DVR values between baseline and treatment and treatment and washout timepoints.
Figure 3. Binding of $[^{18}\text{F}]$ FCP to dopamine D2/D3 receptors in the striatum decreases over a 12-month period in control and MPH treated animals. There were no differences in D2/D3 receptor availability between groups at either time point.
DAT availability. Because washout scans could not be completed for cohort 2 due to unavailability of the ligand, results are presented for cohort 1 for three time points (baseline, treatment, and washout) and for both cohorts for two time points (baseline, treatment). In cohort 1, a two-way repeated measures ANOVA (time x drug condition) was run on DVRs measured in the caudate nucleus, putamen, and ventral striatum. There were no significant main effects of time (Caud: $F_{2,10} = 0.118$; Put: $F_{2,10} = 0.645$; VS: $F_{2,10} = 1.643$) or drug condition (Caud: $F_{1,5} = 0.324$; Put: $F_{1,5} = 0.005$; VS: $F_{1,5} = 0.494$) in any brain region and no significant interactions (Table 1). A second ANOVA (time x drug condition) was performed to examine DAT availability in both cohorts, excluding the washout scans. There were again no significant effects of time (Caud: $F_{1,13} = 2.675$; Put: $F_{1,13} = 0.054$; VS: $F_{1,13} = 1.013$) or drug condition (Caud: $F_{1,13} = 0.669$; Put: $F_{1,13} = 0.016$; VS: $F_{1,13} = 0.289$) in any brain region and no significant interactions. Thus, DAT availability did not change over the course of development during the study and was not affected by chronic MPH treatment.
Table 1: Effects of methylphenidate treatment on $[^{18}F]FCT$ distribution volume ratios. Dopamine transporter availability was measured prior to drug or vehicle treatment, after 12 months of treatment and 3-5 months after treatment cessation. One MPH-treated animal was removed from analysis due to a technical error during the baseline scan.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Placebo-treated (N=8)</th>
<th>Methylphenidate-treated (N=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Treatment</td>
</tr>
<tr>
<td>Caudate</td>
<td>3.40 ± .20</td>
<td>3.61 ± .11</td>
</tr>
<tr>
<td>Putamen</td>
<td>3.99 ± .12</td>
<td>3.94 ± .23</td>
</tr>
<tr>
<td>Ventral striatum</td>
<td>2.29 ± .15</td>
<td>2.27 ± .11</td>
</tr>
</tbody>
</table>

*Data represent distribution volume ratios expressed as means ± S.E.M.*
Self-administration. There were no differences in baseline rates of food-reinforced responding between MPH- and control-treated monkeys (Table 2). After surgery and return to stable food-reinforced responding, saline was substituted for food presentation. There were no differences in the number of sessions required for responding to extinguish between MPH- and control-treated monkeys (Table 2). After a return to food-reinforced responding, acquisition of cocaine reinforcement was examined using ascending doses of cocaine, beginning with 0.001 mg/kg. There were no differences in the number of monkeys that acquired cocaine reinforcement at each dose of cocaine between MPH- and control-treated monkeys (Fig. 4A). Complete cocaine dose-response curves showed that, for both MPH- and control-treated monkeys, response rates ($F_{5, 70} = 4.30; P<0.01$) and cocaine intake ($F_{4,56} = 58.11; P<0.0001$) varied significantly as a function of cocaine dose (Fig. 4B,C). Response rates were characterized as an inverted U-shaped function of dose (Fig. 4B). There was not a significant main effect of Treatment (MPH vs. Control). Cocaine intake increased monotonically as a function of dose in all monkeys and was not different in MPH- and control-treated monkeys (Fig. 4C).
Table 2. Baseline food-maintained responding and number of sessions for response extinction§.

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Food Rates (resp/sec)</th>
<th># Extinction sessions</th>
<th>Monkey</th>
<th>Food Rates (resp/sec)</th>
<th># Extinction sessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALINE-TREATED CONTROLS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-1545</td>
<td>0.73 (.26)</td>
<td>6</td>
<td>R-1546</td>
<td>0.47 (.05)</td>
<td>7</td>
</tr>
<tr>
<td>R-1547</td>
<td>2.43 (.57)</td>
<td>5</td>
<td>R-1548</td>
<td>0.33 (.22)</td>
<td>5</td>
</tr>
<tr>
<td>R-1549</td>
<td>0.93 (.13)</td>
<td>5</td>
<td>R-1550</td>
<td>0.23 (.06)</td>
<td>5</td>
</tr>
<tr>
<td>R-1551</td>
<td>0.56 (.15)</td>
<td>7</td>
<td>R-1552</td>
<td>2.05 (.52)</td>
<td>6</td>
</tr>
<tr>
<td>R-1616</td>
<td>1.09 (.14)</td>
<td>5</td>
<td>R-1620</td>
<td>1.94 (.28)</td>
<td>5</td>
</tr>
<tr>
<td>R-1617</td>
<td>2.86 (.12)</td>
<td>5</td>
<td>R-1621</td>
<td>2.19 (.10)</td>
<td>6</td>
</tr>
<tr>
<td>R-1618</td>
<td>0.50 (.11)</td>
<td>6</td>
<td>R-1622</td>
<td>0.49 (.06)</td>
<td>7</td>
</tr>
<tr>
<td>R-1619</td>
<td>2.67 (.62)</td>
<td>15</td>
<td>R-1623</td>
<td>1.63 (.11)</td>
<td>5</td>
</tr>
</tbody>
</table>

Mean (SEM) 1.41 (.38) 6.75 (1.29) 1.15 (.32) 5.75 (.34)

§ Data represent the mean ± SD of the last 3 sessions of food-reinforced responding prior to saline substitution
Figure 4.  

A. Acquisition of cocaine reinforcement in MPH-treated and control monkeys. Percentage of MPH- (open symbols) and control-treated (closed symbols) monkeys that reached criteria to acquire cocaine self-administration at various doses of cocaine available under an FR 30 schedule of reinforcement.  

B. Mean (±SEM) rate of responding (responses/sec) when saline or various doses of cocaine were available per session for MPH- and control-treated monkeys.  

C. Mean (±SEM) cocaine intake (mg/kg/session). Each dose was available for at least 5 sessions and until responding was stable; data represent the mean last 3 days of availability for each animal.
Discussion

The results of the present study demonstrate that prolonged treatment with therapeutic-level doses of an extended release formulation of MPH does not have a significant influence on physical growth or on the development of dopamine D2/D3 receptors or DAT in a nonhuman primate model. Additionally, MPH treatment did not increase vulnerability to the reinforcing effects of cocaine when tested after the cessation of drug treatment in adolescence. These findings directly address the impact of stimulant medication on three major areas of concern that have been identified in clinical studies of children treated for ADHD: physical growth, DA system development, and future vulnerability to substance abuse. The results of this investigation provide support for the hypothesis that the use of MPH for the treatment of childhood ADHD does not lead to long-term adverse developmental, neurobiological, or behavioral consequences over the course of treatment or later in adolescence.

There are several important features of the current study. First, nonhuman primates provide exceptional models for developmental research because they undergo relatively long childhood and adolescent periods marked by hormonal and physiological maturation, as well as the development of complex social behavior and cognitive abilities that are similar to humans. Nonhuman primates in the current study were between 37- and 54 months of age during MPH treatment, corresponding to approximately 9-13 years of age in children (Knickmeyer et al, 2010), well within the range at which treatment is often initiated in children. Another important feature was the use of an extended release formulation of MPH. The main advantage of these formulations is that they contain an immediate release dose intended to act as an initial bolus and extended MPH release designed to maintain plasma concentrations for up to 6 hours (Patrick et al, 2005), circumventing the need for multiple doses over the course of the day. The use of the
extended release formulation, the most commonly prescribed form of MPH, provides greater translational value to clinical practice.

**Growth**

In the present investigation of juvenile/adolescent nonhuman primates, there were no significant effects of extended release MPH treatment on measures of growth. Likewise, there were no differences between groups in total food consumption. In clinical literature, there has been considerable debate about the effects of stimulant therapy on growth rates. There have been reports of the suppression of the normal trajectory of height and weight gain with stimulant treatment (Safer *et al.*, 1972; Zhang *et al.*, 2010). A recent large multicenter study of the effects of extended release formulations of MPH reported significant suppression of growth rates for both height and weight over a 2 year treatment period, persisting over a 3 year follow-up (Swanson *et al.*, 2007). However, other reports suggest that these effects on growth may attenuate over time and that final adult height is not impacted (Rapoport and Inoff-Germain, 2002). Another recent study with longer follow-up times reported no significant drug effects of growth (Biederman *et al.*, 2010), consistent with an absence of effects in adulthood. In the current study, the absence of any growth suppression may be due to the timing of treatment with respect to the periods of rapid growth. Environmental conditions that suppress growth may not have long-term consequences (Tanner, 1986) when growth rebound occurs after the precipitating condition is removed. It is possible that the washout period obscured any growth effects that would have emerged with continued treatment. Another possible reason for the absence of significant growth suppression was the treatment dose, as dose adjustment has been reported to rectify suppression in
some children (Rapoport et al, 2002). Higher doses may have resulted in more profound effects. Further investigation of the role of dose and duration of treatment is certainly warranted.

**Dopamine D2/D3 receptors and DATs**

As with growth, there was no overall effect of MPH on D2/D3 receptor availability or DAT availability in striatal brain regions as measured by PET with \(^{18}\)FFCP and \(^{18}\)F\]FCT, respectively. Although there were no differences in D2/D3 receptor availability between groups, there was a decrease in binding over time within the entire striatum. This decline in D2/D3 receptor availability during this developmental stage is well-documented in humans (Meng et al, 1999; Seeman et al, 1987) and rats (Teicher et al, 1995) and is thought to be an effect of synaptic pruning. In a large post mortem study, Seeman et al (1987) reported peak levels of D2/D3 receptor density at 5 years of age, followed by a sharp decline into early adolescence (14 yo), and a gradual decrease throughout life. The ages of the monkeys in this study correspond well with the time period in human development of the steepest decline in striatal D2/D3 receptors.

Unlike the D2/D3 receptor family, there was no effect of development on the DAT, despite the fact that numerous studies have documented declines in DAT expression with age in primates and rodents (Hebert et al, 1999; Ma et al, 1999; Volkow et al, 1996). However, the decline in DAT seems to begin later in life than the decline in D2/D3 receptors (Volkow et al, 1996). In human post-mortem tissue, DAT expression in the basal ganglia peaked during adolescence (14-15 yo), then slowly declined (Meng et al, 1999). The developmental window in the present study was likely too early to detect the eventual decline in DAT density.
Methylphenidate did not alter the normal course of DA system development in our study sample. These results are in contrast to rodent studies which have shown effects of MPH on both D2/D3 receptors and DAT measures in the striatum. In rats, oral doses of MPH reportedly decrease D2/D3 receptor availability after two months, and increase availability after 8 months (Thanos et al, 2007), whereas other studies have reported sustained decreases in DAT density after chronic treatment (Izenwasser et al, 1999; Moll et al, 2001; Simchon et al, 2010). Interestingly, one study reported decreases in DAT density with twice-a-day injection of MPH, but not with sustained release via osmotic mini-pump (Izenwasser et al, 1999), highlighting the importance of the drug’s pharmacokinetics and the use of the controlled release formulation. Thus, discrepancies between the results of the rodent models and the nonhuman primate model presented here could be due to differences in dosing regimens and pharmacokinetic profiles of the drug in the different species. Previous studies document intravenous MPH half-life in rats between 25 minutes (Gal and Ames, 1977) and 105 minutes (Segal et al, 1976), while half-life in nonhuman primates has been reported to be 2.8 hours (Wargin et al, 1983), though no recent studies directly compare the two species. The age of the animal at the time of treatment and the duration of treatment may also explain the inconsistent results. The periadolescent/adolescent period in a rodent is brief and difficult to target, yet studies in adult rodents miss the critical window of development during which this drug is most commonly administered.

Despite finding no overall effect of MPH on D2/D3 receptor availability, there was a significant interaction between drug and time. On further analyses, the MPH-treated animals had unchanged D2/D3 receptor availability after washout, while the control animals had continued to decrease. Although the absolute levels of radiotracer binding were not significantly different between groups at washout, the magnitude of the change
between treatment and washout time points in the putamen did differ significantly between MPH- and control-treated animals. Similar trends were seen in the ventral striatum and caudate. These data indicate that, in the MPH-treated animals, there was a change in the trajectory of development to adult levels, which may suggest an interruption of the normal reduction of D2/D3 receptor concentration over time. As D2/D3 receptor availability is regularly associated with vulnerability to drugs of abuse (Dalley et al., 2007; Nader et al., 2006; Volkow et al., 1999), differences in this system may be important for understanding vulnerability to substance abuse after treatment for ADHD. Further investigation is needed to determine if this effect persists after extended periods of abstinence from MPH.

**Cocaine Reinforcement**

In contrast to the present results showing no differences in rates of acquisition to cocaine reinforcement, there have been several preclinical studies demonstrating that MPH treatment enhanced the reinforcing effects of cocaine (Crawford et al., 2011; Harvey et al., 2011; Schenk and Izenwasser, 2002). There are several reasons for the discrepant results, but perhaps most relevant is the dose of cocaine initially made available to the animals. In these three studies, rats were initially exposed to relatively high cocaine doses (0.25-0.75 mg/kg), whereas the present study began with doses well-below reinforcing doses. In the present study, acquisition of reinforcement was operationally defined as response rates higher than when responding resulted in saline injections, whereas most other studies consider acquisition of a performance level (e.g., the dose in which the animal receives 30 injections). Although we did not see differences in response rates across a wide-range of cocaine doses, it is possible that if
we had simply started with high doses, group differences would have emerged. Our current results support the observation that MPH treatment does not make adolescents more vulnerable to the reinforcing effects of cocaine.

These data in nonhuman primates support recent findings that have shown no increase in the rates of substance abuse among adults and adolescents treated as children with stimulant medications for ADHD. Although there is a strong association between a diagnosis of ADHD and higher rates of substance abuse in adolescents and adults (Biederman et al, 1998; Gittelman et al, 1985; Mannuzza et al, 1998; Milberger et al, 1997; Molina et al, 2009; Wilson and Levin, 2005), treatment itself does not necessarily appear to increase the likelihood of drug use in adolescence and/or adulthood. Among young adults treated as children or currently in treatment, rates of drug, alcohol or nicotine abuse have been reported to not be significantly different from rates in a sample of healthy untreated controls (Biederman et al, 2008; Mannuzza et al, 2008). In these studies, there was some suggestion that the earlier treatment is initiated the smaller the likelihood of substance abuse in young adulthood. Although the current study cannot directly address this issue, as there was only a limited range of ages tested, this is potentially an important avenue for future investigations.

Limitations

The study is limited by a relatively small sample size, making it difficult to fully evaluate some variables. Trends suggesting that MPH treatment may have influenced some growth measures might have reached significance with larger numbers of animals, for example. Another consideration is that although all animals received doses of MPH designed to maintain serum levels within an effective therapeutic range in children, the
doses required to achieve these levels were significantly higher on a mg/kg basis than those in children. This may be due to higher rates of drug metabolism in nonhuman primates than in children (Wargin et al, 1983). Additionally, because cognitive and/or attentional outcomes were not measured in the present study, it cannot be determined if these doses in nonhuman primates are sufficient to alter behavior as intended for treatment of ADHD in children. It is possible that even higher doses might be necessary to produce an effect in nonhuman primates which would result in significant changes in behavior and potentially DA receptor availability and developmental measures. However, the dose range used in the current study produced serum levels in some cases that would be considered on the high end of the therapeutic range in humans. The timing of treatment is also an important factor to consider. While our animals were treated at a common relative age for a child to begin treatment, there is evidence that younger animals are less sensitive to the effects (behavioral) of MPH than older animals, which may be related to the developmental stage of the DA system at time of exposure (Rodriguez et al, 2010). It is certainly possible, therefore, that treating our animals at a different age or for a different duration would have altered our results. A broader range of doses, a wider age range at the initiation of treatment, and variable durations of treatment should be considered in the future. For the self-administration studies, it remains possible that using a different acquisition paradigm may have produced different results.

Finally, when interpreting the results of this study, it is critical to acknowledge that we did not employ a nonhuman primate model of ADHD. The animals treated in this study were “normal” and thus cannot be easily compared to studies in children or adults with ADHD. This fact may explain the discrepancy between our results and recent results in a study of adults with ADHD in which one year of treatment resulted in
significantly decreased D2/D3 receptor availability as measured by PET (Volkow et al, 2012). An improved understanding of the underlying dopaminergic pathophysiology of ADHD and the development of a reliable model of ADHD in nonhuman primates would be invaluable in future studies of long-term prescription stimulant treatments.

**Conclusion**

The current findings show that long-term administration of MPH to juvenile nonhuman primates produced no significant alteration in the regulation of the DA systems as measured with PET, or significantly altered growth. These data support the hypothesis that MPH administered in formulations used therapeutically in children does not have obvious long-term effects. In addition, there was no evidence for an increased vulnerability to the reinforcing effects of cocaine in adolescence as a result of MPH treatment. The absence of any significant long-term developmental, neurobiological, or behavioral consequences provides further support that the use of these medications to treat ADHD will not negatively impact children either during or after treatment.

**Disclosure/Conflicts of Interest**

The authors declare that this work was funded by grants from NIDA, DA 20648 (LJP) and DA 06634 (LJP, MAN), NIAAA, AA 17056 (AJB) and T32-AA00756. We wish to acknowledge the generous gift of UCB S.A., Belgium, for study medication.

The authors have no other financial interests to disclose, except for James Swanson who in the past has received the following: Prior Research Support:

Acknowledgements

The authors are grateful to Maria Blevins, Jessica Christenson, Christopher Corcoran, and Keith Groach for assistance in data collection and study conduct. We also thank Pradeep Garg, Sudha Garg, Huw Davies, Daniel Morton, Kim Black and Holly Smith for their efforts collecting PET data, and Tonya Calhoun for her help with the cocaine self-administration studies.
References


CHAPTER THREE

THE EFFECTS OF REARING ENVIRONMENT AND CHRONIC METHYLPHENIDATE ADMINISTRATION ON BEHAVIOR AND DOPAMINE RECEPTORS IN ADOLESCENT RATS

Kathryn E. Gill
Thomas J.R. Beveridge
Hilary R. Smith
Linda J. Porrino

Department of Physiology and Pharmacology
Center for the Neurobiology of Addiction Treatments
Wake Forest School of Medicine
Winston Salem, NC 27157

The following manuscript was published in Brain Research in August 2013. Stylistic variations are due to the requirements of the journal. Kathryn E Gill designed the experiments, collected and analyzed data, and prepared the manuscript.
Abstract

Rearing young rodents in socially isolated or environmentally enriched conditions has been shown to affect numerous components of the dopamine system as well as behavior. Methylphenidate (MPH), a commonly used dopaminergic agent, may affect animals differently based on rearing environment. Here we examined the interaction between environment and chronic MPH treatment at clinically relevant doses, administered via osmotic minipump. Young Sprague Dawley rats (PND 21) were assigned to environmentally enriched, pair-housed, or socially isolated rearing conditions, and treated with either 0, 2, 4, or 8 mg/kg/day MPH for three weeks. At the end of the treatment period, animals were tested for locomotor activity and anxiety-like behavior. The densities of D1-like and D2-like receptors were measured in the striatum using in vitro receptor autoradiography. Locomotor activity and anxiety-like behavior were increased in isolated animals compared to pair-housed and enriched animals. The density of D1-like receptors was greater in isolated animals, but there were no differences between groups in D2-like receptor density. Finally, there were no effects of MPH administration on any reported measure. This study provides evidence for an effect of early rearing environment on the dopamine system and behavior, and also suggests that MPH administration may not have long-term consequences.

Keywords: Environment, Enrichment, Isolation, Dopamine, Methylphenidate
1. Introduction

Early life experiences can have a significant impact on behavioral and brain development. It has been suggested that children raised in impoverished living conditions are at a greater risk for the development of psychiatric disorders such as anxiety, addiction, and attention deficit hyperactivity disorder (ADHD) than children raised in more positive environments (De Bellis, 2002; Jaffee et al., 2012; Latimer et al., 2012; Solinas et al., 2010). Similarly, rodents raised in isolated/impoverished conditions display greater levels of anxiety-like behavior (Bickerdike et al., 1993; Chappell et al., 2013; Hellemans et al., 2004; Lodge and Lawrence, 2003; Lukkes et al., 2009; McCool and Chappell, 2009; Wright et al., 1991; Yorgason et al., 2013), inattention (Ouchi et al., 2013; Schrijver and Wurbel, 2001), and impulsivity (Baarendse et al., 2008; Lovic et al., 2011; Perry et al., 2008). In contrast, rats reared in more enriched housing and social conditions exhibit, for example, improved performance on learning and memory tasks (Fares et al., 2013; Galani et al., 2007; Pamplona et al., 2009; Pappas et al., 1992), decreased levels of anxiety-like behavior (Fares et al., 2013; Pritchard et al., 2013; Urakawa et al., 2013), and decreased levels of depressive-like behaviors (Brenes Saenz et al., 2006), that have been accompanied by increases in neurogenesis (Fares et al., 2013; Ueda et al., 2005) and dendritic complexity (Wang et al., 2012). Enriched rodents also show reduced effects of repeated stimulant administration (Bardo et al., 1995; Gipson et al., 2011; Puhl et al., 2012) and decreased rates of drug self-administration (Alvers et al., 2012; Bardo et al., 2001; Deehan et al., 2011; Stairs and Bardo, 2009). Contrarily, those raised in isolation exhibit higher rates of stimulant drug and alcohol self-administration (Bardo et al., 2001; Chappell et al., 2013; Deehan et al., 2007; McCool and Chappell, 2009; Schenk et al., 1990; Wolffgramm, 1990), increased drug seeking
behavior (Lynch et al., 2005), and more rapid acquisition of cocaine self-administration (Kosten et al., 2000).

These behavioral distinctions are associated with significant differences in brain neurochemistry, particularly in monoamine systems. For example, environmental enrichment has been shown to result in higher levels of 5-HT concentrations in the prefrontal cortex that are associated with lower levels of depressive-like behavior in rodents (Brenes et al., 2008a), as well as decreases in tryptophan-hydroxylase positive cells in the dorsal raphe nucleus, an effect similar to that seen following anti-depressant treatment (MacGillivray et al., 2012). Norepinephrine has been shown to be decreased in the ventral striatum by social isolation (Brenes et al., 2008b), and increased in the parieto-temporo-occipital cortex by environmental enrichment (Naka et al., 2002). The dopamine system, which is thought to play a fundamental role in psychiatric disorders such as addiction and ADHD, is known to be particularly sensitive to environmental manipulations. For example, long-term isolation has also been shown to reduce dendritic spine density and complexity of dopamine neurons (Wang et al., 2012), and increase basal dopamine concentration in the nucleus accumbens (Miura et al., 2002) and prefrontal cortex (Han et al., 2011). Conversely, environmental enrichment has been shown to decrease basal dopamine concentration in the striatum (Bowling et al., 1993), increase dopamine clearance from the medial prefrontal cortex (Neugebauer et al., 2004), enhance dopaminergic neuron migration from the midbrain to the striatum (Urakawa et al., 2013) and increase glucose utilization in the nucleus accumbens (Lack et al., 2010). Thus, the basal tone of dopaminergic systems can be significantly influenced by environmental variables.

Stimulant drugs that act on the dopamine system such as methylphenidate and amphetamine are the most frequently used psychotropic medications in childhood and
adolescence. Currently, an estimated 6-8% of school-aged children are prescribed methylphenidate (MPH; Trade names Ritalin®, Concerta®, Metadate CD™) to treat ADHD (Biederman, 2005). Prescriptions for MPH and similar stimulants such as amphetamine have increased at a rapid rate, from 2.5 million in 1991 to almost 10 million in 1999 (Safer et al., 1996; Spencer et al., 2000; Zito et al., 2000). MPH exerts its effects in the brain by blocking the dopamine transporter (DAT) (Madras et al., 2005; Volkow et al., 1998), a key regulator of dopaminergic transmission. Rodent studies have documented numerous MPH-induced alterations of the dopamine system including changes in dopamine reuptake rates (Harvey et al., 2011), long-term loss of dopamine neurons (Sadasivan et al., 2012), decreases in DAT density (Simchon et al., 2010), and increased basal dopamine levels in the prefrontal cortex (Koda et al., 2010). Behaviorally, acute MPH treatment at low doses has been shown to be anxiolytic on the elevated plus maze (Gray et al., 2007; Koike et al., 2009; Zhu et al., 2010), improve attention (Zhu et al., 2010), and decrease impulsivity (Perry et al., 2008). Taken together, the literature clearly shows that MPH treatment affects many of the same systems and behaviors as social isolation and environmental enrichment, however few studies have examined the interaction of these two variables. Although there are several reports investigating the effects of the acute administration of MPH on animals reared under differing conditions (Perry et al., 2008; Wooters et al., 2011), the effects of chronic treatment remain largely unexplored. The purpose of this study, therefore, was to determine whether the effects of chronic MPH treatment on striatal dopamine systems would differ depending on environmental rearing conditions. As other psychostimulants, such as cocaine, have been shown to have effects on the concentrations of DA D1-like and D2-like receptors in rats (Kleven et al., 1990; Unterwald et al., 1996), and monkeys (Beveridge et al., 2009; Nader et al., 2002), these two targets were chosen for examination. Additionally, because early rearing conditions profoundly alter the
expression of locomotor (Bardo et al., 1995; Bowling et al., 1993; Fabricius et al., 2011; Hoffmann et al., 2009; Shao et al., 2009; Smith et al., 1997; Varty et al., 2000) and anxiety-like behaviors (Bickerdike et al., 1993; Chappell et al., 2013; Lodge and Lawrence, 2003; Lukkes et al., 2009; McCool and Chappell, 2009; Wright et al., 1991; Yorgason et al., 2013), we hypothesized that chronic MPH treatment would produce differential effects on the expression of these behaviors.

In recent years, it has become common clinical practice to prescribe long-acting formulations of MPH such as Concerta® and Metadate CD™, as opposed to immediate-release formulations such as Ritalin®. These drugs are designed to provide an immediate bolus of drug followed by a steady release phase which maintains drug blood levels around the therapeutic range of 10-15 ng/mL (Volkow and Swanson, 2003). Long-acting formulations are reported to be clinically effective for as long as 12 hours after dosing (Pelham et al., 2001; Swanson et al., 2004). Most rodent studies of chronic treatment use twice daily intraperitoneal injection or oral administration, which more closely models the immediate release formulations of MPH. Therefore, questions remain surrounding the effects of chronic dosing with the extended release formulations of MPH that are most commonly prescribed. Here, we use Osmotic MiniPumps (Alzet©; Durect Corporation, Cupertino, CA) to administer MPH continuously during a chronic treatment period, to model some aspects of long-acting formulations of the drug.

To this end, young rats (postnatal day 21) were housed for four weeks in three distinct environmental conditions: enriched environment, standard pair-housed environment, and isolated environment. They were treated for three weeks with saline or 2, 4, or 8 mg/kg/day MPH delivered subcutaneously via osmotic minipump. At the end of the treatment period, animals were tested for locomotor behavior and anxiety-like behavior. Finally, the concentrations of dopamine D1-like and D2-like receptors were
measured using *in vitro* receptor autoradiography. We hypothesized chronic MPH treatment would differentially affect behaviors and dopaminergic measurements depending on the early rearing environment.

2. Results

2.1 Blood Drug Levels

Three animals were removed from this analysis due to insufficient quantities of blood drawn during the procedure. Thus, 24 animals in the 2 mg/kg/day group, 22 animals in the 4 mg/kg/day group, and 23 animals in the 8 mg/kg/day group were included. Osmotic minipump administration of MPH achieved final blood levels of (mean ± SD) 4.4 ± 2.14 ng/mL in the 2 mg/kg/day group, 8.4 ± 3.32 ng/mL in the 4 mg/kg/day group, and 15.4 ± 5.27 ng/mL in the 8 mg/kg/day group. Blood levels of drug did not correlate with any behavioral or dopaminergic measures (data not shown).

2.2 Locomotor Activity

There was a main effect of housing condition on spontaneous locomotor activity in a novel environment on measures of total distance travelled, horizontal beam breaks and stereotypy (tot dist: $F_{2,84} = 83.297; p < 0.001$; horiz act: $F_{2,84} = 41.153; p < 0.001$; stereo: $F_{2,84} = 51.124; p < 0.001$) (Figure 1 A-C). In contrast, there was no effect of MPH treatment (tot dist: $F_{3,84} = 0.399$; horiz act: $F_{3,84} = 1.781$; stereo: $F_{3,84} = 1.732$) and no interaction (tot dist: $F_{6,84} = 0.104$; horiz dist: $F_{6,84} = 0.463$; stereo: $F_{6,84} = 0.317$). Post-hoc analysis (Bonferroni) confirmed that isolated animals exhibited significantly higher levels of locomotor activity (distance traveled) than either paired ($p < 0.001$) or enriched animals ($p < 0.001$) over the one hour test. Higher levels of locomotor activity were also measured in pair-housed animals when compared to environmentally enriched animals ($p < 0.001$). On the measure of horizontal activity, isolated animals had
significantly higher beam breaks than both paired \((p < 0.001)\) and enriched animals \((p < 0.001)\), and enriched animals were also significantly different from paired animals \((p < 0.02)\). Finally, enriched animals exhibited significantly reduced levels of stereotypy as compared with either paired \((p < 0.001)\) or isolated animals \((p < 0.001)\). MPH treatment did not significantly alter measures of spontaneous locomotor behavior in a novel environment.
Figure 1.

A  Total Distance  

B  Horizontal Activity

C  Stereotypy

* p < 0.001
Figure 1. Behavior in the locomotor chamber

A) There was a main effect of housing condition on total distance travelled ($p < 0.001$). Isolated animals travelled the greatest distance in one hour, followed by paired and then enriched animals. B) There was a main effect of housing condition on the measure of horizontal activity ($p < 0.001$). Isolated animals had the highest number of broken beams in the horizontal plane, followed by paired, and then enriched animals. C) Enriched animals had significantly lower expression of stereotypical behavior than paired or isolated animals ($p < 0.001$). Paired and isolated animals were not significantly different from each other.
2.3 Elevated Plus Maze

On the elevated plus maze, greater amounts of time spent on the open arms indicates lower levels of anxiety-like behavior. Over the course of a 5 minute test, there was a main effect of housing condition ($F_{2,84} = 5.502; \ p < 0.01$), no effect of MPH treatment ($F_{3,84} = 1.646$) and no interaction ($F_{6,84} = 0.521$) on time spent in the open arms (Figure 2). Isolated animals spent significantly less time in the open arms than pair-housed ($p < 0.01$) or enriched animals ($p < 0.002$). Paired and enriched animals did not differ from each other in time spent in the open arms.
Figure 2. Time spent in the open arms of the elevated plus maze

There was a main effect of housing condition on anxiety-like behavior as measured by time spent in the open arms of the elevated plus maze ($p < 0.01$). Post hoc tests confirmed that isolated animals spent significantly less time in the open arms than pair-housed ($p < 0.01$) or enriched animals ($p < 0.002$). There was no difference between enriched and paired animals, there was no effect of MPH, and there was no significant interaction.
2.4 Dopamine D1-like Receptor Density

Three animals were removed from analysis due to problems arising from the tissue processing. Thus, all groups were n = 8 except PH 2 mg/kg, PH 4 mg/kg, and SI 4 mg/kg, which each had 7 animals. In all three brain regions there was a significant main effect of housing condition (CPu: $F_{2,81} = 12.050, p < 0.001$; Core: $F_{2,81} = 10.538, p < 0.001$; Shell: $F_{2,81} = 10.966, p < 0.001$) on D1-like receptor density. However, there was no effect of MPH (CPu: $F_{3,81} = 0.488$; Core: $F_{3,81} = 0.463$; Shell: $F_{3,81} = 0.209$) and there were no significant interactions (CPu: $F_{6,81} = 0.493$; Core: $F_{3,81} = 0.207$; Shell: $F_{3,81} = 0.358$) (Table 1). Planned comparisons showed that there was significantly greater D1-like receptor density in all three brain regions among isolated animals when compared to both paired (CPu: $p < 0.005$; Core: $p < 0.05$; Shell: $p < 0.02$) and enriched animals (CPu: $p < 0.001$; Core: $p < 0.001$; Shell: $p < 0.001$). There were, however, no differences in D1-like receptor density between paired and enriched animals (Table 1).
Table 1. The density of D1-like receptors in the striatum, fmol/mg wet weight tissue (mean ± SEM)

<table>
<thead>
<tr>
<th>Housing Condition</th>
<th>Dose (mg/kg/day)</th>
<th>Enriched</th>
<th>Paired</th>
<th>Isolated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPu</td>
<td>0</td>
<td>127.0 ± 5.3</td>
<td>134.1 ± 9.5</td>
<td>162.2 ± 14.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>120.6 ± 3.7</td>
<td>129.6 ± 11.3</td>
<td>147.9 ± 16.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>126.3 ± 7.0</td>
<td>128.1 ± 11.9</td>
<td>176.0 ± 14.9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>113.7 ± 3.8</td>
<td>140.2 ± 13.4</td>
<td>156.6 ± 15.9</td>
</tr>
<tr>
<td>Core</td>
<td>0</td>
<td>120.4 ± 3.2</td>
<td>134.7 ± 11.6</td>
<td>162.8 ± 14.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>118.3 ± 4.3</td>
<td>129.0 ± 10.9</td>
<td>145.3 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>122.4 ± 8.7</td>
<td>136.2 ± 13.6</td>
<td>165.5 ± 14.9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>111.5 ± 2.8</td>
<td>138.8 ± 12.8</td>
<td>152.5 ± 17.1</td>
</tr>
<tr>
<td>Shell</td>
<td>0</td>
<td>107.4 ± 2.3</td>
<td>119.8 ± 9.7</td>
<td>149.4 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>110.8 ± 4.1</td>
<td>116.3 ± 10.6</td>
<td>133.2 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>110.3 ± 9.6</td>
<td>120.2 ± 11.5</td>
<td>150.6 ± 14.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>99.8 ± 3.5</td>
<td>128.4 ± 12.2</td>
<td>141.6 ± 16.9</td>
</tr>
</tbody>
</table>

* p <0.05, There was a main effect of housing on D1-like receptors. Isolated animals had significantly greater concentrations of D1-like receptors than pair housed or enriched animals.
2.5 Dopamine D2-like Receptor Density

As above, two-way ANOVAs were employed to compare the density of D2-like receptors in each region of interest. Again, one animal was removed from analysis due to errors in tissue processing and so the pair-housed 4 mg/kg group had only 7 animals. There were no significant effects of housing condition (CPu: $F_{2,83} = 0.375$; Core: $F_{2,83} = 0.483$; Shell: $F_{2,83} = 0.209$) or drug (CPu: $F_{3,83} = 0.220$; Core: $F_{3,83} = 0.320$; Shell: $F_{3,83} = 0.572$), and no significant interactions (CPu: $F_{6,83} = 0.228$; Core: $F_{6,83} = 0.275$; Shell: $F_{6,83} = 0.458$) on D2-like receptor density in any region of the striatum (Table 2).
Table 2. The density of D2-like receptors in the striatum, fmol/mg wet weight tissue (mean±SEM)

<table>
<thead>
<tr>
<th>Housing Condition</th>
<th>Dose (mg/kg/day)</th>
<th>Enriched</th>
<th>Paired</th>
<th>Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPu</td>
<td>0</td>
<td>104.6 ± 3.9</td>
<td>103.9 ± 3.8</td>
<td>109.6 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>111.4 ± 5.3</td>
<td>103.3 ± 2.5</td>
<td>119.0 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>104.8 ± 5.7</td>
<td>116.2 ± 6.3</td>
<td>113.5 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>108.2 ± 4.4</td>
<td>106.3 ± 3.0</td>
<td>100.4 ± 3.4</td>
</tr>
<tr>
<td>Core</td>
<td>0</td>
<td>62.4 ± 3.8</td>
<td>62.8 ± 2.9</td>
<td>66.6 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>69.5 ± 4.2</td>
<td>59.9 ± 3.8</td>
<td>77.4 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>64.1 ± 6.7</td>
<td>78.7 ± 7.5</td>
<td>71.3 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>65.7 ± 4.1</td>
<td>64.8 ± 2.9</td>
<td>64.6 ± 4.4</td>
</tr>
<tr>
<td>Shell</td>
<td>0</td>
<td>50.9 ± 4.1</td>
<td>50.5 ± 2.4</td>
<td>59.5 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59.7 ± 3.8</td>
<td>45.9 ± 4.0</td>
<td>66.7 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>54.1 ± 6.9</td>
<td>64.4 ± 8.7</td>
<td>64.6 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>55.8 ± 4.6</td>
<td>56.7 ± 4.4</td>
<td>56.7 ± 4.6</td>
</tr>
</tbody>
</table>
3. Discussion

The results of these studies demonstrate that rearing environment during early life has significant effects on both spontaneous behaviors and the dopamine system when measured late in adolescence. Socially isolated animals displayed higher levels of locomotor activity and greater levels of anxiety-like behavior on the elevated plus maze. Additionally, these animals had significantly higher D1-like receptor density throughout the entire striatum. In contrast, chronic MPH, administered via osmotic minipump at clinically relevant doses, had no effect on the behaviors or the dopamine systems of these animals. Perhaps more important, there were no interactions of chronic methylphenidate treatment with housing conditions on any of the measures of behavior or dopamine system regulation. These data suggest that chronic MPH treatment, particularly with extended-release formulations, may not have consequences for the dopamine system or effects on behavioral outcomes, a finding that is supported by recent studies in nonhuman primates (Gill et al., 2012; Soto et al., 2012).

Despite the absence of significant effects of MPH treatment observed here, there were substantial effects of rearing condition on both locomotor activity and anxiety-like behaviors. Rats reared in isolation had higher rates of spontaneous locomotor activity in the open field as compared to the pair-housed animals and those raised in enriched environments when tested after 4 weeks of exposure to the rearing conditions. These data are in concert with numerous previous studies that have clearly shown greater baseline activity levels in rodents following early life isolation (Bardo et al., 1995; Bowling et al., 1993; Fabricius et al., 2011; Hoffmann et al., 2009; Shao et al., 2009; Smith et al., 1997; Varty et al., 2000). Additionally, enriched animals had significantly lower measures of total distance travelled, horizontal activity, and stereotypy than paired animals and isolated animals. This most likely indicates a reduced response to novelty
in this group, again consistent with a number of previous studies (Cain et al., 2006; Elliott and Grunberg, 2005). As opposed to our hypothesis, however, MPH treatment had no effect on levels of locomotor activity in any of the rearing conditions. This finding is similar to a recent study in which chronic oral MPH did not have any effect on activity levels in adolescent rats (Yates et al., 2012). However, these data are contradictory to other studies that have shown decreases in locomotor activity after chronic MPH treatment by intraperitoneal injection (Bolanos et al., 2003) or by oral administration (Bethancourt et al., 2011). In addition, repeated stimulant treatment has been shown to have a greater locomotor sensitizing effect in animals that have been reared in isolated conditions as compared to enriched animals (Bardo et al., 1995). There are considerable differences between the current study and others that have shown significant effects of stimulant treatment in adolescent animals, including the use of doses within the range of those considered clinically relevant in children, rat strain, duration of rearing conditions, and age at testing, which all may have contributed to the discrepant results. Additionally, the continuous infusion of drug via osmotic minipump may have led to behavioral tolerance to the effects of MPH that is not present when using non-continuous routes of administration such as injection and oral dosing. Previous studies comparing continuous and non-continuous administration of other psychostimulants, cocaine and d-amphetamine, have documented differences in locomotor behavior and stereotypy after drug injections that are not present after continuous administration (Nelson and Ellison, 1978; Zeigler et al., 1991).

In addition to greater locomotor activity in the open field, isolated rats displayed higher levels of anxiety-like behavior as measured on the elevated plus maze. This is consistent with previous studies that have demonstrated similar findings in a number of paradigms (Bickerdike et al., 1993; Chappell et al., 2013; Lodge and Lawrence, 2003;
Lukkes et al., 2009; McCool and Chappell, 2009; Wright et al., 1991; Yorgason et al., 2013). However, in the present study on the elevated plus maze, the enriched animals were not different from pair-housed animals, most likely because of the high degree of variability in time spent on the open arms in these two groups. Again, there was an absence of any significant effects of MPH treatment on anxiety-like behaviors. Despite the absence of significant drug effects, previous reports have shown anxiolytic effects of MPH on the elevated plus maze (Koike et al., 2009). In our data, there is some indication that isolated animals that were treated with the highest dose of MPH (8 mg/kg/day) spent more time in the open arms than saline- or low-dose-treated isolated animals. This interaction did not reach statistical significance, but may have with higher doses of MPH.

Isolation rearing was also associated with higher levels of D1-like receptor density throughout the striatum, regardless of MPH dose. D1-like receptor activation in the striatum is known to influence spontaneous locomotor activity in juvenile as well as adult rats, with agonists generally increasing (Charntikov et al., 2011; Desai et al., 2005), and antagonists decreasing (Peters et al., 2007; Schindler and Carmona, 2002) levels of locomotor activity. In addition, D1-like receptor knock-out mice show alterations in locomotor activity compared to controls (Tran et al., 2005) similar to those seen in other studies of isolated animals. It is possible that the altered levels of D1-like receptor density in isolated animals influenced their locomotor activity in the open field in this study.

There are only a few previous reports of alterations to D1-like receptors that have been associated with rearing condition. In mice, D1-like receptors have been shown to be elevated after social isolation (Gariepy et al., 1995), while in rats, another study documented decreases in D1-like receptor density associated with housing in an
enriched environment (Del Arco et al., 2007). Yet, another study reported no changes to D1-like or D2-like receptors associated with rearing condition (Bardo and Hammer, 1991). As a model of early life stress, however, social isolation has been associated with numerous changes to other aspects of the dopamine system, such as reduced dendritic spine density and complexity of dopamine neurons (Wang et al., 2012), increased basal dopamine concentration in the nucleus accumbens (Miura et al., 2002) and prefrontal cortex (Han et al., 2011), increased dopamine turnover (Hall et al., 1998; Heidbreder et al., 2000), and increased evoked dopamine overflow and reuptake rates (Yorgason et al., 2013). Social isolation has also been shown to alter corticosterone levels (Miachon et al., 1993; Rivier and Vale, 1987; Sandstrom and Hart, 2005), indicating that it does, indeed, modulate neural stress systems such as the hypothalamic-pituitary-adrenal axis. Other methods of early life stress, such as chronic exposure to restraint stress, have also been shown to result in increased D1-like receptor density in the prefrontal cortex (Mizoguchi et al., 2000). Additionally, D1-like receptors were increased in the monkey striatum following long-term cocaine self administration (Nader et al., 2002), a pharmacological stressor, and were even further increased after a 30 day period of abstinence (Beveridge et al., 2009). Thus, there is evidence that stressful experiences in general may increase D1-like receptor density, whether in the form of early social isolation, chronic restraint stress, or pharmacological stress.

While D1-like receptor densities were elevated in socially isolated animals, D2-like receptors were unaffected by housing condition. Previous reports have documented differences in the concentration of D2-like receptors associated with rearing condition. However, the direction of the changes is unclear, with reports of both increases (Djouma et al., 2006; King et al., 2009) and decreases (Hall et al., 1998) after social isolation.
There are also several other studies that have failed to see effects of environment on D2-like receptors, similar to the present data (Bardo and Hammer, 1991; Del Arco et al., 2004; Jones, 1992; Malone et al., 2008; Rilke et al., 1998). Additionally, there were no differences in D₂ autoreceptor activity in the nucleus accumbens between isolated and group housed animals, despite increases in dopamine release and reuptake in this region (Yorgason et al., 2013). Behaviorally, isolated animals have been shown to exhibit profiles consistent with lower levels of D2-like receptors, most notably in their vulnerability to drugs and alcohol (McCool and Chappell, 2009; Schenk et al., 1986; Schenk et al., 1987; Stairs and Bardo, 2009). Vulnerability to substance abuse is clearly associated with low levels of D2-like receptor density, as has been shown in nonhuman primates (Morgan et al., 2002) and humans (Dalley et al., 2007; Fehr et al., 2008; Volkow et al., 1996; Volkow et al., 2001; Wang et al., 1997). Conversely, enrichment has been shown to have a protective effect against substance abuse (for review see Stairs and Bardo, 2009), which is associated with higher D2-like receptor density (Morgan et al., 2002; Thanos et al., 2001; Thanos et al., 2004; Volkow et al., 2006). Despite the behavioral profiles that would suggest otherwise, it remains that D2-like receptors have been unaffected by housing condition in numerous studies, including the present one.

In contrast to the effects of rearing conditions on the dopamine system, chronic MPH treatment did not significantly alter the concentrations of either dopamine D1-like or D2-like receptors in the striatum. The present data draw a distinction from work by Thanos and colleagues who showed that chronic, oral MPH treatment was associated with a decrease in D2-like receptor availability after two months, and an increase in availability after eight months of exposure (Thanos et al., 2007). MPH treatment has also been shown to have a lasting effect on presynaptic striatal dopamine function.
(Sproson et al., 2001), and to increase dendritic spine density on both D1- and D2-expressing medium spiny neurons in the shell of the nucleus accumbens (Kim et al., 2009). The methodologies of these studies differ from the current investigation in both duration of treatment (2 or 8 months versus 3 weeks), dose of MPH (15 mg/kg/day vs 2-8 mg/kg/day), and route of administration (oral or intraperitoneal injection versus osmotic minipump), among other factors. As mentioned above, it is additionally possible that the constant infusion of MPH via osmotic minipump over the course of the study resulted in the development of tolerance to the drug that would not occur with non-continuous administration.

One major goal of this study was to explore the effects of chronic MPH treatment at clinically equivalent doses to those used in children in adolescent rodents. To determine whether this dosing regimen was accurately modeling therapeutic dose ranges as found in children of 10-15 ng/ml (Swanson et al., 2004), we tested blood levels of MPH at the conclusion of the study. Blood levels ranged from an average of 4.4 ± 2.14 ng/mL in the 2 mg/kg/day group, to an average of 15.4 ± 5.27 ng/mL in the 8 mg/kg/day group. There were no correlations between blood levels of drug and any behavioral or dopaminergic measures. Importantly, by administering the drug in an osmotic minipump, drug was infused constantly throughout the study. This type of administration differs from the clinical scenario where extended-release formulations are taken once daily and last for 12 hours. Thus, while blood levels reflected therapeutic dose ranges in children, there are differences in the pattern of dosing between this rodent model and MPH treatment in children.

This dosing regimen is also different from the majority of other rodent studies of MPH treatment and it is likely the primary factor underlying the discrepancies between the results here and the results of previous studies. Indeed, one previous study that
directly compared intraperitoneal injection to minipump administration found that acutely injected animals were hyperlocomotive and had enhanced reactions to cocaine, while animals receiving a constant infusion of MPH via minipump exhibited less locomotor activity and were less responsive to cocaine (Griggs et al., 2010). Similarly, behavioral profiles have been shown to vary after the chronic administration of cocaine and d-amphetamine depending on whether the drug was administered continuously or intermittently (Nelson and Ellison, 1978, Zeigler et al., 1991). In humans, pharmacokinetic studies have shown much slower absorption and much longer duration of action with extended-release formulations of MPH when compared to immediate-release forms, though peak concentrations are equivalent (Spencer et al., 2006). Though animal studies of the pharmacokinetics of extended-release MPH treatment or continuous administration are lacking, it is likely that extended absorption and longer duration of action account for differences between continuous and intermittent treatment regimens.

The findings of the present study agree with a recently published study in nonhuman primates that utilized an extended release formulation of MPH which was carefully controlled to maintain doses in the clinically-relevant range as used in children (Gill et al., 2012). In that study, and another study in rhesus monkeys that also titrated doses to the clinically-relevant range (Soto et al., 2012), there were no effects of MPH on growth, availability of dopamine D2-like receptors or DATs, or future vulnerability to cocaine self-administration. All of these results support the notion that chronic MPH treatment in extended release formulations may not have long-term consequences. However, despite the fact that these doses were chosen to replicate clinically-relevant doses in children based upon blood drug levels, it is important to note that it is impossible to determine whether the doses utilized here are behaviorally effective in a
rodent. We did not investigate changes in measures of attention or impulsivity that are the primary targets of MPH treatment. Therefore we cannot state for certain that these doses would be behaviorally effective in a rat as they are in children at these blood levels. It remains possible that higher doses must be used in rodent models to achieve clinical relevance.

In conclusion, there were significant effects of early rearing environment on the dopamine system, specifically on the concentrations of D1-like receptors in striatal brain regions, which were greater in socially isolated animals when compared to paired or enriched animals. Similar to other stressors that are environmental or pharmacological, this finding highlights the impact of early life isolation as a form of stress that can significantly affect the dopamine system and associated behaviors. As disturbances to the dopamine system have been associated with disorders such as ADHD and substance abuse, knowledge of how early life influences can alter this system is critical for understanding the development of these pathologies.

Contrary to our hypothesis, MPH treatment did not differentially affect the behavioral outcomes or dopamine system changes that were associated with rearing environment. This study focused on clinically-relevant doses that were administered via osmotic minipump to model the extended release formulations of MPH that are most commonly prescribed. In agreement with our recent findings in nonhuman primates (Gill et al., 2012), this study provides additional support for the lack of long-term dopaminergic effects associated with chronic MPH treatment.
4. Experimental Procedures

4.1 Subjects and Housing Conditions

Ninety-six male Sprague Dawley rats were acquired at postnatal day (PND) 21 from Harlan Industries. Upon arrival, they were immediately placed into one of three housing conditions (32 rats per housing condition): environmentally enriched, pair-housed, or socially/environmentally isolated. Enriched animals were housed 4 animals per cage in large (280 square inches floor, 8 inches high) clear plastic cages. They were furnished with multiple toys including rodent houses, climbing structures, wooden block chew toys, and kong toys, for example. Toys were rotated twice weekly and all animals were handled daily by the experimenter. Pair-housed animals were housed 2 animals per cage in standard size (142 square inches floor, 8 inches high) clear plastic cages. They did not have any toys and were handled on a limited basis, approximately twice per week. Isolated animals were housed singly in standard size opaque cages. They did not receive toys and were only handled once per week for weighing immediately prior to surgeries. Enriched and paired animals were housed in one satellite housing unit, while isolated animals were housed in a separate unit. Thus, isolated animals were not exposed to any form of experimenter interaction except during daily food and water maintenance, 3 minor surgeries, and final behavioral testing. All animals had 24 hour access to food and water and lights were maintained on a 12 hour on/off schedule with lights coming on at 8:00 am. All studies were carried out in accordance with the guidelines of the Guide for Care and Use of Laboratory Animals, National Research Council, and were approved by the Wake Forest University Institutional Animal Care and Use Committee.
4.2 Drug Dosing

Methylphenidate hydrochloride was obtained from Mallinckrodt (Covidien Pharmaceuticals; Hazelwood, MO). After one week of habituation to the housing conditions, animals were assigned to one of four conditions: 0, 2, 4, or 8 mg/kg/day MPH (8 animals per housing condition/drug dose). Animals that were housed together in the same cage were assigned to the same drug condition. On the morning of the scheduled surgery, each animal was weighed and its dose for the week was calculated. As young rats grow rapidly in this stage, end of the week body weights were estimated using normative growth charts for Sprague Dawley rats published by Harlan Industries (2008). The estimated end of the week weight was, on average, within 4% of the actual measured weight at the end of the week. The “dose weight” was the average of the current body weight and the estimated end of the week weight. After dose weights were calculated, the correct amount of MPH for each animal was mixed into a sterile saline solution (0.9% NaCl) and loaded into osmotic minipumps.

4.3 Surgical Procedures

Osmotic minipumps were implanted subcutaneously to deliver a steady dose of MPH over the drug administration period. Animals were anesthetized with 3-4% isoflurane and given a dose of ketoprofen (5 mg/kg; s.c.) for pain relief. The skin on the animal’s back was shaved and prepared with a three stage wash of betadine surgical scrub, 70% isopropyl alcohol, and betadine surgical solution. A small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissue apart. The pump was inserted into the pocket and the skin was closed with Gluture® (Abbott Animal Health; Abbott Park, IL) tissue adhesive. Each minipump lasted for 7 days, at which point, old pumps were
removed and replaced with new pumps in a separate surgery. Drug treatment lasted for 21 days so each animal had a total of three surgeries, with each surgery lasting under 5 minutes.

4.4 Locomotor behavior

Near the end of the drug treatment period, on PND 48 or 49, spontaneous locomotor activity was measured in all animals. Animals were removed from their home cages and placed into novel locomotor chambers (Med Associates; St. Albans, Vermont) constructed of acrylic glass measuring 43x43x30 cm and containing two infrared beam arrays. Beam breaks were recorded by a computer for one hour. The following measures were calculated: total distance (cm) travelled, horizontal activity (the number of beam breaks in the horizontal plane), and stereotypy. Following the session, rats were immediately returned to their home cages and housing room.

4.5 Elevated Plus Maze

Also on PND 48 or 49 (order of tests counterbalanced among drug and housing conditions), rats were tested for anxiety-like behavior on the elevated plus maze (Med Associates; St. Albans, VT). The elevated plus mazes consisted of two opposite open arms and two opposite closed arms that were elevated 40 cm above the floor. Photosensors on each arm recorded the entries and time spent in each individual arm. At the beginning of the assay, animals were placed in the center of the plus maze facing an open arm. The session lasted for 5 minutes during which entries into each arm and the total time spent in each arm were recorded. Similar parameters on this test have been effective in revealing differences in anxiety-like behavior between rats reared in different environments in previous studies (Chappell et al., 2013; McCool and Chappell, 2009). At the end of 5 minutes, animals were immediately returned to their home cages.
4.6 Blood Sampling

On PND 50, animals were sacrificed by an overdose of sodium pentobarbital (100 mg/kg) by intraperitoneal injection. Approximately 3 mLs of blood was drawn from the cardiac chambers and placed into EDTA treated tubes and frozen at -20°C. Analysis for blood MPH levels was performed using gas chromatography (Medtox Laboratories, St. Paul, MN).

4.7 In vitro receptor autoradiography

Immediately after bloods were taken, brains were harvested, flash frozen in isopentane, and stored at -80°C until sectioning. Coronal sections (20μm) were cut in a cryostat maintained at -22°C. Sections were picked up on charged slides, and then stored at -80°C. Assays included striatal regions that have been shown to be sensitive to environmental manipulation and MPH including the nucleus accumbens (core and shell regions) and caudate putamen (CPu). In vitro receptor autoradiography methods were adapted from Lidow et al. (1991) and Bardo and Hammer (1991).

Dopamine D1-like receptor binding site densities were determined with [³H]SCH 23390 (specific activity 85 Ci/mmol; PerkinElmer, Boston, MA). Sections were preincubated for 20 min in buffer (50 mM Tris, 120 mMNaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, pH 7.4, 25°C) to remove endogenous dopamine. Sections were then incubated for 30 min in the same buffer, pH 7.4, 25°C, containing 1 mM ascorbic acid, 40 nM ketanserin, and 1 nM [³H]SCH 23390. After incubation, sections were rinsed twice for 20 s in buffer containing 1 mM ascorbic acid at pH 7.4, 4°C, then dipped in distilled water at 4°C, and dried under a stream of cool air. Nonspecific binding was defined by incubation of adjacent sections in the incubation solution in the presence of 5 mM (+)-butaclamol.
The density and distribution of dopamine D2-like receptor binding sites was determined with [3H]raclopride (specific activity, 74.4 Ci/mmol; PerkinElmer, Boston, MA). Sections were preincubated for 20 min in buffer (50mM Tris, 120mM NaCl, 5mM KCl, pH 7.4, 25°C) to remove endogenous dopamine. Slides were then incubated for 30 min in the same buffer, containing 5mM ascorbic acid and 2 nM [3H]raclopride. Sections were rinsed 3x2 min in buffer at pH 7.4, 4°C, then dipped in distilled water at 4°C, and dried under a stream of cool air. Nonspecific binding was defined by incubation of adjacent sections in the incubation solution in the presence of 1 mM (+)-butaclamol.

For all experiments, sections, along with calibrated [3H] autoradiographic standards, were exposed to Kodak Biomax MR film for 6 weeks. Films were developed with Kodak GBX developer, stopbath and Rapid Fixer (VWR; West Chester, PA), and then rinsed. Analysis of autoradiograms was conducted by quantitative densitometry with a computerized image processing system (MCID, Imaging Research; InterFocus Imaging Ltd, Cambridge, UK). Optical density values were converted to fmol/mg (of wet-weight tissue) by reference to calibrated [3H] standards. Specific binding was determined by digitally subtracting images of nonspecific binding from superimposed adjacent images of total binding.

4.8 Statistical Analysis

Pearson Product-Moment correlations were used to assess the relationship between drug blood levels and the following measures: time spent in the open arms on the elevated plus maze, total distance travelled, horizontal activity, and stereotypy in the locomotor chamber, D1-like receptor density in the CPU and nucleus accumbens core and shell, and D2-like receptor density in the CPU and nucleus accumbens core and shell. For correlations, blood levels of drug for rats in all housing groups and dosing
conditions were combined to evaluate the relationship between blood levels and other end points. To examine the effects of, and interactions between, MPH and rearing condition on dopamine receptors and behavior, two-way analyses of variance (ANOVAs) (drug x housing condition) were performed on the following measures: time spent in the open arms on the elevated plus maze, total distance travelled, horizontal distance, and stereotypy in the locomotor chamber, and D1-like and D2-like receptor density in the CPu and nucleus accumbens core and shell. For all tests, SPSS software (IBM; Armonk, NY) was used and effects were considered significant if \( p < 0.05 \). Post-hoc tests with a Bonferroni correction were used for planned comparisons between housing conditions where there were significant effects as revealed by ANOVAs. Again, \( p < 0.05 \) was considered statistically significant.

Acknowledgements

We would like to thank Mack Miller and Ann Chappell for their technical assistance with these studies. These studies were funded by grants from NIDA and NIAAA, DA 06634 (LJP, TJRB), DA 20648 (LJP), and T32-AA00756.

Author Roles and Conflict of Interest

Kathryn Gill contributed to study design, performed data collection and data analysis, interpreted results, and prepared the manuscript. Thomas Beveridge contributed to study design and manuscript preparation. Hilary Smith contributed to data collection and manuscript preparation. Linda Porrino contributed to study design,
interpretation of results, and manuscript preparation, and had oversight of the research implementation. None of the authors declare any conflicts of interest.
References


Chappell, A.M., Carter, E., McCool, B.A., Weiner, J.L., 2013. Adolescent rearing conditions influence the relationship between initial anxiety-like behavior and...


Harlan Industries, 2008. Sprague Dawley Growth Curve
http://www.harlan.com/products_and_services/research_models_and_services/research_models/sprague_dawley_outbred_rat.hl


dopamine D2 receptors in unaffected members of alcoholic families: possible protective factors. Arch Gen Psychiatry. 63, 999-1008.


CHAPTER FOUR

CHRONIC METHYLPHENIDATE TREATMENT DURING EARLY LIFE IS ASSOCIATED WITH GREATER ETHANOL INTAKE IN SOCIALLY ISOLATED RATS

Kathryn E Gill
Ann Chappell
Thomas J R Beveridge
Linda J Porrino
Jeff L Weiner

Department of Physiology and Pharmacology
Wake Forest School of Medicine
Winston Salem, NC 27157

The following manuscript will be submitted to Alcoholism: Clinical and Experimental Research in October 2013. Stylistic variations are due to the requirements of the journal. Kathryn E Gill designed the experiments, collected and analyzed data, and prepared the manuscript.
Abstract

Background: Methylphenidate is a stimulant prescribed to treat Attention Deficit Hyperactivity Disorder. Its primary mechanism of action is in the dopamine system, alterations of which are associated with vulnerability to alcohol abuse. There are concerns that juvenile chronic MPH treatment may influence adult drinking behavior. This study examined the interaction of MPH treatment and environmental rearing conditions, which are known to independently influence ethanol (EtOH) drinking behavior, on anxiety-like behavior and vulnerability to alcohol abuse in a juvenile rodent model.

Methods: Male Sprague Dawley rats were housed in enriched, standard, or isolated conditions for four weeks, starting at postnatal day 21. Rats were concurrently treated with 8 mg/kg/day MPH or saline, delivered via osmotic minipump. Anxiety-like behavior was determined at the end of the treatment session, and 5 weeks later. After MPH treatment, rats were exposed to a two-bottle choice EtOH drinking procedure that lasted three weeks.

Results: Early life chronic MPH treatment was associated with greater EtOH intake and greater EtOH preference, but only in socially isolated animals. Isolated animals had greater levels of anxiety-like behavior than standard-housed or enriched animals after 4 weeks of exposure to the housing conditions, a difference that persisted even after all animals had been individually housed for 5 weeks and exposed to EtOH.

Conclusions: These results suggest that early life MPH treatment may increase vulnerability to EtOH drinking in adulthood in a subset of the population. Additionally, this study highlights the importance of early rearing condition for establishing long-lasting behavioral phenotypes. Environmental histories should be considered when prescribing MPH treatment to young children.
1. Introduction

Methylphenidate (MPH) is a stimulant medication that is prescribed to treat Attention Deficit Hyperactivity Disorder (ADHD), the most common psychological disorder of childhood. It has been shown to be extremely effective in acutely reducing the hallmark symptoms of ADHD including inattention, hyperactivity, and impulsivity (for review see Findling, 2008). As ADHD diagnoses have dramatically increased over the past several decades (Olfson et al., 2002, Zuvekas et al., 2006), so have prescriptions for stimulant drugs such as MPH.

MPH exerts its actions on the brain by blocking the dopamine transporter (DAT), a key regulator of dopamine transmission in areas of the brain that are associated with motivation and reward (Patrick et al., 1987, Volkow et al., 1995). Alterations of the dopamine system are regularly associated with vulnerability to substance abuse and the development of addiction. Thus, the potential for MPH treatment to have dopaminergic consequences, coupled with the increase in use, has led to concerns about the possibility for chronic MPH treatment to alter vulnerability to substance abuse.

Recent clinical studies have addressed this question and have suggested that early stimulant medication for ADHD may have a protective effect against the development of substance abuse disorders in adulthood (Wilens et al., 2003, Mannuzza et al., 2003, Volkow and Swanson, 2008, Biederman, 2003, Fischer et al., 2002, Katusic et al., 2005). However, varying treatment histories and differing diagnostic criteria in ADHD patients, among other factors, can produce selection bias and make interpretation of these studies difficult (Volkow and Insel, 2003). Furthermore, ADHD itself is associated with a two-fold greater risk for the development of a substance abuse disorder (Gittelman et al., 1985, Biederman et al., 1995, Biederman et al., 1997, Milberger et al., 1997). In contrast, animal studies afford careful control over factors that
can introduce bias or variability into epidemiologic studies, allowing direct examination of
the variable of interest, in this case exposure to methylphenidate.

There are very few studies in animal models investigating the effects of chronic
MPH treatment on future intake of EtOH, and of those that are available, results are
conflicting (Vendruscolo et al., 2008, Soeters et al., 2008). This question is critical as
alcohol is the most commonly abused drug in age groups that are likely to have a history
of MPH prescription, such as adolescents and college students (Johnston et al., 2013).
There is also a clear association between ADHD diagnosis and alcohol dependence
(Edwards and Kendler, 2012, Ameringer and Leventhal, 2013), which may or may not be
related to stimulant treatment. Thus, one goal of the present study was to examine the
effects of chronic MPH treatment on vulnerability to EtOH abuse in a rodent model.

Most studies of the effects of chronic MPH treatment on vulnerability to
substance abuse have not taken into account the interacting effects of environmental
conditions. However, it is known that environment can shape future behaviors, as well
as vulnerability to substance abuse. For example, children who are exposed to early life
stress have higher rates of depression, anxiety, and substance abuse and addiction as
adults (Nugent et al., 2011, Turner and Lloyd, 2004). Likewise, rodents who are exposed
to early life stress in the form of social isolation have greater levels of anxiety-like
behavior (Bickerdike et al., 1993, Hellemans et al., 2004, McCool and Chappell, 2009,
Chappell et al., 2013, Yorgason et al., 2013, Lodge and Lawrence, 2003, Lukkes et al.,
2009) et al., 2004; et al., 2009 et al., 2013, Chappell 2013, et al 2003, JL 2009) and
depressive-like behavior (Brenes and Fornaguera, 2008, Brenes et al., 2008, Brenes
Saenz et al., 2006). Notably, socially isolated animals have been previously shown to
have greater EtOH intakes than environmentally enriched animals or group housed
animals (Chappell et al., 2013, McCool and Chappell, 2009, Sanna et al., 2011, Deehan
et al., 2007, Deehan et al., 2011, Ehlers et al., 2007, Lodge and Lawrence, 2003, Hall et
al., 1998, Wolffgramm, 1990, Advani et al., 2007, Juarez and Vazquez-Cortes, 2003). Contrarily, rodents raised in enriched environments have been shown to be less sensitive to the rewarding effects of drugs of abuse (Bardo et al., 1995, Puhl et al., 2012, Gipson et al., 2011) and have lower drug intakes (Bardo et al., 2001, Alvers et al., 2012, Deehan et al., 2011).

Previous studies have shown that MPH, when given acutely, interacts with rearing condition to improve the behavioral deficits, such as impulsivity, associated with social isolation (Perry et al., 2008). Additionally, MPH, when administered during a drinking procedure, has been shown to decrease EtOH intake in rodents (Griffin et al., 2010). However, the effects of prior chronic MPH treatment on future EtOH drinking remain unclear. The purpose of the present study was to examine the interaction of chronic MPH and postweaning rearing environment on anxiety-like behavior and future EtOH drinking in young rats. To study chronic treatment, osmotic minipumps (Alzet©; Durect Corporation, Cupertino, CA) were used to administer MPH continuously, keeping blood levels of drug relatively stable. As adults, animals were tested for anxiety-like behavior and EtOH drinking to determine if MPH differentially influenced these behaviors as a function of environment.

2. Materials and Methods

Subjects and Housing Conditions

Forty-eight male Sprague Dawley rats were acquired at postnatal day (PND) 21 from Harlan Industries and immediately placed into one of three housing conditions (16 rats per condition): environmentally enriched, pair-housed, or socially/environmentally isolated. Enriched animals were housed 4 animals per cage in large (280 square inches floor, 8 inches high) clear plastic cages with multiple toys and climbing structures. Toys
were rotated twice weekly and all animals were handled daily. Paired animals were housed 2 animals per cage in a standard size (142 square inches floor, 8 inches high) clear plastic cage without toys and with only limited handling. Isolated animals were housed singly in standard size opaque cages, without toys and were not handled except for weighing immediately prior to surgeries. All animals had 24 hour access to food and water and lights were maintained on a 12 hour on/off schedule with lights coming on at 8:00 am. At PND 50, all animals were individually housed in standard-size cages for the remainder of the study. All studies were carried out in accordance with the guidelines of the Guide for Care and Use of Laboratory Animals, National Research Council, and experimental procedures were approved by the Wake Forest University Institutional Animal Care and Use Committee.

Drugs and Surgeries

Methylphenidate hydrochloride was obtained from Mallinckrodt (Covidien Pharmaceuticals; Hazelwood, MO). After one week of habituation to the housing conditions, animals were assigned to one of two conditions: 0 or 8 mg/kg/day MPH (8 animals per housing/drug dose). Animals that were housed together in the same cage were assigned to the same drug condition. On the morning of the scheduled surgery, each animal was weighed and its dose for the week was calculated. As young rats grow rapidly in this stage, end of the week body weights were estimated using normative growth charts for Sprague Dawley rats published by Harlan Industries (2008). The estimated end of the week weight was, on average, within 5% of the actual measured weight at the end of the week. The “dose weight” was the average of the current body weight and the estimated end of the week weight. After dose weights were calculated, the correct amount of MPH for each animal was mixed into a sterile saline solution (0.9%) and loaded into an osmotic minipump.
Osmotic minipumps were implanted subcutaneously to deliver a steady dose of MPH over the drug administration period. Animals were anesthetized with 3-4% isoflurane and given a dose of ketoprofen (5 mg/kg; s.c.) for pain relief. The skin on the animal’s back was shaved and washed in three stages with betadine surgical scrub, 70% isopropyl alcohol, and betadine surgical solution. A small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissue apart. The pump was inserted into the pocket and the skin was closed with Gluture® (Abbott Animal Health; Abbott Park, IL) tissue adhesive. The minipumps lasted for 7 days, at which point, old pumps were removed and replaced with new pumps. Drug treatment lasted for 21 days, and the final pumps were removed at the end of the third week of treatment. Each animal had a total of four surgeries (three implants, and a final removal), with each lasting under 5 minutes.

Ethanol Drinking

Animals were individually housed for the EtOH self-administration portion of the study, starting at PND 50. Animals were weighed daily between 8:00 and 9:00am and weights were recorded. At 9:00am regular water bottles were removed and EtOH/sucrose and water sipper tubes were hung on the cage side by side and were available for one hour. EtOH/sucrose and water tube sides were changed daily to eliminate side preference. At 10:00am, bottles were removed and measured and regular water bottles were replaced on cages.

A modified Samson sucrose-fade technique was used to eliminate inherent problems of taste aversion to EtOH in rodents (Samson, 1986). Each day animals were given identical bottles containing two different solutions, one being a mixture of EtOH/sucrose and the other a plain water bottle. On day 1, animals were given a bottle of 10% sucrose (10S) mixed into water. On following days the solution was adjusted to
decrease the sucrose concentration and increase the EtOH concentration. Concentrations were as follows: 2% EtOH/7% sucrose (2E7S), 2E5S, 4E5S, 4E3S, 4E3S, 5E2S, 5E1S, 6E2S, 6E1S, 7E2S, 8E1S. Removing sucrose from the solution entirely and testing only a concentration of 8% EtOH eliminated drinking behavior, thus the final concentration used for the remainder of the study was 8E1S. Animals had access to the 8E1S solution one hour per day for 21 consecutive days. Each day, mLs consumed (mLs pre drinking session – mLs post drinking session), g/kg EtOH intake, and % EtOH preference were calculated. G/kg EtOH intake was calculated using the following equation: g/kg EtOH = 1000/(animal’s weight*(5*0.0397*EtOH concentration)). Percent EtOH preference was calculated as the mLs EtOH consumed over the total liquid consumed, thus preference scores greater than 50% reflected a preference for the EtOH/sucrose solution over water.

Anxiety-like behavior

On PND 48 or 49 (Pre-EtOH), rats were tested for anxiety-like behavior on the elevated plus maze (Med Associates; St. Albans, VT). The elevated plus maze consisted of two opposite open arms and two opposite closed arms that were elevated 40 cm above the floor. Photosensors recorded the entries and time spent in each individual arm. At the beginning of the assay, animals were placed in the center of the plus maze facing an open arm. After 5 minutes, animals were immediately returned to their home cages. Animals were re-tested on the elevated plus maze near the conclusion of the study, at PND 84-85 (Post-EtOH), after approximately five weeks of daily EtOH exposure (sucrose fade and then three weeks of two bottle choice at 8E1S). For the Post-EtOH run, animals had their normal EtOH drinking session in the morning, and then sufficient time was allowed to pass for EtOH clearance before testing on the plus maze.
**Blood EtOH concentrations**

On two difference occasions during the 8E1S two-bottle choice study, animals’ blood ethanol concentrations (BECs) were tested at the end of the one hour drinking session. A tail-snip procedure was used to collect approximately 15 µL of blood from each rat. The tail tip was cleaned, sterilized, and treated with analgesic cream. Using a sharp pair of sterile surgical scissors, the very tip of the tail below the tail vertebrae was lacerated and blood was collected. Blood EtOH concentrations were determined using a commercially available alcohol dehydrogenase/NADH enzymatic assay kit (Diagnostic Chemicals, Oxford, CT).

**Sucrose Preference**

In order to assess the impact of the addition of sucrose to the EtOH solution, a 1% sucrose solution, without EtOH, was presented with a water bottle for three consecutive days following the three week EtOH/sucrose exposure. Procedures were identical to those of the EtOH/sucrose drinking. Sucrose consumption was compared between housing and drug treatment groups.

**Statistical Analysis**

Mixed-model three-way analyses of variance (ANOVAs) were used to examine the effects of day, MPH, and housing on the following measures: daily g/kg EtOH intake over three weeks of 8E1S drinking, and mLs sucrose consumed during the sucrose preference test. Post-hoc (Bonferroni) tests were used where significant interactions were present. Where data violated assumptions of sphericity, appropriate Greenhouse-Geisser corrections were used. Two-way ANOVAs (drug x housing) were used to compare average intakes and average preferences over the three-week drinking procedure. T-tests with a Bonferroni correction were used for planned comparisons.
between housing and drug groups where appropriate. In all cases, \( p < 0.05 \) was considered statistically significant.

Two-way ANOVAs (drug condition x housing condition) were run on the following measures: Time spent in the open arms, and number of closed arm entries on Pre-EtOH and Post-EtOH runs of the elevated plus maze. Student’s t-tests were used for planned comparisons between housing groups and drug groups where there were significant main effects on the plus maze. In all cases, \( p < 0.05 \) was considered statistically significant.

On days 11 and 20 of the drinking procedure, BECs were measured and Pearson’s correlations were used to compare g/kg EtOH intake with the BEC across all animals, regardless of drug or housing condition. Again, \( p < 0.05 \) was considered statistically significant.

Results

Drinking

The final concentration of EtOH/sucrose was 8% EtOH and 1% sucrose. A mixed model three-way ANOVA (day x drug x housing) violated assumptions of sphericity and so a Greenhouse-Geisser Correction factor was used on the following data. Using this correction, there was a main effect of day (\( F_{10,413} = 4.924; \ p < 0.001 \)), but no interactions between day and any of the other variables. There were no main effects of drug (\( F_{1,42} = 3.554 \)) or housing (\( F_{2,42} = 0.523 \)), but there was a significant interaction between drug and housing (\( F_{2,42} = 4.457, \ p < 0.02 \)) (Figure 1 A-C). Post-hoc testing showed that isolated, MPH-treated rats drank more than isolated, saline-treated rats. There were no differences between paired and enriched animals, regardless of drug treatment. Likewise, MPH-treated, socially isolated animals had greater average
EtOH intakes than untreated socially isolated animals ($t_{14} = 2.71, p < 0.02$), but there were no differences between the drug treatment groups within enriched ($t_{14} = 1.03$) and paired housing groups ($t_{14} = 1.44$).

As EtOH intakes were only different in the isolated group, further analysis for EtOH/sucrose preference over water was conducted only within the isolated group. A mixed-model two-way ANOVA (day x drug) showed a significant main effect of day ($F_{4,58} = 3.464$, $p < 0.02$; Greenhouse-Geisser correction) and a significant main effect of drug ($F_{1,14} = 4.772$, $p < 0.05$), but no interaction on EtOH preference (Figure 2). MPH-treated, isolated animals showed greater average preference ($t_{14} = 2.17$, $p < 0.05$) for EtOH/sucrose versus water than saline-treated, isolated animals.
Figure 1.

A  Enriched Animals

B  Pair Housed Animals

C  Isolated Animals
Figure 1. *Daily g/kg EtOH intakes.*

MPH- and saline-treated animals within the enriched (A) and pair-housed (B) groups did not differ in their daily intakes of an EtOH/sucrose solution over a three week drinking period. MPH-treated isolated animals had significantly greater average EtOH/sucrose intakes than saline-treated isolated animals (C).
Figure 2.

**Figure 2. Ethanol Preference in Isolated Animals**

MPH-treated isolated animals had significantly greater EtOH preference over water than saline-treated isolated animals.
Anxiety-like behavior

On the pre-EtOH run on the elevated plus maze, there was a main effect of housing condition on time spent in the open arms ($F_{2,42} = 8.961; p < 0.002$), but no effect of MPH ($F_{1,42} = 0.943$) and no interaction ($F_{2,42} = 1.215$) (Figure 3A). Post-hoc t-tests indicated that isolated animals spent significantly less time on the open arms of the plus maze than paired ($p < 0.05$) or enriched animals ($p < 0.01$). The difference between paired and enriched animals was not statistically significant. There were no differences between groups on closed arm entries (Housing effect: $F_{2,42} = 2.410$; MPH effect: $F_{1,42} = 2.920$; Interaction: $F_{2,42} = 0.717$) (Figure 3B), a measure of general locomotor activity.

On the post-EtOH run of the elevated plus maze, there was again an effect of housing on time spent in the open arms ($F_{2,42} = 3.724; p < 0.05$), as well as an effect of MPH ($F_{1,42} = 6.827; p < 0.02$), but no interaction ($F_{2,42} = 0.440$) (Figure 3C). Post-hoc t-tests confirmed that enriched animals still spent significantly more time on the open arms than isolated animals ($p < 0.05$) after EtOH exposure. There was no significant difference between isolated and paired animals, or paired and enriched animals. Although there was a main effect of MPH on the time spent in the open arms on the Post-EtOH run, post-hoc t-tests between the individual MPH- and saline- treated housing groups did not reach significance. Post-EtOH, again, there were no differences on closed arm entries (Housing effect: $F_{2,42} = 1.042$; MPH effect: $F_{1,42} = 0.167$; Interaction: $F_{2,42} = 1.042$) (Figure 3D). The Post-EtOH run on the elevated plus maze was completed at least six hours after the end of that days drinking session. Based on calculated g/kg EtOH intakes for that day, this was ample time to allow for EtOH clearance, without disrupting behavior by omitting the drinking session entirely on plus maze testing day.
Figure 3. Elevated Plus Maze Behavior

In the Pre-EtOH run, there was a main effect of housing condition, but no effect of MPH and no interaction between MPH and housing on open arm time (A), and there were no effects of housing, MPH and no interactions on closed arm entries (B). In the Post-EtOH run, there was still a main effect of housing condition, as well as an effect of MPH, but no interaction (C). Again, there were no effects of housing, MPH, and no interactions on closed arm entries (D).
Blood Ethanol Concentrations

BECs were determined twice during the study, on days 11 and 20 of the drinking procedure. On both days, BECs correlated with EtOH intake for that day (day 11: $r = 0.88$, $p < 0.0001$; day 20: $r = 0.89$, $p < 0.0001$). This test verified that the animals were ingesting measurable quantities of ethanol and that their BECs reflected that intake (Figure 4).
Figure 4. Blood Ethanol Concentrations

BECs taken on two separate drinking days correlated with g/kg EtOH intake on that day. Animals were consuming measurable quantities of EtOH.
Sucrose Drinking

In order to control for the presence of sucrose in the EtOH solution, the animals were given 1-hour per day access to a 1% sucrose solution and water for three days. A three way mixed model ANOVA showed a main effect of day ($F_{2,84} = 5.543$, $p < 0.01$), a main effect of housing ($F_{2,42} = 4.699$, $p < 0.02$), and a main effect of drug ($F_{1,42} = 5.649$, $p < 0.03$) (Figure 5). There were no significant interactions. All animals increased their intake of the 1% sucrose solution (mLs) over the three day period. Enriched animals drank the greatest amount of sucrose, followed by paired housed animals, and isolated animals drank the least. MPH-treated animals drank more sucrose than saline-treated animals in all three housing conditions.
Figure 5. Sucrose drinking

There was a main effect of housing condition and a main effect of MPH treatment on sucrose intake over a three-day sucrose preference test (A-C). Enriched animals drank the greatest amount of the 1% sucrose solution, while isolated animals drank the least. In all three housing groups, MPH-treated animals consumed more sucrose than saline-treated animals. There was no interaction between housing and MPH treatment. * p < 0.05.
Discussion

Here, we report that three weeks of chronic MPH treatment was associated with greater EtOH/sucrose intake and preference, but only in socially isolated animals, suggesting that MPH treatment may increase vulnerability to alcohol abuse in adulthood in a subset of the population. Additionally, we show an enduring effect of early environmental enrichment on anxiety-like behavior. Early enriched animals continued to spend more time on the open arms of the elevated plus maze than early isolated animals even after 5 weeks of individual housing. This suggests that housing conditions, specifically during the pre-adolescent/adolescent ages of PND 21-50, can have long enduring effects on adult behaviors, regardless of adult environment.

While there was no main effect of housing condition or MPH treatment on EtOH intake over the course of the study, there was an interaction between the two variables. MPH-treated isolated animals had greater EtOH intake than all other groups and greater EtOH/sucrose preference than saline-treated isolated animals. Greater levels of EtOH drinking after MPH treatment are consistent with a previous report which documented greater EtOH intakes among adult rodents that had been treated with MPH during adolescence, even though those animals were group housed at the time. (Vendruscolo et al., 2008). Notably this effect was in female Spontaneously Hypertensive Rats (SHRs), a strain that is a presumed model of ADHD (Sagvolden, 2000), and shares several behavioral characteristics with socially isolated animals including hyperactivity and impulsivity (Sagvolden et al., 1993). Yet, another study using the SHR strain documented no increases in EtOH consumption after chronic MPH treatment (Soeters et al., 2008), though the sex of the animals and the housing conditions were not specified. While doses of MPH (2 mg/kg/day) were the same and the duration of dosing was similar in the two studies, one study administered MPH orally by mixing drug into condensed milk and allowing the animal to drink voluntarily (Soeters et al., 2008), and
the other study administered drug via intraperitoneal injection (Vendruscolo et al., 2008). Additionally, the EtOH was presented differently between the two studies, with one study using ascending concentrations of EtOH over several days to a maximum of 6% EtOH solution (Soeters et al., 2008), and the other using a 10% solution throughout the study (Vendruscolo et al., 2008). Thus, there are several variables that may explain the discrepant results between the two studies. While the present study utilized a different strain of animal, these results are in line with those of Vendruscolo and colleagues (2008), indicating that chronic MPH treatment during early life is associated with greater EtOH drinking in adulthood.

However, it is important to note that MPH treatment was associated with greater EtOH intakes only in isolated animals. There was no effect of drug treatment on the drinking behavior of paired or enriched animals. Social isolation during early life has been shown previously to be associated with greater intake of a number of drugs of abuse (McCool and Chappell, 2009, Schenk et al., 1990, Schenk et al., 1987, Gipson et al., 2011, Green et al., 2002, Bardo et al., 2001, Deehan et al., 2007, Wolffgramm, 1990), an effect that may be due to substantial differences in dopaminergic neurochemistry between isolated and enriched animals in reward-related brain areas, such as the nucleus accumbens (NAcc). For example, isolation rearing has been associated with reduced dendritic spine density and neuronal complexity (Wang et al., 2012), and increased dopamine release and dopamine transporter activity in the NAcc (Yorgason et al., 2013). Enriched animals have been shown to have greater dopamine synthesis in the NAcc in response to amphetamine injections (Bowling et al., 1993), and higher levels of glucose utilization in the NAcc core and shell (Lack et al., 2010). Thus, in the current study, the effects of chronic MPH were prominent only in a population that may have had an underlying vulnerability to the reinforcing effects of drugs of abuse related to alterations within the dopamine system.
The rearing environments used here significantly influenced the expression of anxiety-like behavior, without altering locomotor behavior, on the elevated plus maze. These results demonstrate that the environmental conditions in the present study were sufficient to produce the expected behavioral phenotype, despite the lack of a main effect of housing on drinking behavior. Additionally, this study extends previous work by re-examining anxiety-like behavior after changing housing conditions in adulthood. At PND 50 all enriched and paired animals were separated into individual housing, identical to the isolated condition, for the remainder of the study. All animals were retested on the elevated plus maze on PND 84-85 after 5 weeks of individual housing, daily handling, and daily exposure to EtOH. Despite the changing environmental conditions, the significant difference between isolated and enriched animals persisted. Animals that were previously enriched and became individually housed still spent significantly greater time on the open arms of the elevated plus maze than previously paired or consistently isolated animals. This data is in accordance with a recent publication by Yorgason and colleagues (2013), which reported enduring lower levels of anxiety-like behavior in Long Evans rats that had been group housed postweaning for 7 weeks and were subsequently isolated for an additional 4 months, when compared to animals that had been constantly isolated. The results of that work and the current study highlight the importance of the postweaning environment, which can have a profound and enduring impact on adult behavior, regardless of the adult housing environment.

In addition to the persistent effect of housing on the second run of the elevated plus maze, there was an effect of early MPH treatment that was not present in the first exposure to the plus maze, despite cessation of drug infusion approximately 5 weeks earlier. Previous reports of the effects of chronic MPH treatment and subsequent drug washout on anxiety-like behavior are mixed, with some studies showing increases in anxiety-like behavior (Bolanos et al., 2003, Bolanos et al., 2008), and others showing
decreases (Gray et al., 2007). It is also possible that EtOH exposure influenced the behavior on the elevated plus maze on the Post-EtOH run as all animals had been exposed to EtOH for 5 weeks by that time.

Most studies of EtOH drinking in rodents do not use a sweetened EtOH solution, and it is possible that the sucrose addition affected the drinking patterns in these animals. In order to control for the presence of sucrose, we did a three day sucrose preference test at the conclusion of the drinking period. When exposed to an EtOH-free 1% sucrose solution, enriched animals had the greatest intakes, followed by paired and then isolated animals. Additionally, MPH treatment was associated with greater intakes of sucrose in enriched, paired, and isolated animals, when compared to saline controls. However, when sucrose was combined with the pharmacological effects of EtOH, only the isolated animals were affected by prior MPH treatment. Thus, it is likely that the reinforcing effect of EtOH, rather than the sucrose, was responsible for the increased intake and preference in MPH-treated, isolated animals.

Notably, the present findings conflict with a large portion of current literature in that isolation alone was not sufficient to elevate EtOH intakes in those rats over the intakes of pair-housed and enriched animals. This is in contrast to many reports documenting greater EtOH consumption in isolated animals when compared to group-housed or environmentally enriched animals (Chappell et al., 2013, McCool and Chappell, 2009, Sanna et al., 2011, Deehan et al., 2007, Deehan et al., 2011, Ehlers et al., 2007, Lodge and Lawrence, 2003, Hall et al., 1998, Wolffgramm, 1990, Advani et al., 2007, Juarez and Vazquez-Cortes, 2003). One potential reason for the discrepancy between the results presented here and previous work is the strain of rat. None of the above cited studies used outbred Sprague Dawley rats in their drinking experiments. Another study that did use Sprague Dawley rats also found that animals that were isolated for 30 days postweaning did not have greater EtOH intake than their group-
housed counterparts, in concert with the present results (Pisu et al., 2011). There is also data that suggests that extended enrichment rearing (over 90 days) in Sprague Dawley rats will actually enhance voluntary ethanol intake when compared to extended isolation rearing (Rockman et al., 1989).

In summary, chronic MPH treatment interacted with rearing environment to increase EtOH intake and preference in animals that were socially isolated in early life. This suggests that MPH treatment may increase vulnerability to future alcohol abuse in a subset of the population. In addition, here we show that postweaning rearing conditions of isolation and enrichment are associated with enduring effects on anxiety-like behavior, regardless of adult housing conditions. This highlights the importance of early life experience in young rodents for establishing long-term behavioral phenotypes. Overall, the current findings emphasize the importance of considering previous environmental history when prescribing stimulants as a treatment for ADHD.

Acknowledgements: We would like to thank Hilary Smith, Amanda Gogolak, and Mack Miller for their help with these experiments. These studies were funded by grants from NIAAA, AA 21099 (JLW), AA 17531 (JLW), and T32-AA00756.
References


Biederman J (2003) Pharmacotherapy for attention-deficit/hyperactivity disorder (ADHD) decreases the risk for substance abuse: findings from a longitudinal follow-up of
youths with and without ADHD. The Journal of clinical psychiatry 64 Suppl 11:3-8.


adults ages 19-50, in Series Monitoring the Future national survey results on
drug use, 1975-2012. Volume II: College students and adults ages 19-50, pp 400

Juarez J, Vazquez-Cortes C (2003) Alcohol intake in social housing and in isolation
before puberty and its effects on voluntary alcohol consumption in adulthood.
Developmental psychobiology 43:200-207.

Psychostimulant treatment and risk for substance abuse among young adults
with a history of attention-deficit/hyperactivity disorder: a population-based, birth

Lack AK, Gill KE, Porrino LJ (2010) Local cerebral glucose utilization in rats exposed to
an enriched environment: A comparison to impoverishment. Pharmacol Biochem
Behav. 96, 521-5.

consumption and central CCK/dopamine systems in Fawn-Hooded rats. Behav
Brain Res 141:113-122.

isolation exhibit increased anxiety and conditioned fear behavior, and altered

at risk for adult substance abuse? A controlled, prospective follow-up study. J

McCool BA, Chappell AM (2009) Early social isolation in male Long-Evans rats alters
both appetitive and consummatory behaviors expressed during operant ethanol


CHAPTER FOUR
SUPPLEMENTARY MATERIAL

PERFORMANCE ON THE ELEVATED PLUS MAZE IS ASSOCIATED WITH
DRINKING BEHAVIOR
Introduction

In Chapter Four, there was a main effect of MPH on the Post-EtOH exposure of the elevated plus maze that was not present in the Pre-EtOH test, despite the fact that drug administration had ceased 5 weeks earlier. During that time, all animals had been exposed to EtOH daily. As many of the MPH-treated animals, and particularly the socially isolated animals, had greater intakes of EtOH over the course of the study than saline-treated animals, we hypothesized that animals that were heavier drinkers also spent more time in the open arms of the elevated plus maze than animals that did not drink, or were light drinkers.

Methods

In order to examine the influence of EtOH intake on elevated plus maze behavior at the end of the study, we divided animals into “drinkers” and “non-drinkers” based upon their average daily EtOH intake (g/kg) in the last 11 days of the drinking period, when intakes had stabilized. Based on BECs, 0.50 g/kg was chosen as the cut-off point to determine “drinkers” versus “non-drinkers” as rats that consumed more than 0.50 g/kg per day had measurable quantities of EtOH in the blood (see Chapter Four, Figure 4). Animals that drank more than 0.50 g/kg per day or more were defined as “drinkers” (n = 26), while animals that drank less than 0.50 g/kg per day were defined as “non-drinkers”. Student’s t-tests were also used to compare “drinkers” and “non-drinkers” on average time spent in the open arms on the Pre-EtOH and Post-EtOH runs and the average change in time spent in the open arms between the Pre- and Post-EtOH runs.

Results

On the Pre-EtOH run, animals that went on to become “drinkers” were not significantly different on open arm time than animals who were defined as “non-drinkers”
at the end of the study ($t_{46} = 0.013$) (Figure 1A). However, on the Post-EtOH run, there was a significant difference between “drinkers” and “non-drinkers” ($t_{42} = 3.033; p < 0.005$), with “drinkers” spending significantly more time on the open arms than “non-drinkers” (Figure 1B). Additionally, there was a significant difference in change in time spent in the open arms between “drinkers” and “non-drinkers” ($t_{46} = 3.237; p < 0.002$), with “drinkers” increasing their time on the open arms by an average of $22.5 \pm 8.12$ (mean ± SEM) seconds, and “non-drinkers” decreasing their time on the open arms by an average of $8.0 \pm 4.8$ (mean ±SEM) seconds (Figure 1C).
A  Pre-EtOH

Open Arm Time (s)

Drinkers

Non-drinkers

B  Post-EtOH

Open Arm Time (s)

C  Difference

Change in time (s)

*
Figure 1. Drinkers versus Nondrinkers on the Elevated Plus Maze

On the Pre-EtOH run, there were no differences in open arm time between “drinkers” and “non-drinkers” (A). On the Post-EtOH run, “drinkers” spent significantly more time in the open arms than “non-drinkers” (B). The difference in open arm time between the Pre- and Post-EtOH runs was significantly different between drinking and non-drinking groups (C). * p < 0.005
Discussion

Rats were divided into “drinkers” and “non-drinkers” based upon their individual average daily intake over the second half of the EtOH drinking procedure. Importantly, there was no difference on time spent in the open arms between “drinkers” and “non-drinkers” on the run prior to the EtOH exposure. However, on the Post-EtOH run, “drinkers” spent significantly more time on the open arms than “non-drinkers”, exhibiting far lower levels of anxiety-like behavior on this particular task. The change in open arm time between runs was also significantly different between groups, with “drinkers” spending greater time on the open arms during the second exposure to the plus maze, and “non-drinkers” spending less time. That the difference between the drinking and non-drinking groups did not exist prior to EtOH exposure suggests that it may be EtOH intake, rather than the MPH treatment, that is driving this effect. However, an anxiolytic effect of prior MPH treatment and drug washout cannot be entirely ruled out and this is an important area for future investigation.
CHAPTER FIVE
DISCUSSION

These studies were designed to address the concerns of physicians, teachers, and most importantly, parents, surrounding the ever expanding use of methylphenidate (MPH) to treat Attention Deficit Hyperactivity Disorder (ADHD) in the pediatric population. These concerns are justified for a number of reasons, the first of which being the current prevalence of ADHD diagnosis and prescription in the United States and elsewhere. Estimates as of 2007 suggest that 4.1 million children (7.2%) ages 4-17 had a diagnosis of ADHD in the United States alone. Of those with ADHD, 2.7 million (66.3% of those with a diagnosis) were taking medication to treat the condition (Visser et al., 2013). Rates of ADHD diagnosis are positively associated with age and with sex, with teenage boys having the highest rates of diagnosis and medication (Visser et al., 2013). Figure 1 (A, B) (adapted from Visser et al., 2013) depicts the rates of ADHD diagnosis and medication in boys and girls across the surveyed age range in 2007-2008. These data are the most recent estimates that have been published by the Center for Disease Control and Prevention (CDC). However, in March of 2013, the New York Times reported that new data, as yet unpublished by the CDC, documented ADHD in 11% of children ages 4-17 and in nearly 20% of high school age boys, which is a 16% increase from the data reported in 2007, and a 41% rise over the past decade. Of the 6.4 million children who had received an ADHD diagnosis at some point in their lives, two-thirds were taking stimulant medication to treat the disorder (Schwarz and Cohen, 2013). Thus, the most recent evidence suggests that the trend of increasing diagnosis and medication is continuing at the present time, contrary to the suggestion that rates had slowed in the early 2000’s (Zuvekas et al., 2006). MPH continues to be the drug of choice in the treatment of ADHD (Brown et al., 2005).
Figure 1. Percentage of boys and girls ages 4-17 who were diagnosed with ADHD and taking medication to treat ADHD as of 2007-2008. Adapted from Visser et al., 2013.

A second reason for concern is that MPH is a drug that affects the dopamine system, specifically by blocking the re-uptake of dopamine into the presynaptic terminal, thereby enhancing dopaminergic transmission (Volkow et al., 1995). It is well known that the dopamine system is undergoing substantial development during the stages of life when MPH is most commonly prescribed (Andersen, 2005), as detailed in Chapter One. Chronic treatment with MPH, therefore, has potential to alter the course of normal dopaminergic development. Alterations of the dopamine system have been associated with numerous psychopathologies including schizophrenia, Parkinson’s disease, and substance abuse. ADHD itself is associated with greater rates of substance abuse (Biederman et al., 1995; Biederman et al., 1997; Gittelman et al., 1985; Milberger et al., 1997a; Milberger et al., 1997b) and it is unclear whether or not that association is related to stimulant medication.
Therefore, taken together, almost 4 million children are taking psychostimulant drugs that have potential to cause long term alterations to the dopamine system, a neural system which is associated with vulnerability to substance abuse and addiction. Thus, the concerns expressed by parents, teachers, physicians, and public health officials surrounding MPH treatment are highly justifiable.

Another factor that has been shown to influence vulnerability to substance abuse is early environmental condition. Early childhood adversity has been associated with increased substance abuse vulnerability in a number of studies (Dube et al., 2003; Gerra et al., 2009; Gerra et al., 2010; Schafer et al., 2010). Exposure to stress at a young age contributes to increased severity and duration of addiction, potentially due to developmental modifications in the dopamine reward system (Andersen and Teicher, 2009). Interestingly, studies have also shown that “negative” environmental conditions are associated with greater rates of ADHD. Children exposed to maladaptive parenting in the form of abuse, inconsistent or excessively harsh punishment, marital conflict between parents, and parental antisocial behavior are more likely to develop several behavioral disorders including ADHD, conduct disorder, oppositional defiant disorder, and substance abuse (Bornovalova et al., 2010; Bornovalova et al., 2013; Caspi et al., 2004; Stanger et al., 2004; Stormshak et al., 2000). Recently, Pires and colleagues (2013) reported that “precariously functioning families”, as indicated by a lack of social support, adverse life events, discord during pregnancy, parent’s loss of employment, or death in the family, had greater rates of children diagnosed with ADHD. Thus, there is an association between ADHD and early life stress. It can be assumed that many children who have been exposed to adversity have been treated with methylphenidate and other stimulants.
This research was conducted to address these issues by examining the effects of early chronic methylphenidate treatment, alone and in combination with environmental manipulations, on the dopamine system and vulnerability to substance abuse. The major findings of this series of studies are documented in the preceding chapters and will be summarized again here in three sections: Main effects of methylphenidate, Main effects of environment, and Interaction between environment and methylphenidate. In addition, this section will serve to put these findings into a broader context, discussing potential mechanisms as well as the clinical significance of these results.

MAIN EFFECTS OF METHYLPHENIDATE

1. Chronic treatment with methylphenidate did not impact dopamine receptors or dopamine transporters in nonhuman primates or rodents

Perhaps the most important finding in this series of studies is the lack of effects of chronic MPH administration on almost all of the dopaminergic measures. In AIM 1, we hypothesized that MPH treatment would be associated with changes in the availability of D2-like receptors and DATs in treated monkeys when compared to untreated monkeys. Based upon prior studies in rodents (Moll et al., 2001; Thanos et al., 2007), we expected that chronic MPH treatment would be associated with increased availability of D2-like receptors and decreased availability of DATs. An increase in D2-like receptors in treated animals would also be expected based upon clinical literature that reports a lower level of substance abuse disorders in adults who were treated with MPH as children (Biederman, 2003; Biederman et al., 2009). Studies in humans and animals have consistently shown greater levels of D2-like receptor availability to be
associated with lower vulnerability to substance abuse (Morgan et al., 2002; Volkow et al., 1999; Volkow et al., 2006).

Contrary to our hypotheses, there were almost no effects of chronic MPH treatment on dopaminergic measures in nonhuman primates. PET measurements of the availability of DATs and D2-like receptors were not significantly different between treated and untreated animals, with both groups showing the expected decline in D2-like receptor availability over development, and no change in DAT availability, after one year of chronic treatment.

The results of this study in nonhuman primates are supported by those in another study of the effects of chronic stimulant treatment on D2-like receptor and DAT availability done by a group at John's Hopkins University (Soto et al., 2012). In that study, which was co-published with our own, juvenile nonhuman primates were treated for 18 months with a twice-daily immediate-release formulation of methylphenidate or dl-amphetamine, delivered orally, also titrated to reach plasma levels that are associated with clinical efficacy in human populations. The availability of D2-like receptors and DATs was quantified at four time points: after 6 months of treatment, after 12 months of treatment, after 18 months of treatment, and after 6 months of drug washout. At all four time points, there were no differences in dopaminergic measures between MPH-treated, dl-amphetamine-treated, or control animals (Soto et al., 2012), similar to the present results.

The results of AIM 2, in which we investigated the effects of chronic MPH treatment on the dopamine system of adolescent rats, corroborate our findings in the nonhuman primate model. We hypothesized that there would be a significant interaction effect between MPH and environment on the dopamine receptors of adolescent rats. As
several studies have shown that the behavioral effects of acute administration of MPH vary depending on rearing environment (Hill et al., 2012; Perry et al., 2008), we hypothesized that chronic MPH treatment would interact with environment in its actions on the dopamine system as well. First, we hypothesized that socially isolated animals would have lower baseline levels of D2-like receptors than environmentally enriched animals. Subsequently, we expected that MPH treatment would not affect the dopamine receptors of enriched animals, but that treatment would be associated with greater density of D2-like receptors in socially isolated animals, when compared to untreated isolated animals.

Again contrary to our hypothesis, there was no effect of three weeks of chronic MPH treatment on the density of D2-like dopamine receptors in the striatum of young-adult rats as measured by in vitro autoradiography. Additionally, there were no significant differences in the density of D1-like receptors between treated and untreated animals. Thus, MPH did not impact the dopamine systems of adolescent rats, regardless of housing condition. Contrary to these results, prior studies in rodents have documented alterations in D1-like and D2-like receptors that were associated with chronic MPH treatment (Kim et al., 2009; Thanos et al., 2007). However, these studies differed from the present work in a number of important ways. While Thanos and colleagues (2007) also administered low doses of MPH, they treated animals for eight months, which is well into adulthood in a rodent. Kim and colleagues (2009) saw increases in dendritic spine density of D1-like and D2-like receptor expressing medium spiny neurons, but that was after daily doses of 15 mg/kg MPH in adult mice. The targeting of the adolescent period of PND 28-49, as well as the use of doses that led to blood plasma levels of MPH that are associated with clinical efficacy separate the present work from prior studies and add translational value.
In summary, the results of these studies provide evidence that MPH may not affect dopamine receptors and DATs. While there are no clinical studies of this subject in children to compare to, several studies have looked at the effects of MPH treatment on the dopamine system in adult ADHD patients. Individuals with ADHD were scanned using PET and compared to age-matched healthy control subjects on the availability of D2-like receptors and DATs in striatal regions of interest, including the caudate nucleus, putamen, and ventral striatum (Volkow et al., 2009). It was reported that there were significantly lower levels of both D2-like receptor availability and DAT availability in adults with ADHD when compared to normal, healthy persons (Volkow et al., 2009). The same individuals, but only the ADHD patients, were then treated with long-acting MPH for one year. The doses were optimized based on behavioral improvement using several scales designed to assess the severity of ADHD symptoms, rather than blood plasma levels. After one year of treatment, there was a significant increase in the availability of DATs in the ventral striatum of the ADHD patients, but there was no change in untreated healthy control subjects (Wang et al., 2013). Effects were not reported for the availability of D2-like receptors after treatment in this population. This indicates that DATs may upregulate in human ADHD subjects in response to higher-than-normal levels of extracellular dopamine caused by MPH treatment, which is in contrast to our findings in nonhuman primates.

Importantly, the studies described above used adult ADHD subjects and reported that baseline levels of DAT availability were lower in adults with ADHD than in healthy controls (Volkow et al., 2009). In children, however, it has been reported that specific binding to the DAT is increased in ADHD patients when compared to normal children (Cheon et al., 2003). It is well known that childhood and adolescence are a dynamic period of dopamine system development, and therefore ADHD may manifest itself
differently, pathologically, in adults versus children, even while behavioral symptoms are similar. Consequently, it is possible that MPH may have different dopaminergic effects in children when compared to adults. Accordingly, we may have noticed differences between treated and untreated animals in dopamine receptor and transporter availability had we used adult animals. However, while MPH use in adults is increasing, it is still most commonly prescribed to children, and thus it is important to perform preclinical studies in juvenile animals.

In these studies, in both nonhuman primates and rodents, the doses of MPH reflected blood plasma levels that are associated with clinical efficacy in children. However, it is important to note that we did not examine cognitive measures such as attention or behavioral inhibition that are normally improved in children with ADHD by MPH treatment. Unfortunately, recently published studies have not been able to establish an optimal behaviorally effective dose in young animals, based on cognitive tasks. Oral doses of immediate release MPH at 2.5 to 3.0 mg/kg b.i.d. (Rodriguez et al., 2010), or 10 to 12 mg/kg b.i.d. (Soto et al., 2012), did not have a significant effect on cognitive behavior in juvenile nonhuman primates (Rodriguez et al., 2010; Soto et al., 2012). Thus, the doses used here, which averaged 6.8 mg/kg in nonhuman primates and ranged from 2 to 8 mg/kg in rodents may not have been behaviorally effective in these animals. Indeed, MPH has been shown to be far less potent in nonhuman primates and rodents at occupying the dopamine transporter, due to dramatic differences in pharmacokinetics between species (Wilcox et al., 2008), hence greater doses were used here, and in other animal studies, than are normally used in humans. However, these studies could still be strengthened by the addition of several cognitive tasks measuring constructs such as response inhibition or impulsivity to identify behaviorally effective doses. It is possible that higher doses are necessary in animals to
evoke behavioral changes and that those doses may also lead to dopaminergic alterations.

Blood plasma levels of 10-15 ng/mL have been shown to occupy about 50% of dopamine transporters in humans (Volkow et al., 1998) and there are strong correlations between blood plasma level and dopamine transporter occupancy (Leonard et al., 2004). Therefore, it stands to reason that the doses used in the present studies, which produced blood levels around 10-15 ng/mL should be translatable to the human condition. Nonetheless, the results of this work were clear in that the availabilities of dopamine receptors and transporters, when quantified immediately following the treatment period, were not affected by MPH treatment in nonhuman primates or rats. Largely, these data can be considered reassuring to the public health community, as it appears that MPH, in an extended release formulation, has little impact on the dopamine system.

2. Chronic MPH treatment and washout impacted the trajectory of D2-like receptor binding

Interestingly, chronic MPH administration plus four months of drug washout resulted in a change in the trajectory of D2-like receptor development in the treated nonhuman primates. Whereas D2-like receptor availability continued to decrease at the washout point in the control animals, there was a slight rebound of receptor availability in treated animals. Considering the importance of the D2 receptor family in substance abuse vulnerability, this is an important area for follow up. Although this is speculation, it is possible that increases in D2-like receptor availability after drug washout may contribute to the finding that adults who were treated with MPH as children have lower
levels of substance abuse than untreated adults with ADHD (Biederman, 2003; Biederman et al., 2009).

There was also a main effect of MPH on the Post-EtOH run of the elevated plus maze after 5 weeks of drug washout. However, this finding is naturally confounded with ethanol exposure as the socially isolated animals that were treated with MPH had significantly greater EtOH intakes than other animals. The analysis described in the Chapter Four supplement shows that animals that became “drinkers” spent more time on the open arms of the plus maze than animals that were “non-drinkers”. Thus, it is impossible to say whether the main effect of MPH on the Post-EtOH run of the plus maze was a result of MPH treatment and washout, or of EtOH exposure. An important control to include in a future study would be a non-drinking group that is exposed to the same environmental, MPH treatment, and EtOH drinking protocols, but substituting water for EtOH. Having this group would enable us to separate the effects of environment and MPH treatment/washout from the effects of ethanol. Additionally, we cannot say whether or not there were dopaminergic changes after drug washout in the rodents. With a non-drinking control group, it would be possible to see if there were any effects of MPH and washout on the dopaminergic measures in rodents, similar to those observed in the nonhuman primate study.

3. Chronic methylphenidate treatment did not alter vulnerability to the acquisition of cocaine self-administration in nonhuman primates

A major goal of this dissertation work was investigating the contribution of MPH treatment to vulnerability to substance abuse. Substance abuse and addiction are devastating to the people they affect, their families and friends, and to society in general.
The most recent estimates suggest that abuse of illegal drugs, tobacco and alcohol costs the United States $524 billion annually (Harwood 2000; ONDCP 2004). As a comparison, heart disease, the nation’s leading killer, cost $444 billion in 2010. As with heart disease, prevention of substance abuse and addiction is a primary goal for reducing its impact on society.

While ADHD patients are known to have greater rates of tobacco and alcohol use (Biederman et al., 1995; Biederman et al., 1997; Gittelman et al., 1985; Milberger et al., 1997a; Milberger et al., 1997b), most recent clinical literature suggests that treated children are less likely to develop substance abuse disorders than untreated children (Biederman, 2003; Fischer and Barkley, 2003; Katusic et al., 2005; Mannuzza et al., 2003; Volkow and Swanson, 2008; Wilens et al., 2003). Thus, we hypothesized that nonhuman primates treated with MPH would be less vulnerable to the reinforcing effects of cocaine than untreated animals. Contrary to our expectations, there were no differences between the untreated and treated animals on vulnerability to cocaine reinforcement as measured by cocaine acquisition, defined as the dose at which responding for cocaine was greater than responding for saline. There were no differences between groups in cocaine response rate or cocaine intake across a range of doses.

In agreement with these findings, an excellent meta-analysis that was recently published used the results from studies done in the past 22 years which were longitudinal in design and in which the ADHD medication period preceded the measurement of substance use and abuse (Humphreys et al., 2013). Using these criteria, 2,565 subjects from 15 studies including stimulant treated and untreated subjects were examined for lifetime use and abuse of alcohol, cocaine, marijuana, nicotine and other drugs. No association was found between prior stimulant drug
treatment and substance use or dependence, leading the authors to conclude that ADHD treatment neither protects against nor increases the risk of substance abuse disorders (Humphreys et al., 2013). The results of this study in young nonhuman primates agree with the findings in the meta-analysis by Humphreys and colleagues (2013), and suggest that chronic MPH treatment does not have any impact on vulnerability to cocaine abuse.

Extending the present findings, Martelle and colleagues (2013) recently tested the same nonhuman primates for MPH self-administration. The study was performed as MPH has been shown to function as a reinforcer in animal models (Alvers et al., 2012; Johanson and Schuster, 1975; Lile et al., 2003). Additionally, there is substantial evidence that adolescents and young adults mis-use MPH and other stimulants for recreational purposes as well as for cognitive enhancement (Boyd et al., 2006; McCabe et al., 2009). Martelle and colleagues (2013) reported that early life MPH treatment does not affect future MPH self-administration, though it is important to note that all animals had a history of cocaine self-administration prior to MPH self-administration testing.

In conclusion, we found that treatment with an extended release formulation of MPH at doses that produced blood plasma levels in the range that is associated with clinical efficacy did not have any impact on future vulnerability to cocaine abuse in nonhuman primates. In addition to the lack of effects of MPH on the dopamine system, these results support the use of MPH as a safe treatment for ADHD in children. The effects of MPH on vulnerability to EtOH drinking in rodents will be discussed below as there was a specific interaction between MPH and environment on that measure.
MAIN EFFECTS OF ENVIRONMENT

4. Environmental conditions had a significant impact on the density of D1-like receptors in the striatum

In Chapter Three, we investigated the effects of environmental conditions on dopamine receptors. Several previous studies have reported lower levels of D2-like receptors in animals raised in isolated environments (Fitzgerald et al., 2013; Hall et al., 1998). Additionally, it is well documented that socially isolated animals have greater vulnerability to many drugs of abuse (for examples see Bardo et al., 2001; McCool and Chappell, 2009; Zhang et al., 2005). Thus, as mentioned above, we expected isolation housing to be associated with lower levels of D2-like receptor density than enrichment housing or standard, pair-housing.

There were no effects of housing environment on the density of D2-like receptors in young adult rats. However, there was an effect of environmental condition on the density of D1-like receptors in the striatum, which we had not hypothesized. Specifically, D1-like receptors were increased in animals that had been socially isolated from PND 21 to 49, significantly above the levels of both environmentally enriched rats and pair-housed rats, regardless of MPH treatment. In previous studies, greater levels of D1-like receptor density have been reported after exposure to a variety of stressors including social isolation (Gariepy et al., 1995), chronic restraint stress (Mizoguchi et al., 2000), and cocaine withdrawal (Beveridge et al., 2009). As a form of early life stress, social isolation has been shown to increase corticosterone levels (Miachon et al., 1993; Miyazaki et al., 2012; Rivier and Vale, 1987; Sandstrom and Hart, 2005; Stairs et al., 2011) indicating that it does, indeed, modulate neural stress systems such as the hypothalamic-pituitary-adrenal (HPA) axis.
The modulation of the HPA axis due to early life stress, and particularly greater levels of corticosterone, may be the mechanism by which D1-like receptors were greater in striatal brain regions of isolated rats. D1-like receptor density, as measured by *in vitro* autoradiography, has been shown to be significantly lower in the caudate putamen of adrenalectomized (ADX) rats (Biron et al., 1992), and rats treated with metyrapone, a corticosterone synthesis inhibitor (Czyrak et al., 1997). Administration of corticosterone to ADX rats has been shown to increase D1-like receptor density to pre-operative levels (Biron et al., 1992). There is also anatomic evidence for the regulation of D1-like receptors by corticosterone as D1-like receptors and Type II glucocorticoid receptors, for which corticosterone is the endogenous ligand, are co-localized on neurons in the rat striatum (Czyrak and Chocyk, 2001). Finally, our studies reported that socially isolated rats had greater locomotor activity than environmentally enriched and pair-housed animals, and administration of exogenous corticosterone, at physiologically relevant levels, has been shown to increase locomotor activity (Diaz et al., 1997; Piazza et al., 1996; Wolkowitz et al., 1986) and stimulate dopamine release in the nucleus accumbens (Piazza et al., 1996).

We did not quantify corticosterone in these animals, and thus we cannot make any conclusions about the effects of glucocorticoids on these dopaminergic or behavioral outcomes. However, it is possible that the isolated animals had greater levels of circulating corticosterone than enriched or paired animals, and that difference may underlie the higher levels of D1-like receptor density and greater locomotor behavior observed in this group. An interesting follow-up study would quantify corticosterone levels in these animals and then use a Type II glucocorticoid receptor antagonist to block its action prior to locomotor testing. If the increased levels of locomotor activity in
isolated animals are a result of corticosterone action then antagonizing that system should result in levels of locomotor activity that are similar to enriched animals.

INTERACTION BETWEEN ENVIRONMENT AND METHYLPHENIDATE

5. Chronic methylphenidate treatment was associated with greater EtOH intake and preference in animals that were socially isolated early in life

In Chapter Four, we reported a significant interaction between chronic MPH treatment and environmental condition on EtOH drinking in young adult rats. Specifically, MPH treatment was associated with greater levels of EtOH intake and preference only in socially isolated animals. This effect was, in fact, the opposite of what was hypothesized. We expected isolated animals to have greater levels of EtOH intake and preference compared to enriched and pair-housed animals. We also expected MPH treated socially isolated animals to drink less EtOH than untreated socially isolated animals. Instead, MPH treated isolated rats drank significantly more EtOH, and had greater EtOH preference, than any other group. There were no differences in EtOH intake or preference between saline-treated, isolated animals and environmentally enriched or pair-housed animals, regardless of treatment.

These results are in contrast to the results of the nonhuman primate study in which MPH treatment did not alter vulnerability to substance abuse, though in that case it was cocaine. They are also in contrast to much of the clinical literature which suggests that children with ADHD that are treated with MPH are less likely to abuse alcohol in adulthood (Biederman, 2003; Biederman et al., 2009). However, to the best of our knowledge, there have been no clinical studies that examined the effects of stimulant treatment for ADHD on future substance abuse within a population of children that had
been exposed to childhood adversity. It is possible that the combination of early life adversity, or social isolation in the rodent model, and MPH treatment may result in increased likelihood of adult alcohol abuse.

Increased levels of corticosterone may help to explain these results as well. Multiple studies have documented enhancement of EtOH drinking with increased levels of circulating corticosterone in rodents, whether induced by stress or administered directly to the animal (Fahlke et al., 1994a). Both adrenalectomy and metyrapone have been shown to decrease EtOH drinking (Fahlke et al., 1994b) and a glucocorticoid Type II receptor antagonist has been shown to reduce EtOH intake by 10% in a limited-access paradigm (Koenig and Olive, 2004). As isolated animals that were treated with saline did not drink more than enriched or pair-housed animals in the present study, it appears that corticosterone was not increased enough in these animals to alter drinking behavior. MPH administration, though, is also known to increase corticosterone in rodents (Ferguson and Boctor, 2010; Schaefer et al., 2006) and cortisol, the human equivalent to corticosterone, in children (Chen et al., 2012). Ferguson and Boctor (2010) treated adolescent rats with low, oral doses of MPH for three weeks from PND 29-50, which is the age range for treatment used in the present study, and found that corticosterone levels were 63% greater in MPH treated animals than controls at PND 90. Most importantly, there is evidence that early corticosterone treatment sensitizes animals to the effects of acute MPH treatment. Increased corticosterone levels with the addition of MPH administration enhanced locomotor activity of young rats in the open field above that of either substance administered alone (Juarez and Vazquez-Cortes, 2010).

Thus, as with the greater levels of locomotor activity and D1-like receptor density reported in Chapter Three, it is possible that corticosterone levels played a significant role in the interaction between MPH and environment on EtOH intake and preference.
Potentially, social isolation and chronic MPH treatment increased corticosterone substantially enough to enhance EtOH intake only in those animals that were subjected to both conditions. This is an important avenue for follow up studies as stress (Hunter et al., 2011) and MPH treatment (Chen et al., 2012) have both been shown to increase cortisol in children. As the behavioral consequences of developmental stress exposure in rodents are similar to those seen in adolescents who were victims of childhood abuse (Teicher et al., 2006), it is possible that MPH treatment may increase vulnerability to alcohol abuse within the subset of treated children with a negative environmental history.

**FINAL THOUGHTS**

These studies were carefully controlled to model the human condition as closely as possible in important ways, including the formulation of methylphenidate and route of administration, the titration of doses to produce plasma levels associated with clinical efficacy in human populations, and the age of the animals during treatment. These controls add strength to this data and separate these studies from previous examinations of the long-term effects of methylphenidate, most of which used adult animals and an immediate release formulation of drug or drug injections. Additionally, at the time of the conception of this study design, there had been no previous examinations of chronic MPH treatment in nonhuman primates. Because of their close genetic homology to humans, as well as their long developmental periods, the use of rhesus macaques in the study presented here is of great translational value. In the rodent model, the combination of exposure to differing environments with simultaneous MPH administration is innovative and reflects potential clinical scenarios that might occur
when treating children. The strengths of this data set are many, yet there are also limitations and areas that could be improved.

We limited our assessments of the dopamine system to the striatum in both nonhuman primates and rodents. There was rationale for doing so as the highest area of binding of MPH is in the striatum. However, we may have missed effects of drug treatment in other areas of the brain, particularly within the prefrontal cortex (PFC). The PFC is known to regulate many of the executive functions that are implicated in the pathogenesis of ADHD and substance abuse, including behavioral inhibition and impulsivity (for review see Arnsten and Rubia, 2012). Additionally, MPH has been shown to have long lasting effects on PFC function (Salek et al., 2012; Urban et al., 2012). There is also some evidence that MPH elevates dopamine in the PFC, but not in the striatum, at low doses that are associated with clinical efficacy (Koda et al., 2010; Kuczenski and Segal, 2002). Thus, the PFC is an important brain region to evaluate when considering both acute and chronic effects of MPH, and should be investigated in future studies.

One of the most frequent questions that has been asked while discussing this work is, “Why didn’t you use an animal model of ADHD?” Indeed, it is likely that the dopamine systems of children with ADHD are different from those of normal children and we were attempting to model the clinical environment as closely as possible. There are several reasons that we chose to perform these studies in “normal” animals. First, there is not, currently, a well-validated model of ADHD in nonhuman primates and using a nonhuman primate model was important for measuring many aspects of physical development and an extended duration of MPH treatment. The use of nonhuman primates enabled us to look longitudinally at the development of the dopamine system and physical growth and how MPH affected those variables. In rodents, the
spontaneously hypertensive rat (SHR) is the most commonly used model of ADHD and reflects many of the features of ADHD that are seen in children, including hyperactivity, behavioral disinhibition, and impaired sustained attention (see Sagvolden et al., 2005 for review). Additionally, these behaviors in SHR rats are improved by methylphenidate administration (Sagvolden et al., 1992; Wultz et al., 1990). Yet the purpose of the present set of studies was not to examine the effects of MPH on behavior, but on the development of the dopamine system and vulnerability to substance abuse. In those respects, it is unknown whether the SHR rat reflects the clinical condition, particularly considering the fact that there is no consensus on the exact dopaminergic disturbances that are associated with ADHD in children.

Finally, as mentioned previously (Chapter One), there is considerable controversy surrounding the high rates of ADHD diagnoses and many physicians and public health officials have suggested that children are being diagnosed inaccurately. For example, recent studies have suggested that sleep disorders may frequently be diagnosed as ADHD, as sleep deprived children show problems with attention, inability to focus, and hyperactivity (Avior et al., 2004). Chervin and colleagues (2006) measured ADHD symptoms in 100 children who were scheduled for tonsillectomies to treat sleep-disordered breathing problems. Twenty-eight percent of those children were diagnosed with ADHD prior to their surgery. At a one-year follow-up post surgery, ADHD symptoms had completely resolved in half of the ADHD group, indicating that the “ADHD” in these children was mostly likely due to a sleep disturbance (Chervin et al., 2006). There is also reason to believe that parents and children are seeking a diagnosis of ADHD to obtain stimulant medication for the purposes of “cognitive enhancement”, and that some physicians are also readily prescribing it for that purpose (Graf et al., 2013). Thus, it is
likely that there is a substantial portion of the population that is taking MPH, or other stimulants, without a valid diagnosis of ADHD.

The lack of a well-validated nonhuman primate model of ADHD, the focus of these studies on the dopamine system and substance abuse rather than cognitive behavior, and the suggestion that many children who are treated with stimulants are misdiagnosed with ADHD led to the decision to use “normal” animals rather than ADHD models. Although it remains possible that the results of this series of studies might have been different if we had used an animal model of ADHD, our findings are still highly relevant as well as translatable to current practice.

These data largely suggest that MPH treatment does not have a long term impact on dopamine receptors and transporters or on vulnerability to substance abuse and thus provide evidence that MPH use is safe. However, it is extremely important to note that these studies were designed to reflect a treatment regimen as would be prescribed by a physician for the treatment of ADHD. The results of this work are not applicable to conditions of MPH, or other stimulant, mis-use and abuse. Recent scientific reports, as well as media stories, have highlighted the dangers of prescription stimulant abuse, which is an increasing phenomenon (Sherman et al. 1987; Morton & Stockton 2000; Rush & Baker 2001; Teter et al. 2006). In humans, we found that college students who reported mis-using prescription stimulants without an ADHD diagnosis for the purposes of “getting high” and to improve academic performance were at greater risk for the use of many other drugs, including cocaine, tobacco, and ecstasy (Gill et al., in preparation). In animals, recent work has suggested that dopamine transporter density is increased and the potency of MPH to elevate extracellular dopamine is increased following extended, high-dose, MPH self-administration (Calipari, et al., 2012). Thus, it appears that abuse level doses of MPH have significant effects of
dopamine system physiology, in contrast to the present work using “therapeutic-level” doses. Using MPH at higher than normal doses, or for motives other than treating ADHD, therefore, may have adverse consequences. In conclusion, chronic MPH treatment was not associated with alterations to D2-like receptors, D1-like receptors or DATs in nonhuman primates or rodents. MPH treatment did not alter vulnerability to cocaine abuse in nonhuman primates. Yet, MPH did increase EtOH intake and preference, but only in a group of animals that may have had an underlying vulnerability to alcohol abuse based upon a history of social isolation. In addition, social isolation was associated with a significant upregulation of dopamine D1-like receptors in young rats. While it is possible that differences in corticosterone, a neuroendocrine hormone that is involved in the stress response, may have influenced the dopamine systems and EtOH intakes of socially isolated animals, future studies are necessary to parse out these mechanisms.

Overall, these data should be largely reassuring to parents, teachers, physicians, and public health officials who are concerned about the long term effects of stimulant drugs on children with ADHD. In these studies, which were designed to approximate the clinical condition as closely as possible, there were very few effects of MPH on the dopamine system or vulnerability to substance abuse. The one exception was among animals with a negative environmental history in the form of social isolation, where MPH treatment was associated with greater EtOH intake. Thus, these results suggest that MPH does not have a long term impact on the dopamine system in children with ADHD, but they also indicate that attention should be paid to home and school environments of children with ADHD, as the combination of negative environment and MPH may increase vulnerability to alcohol abuse.
References


cocaine? Studies on their pharmacokinetics and distribution in the human brain. Arch Gen Psychiatry. 52, 456-63.


methylphenidate in juvenile rhesus monkeys measured by high resolution PET.

Synapse. 62, 950-2.


KATHRYN E. GILL

1710 Yorktown Rd
Lexington, KY 40504
kategill0@gmail.com
(610) 716-0685

Education

Ph.D., October, 2013  Wake Forest University School of Medicine
Department of Physiology and Pharmacology
Winston-Salem, North Carolina
Advisor: Linda J. Porrino, Ph.D.
Dissertation: Methylphenidate Treatment and Rearing Environment: Effects on the Dopamine System and Vulnerability to Substance Abuse in Animal Models

B.A., December 2004  Wake Forest University.
Winston-Salem, North Carolina
Majors in Psychology and French
Cumulative GPA: 3.33
Dean’s List five semesters.

Employment History

July 2007- August 2008  Laboratory Technician III,
Wake Forest University School of Medicine
Winston-Salem, North Carolina.
Performed research on the neurological effects of substance abuse using imaging techniques such as Positron Emission Tomography, functional Magnetic Resonance Imaging, and Autoradiography. Contributed to scientific publications, data analyses, and experimental design.

October 2004- October 2006  Laboratory Technician II,
Wake Forest University School of Medicine
Winston-Salem, North Carolina.
Performed research on the neurological and behavioral effects of sleep deprivation and stimulant drugs in rhesus monkeys. Responsible for animal care, collection of behavioral data and Positron Emission Tomography data, literature searches and design of imaging experiments.
September 1999-August 2000  **Laboratory Intern**, SmithKline Beecham Pharmaceuticals. Upper Merion, PA.
Responsible for synthesis of DNA and genotyping of samples to be used in pharmaceutical research.

**Awards**

2009-2011  **National Institute of Alcoholism and Alcohol Abuse.** Awarded a position on a T-32 Training grant that covered graduate student tuition and stipend and additional research support for research investigating factors influencing vulnerability to alcohol abuse.

2010  **Competitive Travel Award.** To attend a symposium entitled “Application of Genetic Approaches to Understand Drug Abuse and Addiction” at the 2010 College on Problems of Drug Dependence.

**Research Interests**

My dissertation work examined the effects of methylphenidate, a drug commonly used to treat attention deficit hyperactivity disorder (ADHD), on the developing brain and future vulnerability to cocaine and alcohol abuse. I explored this complex interaction by using multiple species (rat, monkey, human) and multiple techniques including receptor autoradiography, in situ hybridization, positron emission tomography, and epidemiological survey.

**Teaching Experience**

During graduate school, I volunteered to teach masters-level courses in physiology and pharmacology to physical therapy students at Winston-Salem State University. I was responsible for preparing classes including lectures and case studies, designing and administering assessments and aiding in laboratory work. In addition, I took two semesters of formal coursework designed to develop skills for undergraduate and graduate level instruction.

**Publications**


Abstracts


Presentations


Gill KE. The effects of chronic treatment with methylphenidate on growth, dopaminergic development, and vulnerability to cocaine abuse in juvenile rhesus monkeys. Physiology and Pharmacology Departmental Student Seminar Series, April 2010

Gill KE. Chronic methylphenidate treatment does not affect the course of dopaminergic development in juvenile rhesus monkeys. Graduate Student Research Day, Wake Forest University, 2010.

Gill KE. Methylphenidate treatment and vulnerability to substance abuse: Humans and monkeys and rats (oh my!). National Institute of Alcoholism and Alcohol Abuse training grant data presentation, 2010.

Gill KE. Developmental changes in the dopamine system: Effects of methylphenidate. Emory University/Wake Forest University Laboratory Exchange, September 2009

Gill KE. Decreases in dopamine D2 receptor availability are associated with age in juvenile rhesus monkeys. Western North Carolina Society for Neuroscience Neuroscience Research Day 2009.


Outreach Activities

2009-2011  

Brain Awareness Council: Organized by graduate students across multiple disciplines at Wake Forest University, the BAC visits local elementary, middle, and high schools, and coordinates with Winston Salem’s SciWorks and the Children’s Museum, to educate children in the areas of comparative brain anatomy, drug addiction, basic neurology, and career opportunities in science.
2009-2011 Neurotransmitter Newsletter: Contributed to this online internal newsletter written by graduate and students and faculty in the neurosciences at Wake Forest to inform and educate the scientific communities on local and national happenings in the Neuroscience world.

2010-2011 Kernersville Cares for Kids: Helped organize visits by local elementary and middle school children to the drug abuse research laboratories at Wake Forest to educate them about drug and alcohol use.

2009-2011 Education on Animal Research: Volunteered to lead tours of middle and high school students around the nonhuman primate research facilities at Wake Forest to educate young students about humane animal research.