EFFECTS OF CHRONIC METHYLPHENIDATE ON DOPAMINE/SEROTONIN
INTERACTIONS IN THE MESOLIMBIC DA SYSTEM OF THE MOUSE

BY

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LIST OF ABBREVIATIONS

[DA]p
5-HT: 5-Hydroxytryptamine, Serotonin
5-HT1A, 1A: Serotonin 1A receptor subtype
5-HT1B, 1B: Serotonin 1B receptor subtype
5-HT1B KO: Serotonin 1B knockout mouse
5-HT1D, 1D: Serotonin 1D receptor subtype
5-HT2A, 2A: Serotonin 2A receptor subtype
5-HT2C, 2C: Serotonin 2C receptor subtype
5-HT6, 6: Serotonin 6 receptor subtype
8-OH-DPAT: R(+)-8-hydroxy-2-(di-n-propylamino)-tetralin
ADHD: Attention-deficit/hyperactivity disorder
ANOVA: Analysis of variance
BCA: Bicinchoninic Acid
CGRP: Calcitonin gene-related peptide
CNS: Central nervous system
CPBG: 1-(m-chlorophenyl)-biguanide
CPP: Conditioned place preference
CPu: Caudate putamen
DA: Dopamine
DAp: Dopamine release per stimulus
DAT: Dopamine transporter
DAT-KO: Dopamine transporter knock-out mouse
DHBA: 3,4-Dihydroxybenzylamine

DOI: 2,5-Dimethoxy-4-iodoamphetamine

DRD4: Dopamine D4 receptor subtype

DRN: Dorsal raphe nucleus

ENU: N-ethyl-N-nitrosourea

ERK-2: Extracellular signal-regulated kinase 2

GABA: γ-Aminobutyric acid

GBL: Gamma-butyrolactone

GR 127, 935: N-[4-methoxy3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-[(5me thyl-1,2,4-oxadiazol-3-yl)-biphenyl-4-carboxamide

GR 55562: 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyridin-4-ylphenyl)-benzamide hydrochloride

HPLC: High performance liquid chromatography

L-DOPA: L-3,4-dihydroxyphenylalanine

mCPP: meta-Chlorophenylpiperazine

MDL 100,907: (R)-(+)-α-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol

MDMA: 3,4-Methylenedioxymethamphetamine

MK 212: agonist 6-chloro-2-(1-piperazinyl)pyrazine HCl

MPH: Methylphenidate

NAc: Nucleus accumbens

NE: Norepinephrine

NET: Norepinephrine transporter
NSD-1015: 3-Hydroxybenzylhydrazine

PFC: prefrontal cortex

p-MPPF: 4-(2'-methoxy-)-phenyl-1-[2'-(N-2''-pyridinyl)-p-fluorobenzamido]-ethyl-piperazine

RU 24969: 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole

SR 46349B: 1(Z)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(E)-propene

SERT: serotonin transporter

SSRI: selective serotonin reuptake inhibitor

Vmax: rate of transporter uptake

VTA: ventral tegmental area

WAY 100,635: N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- N-(2-pyridyl)cyclohexanecarboxamide
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Methylphenidate (MPH) is one of the most commonly prescribed drugs in the United States. MPH is a psychostimulant that inhibits the dopamine (DA) and norepinephrine (NE) transporters to increase extracellular monoamines. Abuse rates of MPH are increasing as prescription rates increase, but the effects of high-dose, chronic MPH exposure have not been well defined. In particular, the effects of MPH on serotonin (5-HT) systems have not been well investigated. While MPH itself does not have affinity for the 5-HT transporter, DA and 5-HT systems have many points of interaction, particularly in the mesolimbic DA system, an area associated with the rewarding and reinforcing properties of drugs. Thus, our studies focused on the interactions of DA and 5-HT systems at the level of the ventral tegmental area (VTA) and nucleus accumbens (NAc) following chronic MPH treatment in mice. The experiments utilized locomotor activity measures, conditioned place preference, single and dual probe in vivo microdialysis, radioligand binding, and behavioral tests for depressive like behaviors to examine the effects of chronic MPH.

Our studies showed that chronic MPH alters DA/5-HT system interactions in the mesolimbic DA system. The alterations are such that elevations in 5-HT produced a stronger influence over DA neuron firing. Specifically, we found that the serotonin agonist fluoxetine increased DA in the NAc and produced place preference in MPH treated animals. Further investigations traced these changes to the VTA, where increasing 5-HT in the VTA produced increased DA cell firing when stimulated. Our studies subsequently examined which 5-HT receptors are responsible for this change. Of
the five 5-HT receptors examined using locomotor activity, conditioned place preference, and in vivo microdialysis, the 5-HT1A and 1B receptors showed sensitization, and it appears that alterations in these receptors are primarily responsible for the influence of 5-HT over the DA system following MPH exposure. Finally, these studies have determined that chronic MPH produced depressive-like effects during withdrawal, and behavioral and neurochemical sensitization following exposure. Taken together, the investigations conducted in this thesis showed novel effects of MPH on 5-HT/DA interactions, with implications for the consequences of MPH abuse in humans.
CHAPTER I

EFFECTS OF ACUTE AND CHRONIC METHYLPHENIDATE ON THE 
NEUROBIOLOGY AND BEHAVIOR OF RODENTS

Brookshire, BR, and Jones, SR

Introduction

Methylphenidate (MPH, Ritalin), prescribed in children and adults for treatment of attention-deficit/hyperactivity disorder (ADHD), is one of the most common prescription stimulants currently in use (Accardo et al, 2001; Goldman et al, 1998; Levin et al, 1995; Solanto, 1998). ADHD is the most common psychiatric disorder diagnosed in children, and stimulant treatment using MPH or amphetamine is considered the first line of treatment for this disorder (Challman et al, 2000; Swanson et al, 2003). Additionally, ADHD often continues well into adulthood, and prescription stimulants remain the drug treatment of choice for this disorder (Eichlseder, 1985; Fone et al, 2005; Kollins et al, 2001; Kollins, 2003). Unfortunately, MPH is a psychostimulant with significant reinforcing and rewarding properties, raising many concerns regarding its widespread use in child and adult populations. There are concerns about the diversion and abuse of methylphenidate, particularly in young adult populations (Wilens et al, 2006). There are additional worries that exposure to prescription stimulants in children and adults may increase the propensity for drug abuse, particularly abuse of other stimulants such as cocaine. Many animals and human studies have attempted to address this issue, with variations in time of treatment, age of subject, treatment dose, and follow-up measures. By examining the abundant literature on acute and chronic MPH administration in animals and the effects on neurobiology and reward-related behaviors,
it may be possible to draw conclusions as to the effects of MPH on developing and mature brain systems, and thus make correlations regarding the possible effects of chronic MPH administration in humans.

**ADHD**

ADHD is a disorder that is characterized in children and adults by deficits in executive function including vigilance, response inhibition, and planning, as well as deficits in motivation and reward responses (Karande, 2005; Volkow et al, 2009). These behavioral deficits can produce significant problems with school and work performance as well as successful social interactions (Karande, 2005). Symptoms of ADHD are thought to be present in up to 10% of the child population (Faraone et al, 2003). Some reports have shown that up to 70% of cases will disappear in adulthood, while others have shown that 60% of cases will continue to suffer symptoms throughout the lifespan (Taylor et al, 2001). There are some suggestions that ADHD is currently underdiagnosed, and that up to 20% of school children may meet criteria for ADHD (Rocha et al, 1998; Wilens et al, 2002; Zito et al, 2000), with a reported prevalence in the overall population at 12% (Brown et al, 2001; Swanson et al, 1998a). Other published reports, however, demonstrate the prevalence to be much lower (4.4%) in the adult population (Kessler et al, 2006).

The causes of ADHD remain poorly understood. There is a strong genetic contribution, with up to 0.8 heritability in twin studies (Biederman et al, 1990; Biederman et al, 1992; Faraone et al, 2001; Kieling et al, 2008). The dopamine (DA) D4 and D5 receptors have been implicated in the disorder, as well as the DA transporter (DAT) and
serotonin (5-HT) transporter (SERT) (Brookes et al, 2006; Gill et al, 1997; Swanson et al, 1998b; Swanson et al, 2000), and the genes for dopamine beta-hydroxylase, HTR1B, and SNAP-25 have also been implicated (Faraone et al, 2005; Gizer et al, 2009). Neurobiologically, it appears that children and adults with ADHD have higher levels of striatal DAT binding and availability than normal subjects (Cheon et al, 2003; Dougherty et al, 1999; Dresel et al, 2000; Krause et al, 2000; Spencer et al, 2005b), although other reports have found decreased DAT levels as well (Volkow et al, 2007). The conclusions of these studies have often been contradictory, suggesting either hyper- or hypodopaminergic systems in patients with the disorder, with a hyperdopaminergic system characterized by reduced DAT binding, What is certain is that the most effective treatments for ADHD symptoms have focused on the DA system and have been psychostimulant drugs, with the two most common treatments being formulations of amphetamine and MPH.

Methylphenidate

The use of psychostimulants to treat symptoms of ADHD is not recent. Original proposals for psychostimulant treatment of hyperactivity were made as early at 1937 (Eichlseder, 1985). MPH itself was first isolated and patented for use in 1954 and is a close structural relative of the dextroamphetamine isomer of amphetamine (Kallman et al, 1975). MPH is a psychostimulant that behaves in a manner similar to cocaine, blocking the reuptake of the monoamines norepinephrine (NE) and dopamine (DA) from the synapse (Volkow et al, 1999; Volkow et al, 2004). This results in increases in DA and NE in the extracellular space (Leonard et al, 2004), which are believed to improve
attention and focus in patients with ADHD. MPH is also commonly prescribed for the treatment of narcolepsy and chronic fatigue syndrome (Harris, 2008; Leonard et al, 2004; Niederhofer, 2009). MPH easily crosses the blood-brain barrier, and peak concentrations in human occur at 1-1.5 hours following oral administration, while onset following i.v. administration occurs in a few seconds (Volkow et al, 2002). It is metabolized and excreted in the urine in 48 hours (Faraj et al, 1974; Kollins, 2003; Leonard et al, 2004; Swanson et al, 1999). MPH and cocaine have similar rates of diffusion into the brain, and block DA uptake with similar affinity, although MPH has a longer duration of effects (Kollins, 2003; Volkow et al, 1999).

Methylphenidate in Humans

MPH is a highly effective prescription medication, attenuating the symptoms of ADHD in 70% of pediatric patients, with similar efficacy estimated in adults (Greenhill et al, 2002; Swanson et al, 2002). Prescription rates of MPH in humans vary widely across demographics. Some published findings estimate that between 3 and 6% of children under the age of 18 are on prescription MPH (Kuczenski et al, 2005), while others estimate that up to 15% of the US population (both juvenile and adult) may be treated with MPH in some areas (Barbaresi et al, 2002). Prescription rates for MPH have tripled since the 1990’s (Safer et al, 1996; Wu et al, 2007; Zito et al, 2000). MPH now accounts for 90% of stimulant prescriptions used to treat ADHD (Goldman et al, 1998; Zito et al, 2000), and 90% of MPH prescriptions are among children and adolescents (Wilens et al, 2002). Treatment is continued throughout childhood and often through the lifespan if symptoms fail to resolve upon maturation (Heiligenstein et al, 1999). Doses
are given orally in either immediate or extended release tablets (with extended release being a more popular prescription choice) in doses of 1 mg/kg/day on average (Spencer et al, 2005a).

Despite evidence for safety and efficacy in treatment of ADHD symptoms (Fone et al, 2005; Greydanus et al, 2007). MPH is a psychostimulant with significant abuse liability. The abuse of prescription stimulants to improve attention and focus has been increasing dramatically (Teter et al, 2006), with widespread abuse noted for the first time in the 1980s (Haglund et al, 1982). After marijuana, prescription drugs, including stimulants such as MPH, are the most common drugs in use among adolescents (Johnson et al, 2005). Abuse of ADHD medications has been increasing in recent years along with the increased prescription rates for these drugs (Setlik et al, 2009). This effect may be due to the view that prescription drugs are safer because they are approved for clinical use (Friedman, 2006). By the sophomore year of college, 50% of students have had the opportunity to abuse prescription stimulants (Arria et al, 2008). While clinical doses of MPH are not associated with drug “liking” (Chait, 1994), abused doses can be between 2-10 times those prescribed, and are associated with a “high” similar to that of cocaine, especially when administered intranasally or i.v (Barrett et al, 2005). Immediate release MPH (compared to extended release) yields higher plasma concentration and DAT occupancy, and only immediate release formulas elicit subjective effects related to drug abuse (Spencer et al, 2006), including drug “liking”, increased alertness, and feelings of stimulation (Volkow et al, 2009). Indeed, misuse and diversion of MPH is reported only by subjects using immediate release formulas (Wilens et al, 2006). These observations have led many researchers to study the effects of injected and immediate MPH dosing in
animal models without ADHD, to better achieve the effects associated with abuse. Abuse of MPH is most common among young adults, both to get high and to increase concentration (McCabe et al, 2005), and 8-16% of college students have admitted to use of prescription stimulants for non-clinical purposes (Babcock et al, 2000; Teter et al, 2006). While most abuse is oral, routes of administration also include inhalation or i.v. injection (McCabe et al, 2004; Teter et al, 2006). Further, some work has indicated that use of stimulant medications such as MPH may increase the likelihood of later abuse of psychostimulants such as cocaine (Lambert et al, 1998).

MPH use is common among patients with ADHD as well as those who do not fulfill the criteria for ADHD symptoms, due to diversion of the drug from clinical sources (Wilens et al, 2006). A significant portion of people without ADHD using prescription stimulants non-medically develop problems with the use of MPH or amphetamine, and abuse of MPH may increase further psychostimulant abuse (Lambert et al, 1998; McCabe et al, 2005). In addition to psychostimulant abuse, MPH abuse is highly correlated with the use of non-psychostimulant drugs, particularly alcohol, nicotine, and marijuana (Lambert et al, 1998; McCabe et al, 2005). Thus it is of particular importance to come to a full understanding of the effects of MPH abuse, both acute and chronic, as well as the effects of MPH abuse on subsequent exposure to psychostimulants and other drugs of abuse.

Methylphenidate in Animals: Acute Effects

While a large body of work has been performed on the epidemiology of MPH use and abuse in humans, studies in humans have inherent limitations. It is extremely
difficult to fully control for previous drug use and the effects of other drugs in humans, and humans are often polydrug users, making it difficult to determine the specific effects of any one drug. Additionally, many manipulations and investigations must be conducted in the living brain or post-mortem, which is a difficult proposition in human subjects. Thus, studies on the behavioral and neurobiological effects of MPH exposure in animals have been conducted in the hopes of elucidating the acute and chronic effects of MPH abuse.

Neurobiological Responses to Acute MPH. Acute exposure to MPH in rodents is associated with significant increases in DA and norepinephrine (NE) in the extracellular space, particularly in the striatum and prefrontal cortex (Bymaster et al, 2002; Kuczenski et al, 2002; Volkow et al, 2004). MPH prevents the reuptake of DA and NE, resulting in a prolonged lifetime of these neurotransmitters in the extracellular space (Leonard et al, 2004). This increase in extracellular monoamines amplifies the intensity and duration of neuronal signaling. It is also accompanied by a temporary decrease in VTA DA neuron firing (Brandon et al, 2003a) associated with the activation of inhibitory presynaptic receptors on DA neurons (Welch et al, 1996; White, 1996). Acute MPH administration also decreases DARPP-32 levels in the hippocampus, the phosphorylation of which is strongly affected by stimulation of D1 and D1 receptors (Souza et al, 2009; Svenningsson et al, 2003). As MPH also acutely increases NE and activates autoreceptors in the locus coeruleus, there is also a decrease in locus coeruleus NE neuron firing (Devilbiss et al, 2006).
MPH thus shares neurochemical effects with other psychostimulants such as cocaine and amphetamine, including increases in extracellular DA and NE (Swanson et al, 2003). MPH and cocaine have similar potencies at the DAT (Volkow et al, 1999). In addition, acute MPH administration increases levels of the immediate early gene cFos and the transcription factor zif268 in the striatum, in a manner similar to cocaine (Brandon et al, 2003b). But unlike cocaine and amphetamine, MPH has low affinity for the SERT, and does not increase extracellular 5-HT levels (Gatley et al, 1996), although recent results indicate that MPH can act as an agonist at the 5-HT1A receptor (Markowitz et al, 2009). Thus, acute doses of MPH increase DA levels in the prefrontal cortex (PFC), striatum, and nucleus accumbens (NAc), and increase levels of NE in the PFC (Andrews et al, 2006; Balcioglu et al, 2009; Berridge et al, 2006; Bymaster et al, 2002; Kuczenski et al, 2002), leading to excitation of cortical pyramidal neurons (Andrews et al, 2006).

Behavioral Responses to Acute MPH. MPH shares many similarities with other psychostimulants, in particular increases in DA in the NAc. These increases are associated with the initial rewarding and reinforcing effects of drugs (Wise, 1987), and it is not surprising that acute MPH administration results in behavioral effects similar to those following acute cocaine. In rodents, low doses of MPH (0-5 mg/kg in rats) stimulate locomotor activity, while higher doses (10 mg/kg and above in rats) produce stereotypies (Brandon et al, 2001; Gaytan et al, 1996; Gaytan et al, 2000; Kollins et al, 2001; Solanto, 1998). MPH produces reinforcing effects and can substitute for cocaine in humans, rats, mice, dogs, and primates (Czoty et al, 2004; Li et al, 2006; Lile et al, 2003;
Risner et al, 1975; Stoops et al, 2005). Furthermore, MPH is readily self-administered by rodents and produces conditioned place preference (CPP) (Kankaanpaa et al, 2002; Martin-Iverson et al, 1991; Meririnne et al, 2001; Schenk et al, 2002; Sellings et al, 2006), which supports the role of MPH as a drug with significant rewarding and reinforcing properties. MPH also shows subjective similarity to other psychostimulants, and can decrease latency to self-administration of cocaine, as well as reinstating CPP for cocaine, which preference for the drug associated chamber is extinguished, and then reinstated by a challenge dose of drug (Itzhak et al, 2002; Schenk et al, 2002).

Taken together, the data on acute MPH administration show neurobiological and behavioral effects that are strikingly similar to that of other psychostimulants such as cocaine and amphetamine (Swanson et al, 2003). These studies suggest that chronic, high doses of MPH might produce neurochemical and behavioral adaptations consistent with addiction-like effects in rodents.

Methylphenidate in Animals: Chronic Effects

Published results with acute MPH have focused on initial rewarding effects and the neurobiological similarity to other psychostimulants. However, the use of MPH in the clinic is at lower doses and given over months or even years of treatment (Eichlseder, 1985; Kollins et al, 2001; Kollins, 2003). Thus, the scientific community has deemed it of particular importance to look at the effects of chronic MPH exposure, both at clinical and abused doses. In addition, MPH is often prescribed to juveniles and adolescents (Faraone et al, 2003; Schubiner, 2005). Thus the effects of chronic MPH treatment at clinical and abuse-related doses during development are of particular interest.
Unfortunately, in rodent models, doses that would be considered clinically relevant or abusive have not been established. Thus, an extremely wide range of doses and treatment regimens has been used, with varying effects on subsequent neurobiological and behavioral measures.

**Neurobiological responses to chronic MPH treatment.** Some neurobiological effects of chronic MPH exposure appear to be consistent with those of other psychostimulants. Chronic MPH treatment and the resulting striatal accumulation of DA increase activation of presynaptic D2 receptors, reducing DA neuron firing in the ventral tegmental area (VTA) (Brandon et al, 2003a; Seeman et al, 1998). This results in decreases in D1 and D2 receptors following chronic stimulation (Seeman et al, 1998), though reductions in D2 receptors are by no means universal. Other findings have shown that presynaptic DA release is attenuated with repeated MPH treatment (Sproson et al, 2001), and that chronic MPH exposure may reduce DAT levels (Kuhar et al, 1996). However, the age during treatment in these reports has been highly variable, which may have influenced the results of the work. Subsequent findings have shown specific effects of age on the neurobiological response to chronic MPH administration.

*In adolescents:* Many studies have looked at the immediate and long term neurobiological effects of chronic MPH treatment in adolescent and juvenile rats. A summary of the treatment regimens and the immediate and long-term neurobiological effects can be found in Table 1. Overall, it appears that lower doses in the 1.0-2.0 mg/kg range (though 5.0 mg/kg is also sometimes used) are commonly thought to represent clinical treatment in rats, while doses in the 10-20 mg/kg range may be more
representative of abused doses. Results have shown that 10.0 mg/kg MPH, given in shorter periods of 7 days, results in immediate effects of increased dynorphin, and decreased cFos, zif268, and substance P in the striatum, which are drug-induced adaptations similar to those produced by cocaine (Brandon et al, 2003b). Longer term studies have found transient increases in VTA neuron firing rates following lower doses (1.0-2.0 mg/kg for 7 days), which gave way to decreases in VTA neuron firing rate in withdrawal (Brandon et al, 2003a). While these changes in firing rate might be indicative of decreased or increased D2 receptor control over VTA DA neuron firing, no D2 changes were apparent (Brandon et al, 2003a). These results indicate that chronic MPH administration in adolescents at both clinically relevant and higher doses for a relatively short period of time can produce some changes similar to that of other psychostimulants.

However, while 2.0 mg/kg in rats might be close to a clinically-relevant dose, MPH is rarely given for such a short period of time as 7 days. Other reports have looked at the effects of 1.0 and 2.0 mg/kg MPH given in periods of 14-16 days, and 25-28 days. In the case of 2.0 mg/kg doses of MPH given over 14-16 days, immediate effects include decreases in DAT binding as seen in other chronic studies (Kuhar et al, 1996; Moll et al, 2001). Changes in immediate early genes and transcription factors related to drug exposure in reward-related areas like the striatum, including increases in FosB, and decreases in cFos were also observed (Chase et al, 2005). Findings also showed upregulation of genes involves in neuronal migration and axon growth in the striatum, including Homer1b, Shank2, and Mpp3, Grik2, the Htr7, the (Adr)alpha1b, the GabR gamma1, and GabRbeta3 (Adriani et al, 2006a). Interestingly, the long-term neurobiological effects of 14-16 day exposure appear to show augmented early effects
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration</th>
<th>Age at Start</th>
<th>Age At Testing</th>
<th>Immediate Neurobiological Effects</th>
<th>Long Term Neurobiological Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>15 days</td>
<td>p20</td>
<td>p60</td>
<td>-</td>
<td>Increased CREB in NAc</td>
<td>Andersen, 2001</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>14 days</td>
<td>p35</td>
<td>p45 and p70</td>
<td>25% decrease in DAT in striatum</td>
<td>50% decrease in DAT in striatum</td>
<td>Moll, 2001</td>
</tr>
<tr>
<td>10.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p42</td>
<td>Increased dynorphin in striatum, decreased cFos, zif268, substance P</td>
<td>-</td>
<td>Brandon, 2003</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p45</td>
<td>-</td>
<td>Increased VTA neuron firing rate, no D2 differences</td>
<td>Brandon, 2003b</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p53</td>
<td>-</td>
<td>Decreased VTA neuron firing rate, no D2 differences</td>
<td>Brandon, 2003b</td>
</tr>
<tr>
<td>2.0 mg/kg i.p., 2x per day</td>
<td>16 days</td>
<td>p20</td>
<td>p40 and p70</td>
<td>-</td>
<td>Increased CORT response to stress</td>
<td>Bonalos, 2003</td>
</tr>
<tr>
<td>2.0 or 10.0 mg/kg i.p.</td>
<td>14 days</td>
<td>p28</td>
<td>p38 and p66</td>
<td>Increased FosB and decreased cFos in striatum</td>
<td>Increased FosB and decreased cFos in striatum following MPH challenge</td>
<td>Chase, 2005</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>16 days</td>
<td>p30</td>
<td>p46 and p60</td>
<td>Upregulation of Homer1b, Shank2, and Mpp3, Grik2, the Htr7, the A德拉pha1b, the GabR gamma1, and the GabRbeta3 in striatum</td>
<td>Upregulation of Homer1a, CREB, Grik2, and 5-HT7 in striatum</td>
<td>Adriani, 2006</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>14 days</td>
<td>p30</td>
<td>p44</td>
<td>Upregulation of genes involved in neuronal migration and axon growth</td>
<td>-</td>
<td>Adriani, 2006</td>
</tr>
</tbody>
</table>

Table 1.
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration</th>
<th>Age at Start</th>
<th>Age At Testing</th>
<th>Immediate Neurobiological Effects</th>
<th>Long Term Neurobiological Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0, 2.0, or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p53</td>
<td>Dose dependent decreases in lipid peroxidation in cortex and striatum, decreases in TBARS in hippocampus, increases in protein carboynlation in striatum</td>
<td>-</td>
<td>Martins, 2006</td>
</tr>
<tr>
<td>5.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p7</td>
<td>p35 and p60</td>
<td>High TH and decreased NET in cortex</td>
<td>Low TH in cortex</td>
<td>Grey, 2007</td>
</tr>
<tr>
<td>1.0, 2.0 or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p53</td>
<td>Dose dependent increases in DNA damage in blood, hippocampus, and striatum</td>
<td>-</td>
<td>Andreazza, 2007</td>
</tr>
<tr>
<td>10 mg/kg i.p.</td>
<td>25 days (degus)</td>
<td>p21</td>
<td>p46</td>
<td>Decrease in basal DA levels in mPFC and NAc, attenuated mPFC DA in response to MPH challenge, no change in NAC</td>
<td>-</td>
<td>Jezierski, 2007</td>
</tr>
<tr>
<td>1.0, 2.0 or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p53</td>
<td>Increased Na(+), K(+) -ATPase activity in hippocampus and prefrontal cortex</td>
<td>-</td>
<td>Scherer, 2009</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p60</td>
<td>Decreased production of superoxides in cerebellum</td>
<td>-</td>
<td>Gomes, 2009</td>
</tr>
<tr>
<td>1.0 or 2.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p53</td>
<td>Decreased DARPP-32 in cerebellum</td>
<td>-</td>
<td>Souza, 2009</td>
</tr>
</tbody>
</table>

Table 1, continued.
Table 1: The effects of chronic MPH treatment on immediate and long-term neurobiological measures in adolescent rats. Findings are listed with regard to dose used for treatment, age of animals at the start of treatment, the duration of treatment, and the age at testing.
similar to results with acute psychostimulants. These include further decreases in DAT binding in the striatum (Moll et al, 2001), increased cortisol responses, increased CREB, Homer1a, Grik2, 5-HT7, and FosB, and decreased cFos (Adriani et al, 2006b; Andersen et al, 2002; Bolanos et al, 2003; Chase et al, 2005). Thus, reports with lower dose MPH administration over intermediate to chronic periods in adolescents show immediate and long term effects similar to those seen with chronic administration of other psychostimulants.

The effects of the longest term treatments of around 28 days have been performed primarily to investigate short term effects following chronic exposure. While 1.0 or 2.0mg/kg are the most common, other work has investigated both 5.0 and 10 mg/kg doses over this chronic regimen in an effort to reflect abused doses (Andreazza et al, 2007; Gray et al, 2007; Jezierski et al, 2007; Martins et al, 2006). Neurobiological work has continued to study reward-related areas and dopaminergic function, and increased interest has also appeared in relation to the development of oxidative stress (Andreazza et al, 2007; Gomes et al, 2009; Martins et al, 2006). Some long-term results (5.0 mg/kg i.p. for 28 days, Table 1) have shown initially high levels of tyrosine hydroxylase, and decreased NET levels (Gray et al, 2007). The decreased NET levels might be expected given similar decreases in DAT levels following chronic MPH treatment (Kuhar et al, 1996; Moll et al, 2001). These results have been accompanied with decreases in basal DA levels in the PFC and NAc at higher doses of MPH (10 mg/kg i.p., 25 days, Table 1) (Jezierski et al, 2007), which may reflect decreases in DA neuron firing. Studies have also shown increased NA(+), K(+) ATPase activity in areas like the PFC (Scherer et al, 2009), and decreased DARPP-32 in the cerebellum (Souza et al, 2009) following 28 day
treatments with 1.0 and 2.0 mg/kg MPH (see Table 1). Both of these changes represent neurobiological indicators of plastic changes in synaptic function following MPH treatment.

The role of MPH treatment in oxidative stress in adolescents has also begun to come under scrutiny. Oxidative stress, while part of normal metabolism, can also result in tissue injury, and is implicated in some psychiatric conditions and in the consequences of drug abuse (Brown et al, 2003; Dal Pizzol et al, 2000; Dal Pizzol et al, 2001; Fukami et al, 2004). In adolescent rats, chronic MPH administration appears to increase some signs of oxidative stress. These signs included increases in protein carbonylation in the striatum, increased DNA damage, and decreased production of superoxides and lipid peroxidation (Andreazza et al, 2007; Gomes et al, 2009; Martins et al, 2006). Thus it appears that chronic MPH treatment may increase signs of oxidative stress in adolescent rats, which may indicate tissue injury.

Taken together, the neurobiological responses to MPH administration in adolescent rats appear to be fairly consistent with those seen in response to administration of other psychostimulants. These results have indicated that plastic responses to MPH are particularly apparent in reward-related areas such as the striatum. It is thus of great interest to observe the possible effects of chronic MPH exposure on reward related behaviors in adolescents, to find possible correlations between neurobiological effects and behavioral responses.

In adults: While many reports have focused on both immediate and long term effects of chronic MPH exposure in adolescents, relatively few have focused on the effects of chronic MPH administration in adults. This is, however, still an important field
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration of Treatment</th>
<th>Immediate Neurobiological Effects</th>
<th>Long Term Neurobiological Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>5, 10, 15, 20 mg/kg i.p.</td>
<td>5 days</td>
<td>Depressed PKA and stimulated adenylyl cyclase, increased basal adenylyl cyclase</td>
<td>-</td>
<td>Crawford, 1998</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>15 days</td>
<td>-</td>
<td>Increased CREB in Nac, increased GluR2 in Nac</td>
<td>Andersen, 2001</td>
</tr>
<tr>
<td>2.0 or 10.0 mg/kg i.p.</td>
<td>14 days</td>
<td>Increased FosB and decreased cFos (at 10 mg/kg dose only) in striatum</td>
<td>-</td>
<td>Chase, 2005</td>
</tr>
<tr>
<td>1.0, 2.0, or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>Decrease in lipid peroxidation in the cerebellum and hippocampus, indicating oxidative damage</td>
<td>-</td>
<td>Martins, 2006</td>
</tr>
<tr>
<td>2.5 mg/kg i.p.</td>
<td>5 days</td>
<td>Decreased amplitude of field evoked responses in VTA and NAc</td>
<td>-</td>
<td>Dafny, 2006</td>
</tr>
<tr>
<td>1.0, 2.0 or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>Dose dependent increases in DNA damage in blood, hippocampus, and striatum</td>
<td>-</td>
<td>Andreazza, 2007</td>
</tr>
<tr>
<td>1.0, 2.0 or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>Increased Na(+), K(+)-ATPase activity in hippocampus, prefrontal cortex, and striatum</td>
<td>-</td>
<td>Scherer, 2009</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>28 days</td>
<td>Decreased production of superoxides in cerebellum</td>
<td>-</td>
<td>Gomes, 2009</td>
</tr>
<tr>
<td>1.0 or 2.0 mg/kg i.p.</td>
<td>28 days</td>
<td>Decreased DARPP-32 in hippocampus, increased in cortex</td>
<td>-</td>
<td>Souza, 2009</td>
</tr>
</tbody>
</table>

Table 2.
Table 2: The effects of chronic MPH treatment on immediate and long-term neurobiological measures in adult rats. Findings are listed with regard to dose used for treatment, the duration of treatment, and the immediate and long-term neurobiological effects of the treatment regimen.
of study for both clinical and nonclinical use of MPH. 50% of ADHD cases are thought to continue into adulthood (Biederman et al, 2000), but there is significant non-medical use of MPH in the adult population for cognitive enhancement and abuse purposes (Frisch et al, 2003; McCabe et al, 2005; Teter et al, 2003). Thus, it is important to study the neurobiological effects of both clinical and abused doses of MPH in adults.

Some published reports focusing on the neurobiology of chronic MPH administration in adults have been listed in Table 2. It is apparent that most studies have focused on the immediate neurobiological effects following administration of MPH in adults, and doses vary widely, as well as duration of treatment. A sensitizing regimen of 2.5 mg/kg over a period of five days resulted in decreased amplitude of evoked responses in the VTA and NAc, accompanied by behavioral sensitization (Dafny et al, 2006). At doses of 1.0 and 2.0 mg/kg, given for 28 days, studies have produced neurobiological results similar to those seen in adolescent reports. In particular, findings in adults have produced indications of changes in neuronal activity with increased Na(+), K(+) ATPase activity in the hippocampus, prefrontal cortex, and striatum (Scherer et al, 2009). Similar experiments have seen decreased DARPP-32 in the hippocampus with increases in the cortex, further indicating changes in synaptic transmission (Souza et al, 2009). A 15 day study at 2.0 mg/kg also found long-term effects of increased CREB and GluR2 in the NAc, an effect similar to that seen with other psychostimulants (Andersen et al, 2002).

Some reports have also investigated the changes in oxidative stress following chronic MPH treatment. Results appear to be similar to the findings seen above, with decreases in lipid peroxidation in the cerebellum and hippocampus (Martins et al, 2006). Similar reports have observed decreased production of superoxides, and increases in
DNA damage in blood, hippocampus, and striatum (Andreazza et al., 2007; Gomes et al., 2009). These are all findings that indicate oxidative damage and potential tissue injury, which could potentially impact baseline behaviors as well as subsequent responses to drug challenge.

Finally, there have been one or two published reports that have specifically investigated higher doses of MPH in the adult rat for their subsequent neurobiological effects. Doses up to 20 mg/kg produce depressed PKA levels and decreased stimulation of adenylyl cyclase in the striatum (Crawford et al., 1998). These decreases were accompanied by increased basal adenylyl cyclase in the striatum (Crawford et al., 1998). Another study has shown increased FosB and decreased cFos in striatum (Chase et al., 2005). All of these findings indicate changes affecting signal transduction in the striatum, an area that plays an important role in responses to psychostimulant exposure.

In sum, the work on the neurobiological effects of chronic MPH exposure in adult animals has not been as extensive and thorough as those in adolescents, but the studies performed so far do indicate that chronic MPH exposure may have similar neurobiological effects in adolescents and adults. These studies show that chronic MPH treatment results in changes in the DA system in a manner similar to that following exposure to other psychostimulants, and that MPH exposure may be associated with increased levels of oxidative stress.

Behavioral responses to chronic MPH exposure.

Like other psychostimulants, repeated MPH is capable of producing sensitization to subsequent MPH exposure and cross-sensitization to other psychostimulants (Brandon
Behavioral sensitization is the augmentation of behavioral responses to stimulant challenge following repeated psychostimulant exposure (Kalivas et al., 1991; Robinson, 1984; Robinson et al., 1993; Vanderschuren et al., 1999; Woolverton et al., 1992). The phenomenon of behavioral sensitization has been associated with drug abuse liability, although the relationship to addiction remains unclear (Lorrain et al., 2000; Robinson et al., 1993). Increases in DA in mesolimbic areas induced by psychostimulant exposure are thought to underlie both the mechanisms of behavioral sensitization and the mechanisms behind drug craving and addiction (Kalivas et al., 1998; Robinson et al., 1993).

Behavioral sensitization as a phenomenon can be divided into two parts, induction and expression, which are believed to involve different mechanisms (Dafny et al., 2006). The initiation of behavioral sensitization is thought to result from molecular changes in the dopaminergic neurons in the ventral tegmental area (VTA) (Kalivas et al., 1993; Pierce et al., 1997). The expression of behavioral sensitization is thought to be mediated by molecular changes in the NAc (Pierce et al., 1995). MPH injections in rodents are known to produce both the induction and the expression of behavioral sensitization (Crawford et al., 1998; Dafny et al., 2006; Gaytan et al., 1996; McDougall et al., 1999; Meririnne et al., 2001; Shuster et al., 1982; Yang et al., 2007), and this behavioral
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration</th>
<th>Age at Start</th>
<th>Age At Testing</th>
<th>Immediate Behavioral Effects</th>
<th>Long Term Behavioral Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>15 days</td>
<td>p20</td>
<td>p60</td>
<td>-</td>
<td>CPA for 10 mg/kg cocaine</td>
<td>Andersen, 2001</td>
</tr>
<tr>
<td>5.0, 10.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p56</td>
<td>-</td>
<td>Locomotor cross sensitization to cocaine</td>
<td>Brandon, 2001</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p56</td>
<td>-</td>
<td>No locomotor cross sensitization to cocaine</td>
<td>Brandon, 2001</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>7 days</td>
<td>p35</td>
<td>p56</td>
<td>-</td>
<td>Increased cocaine self-administration</td>
<td>Brandon, 2001</td>
</tr>
<tr>
<td>4 mg/kg, i.p. 2x per day</td>
<td>4 days</td>
<td>p42</td>
<td>p46 and p58</td>
<td>Mild hyperactivity but no effect on novel object exploration</td>
<td>No long term effect on social interaction</td>
<td>Sproson, 2001</td>
</tr>
<tr>
<td>2.5 mg/kg i.p.</td>
<td>3 days</td>
<td>p28</td>
<td>p39 and p54, 64</td>
<td>-</td>
<td>No locomotor sensitization to MPH, but cross-sensitization to amphetamine</td>
<td>Yang, 2003</td>
</tr>
<tr>
<td>2.5 mg/kg i.p.</td>
<td>8 days</td>
<td>p28 for 2 days, p54 for 5 days</td>
<td>p39 and p54, 64</td>
<td>-</td>
<td>No locomotor sensitization to MPH, but cross-sensitization to amphetamine</td>
<td>Yang, 2003</td>
</tr>
<tr>
<td>2.0 mg/kg i.p., 2x per day</td>
<td>16 days</td>
<td>p20</td>
<td>p40 and p70</td>
<td>p40 no changes in water intake or play behavior</td>
<td>Decreased sucrose preference and loco in a novel environment, decreased open arm time in elevated + maze, increased time grooming, decreased sexual activity, decreased FST struggle</td>
<td>Bonalos, 2003</td>
</tr>
</tbody>
</table>

Table 3.
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration</th>
<th>Age at Start</th>
<th>Age At Testing</th>
<th>Immediate Behavioral Effects</th>
<th>Long Term Behavioral Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/kg i.p.</td>
<td>7 days</td>
<td>p26</td>
<td>p60</td>
<td>Initial decrease in weight gain, but recovered by adulthood</td>
<td>Blunted cocaine CPP, but priming dose produced increased cocaine CPP, loco sensitization to cocaine</td>
<td>Achat-Mendes, 2003</td>
</tr>
<tr>
<td>2 mg/kg i.p.</td>
<td>16 days</td>
<td>p30</td>
<td>p46 and p60</td>
<td>-</td>
<td>Locomotor sensitization to cocaine, blunted CPP for cocaine, increased choice flexibility</td>
<td>Adriani, 2006</td>
</tr>
<tr>
<td>0.6, 2.5, or 10.0 mg/kg i.p.</td>
<td>5 days</td>
<td>p34-p41</td>
<td>p44-50</td>
<td>-</td>
<td>No behavioral sensitization or tolerance to subsequent MPH</td>
<td>Yang, 2006</td>
</tr>
<tr>
<td>2 and 10 mg/kg i.p.</td>
<td>14 days</td>
<td>p15</td>
<td>p56</td>
<td>-</td>
<td>No locomotor cross sensitization to cocaine, MPH did not augment cocaine locomotor responses</td>
<td>Guerriero, 2006</td>
</tr>
<tr>
<td>2 and 10 mg/kg i.p.</td>
<td>14 days</td>
<td>p36</td>
<td>p56</td>
<td>-</td>
<td>No locomotor cross sensitization to cocaine</td>
<td>Guerriero, 2006</td>
</tr>
<tr>
<td>2.0 or 5.0 mg/kg i.p.</td>
<td>9 days</td>
<td>p11</td>
<td>p60</td>
<td>-</td>
<td>Increased magnitude of morphine place preference</td>
<td>Crawford, 2007</td>
</tr>
<tr>
<td>1.0, 2.0, or 10.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p25</td>
<td>p67</td>
<td>-</td>
<td>Locomotor cross sensitization to amphetamine, but no locomotor sensitization to MPH</td>
<td>Valvassori, 2007</td>
</tr>
<tr>
<td>5.0 mg/kg i.p.</td>
<td>28 days</td>
<td>p7</td>
<td>p35 and p60</td>
<td>-</td>
<td>Decreased anxiety responses</td>
<td>Grey, 2007</td>
</tr>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>15 days</td>
<td>p20</td>
<td>p60</td>
<td>-</td>
<td>Less responsive to sucrose and other reward, more sensitive to stress and anxiety</td>
<td>Bolanos, 2007</td>
</tr>
</tbody>
</table>

Table 3, continued.
Table 3: The effects of chronic MPH treatment on immediate and long-term behavioral measures in adolescent rats. Findings are listed with regard to dose used for treatment, the duration of treatment, age at the start of treatment, age at testing, and the immediate and long-term neurobiological effects of the treatment regimen.
sensitization can cross over to other psychostimulant drugs such as cocaine (Brandon et al, 2001) and amphetamine (Valvassori et al, 2007).

MPH is also known to exhibit behavioral changes following chronic treatment that are similar to that of other psychostimulants (Gaytan et al, 1997). Some reports have also shown that MPH can elicit locomotor tolerance (Crawford et al, 1998; Izenwasser et al, 1999; Kuczenski et al, 1997; McNamara et al, 1993). Given the significant abuse liability of psychostimulants such as cocaine and amphetamine, the similarity of MPH in its acute neurobiology is of concern, and thus the effects of chronic treatment on subsequent psychostimulant exposure are an important area of investigation.

In adolescents: The effects of chronic MPH administration in adolescents appear to vary widely, depending upon age at time of treatment, the dose used, the duration of treatment, and the time between treatment and post-treatment testing. Published reports on chronic MPH exposure in adolescent animals have often focused on the effects of MPH treatment on cocaine self-administration and reward-related behaviors. A summary of the findings of the behavioral effects of chronic MPH treatment in adolescents has been provided in Table 3. Many adolescent experiments have chosen doses of 2.0 mg/kg in rats and 5.0 mg/kg in mice in an effort to mimic clinical dosing (Adriani et al, 2006a; Andersen et al, 2002; Bolanos et al, 2003; Brandon et al, 2001; Brandon et al, 2003a; Guerriero et al, 2006). However, doses that best represent human exposure have not yet been established, and doses as high as 10 or even 20 mg/kg are used (Achat-Mendes et al, 2003; Brandon et al, 2003b; Crawford et al, 1998; Guerriero et al, 2006).
Conversely, some results have indicated that doses as low at 0.75 mg/kg in mice may be better associated with the oral doses used in humans (Balcioglu et al, 2009).

Treatment regimens for MPH have varied widely, from dosing regimens as long as 28 days, to as short as three days. As might be expected, the behavioral results also vary widely. In cases where the lower doses of 2.0 to 2.5 mg/kg have been used for periods of less than 10 days, effects appear to be relatively consistent. Adolescent rats treated with MPH (2.0 mg/kg i.p. 7 days, treated post-natal day 35-p42) show increased cocaine self-administration in adulthood (Brandon et al, 2001). Interestingly, although locomotor sensitization to MPH is not present, there is often cross sensitization to amphetamine (Yang et al, 2003; Yang et al, 2006b). Short term dosing at higher doses of 4.0 to 10.0 mg/kg appears to result in a stimulant-like effect in adulthood, with blunted place preference for cocaine, which may indicate tolerance to the rewarding effects of cocaine (Achat-Mendes et al, 2003). Interestingly, other findings have shown increased CPP responses to priming doses of cocaine and for drugs such as morphine, which would seem to suggest increased sensitivity to the rewarding aspects of these drugs (Crawford et al, 2007). The effects on locomotor sensitization appear to be varied, with sensitization present with some regimens and absent in others (Brandon et al, 2001; Yang et al, 2006a). Thus it appears that short term dosing of MPH in adolescence results in behavioral adaptations resembling those of other psychostimulants, and that appear to increase the rewarding and reinforcing effects of cocaine in adulthood.

While the findings above showed increased rewarding and reinforcing effects following chronic MPH exposure in adolescence, the treatment regiments used were short and not necessarily clinically representative. Other behavioral reports have looked at low
and high doses of MPH over longer periods of time, for up to 28 days, and results differ dramatically from the short-term administration regimen. When using 2.0 mg/kg and 10 mg/kg over a 15 day span, it appears that behavioral effects may in fact be reversed at both doses, possibly due to the longer duration of treatment. These treatment paradigms produce aversion for cocaine in adulthood, and no locomotor cross sensitization to cocaine (Andersen et al, 2002; Guerriero et al, 2006). These animals also exhibit more symptoms of anxiety, including decreased time in open arms of the elevated plus maze and increased (Bolanos et al, 2003; Bolanos et al, 2008). In addition, these animals display a depressive-like phenotype, with decreases in sucrose preference and in struggle time in the forced swim test (Bolanos et al, 2003; Bolanos et al, 2008). These findings suggest that longer treatment regimens with MPH may result in decreases in the rewarding effects of psychostimulants in adulthood, in contrast to effects seen with shorter administration regimens, which appear to show increases in the rewarding effects of subsequent psychostimulant challenge.

Finally, a set of published reports has examined the effects of both low and high doses of MPH during treatment regimens of up to 28 days. While just a few experiments have been performed for this length of time, the results are again different from shorter regimens. In a manner similar to that seen with doses of MPH over fewer than 10 days, 28 days of 1.0, 2.0, or 10.0 mg/kg of MPH produces locomotor cross-sensitization to amphetamine at adulthood, but no sensitization to MPH itself (Valvassori et al, 2007). At 5.0 mg/kg over 28 days, animals show decreased anxiety responses, in contrast to studies that administered MPH for 15 days (Gray et al, 2007). It should be noted, however, that the study finding decreased anxiety responses at adulthood also
began administration of MPH at the earliest time point, when animals were 7 days old (Gray et al, 2007), and this may play an important factor in the development of anxiety responses.

Taken together, the current data on the effects of adolescent administration of MPH on subsequent behaviors, in particular reward-associated behaviors, appear varied. When broken down into length of treatment and the dose of treatment, however, the findings on chronic behavioral affects in fact are relatively consistent. It appears that dosing for a period of less than 10 days results in some cross-sensitization to cocaine and other effects associated with sensitizing regimens of psychostimulants (Brandon et al, 2001; Yang et al, 2003; Yang et al, 2006a). In contrast, longer exposure for around 15 days results in increases in anxiety-like behavior, depressive-like behavior, and some increased sensitivity to the aversive effects of psychostimulants (Andersen et al, 2002; Bolanos et al, 2003; Bolanos et al, 2008; Guerriero et al, 2006). Finally, the longest treatments produce cross-sensitization to psychostimulants (Valvassori et al, 2007), although there have been very few reports assessing these time points. Thus, it appears that the effects of chronic MPH administration on behavior are dependent not only on dose, but on the length of time they are given.

In adults: Given the similarity of MPH to other psychostimulants such as amphetamine and cocaine with regard to neurobiology, it would be logical to assume that the effects of chronic MPH treatment in the fully developed, adult rodent would prove
<table>
<thead>
<tr>
<th>Dose of MPH</th>
<th>Duration of Treatment</th>
<th>Immediate Behavioral Effects</th>
<th>Long Term Behavioral Effects</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 mg/kg i.p.</td>
<td>15 days</td>
<td>-</td>
<td>Normal locomotor response to cocaine, blunted cocaine CPP</td>
<td>Andersen, 2001</td>
</tr>
<tr>
<td>1.0 mg/kg i.p. 2x per day</td>
<td>11 days</td>
<td>Locomotor sensitization to MPH, cross sensitization to AMPH</td>
<td>-</td>
<td>Kuczenski, 2001</td>
</tr>
<tr>
<td>5.0 mg/kg i.p.</td>
<td>9 days</td>
<td>No change in latency to self-administer cocaine</td>
<td>-</td>
<td>Schenk, 2002</td>
</tr>
<tr>
<td>20 mg/kg i.p.</td>
<td>9 days</td>
<td>Decreased latency to self-administer cocaine</td>
<td>-</td>
<td>Schenk, 2002</td>
</tr>
<tr>
<td>0.24, 2.5 or 10.0 mg/kg i.p.</td>
<td>6 days</td>
<td>-</td>
<td>Following 3 days washout, got locomotor sensitization at 2.5 mg/kg and tolerance at 10 mg/kg</td>
<td>Yang, 2003</td>
</tr>
<tr>
<td>2.5 mg/kg i.p.</td>
<td>5 days</td>
<td>-</td>
<td>No locomotor sensitization to MPH, but cross-sensitization to amphetamine</td>
<td>Yang, 2003</td>
</tr>
<tr>
<td>10 mg/kg i.p.</td>
<td>4 weeks</td>
<td>Increased anxiogenic phenotype</td>
<td>-</td>
<td>LeBlanc-Duchin, 2004</td>
</tr>
<tr>
<td>2.5 mg/kg i.p.</td>
<td>5 days</td>
<td>Locomotor sensitization to MPH</td>
<td>-</td>
<td>Dafny, 2006</td>
</tr>
</tbody>
</table>

Table 4.
Table 4: The effects of chronic MPH treatment in adult rats on immediate and long-term behavioral measures. Findings are listed with regard to dose used for treatment, the duration of treatment, and the immediate and long-term neurobiological effects of the treatment regimen.
similar to the effects of these drugs. Many of the initial reports in adult animals with MPH focused on measures of initial reward, and there has therefore been a good deal of work done on self-administration of MPH (Li et al, 2006; Lile et al, 2003; Risner et al, 1975; Stoops et al, 2005), and on its ability to elicit conditioned place preference (Kankaanpaa et al, 2002; Martin-Iverson et al, 1991; Meririnne et al, 2001; Schenk et al, 2002; Sellings et al, 2006).

The effects of chronic MPH treatment on subsequent reward-related behaviors have been summarized in Table 4. Most investigations into the effects of chronic MPH exposure on subsequent behaviors have focused on sensitizing regimens of MPH administration, lasting for no more than 11 days. Findings have shown that doses as low as 2.5 mg/kg for 5 days are enough to produce locomotor sensitization (Dafny et al, 2006), and cross-sensitization to amphetamine (Yang et al, 2003). Even lower doses of 1.0 mg/kg, when given for 11 days, produce locomotor sensitization to MPH and cross-sensitization to amphetamine (Kuczenski et al, 2001). Higher doses, 5.0 and 10.0 mg/kg for 6-9 days, produce no changes in the latency to self-administer cocaine (Schenk et al, 2002) and produce locomotor tolerance (Yang et al, 2003). In contrast, the highest doses tested, 20.0 mg/kg for 9 days, produces a decreased latency to self-administer cocaine (Schenk et al, 2002). This result could be the result of sensitization, as prior sensitization with psychostimulants can facilitate the acquisition of self-administration behaviors (Dafny et al, 2006; Vezina et al, 2002).

It seems that few reports have looked at the effects of chronic MPH treatment in rats for longer than typical sensitization regimens. Andersen et al (Andersen et al, 2002), found that 15 days of 2.0 mg/kg in adults produced no sensitization and a blunted place
preference response to cocaine, a finding similar to some investigations performed in adolescents (Adriani et al, 2006a; Bolanos et al, 2008). LeBlanc-Duchin (Leblanc-Duchin et al, 2004) and colleagues investigated the effects of a relatively high dose in rats, 10 mg/kg, for 4 weeks, and found increases in anxiogenic phenotypes, which could affect drug-related behaviors. Further studies clearly need to be performed on the effects of chronic MPH administration in adult animals and the long-term effects on reward-related behaviors.

Taken together, the behavioral studies performed using chronic MPH treatment appear to vary widely in their findings, but these variations appear to be clearly related to both the dose used for treatment and the duration of the treatment regimen.

Discussion

In sum, the effects of MPH on the behavior and neurobiology of rodents appear similar in some respects to those of other psychostimulants, while exhibiting significant differences in behavioral effects such as sensitization. Acutely, MPH acts as a traditional psychostimulant, with the behavioral and neurobiological effects associated with other psychostimulants such as cocaine and amphetamine. These effects include drug self-administration, conditioned place preference, increases in extracellular DA and NE, and decreases in VTA DA neuron firing due to stimulation of D2 receptors. Chronically, however, the effects of MPH appear in some cases to be drastically different from the effects of other psychostimulants.

The neurobiological effects of chronic MPH treatment in adolescents may correlate to some extent with the behavioral effects of these same treatments. These
correlations are difficult to tease apart, as most neurobiological reports have looked at the immediate effects of chronic MPH treatment, while behavioral studies have generally waited to look at the effects on reward-related behaviors in adulthood. It appears that early neurobiological effects in adolescents are typical of those associated with chronic psychostimulant administration. Depending on dose and duration of treatment, these neurobiological changes could be associated with either tolerance or sensitization. The behavioral effects studied at later time points reflect cross-sensitization, but this also appears to depend on the dose used and the duration of treatment. It is obvious that further neurobiological studies need to be undertaken at these time points to correlate with the results of behavioral studies.

In contrast, the effects of chronic MPH exposure on neurobiology and reward-related behaviors in adults appear to be very consistent. MPH, when given over periods of 5-7 days, results in neurobiological and behavioral changes associated with sensitization (Crawford et al, 1998; Dafny et al, 2006; Schenk et al, 2002; Yang et al, 2003). These results suggest that in adult models, MPH may act as a traditional psychostimulant, but that the effects of MPH may vary in the developing brain.

In conclusion, the effects of MPH appear to result in acute effects similar to other psychostimulants, but further studies are necessary to determine the neurobiological and behavioral effects following chronic MPH exposure in adolescent and adult animals, both to determine the changes in the behavioral and neurobiological effects of subsequent drug exposure, as well as to fully establish the effects of MPH treatment on other related phenotypes.
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EFFECTS OF SPECIFIC 5-HT RECEPTOR SUBTYPES ON ACTIVATION OF
DOPAMINE SIGNALING AND PSYCHOSTIMULANT RESPONSES IN THE
VENTRAL TEGMENTAL AREA/NUCLEUS ACCUMBENS PATHWAY
Bethany R. Brookshire and Sara R. Jones

Introduction

It is well established that the initial rewarding and reinforcing effects of drugs of abuse require the activation of the mesolimbic dopamine (DA) system (Wise, 1987). Dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) are thought to be involved in many aspects of motivation and reward (Carboni et al, 1989; Di Chiara, 1995). Psychostimulant drugs of abuse activate this pathway via inhibition of the DA transporter, causing increases in extracellular DA, particularly in the NAc (Di Chiara, 1995; Koob et al, 1988; Volkow et al, 1997).

However, the mesolimbic DA system receives inputs from many other systems that may modulate dopaminergic responses to psychostimulant drugs. In particular, serotonin (5-HT) neurons in the dorsal and median raphe nuclei (DRN and MRN, respectively) project to both the VTA and NAc (Jacobs et al, 1992; Kalivas et al, 1991; Shirayama et al, 2006; Vertes et al, 1999). DA and 5-HT systems have extensive
interactions, and the activation of both systems can produce more complex interactions than the activation of one system alone. Many findings suggest that general elevations in 5-HT oppose the reinforcing effects of DA in the mesolimbic DA system (Roberts et al, 1994), but the effects of activation of specific 5-HT receptors on DA-mediated behaviors appear to be more complex (Czoty et al, 2002; Howell et al, 1995).

As many traditional psychostimulants such as cocaine activate both DA and 5-HT systems, the results of increases in extracellular 5-HT, as well as the outcomes following activation of specific 5-HT receptor subtypes, are of interest. The effects of general and specific 5-HT receptor agonists have been studied in some detail with regard to DA-associated behaviors related to incentive salience, motivation, and reward related learning. In addition, 5-HT is known to play an important role in the modulation of locomotor activity, a behavior that requires activation of the DA system (Geyer, 1996; Millan et al, 1997). While general 5-HT agonists do not appear to exhibit reinforcing efficacy (Roberts et al, 1999), studies suggest that DA and 5-HT could play simultaneous but separate roles in salience and motivation that are more complicated than simple positive or negative effects on reinforcement (Sora et al, 2001; Uhl et al, 2002).

It is important to focus on the various roles of 5-HT receptors within the mesolimbic DA system, as the types of receptors present determine local responses to 5-HT signaling and the subsequent effects on mesolimbic DA. There are currently 17 known functional 5-HT receptor variants (Martin et al, 2009), 13 of which have undergone significant physiological and pharmacological investigation. Of these, six receptors have been identified as having strong effects on DA signaling in the NAc-VTA pathway: the 5-HT1A, 1B, 2A, 2C, 3, and 6 receptors. This review will discuss these 5-
HT receptors and their hypothesized roles in mesolimbic DA neurotransmission, as well as their interactions with behavioral and neurobiological psychostimulant responses, to present a comprehensive picture of the role of specific 5-HT receptors in the modulation of the mesolimbic DA pathway.

The 5-HT1A Receptor

The 5-HT1A receptor has been extensively studied with regard to its interactions with the mesolimbic DA system. While the 5-HT1A receptor is expressed peripherally in the kidney and prostate where it plays a role in cell proliferation (Dizeyi et al, 2004; Raymond et al, 1993), the highest expression of this receptor in the rat is in the central nervous system (CNS), where it is present both pre and postsynaptically. Presynaptically the 5-HT1A receptor is an autoreceptor on the 5-HT cell bodies of the DRN and MRN (Gozlan et al, 1983) where it provides feedback inhibition of 5-HT turnover, synthesis, and 5-HT neuron firing rate (Collu et al, 1997; Hamon et al, 1988; Riad et al, 2000; Yoshimoto et al, 1992). Postsynaptically, results have shown high binding levels for the 5-HT1A receptor in rat limbic areas including the hippocampus, lateral septum and amygdala, and cortical areas including the entorhinal and cingular cortices, with moderate binding in the olfactory bulb, thalamus, hypothalamus, brainstem, and neocortex (Albert et al, 1990; Farde et al, 1997; Gozlan et al, 1983; Hall et al, 1985; Hume et al, 2001; Khawaja et al, 1995; Kia et al, 1996; Maeda et al, 2001; Marcinkiewicz et al, 1984; Palacios et al, 1990; Palchaudhuri et al, 2005; Pompeiano et al, 1992; Raurich et al, 1999; Verge et al, 1986). Despite some findings showing low density of 5-HT1A receptors in the basal ganglia (Barnes et al, 1999), other studies have
shown that the 5-HT1A receptor is a postsynaptic receptor in the VTA, and is located on DA cell bodies in the VTA (Doherty et al, 2000; Doherty et al, 2001).

The 5-HT1A receptor has high sequence homology with the rest of the 5-HT1 family, including the 5-HT1B receptor and the human 5-HT1D receptor (van, I et al, 1990). It is a seven transmembrane G-protein coupled receptor with 422 amino acids that couples to Gi/Go to inhibit adenylyl cyclase resulting in the opening of inward K+ channels and neuronal hyperpolarization (De Vivo et al, 1986; Hamon et al, 1990; Innis et al, 1987; Innis et al, 1988; Schoeffter et al, 1988; Weiss et al, 1986). In the DRN, there is also coupling of 5-HT1A receptors to CA2+ channels, stimulation of which produces decreased conductance, resulting in decreases in 5-HT neuron firing (Andrade et al, 1987; Ropert, 1988; Sprouse et al, 1987; Sprouse et al, 1988). The wide limbic expression of the 5-HT1A receptor is consistent with roles in hypocretin/orexin transmission, sleep, epilepsy, pituitary hormone release, autoinhibitory control of 5-HT release, regulation of acetylcholine, glutamate and γ-aminobutyric acid (GABA) transmission, anxiety, locomotor activity, stress responses, aggression, feeding behavior, hippocampal function, and pain (Bortolozzi et al, 2004; Boutrel et al, 2002; Buhot et al, 1995b; Harte et al, 2005; Kusserow et al, 2004; Li et al, 2004; Lopez-Meraz et al, 2005; Madjid et al, 2006; Monti et al, 2003; Monti et al, 2004; Muraki et al, 2004; Osei-Owusu et al, 2005; Pattij et al, 2003; Pesonen et al, 1991; Popova et al, 2005; Shannon et al, 2000).

Effects of 5-HT1A receptor stimulation on the DA system

A. Effects of 5-HT1A receptors in the raphe.
Although the 5-HT1A receptor is present postsynaptically in the VTA on DA and GABA neurons (Doherty et al, 2000; Doherty et al, 2001; Lejeune et al, 1998; Lejeune et al, 2000), most investigators believe that the majority of the effects of 5-HT1A receptors on mesolimbic dopaminergic neurotransmission stem from actions in the raphe (Yoshimoto et al, 1992). The 5-HT1A receptor is expressed as a presynaptic autoreceptor in the raphe, where its stimulation results in reductions in 5-HT neuron firing and reduced 5-HT levels in areas like the DRN, MRN, prefrontal cortex (PFC), and hippocampus (Casanovas et al, 2000; Collu et al, 1997; Yoshimoto et al, 1992). The DRN and MRN both project to the VTA and NAc (Jacobs et al, 1992; Kalivas et al, 1991; Shirayama et al, 2006; Vertes et al, 1999), and it is currently hypothesized that 5-HT from the DRN provides a tonic inhibitory influence over DA neuron firing rate in the VTA (Adell et al, 2004).

Given the extensive inhibitory control that presynaptic 5-HT1A autoreceptors provide over raphe neuron firing (Casanovas et al, 2000; Collu et al, 1997; Yoshimoto et al, 1992), it is not surprising that 5-HT1A receptor modulation in this area can have extensive effects on mesolimbic DA. It is thought that the systemic locomotor effects of 5-HT1A receptor activation may be dominated by the influence of 5-HT1A autoreceptor activation over 5-HT neuron firing (Adell et al, 2004; Gobert et al, 1998; Sakaue et al, 2000; Sharp et al, 1989). Systemic administration of the 5-HT1A receptor agonist R(+)-8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) has been shown to reduce 5-HT and increase DA in the PFC (Gobert et al, 1998), and systemic activation of 5-HT1A receptors results in increased DA release in the NAc (Campbell et al, 1995; Parsons et al, 1993; Yan, 2000). These findings may be the result of 5-HT1A autoreceptor-mediated
decreases in raphe neuron firing, resulting in disinhibition of VTA DA neurons (Adell et al, 2004). Inhibition of 5-HT cell firing has been hypothesized to produce conditioned place preference, presumably via this same mechanism in increasing DA neuron activity (Adell et al, 2004), and this hypothesis has additional support from a number of published results showing that local injection or infusions of 8-OH-DPAT into the raphe produce conditioned place preference (Fletcher et al, 1993; Shippenberg, 1991). However, it should be noted that other studies have found that local administration of 8-OH-DPAT into the DRN cause decreases in 5-HT and DA in the NAc and striatum (Yoshimoto et al, 1992). In addition, it appears that higher doses of 8-OH-DPAT (1.0 mg/kg, i.p.) may have aversive effects (Papp et al, 1991), in contrast to the conditioned place preference found following administration of lower doses (0.1-0.25 mg/kg s.c., Shippenberg, 1991; 65-250 micrograms/kg, i.p., Papp et al, 1991). Taken together, it seems that the influence of 5-HT1A autoreceptors in the DRN over mesolimbic dopaminergic neurotransmission may be an excitatory one, with decreased 5-HT firing rate following 5-HT1A autoreceptor stimulation resulting in disinhibition of DA neuron firing in areas like the VTA.

B. Effects of local postsynaptic 5-HT1A receptors in the mesolimbic DA system.

In addition to the extensive influence of raphe-level 5-HT1A receptors on dopaminergic neurotransmission, there are also postsynaptic 5-HT1A receptors in the VTA and NAc, which can have local effects on DA neuron firing and DA release (Doherty et al, 2000; Doherty et al, 2001; Lejeune et al, 1998; Lejeune et al, 2000). Some findings have suggested that only the interactions in the VTA are of import, however, as 5-HT1A autoreceptors and heteroreceptors are not involved in DA release in the NAc (Boulenguez et al, 1996). Thus far, it appears that 5-HT release is modulated by
presynaptic 5-HT1A autoreceptors in the raphe, while DA release is modulated by postsynaptic 5-HT1A receptors in the VTA (Sakaue et al, 2000; Sharp et al, 1989). It should be noted that some laboratories have also shown that activation of somatodendritic 5-HT1A autoreceptors can also result in decreases in 5-HT release, which appears to be a feedback response in the presence of high levels of extracellular 5-HT, providing another inhibitory mechanism on 5-HT neuron firing in the DRN (Adell et al, 1998; Casanovas et al, 1997).

Previous work has shown that local administration of flesinoxan, a 5-HT1A receptor agonist, had a biphasic effect on VTA DA neuron firing (Lejeune et al, 1998; Lejeune et al, 2000). Low doses of 8-OH-DPAT (0.05 mg/kg, s.c.) appear to stimulate DA neuron firing via direct activation of postsynaptic 5-HT1A receptors on DA neurons, while higher doses of agonists such as flesinoxan (19.5 micrograms/kg, i.v.) activate postsynaptic 5-HT1A receptors on GABA neurons, resulting in inhibition of DA neuron firing (Chen et al, 1995; Lejeune et al, 1998; Lejeune et al, 2000). This biphasic effect could also explain why low doses (0.1-0.25 mg/kg, s.c.) of 8-OH-DPAT cause conditioned place preference (5-HT1A autoreceptor stimulated decreases in 5-HT neuron firing in the DRN may cause disinhibition of VTA DA neuron firing (Fletcher et al, 1993); (Shippenberg, 1991)), while higher doses (1.0 mg/kg, i.p.) can be aversive, (due to activation of postsynaptic 5-HT1A receptors on local GABA neurons in the VTA (Papp et al, 1991)). Interestingly, the 5-HT1A antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide (WAY 100,635) did not appear to modify the activity of DA neurons in the VTA (Lejeune et al, 1998), an effect that may be due to other controls over GABA and DA neuron firing. Overall, the net
effects of postsynaptic 5-HT1A receptor activation in the VTA appear to be biphasic in their influence on DA neuron firing, with low doses (as low as 0.1-0.25 mg/kg 8-OH-DPAT s.c. (Shippenberg, 1991)) activating autoreceptors in the raphe, decreasing 5-HT neuron firing and disinhibiting VTA DA neuron firing, while higher doses (1.0 mg/kg 8-OH-DPAT i.p., (Papp, et al, 1991)) act on postsynaptic 5-HT1A receptors in the VTA directly, activating GABA neurons to inhibit DA activity.

Effects of 5-HT1A receptors on psychostimulant responses.

Extensive work has been performed with regard to the effects of 5-HT1A receptors on the modulation of psychostimulant-induced changes in neurochemistry and behavior. As mentioned above, acute decreases in 5-HT neuron firing in the raphe are thought to disinhibit VTA DA neurons (Adell et al, 2004), and this mechanism may contribute to the many effects of 5-HT1A receptor agonists and antagonists on psychostimulant responses (Muller et al, 2003). In the case of psychostimulant exposure, 8-OH-DPAT (0.2 mg/kg, i.p.) may enhance the reduction in 5-HT cell firing produced by cocaine, potentiating locomotor activity through further disinhibition of VTA DA neurons (Adell et al, 2004; Muller et al, 2003). 5-HT1A receptor agonists do enhance acute cocaine-induced locomotor activity (Carey et al, 2000a; Carey et al, 2005; De La et al, 2000; Muller et al, 2003), while the 5-HT1A receptor antagonist WAY 100,635 (0.05-0.4 mg/kg, i.p.) has been shown in many studies to attenuate the locomotor effects of cocaine (Carey et al, 2000b; Carey et al, 2001; Carey et al, 2005; Muller et al, 2002b; Muller et al, 2003). Additionally WAY 100,635 attenuates the potentiation of cocaine effects resulting from concurrent administration of fluoxetine (Herges et al, 1998) and
citalopram (Weikop et al, 2007), two selective 5-HT reuptake inhibitors (SSRIs). 8-OH-DPAT (0.2 mg/kg, i.p.) and osemozotan (1.0 mg/kg, i.p.) also have been shown to reduce cocaine-induced elevations in 5-HT in the hippocampus, NAc, and PFC and to potentiate DA in the PFC, while WAY 100,635 (1.0 mg/kg, i.p.) potentiates 5-HT in the PFC (Muller et al, 2003; Nakamura et al, 2006a), which could contribute to DA effects seen in other reports.

However, these findings are not universal. Work from Filip and Cunningham groups has shown that 5-HT1A receptor agonists including 8-OH-DPAT (0.2 mg/kg, i.p.) and osemozotan (1.0 mg/kg, i.p.) attenuate amphetamine and cocaine-induced locomotor activity (Przegalinski et al, 1997; Nakamura et al, 2006a; De La Garza et al, 2000) reduce cocaine-induced rearing (De La Garza et al, 2000), while the 5-HT1A receptor antagonist 4-(2’-methoxy-)-phenyl-1-[2’-(N-2”-pyridinyl)-p-fluorobenzamido]-ethyl-piperazine (p-MPPF, 30 mg/kg, i.p.) potentiated cocaine-induced activity, along with DA and 5-HT responses to cocaine in the NAc (Andrews et al, 2005). Additionally, there are indications that 5-HT1A receptor agonists and antagonists do not block the DA effects of cocaine in the NAc (Muller et al, 2002a; Szumlinski et al, 2004). Work in the Huston group (Muller et al, 2002a; Muller et al, 2003) has suggested that the blockade of cocaine-induced stimulant effects by 5-HT1A receptor antagonists is paralleled by potentiation of cocaine-induced increases in 5-HT in the hippocampus and NAc. These effects are dependent on the dose of 5-HT1A receptor antagonist being too low (65-250 micrograms/kg, i.p.) to potentiate further DA increase, activating 5-HT1A autoreceptors in the raphe without concurrent activation of postsynaptic 5-HT1A receptors in the VTA (Muller et al, 2002b). Other groups have also suggested that 5-HT1A receptor antagonists potentiate cocaine-
induced DA and 5-HT in the NAc via decreased inhibitory feedback on 5-HT (Andrews et al., 2005). If these findings are correct, it is possible that differences in the dosing of 5-HT1A receptor agonists and antagonists may be responsible for the varied effects on acute responses to psychostimulants. Additionally, these data indicate that the effects of 5-HT1A receptors on psychostimulant responses are biphasic, as seen above, with low doses (0.25 mg/kg i.p. and below) showing the effects of autoreceptor activity in the raphe, while higher doses (1.0 mg/kg, i.p.) activate postsynaptic 5-HT1A receptors on VTA GABA neurons.

In addition to the effects of 5-HT1A receptors on the modulation of the acute psychostimulant response, 5-HT1A receptors may also play a role in chronic psychostimulant effects. Systemic activation of 5-HT1A receptors enhances cocaine sensitization (De La et al., 2000; Przegalinski et al., 2000). Repeated cocaine sensitizes 5-HT1A receptors (King et al., 1993), and 5-HT1A receptors on raphe cell bodies are increased in amphetamine sensitized rats (Bonhomme et al., 1995). However, other groups have found opposite effects. Higher doses of 8-OH-DPAT (0.25-1.0 mg/kg, s.c.) inhibits both the expression and development of amphetamine sensitization (Przegalinski et al., 2000), and osemozoton (0.1-0.3 mg/kg, i.p.) has also been shown to block methamphetamine (METH) sensitization and attenuate METH-sensitized 5-HT release (Ago et al., 2006). In contrast to the increases in 5-HT1A receptors seen following chronic amphetamine (Bonhomme et al., 1995), other studies have shown that 3,4-methylenedioxymethamphetamine (MDMA) may decrease the sensitivity of 5-HT1A receptors in the hippocampus (Aguirre et al., 1997), and cocaine exposure may result in subsensitive prolactin responses to 8-OH-DPAT challenge, indicating a subsensensitive
system (Baumann et al, 1995). Also, 8-OH-DPAT pretreatment (0.03-1.0 mg/kg, s.c.) decreases self-administration of cocaine and cocaine reinforced responding under a progressive ratio schedule (Parsons et al, 1998; Peltier et al, 1993), while antagonists fail to affect cocaine breakpoints. This indicates that 5-HT1A receptors may not be involved in the potentiation of cocaine reinforcement (Parsons et al, 1998). Additionally, some results have shown that WAY 100,635 (0.1-1.0 mg/kg, i.p.) potentiates cocaine-induced locomotor activity (Nakamura et al, 2006b), and does not substitute for systemic cocaine cues (De La et al, 1998).

While many of the effects of 5-HT1A receptor agonists and antagonists on psychostimulant effects have been investigated using systemic administration, the extensive effects of presynaptic 5-HT1A receptors in the raphe beg the question of how raphe 5-HT1A receptors in particular modulate the dopaminergic effects of psychostimulants. As the effects of 5-HT1A presynaptic autoreceptors on 5-HT neuron firing in the raphe are well known (Casanovas et al, 2000; Collu et al, 1997; Yoshimoto et al, 1992), and the decreases in 5-HT neuron firing are thought to be associated with disinhibition of VTA DA neurons (Adell et al, 2004), it might be expected that 5-HT1A receptors in the raphe might have extensive effects on psychostimulant responses. 5-HT1A receptor agonists infused into the DRN potentiate cocaine-induced locomotor activity in rats and the DA effects of acute cocaine in the NAc, and augment the acute effects of cocaine on glutamate in the NAc (Szumlinski et al, 2004).

However, it should be noted that both the DRN and the MRN have inputs to the mesolimbic DA system (Jacobs et al, 1992; Kalivas et al, 1991; Shirayama et al, 2006; Vertes et al, 1999), and 5-HT1A receptor stimulation in these two areas appears to result
in contrasting effects. While the 5-HT1A receptors in the DRN appear to be involved in the acute effects of cocaine as discussed above, the presynaptic 5-HT1A receptors in the MRN may have a greater influence on long-term exposure to psychostimulants (Szumlinski et al, 2004). 8-OH-DPAT (5-10 ug) administered directly into the MRN have no effect on initial locomotor responses, but potentiate sensitization expression (Szumlinski et al, 2004), including the sensitized effects of cocaine on glutamate in the NAc (Szumlinski et al, 2004). Interestingly, 8-OH-DPAT infused into the MRN blocks the development of 5-HT sensitization following cocaine (Szumlinski et al, 2004), suggesting that the effects of presynaptic 5-HT1A receptors in the MRN augment the expression of psychostimulant sensitization, while having a potential negative effect on sensitization development.

Taken together, the effects of 5-HT1A receptors on mesolimbic DA and responses to psychostimulants appear often contradictory. Further work will be required to form a consensus as to what effects result from different doses of 5-HT1A receptor agonists and antagonists. As so much of the VTA DA responses to 5-HT1A receptor stimulation appears to be biphasic, it is possible that many of the effects noted above fit somewhere on this biphasic curve. In addition, the lack of 5-HT1A receptor antagonist effects in many cases may indicate that these receptors do not contribute to baseline behavioral effects, and that 5-HT1A receptors only modulate mesolimbic DA function when stimulated directly, their effects otherwise being overwhelmed by the influence of other receptors. The significant influence of the DRN and MRN are also important modulatory factors in the control of 5-HT1A receptors over DA system function, and teasing apart
the specific roles and degrees of influence for the different 5-HT1A receptors is an ongoing task.

**The 5-HT1B Receptor**

The 5-HT1B receptor has also been studied extensively with regard to its role in the control of dopaminergic neurotransmission and its effects on psychostimulant reward mechanisms. It is found peripherally in the coronary and cortical cerebral arteries, where it plays a role in vasoconstriction (Nilsson et al, 1999), but the highest expression levels are in the CNS. The 5-HT1B receptor has the highest density of expression in the basal ganglia, with notable densities also in the globus pallidus, substantia nigra, and dorsal subiculum (Bonaventure et al, 1997; Bonaventure et al, 1998; Boulenguez et al, 1992; Palacios et al, 1992; Pazos et al, 1985; Sari et al, 1999; Segu et al, 1991; Varnas et al, 2001). There is moderate 5-HT1B receptor expression in the entopeduncular nuclei, the superior colliculus, and the periaqueductal gray, and light levels of expression in the cerebral cortex, amygdale, hypothalamus, and spinal cord (Bruinvels et al, 1993; Pazos et al, 1985). Overall, the highest expression is in the mesolimbic DA system, where 5-HT1B receptors are known to be localized to the NAc, and to GABA neurons in the VTA (Cameron et al, 1994; Hallbus et al, 1997; O'Dell et al, 2004; Yan et al, 2001a).

Unlike the 5-HT1A receptor, which is found presynaptically at the cell body and in terminal fields (Doherty et al, 2000; Doherty et al, 2001; Gozlan et al, 1983; Riad et al, 2000), the 5-HT1B receptor is primarily presynaptic, where it acts as auto- and heteroreceptors controlling release at the terminal (Auerbach et al, 1991; Bosker et al, 1995; Boulenguez et al, 1996; Martin et al, 1992; Riad et al, 2000; Sari et al, 1997;
Sleight et al, 1989). Like the 5-HT1A receptor, with which it shares significant homology (van, I et al, 1990), the 5-HT1B receptor is a seven transmembrane G-protein coupled receptor. Containing 386 amino acids in the rat and the mouse, it corresponds to the 390 amino acid 5-HT1D receptor in humans (Adham et al, 1992; Demchyshyn et al, 1992; Jin et al, 1992; Maroteaux et al, 1992a; Mochizuki et al, 1992; Pauwels et al, 1996; Weinshank et al, 1992). The 5-HT1B receptor is coupled to $G_i/G_o$ and inhibits adenylyl cyclase, and may also activate MAP-kinase (Hamblin et al, 1991; Pullarkat et al, 1998; Seuwen et al, 1988). Because of its location as an auto and heteroreceptor on neurons terminals, the 5-HT1B receptor has been shown to regulate presynaptic inhibition of GABA release, glutamatergic transmission, pain, aggression, 5-HT release, acetylcholine release, and DA release (Ase et al, 2000; Bartsch et al, 2004; Buhot et al, 1995c; Chadha et al, 2000; Johnson et al, 1992; Lee et al, 2002; Maura et al, 1986; Morikawa et al, 2000; Saudou et al, 1994; Singer et al, 1996; Trillat et al, 1997). Behaviorally, the high expression of the 5-HT1B receptor in the basal ganglia is consistent with behaviors such as impulsivity, learning and memory, regulation of hippocampal function, food intake, pain, and aggression (Ase et al, 2000; Bartsch et al, 2004; Buhot et al, 1995c; Chadha et al, 2000; Johnson et al, 1992).

**Effects of 5-HT1B receptors on the mesolimbic DA system.** The 5-HT1B receptor is the most abundant 5-HT receptor subtype present in the ventral midbrain (Hoyer et al, 1994), and thus would be expected to have a strong influence over mesolimbic DA neurotransmission. Specifically, 5-HT1B receptors are known to be present presynaptically on NAc GABA terminals projecting to the VTA, where their stimulation results in inhibition of GABA release and disinhibition of DA neuron firing (Cameron et
A large number of published reports in the literature concur with and expand upon this mechanism. The systemic activation of 5-HT1B receptors with the agonist 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (RU 24969, 0.2-2 mg/kg i.p.) enhances basal and stimulated extracellular DA in the NAc (Boulenguez et al, 1996; Guan et al, 1989), and direct infusion of 5-HT1B receptor agonists RU 24969 and CP 93,129 (0.15 and 0.45 µM) into the VTA increases DA in the VTA and NAc, with a concurrent decrease in GABA in the VTA (Yan et al, 2001b; Yan et al, 2004), providing further evidence for 5-HT1B receptor activation leading to disinhibition of VTA DA neurons. Behaviorally, the 5-HT1B receptor agonists including RU 24969, CP 94, 253 administered systemically (RU 24969: 0.25-5.0 mg/kg i.p., CP 94,253: 0.3-30 mg/kg i.p.) or infused directly (CP 93129, 100 µM) into the VTA have been shown to increase locomotor activity (Chaouloff et al, 1999; De Souza et al, 1986; Fish et al, 2000; Geyer, 1996; Green et al, 1984; Oberlander et al, 1986; Oberlander et al, 1987; Tricklebank et al, 1986), although the 5-HT1B receptor agonist CP 94,253 (2.5 and 10 mg/kg i.p.) produces conditioned place aversion rather than preference (Cervo et al, 2002). This may be due to dissociation between the locomotor stimulating and motivational or salience-mediating properties of the receptor (Murphy et al, 2001). The 5-HT1B receptor is prominently expressed in the globus pallidus and substantia nigra and cerebellum (Boschert et al, 1994; Maroteaux et al, 1992b; Sari et al, 1999), and effects of 5-HT1B receptor activation on locomotor activity may be mediated via receptor activity in these areas (Oberlander et al, 1986; Oberlander et al, 1987; Tricklebank et al, 1986). Given the strong stimulatory effect of 5-HT1B receptor agonists on locomotor activity (Chaouloff et al, 1999; De
Souza et al, 1986; Fish et al, 2000; Geyer, 1996; Green et al, 1984; Oberlander et al, 1986; Oberlander et al, 1987; Tricklebank et al, 1986), it initially seems surprising that 5-HT1B receptor antagonists have no effect on locomotor activity (Przegalinski et al, 2004). However, extensive research into the 5-HT1B receptor has suggested that the receptor contributes little to behavioral phenotypes in the absence of direct agonist stimulation (Parsons et al, 1998) and that the influence of the 5-HT1B receptor may be overwhelmed by the influence of other receptors during general elevations in 5-HT.

Although the primary effects of 5-HT1B receptor stimulation may occur in the VTA, the influence of 5-HT1B receptors in other regions should not be discounted. The 5-HT1B receptor is present as an autoreceptor on 5-HT terminals extending from the DRN, inhibiting 5-HT release (Sharp et al, 1989), and 5-HT1B receptor stimulation decreases local 5-HT release in the raphe (Moret et al, 1997). Some studies have hypothesized that 5-HT1B receptors may have an excitatory influence over 5-HT neuron firing (Evrard et al, 1999), but this view appears to be in the minority. A large number of results indicate that 5-HT1B receptor activation results in decreases in 5-HT release at the terminal level in areas like the hippocampus, diencephalon, and cortex (Auerbach et al, 1991). Interestingly, 5-HT1B receptor stimulation can enhance DA in the NAc and VTA even at doses too low to affect GABA (Yan et al, 2004), which is the predominant mechanism of 5-HT1B receptor effects in the VTA. It is possible that this enhanced DA at low doses (20 µM CP 93,129) occurs through inhibition of 5-HT release from 5-HT terminals extending toward the VTA from the raphe (Jacobs et al, 1992; Kalivas et al, 1991; Shirayama et al, 2006; Vertes et al, 1999). Thus, the primary effects of 5-HT1B
receptors on mesolimbic DA appear to stem from their effects on GABA neurons in the VTA, with possible secondary autoinhibitory effects at the terminals of raphe neurons.

**Effects of 5-HT1B receptors on psychostimulant responses.** The 5-HT1B receptor is the most extensively studied 5-HT receptor with regard to interactions with drugs of abuse. Given the well-documented increases in VTA DA neuron firing and extracellular NAc DA following 5-HT1B receptor stimulation (Cameron et al, 1994; Hallbus et al, 1997; Iyer et al, 1996; O'Dell et al, 2004; Sari, 2004; Yan et al, 2001a; Yan et al, 2004), modulation of psychostimulant drug responses seemed highly likely (Neumaier et al, 2002). Acutely, 5-HT1B receptor agonists administered systemically (RU 24969, 1-3 mg/kg i.p.) and infused (CP, 93129, 30 and 100 µM) into the VTA increase cocaine-stimulated DA in the NAc (O'Dell et al, 2004; Parsons et al, 1999) and the locomotor effects of 5-HT1B receptor agonists mimic the hyperlocomotive effects of psychostimulants such as MDMA (Fletcher et al, 2002a; McCreary et al, 1999a; Rempel et al, 1993). Thus, it is not surprising that 5-HT1B receptor stimulation potentiates increases in locomotor activity stimulated by cocaine and MDMA (Castanon et al, 2000; Rempel et al, 1993; Scearce-Levie et al, 1999). Increases in 5-HT1B receptor expression on GABA efferents from the NAc to the VTA using viral mediated gene expression produced a similar result, increasing cocaine-induced locomotor activity without affecting baseline activity (Neumaier et al, 2002). In addition, the 5-HT1B receptor agonist CP 94,253 (2.5 and 10 mg/kg i.p.) induces conditioned place aversion, but enhances conditioned place preference for cocaine, while the 5-HT1B receptor antagonist N-[4-methoxy3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5methyl-1,2,4-oxadiazol-3-yl)-biphenyl-4-carboxamide (GR 127, 935, 10 mg/kg s.c.) have no effect on place
preference (Cervo et al, 2002), indicating that this receptor potentiates the rewarding effects of psychostimulants. Finally, the 5-HT1B receptor is believed to be responsible for both psychostimulant and SSRI-induced enhancement of behavioral and neurochemical effects of psychostimulants, as these effects are blocked by the 5-HT1B receptor antagonist 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyridin-4-ylphenyl)benzamide hydrochloride (GR 55562, 1 mg/kg i.p.), which indicates that stimulation of the 5-HT1B receptor may be responsible for these augmented responses (Borycz et al, 2008; Callahan et al, 1997; Lucas et al, 1997). Thus, the literature concerning acute 5-HT1B receptor modulation of psychostimulant responses is remarkably consistent, and points toward 5-HT1B receptor stimulation as potentiating the neurochemical, behavioral, reinforcing, and rewarding effects of psychostimulants (Neumaier et al, 2002; O'Dell et al, 2006; Parsons et al, 1998).

While the effect of 5-HT1B receptor stimulation on the acute response to psychostimulants is a strong one, there is also evidence that 5-HT1B receptors may modulate responses to chronic psychostimulant exposure. Activation of 5-HT1B receptors using intra-VTA injections of CP 93,129 (0.03-1.0 µg) has been shown to enhance the development and expression of cocaine sensitization (De La et al, 2000; Przegalinski et al, 2001; Przegalinski et al, 2004) and 5-HT1B receptor antagonists such as GR 55562 (0.3-3.0 µg acute injection or infusion of 300 µM) can inhibit the development and expression of psychostimulant sensitization (Amato et al, 2007; O'Dell et al, 2004; Przegalinski et al, 2001; Przegalinski et al, 2004), The role of 5-HT1B receptor modulation in sensitization is also supported by evidence that chronic cocaine exposure and subsequent withdrawal increases 5-HT1B receptor expression in the NAc
and VTA between 30 and 60% (Hoplight et al, 2007; Przegalinski et al, 2003; Przegalinski et al, 2007). Increasing 5-HT1B receptor expression via viral–mediated gene expression on GABA neuron terminals has been shown to increase the sensitivity of GABA terminals to VTA 5-HT, resulting in a supersensitive decrease in GABA release when 5-HT1B receptors are subsequently stimulated (Anthony et al, 2000; Hoplight et al, 2007). Taken together, it appears that 5-HT1B receptor stimulation plays a significant role in both the development and expression of psychostimulant sensitization.

Interactions between the 5-HT1B receptor and psychostimulant responses have also been intensely studied with regard to drug self-administration. While 5-HT1B receptor agonists are not self-administered in rats (Cervo et al, 2002; Parsons et al, 1998), they do partially substitute for cocaine in discrimination tasks (Filip et al, 2003). Artificial increases in 5-HT1B receptor expression using viral-mediated gene transfer in the NAc also increase cocaine-associated reward (Barot et al, 2007). Furthermore, systemic 5-HT1B receptor activation with the agonists RU 24969 and CP 94,253 potentiate the reinforcing effects of cocaine (Parsons et al, 1998; Tanaka et al, 1993). They also augment cocaine-reinforced responding in rats and increase cocaine breakpoints on a progressive ratio schedule (Parsons et al, 1998). There is evidence for 5-HT1B receptor involvement in the expression of tolerance during drug self-administration. Locomotor responses to RU 24969 (1.0 mg/kg, s.c.) are reduced in animals self-administering cocaine, and increased in animals in withdrawal (O'Dell et al, 2006). Although local administration of the 5-HT1B receptor agonist CP 93,129 (0.15 and 0.45 µM) into the VTA and NAc increases DA in naïve animals (Yan et al, 2001a), CP 93,129 infused into the VTA (100 µM) did not affect NAc DA in cocaine-
experienced rats (O'Dell et al, 2006). This effect is dramatically reversed in withdrawal, resulting in marked increases in DA in response to CP 93,129, and is accompanied by increased expression of the 5-HT1B receptor (Przegalinski et al, 2003), thus providing further evidence for the role of 5-HT1B receptors in both sensitization and tolerance to psychostimulant effects (Buhot et al, 1995a; O'Dell et al, 2006; Ramboz et al, 1996). It appears, therefore, that the 5-HT1B receptor plays an important role in drug self-administration, although this effect may be masked by the stimulation of multiple 5-HT receptor subtypes during normal drug-taking behavior (Parsons et al, 1996).

The modulation of psychostimulant responses by 5-HT1B receptors is so profound that the 5-HT1B receptor knockout mouse (5-HT1B KO) is used as a model of psychostimulant addiction (Ase et al, 2008). 5-HT1B KO mice exhibit high D2 receptor levels and decreased levels of extracellular DA in the NAc, with increases in tissue DA content, compared to wild type mice (Ase et al, 2000; Ase et al, 2008). This finding is controversial, however, as other work has shown that 5-HT1B KO mice have high extracellular DA in the NAc and increased DA responding to DA efflux in the NAc induced by i.p. cocaine (5.0 and 20 mg/kg i.p.) (Shippenberg et al, 2000). In agreement with these latter findings, other studies have found that 5-HT1B KO mice exhibit increased rates of cocaine self-administration (Castanon et al, 2000) and enhanced reinforcing effects of cocaine indicated by self-administration of low cocaine doses (0.5 mg/kg, i.v. in the mouse) (Rocha et al, 1997; Rocha et al, 1998). 5-HT1B KO mice also show increased 5-HT turnover in the NAc with no difference in extracellular 5-HT concentration (Doherty et al, 2000). However, 5-HT1B KO mice do not appear to display conditioned place preference for cocaine (Belzung et al, 2000). Taken together,
the findings in the 5-HT1B KO mouse appear to indicate increased reinforcing effects of psychostimulants as evidenced by increased rates of cocaine self-administration and increase reinforcing efficacy of cocaine, despite a lack of conditioned place preference, which indicates that these mice are more sensitive to the reinforcing effects of psychostimulants. However, the lack of conditioned place preference does not corroborate other reports. While this may be a function of the 5-HT1B receptor, it may also indicate learning deficits or deficits in a non-reward-related area. Further work will be necessary to understand the full effects of the 5-HT1B receptor knockout with regards to reward-related behaviors.

The extensive literature on 5-HT1B receptors and their roles in normal mesolimbic DA neurotransmission as well as in modulating psychostimulant responses is remarkably consistent. These results point toward a role for the 5-HT1B receptor in the responses to the increased extracellular levels of monoamine resulting from psychostimulant administration, resulting in augmentation of the effects of psychostimulants via disinhibition of VTA DA neuron firing (O'Dell et al, 2004; Parsons et al, 1999). Despite this, the 5-HT1B receptor appears to have few behavioral effects under baseline conditions, as evidenced by antagonist studies (Parsons et al, 1998). Future work will further elucidate the possible role of 5-HT1B receptors in basal dopaminergic neurotransmission, and the pursuit of the 5-HT1B receptor as a target for investigation of psychostimulant effects is a rich field for continuing research into pharmacological treatments for addictive disorders.

The 5-HT2A Receptor
The 5-HT2A receptor is expressed in both the peripheral nervous system (PNS) and central nervous system (CNS). In the periphery it plays a role in blood vessel contraction and is found in the coronary artery, epicardium, and renal arteries (Nilsson et al, 1999; Watts et al, 1995; Watts et al, 2004). Centrally, the highest density of the 5-HT2A receptor is in layer five of the cortex and claustrum, the olfactory bulb, NAc, caudate-putamen, CA3 of the hippocampus, brainstem nuclei, and the cerebellum (Hamada et al, 1998; Lopez-Gimenez et al, 1997; Maeshima et al, 1998; Mijnster et al, 1997; Pazos et al, 1985). In the mesolimbic DA system, the 5-HT2A receptor is expressed in both the NAc and VTA. In the VTA it is found on both DA and GABA neurons, and it is expressed in the core of the NAc, as well as in the caudate-putamen (Lopez-Gimenez et al, 1997; Mijnster et al, 1997; Van Bockstaele et al, 1993).

The 5-HT2A receptor is expressed primarily postsynaptically, particularly on dendrites in the NAc (Barnes et al, 1999; Cornea-Hebert et al, 1999; Doherty et al, 2000; Lopez-Gimenez et al, 2001; Mengod et al, 1990), and there may also be cytoplasmic expression in the VTA (Cornea-Hebert et al, 1999; Doherty et al, 2000). Like other receptors of the 5-HT1 and 5-HT2 classes, it is a seven transmembrane G-protein coupled receptor. It is coupled to $G_{\alpha q/11}$ that activates phospholipase C, leading to downstream effects such as increased intracellular Ca$^{2+}$ and activation of phospholipase A2 to release arachidonic acid, which modulates synaptic transmission (Felder et al, 1990; Hoyer et al, 2002; Raymond et al, 2001). The 5-HT2A receptor also couples secondarily to $G_i/G_o$ to inhibit adenylyl cyclase (Day et al, 2002; Garnovskaya et al, 1995).

**Effects of 5-HT2A receptors on the mesolimbic DA system.** Due to the expression of the 5-HT2A receptor postsynaptically on dendrites in the NAc (Barnes et
al, 1999; Cornea-Hebert et al, 1999; Doherty et al, 2000; Lopez-Gimenez et al, 2001; Mengod et al, 1990; Pompeiano et al, 1992), and the expression of the receptor on DA and GABA neurons in the VTA (Lopez-Gimenez et al, 2001; Mijnster et al, 1997; Van Bockstaele et al, 1993), it might be expected that 5-HT2A receptor stimulation would result in changes in mesolimbic dopaminergic neurotransmission. Published findings to date have suggested that the 5-HT2A receptor is involved primarily in the modulation of stimulated DA transmission (Di Giovanni et al, 2001; Lucas et al, 2000), and that the 5-HT2A receptor has a phasic, excitatory influence over VTA DA neurons (Adell et al, 2004). Activation of 5-HT2A receptors with agonists such as 1-[2,5-dimethoxy-4-iodophenyl-2-aminopropane] (DOI, 40 pMol) increases DA neuronal activity when injected into the VTA (Auclair et al, 2004; Bortolozzi et al, 2005; Doherty et al, 2000; Pessia et al, 1994), and may also enhance local DA transmission and release in the NAc (Cornea-Hebert et al, 1999; Yan, 2000). In correlation with these findings, the 5-HT2A/2C receptor agonist DOI increases locomotor activity (0.57-1.09 mg/kg i.p.), and this is thought to be primarily the result of 5-HT2A receptor activation (Schreiber et al, 1995), suggesting a role for the 5-HT2A receptor in the stimulation of DA cell firing and DA release.

**Effects of 5-HT2A receptors on psychostimulant responses.** The 5-HT2A receptor has been examined somewhat with regard to acute psychostimulant effects, and 5-HT2A receptor stimulation does appear to modulate DA release and locomotor responses to psychostimulants such as amphetamine (Auclair et al, 2004). Similar to the 5-HT1B receptor, 5-HT2A receptor activation with DOI (0.1-2.5 mg/kg s.c.) increases acute amphetamine-induced DA release (Ichikawa et al, 1995), as well as cocaine-induced
locomotor activation (Filip et al, 2004). Also, the 5-HT2A receptor antagonists (R)-(+)\(\alpha\)-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (MDL 100, 907, 0.3-1.0 mg/kg), reduced the effects of cocaine, amphetamine, and MDMA on locomotor activity and extracellular DA (Barr et al, 2004; Carlsson, 1995; Filip et al, 2004; Fletcher et al, 2002b; Kehne et al, 1996; McMahon et al, 2001; Moser et al, 1996; O’Neill et al, 1999; Schmidt et al, 1994; Yamamoto et al, 1995). Thus, 5-HT2A receptors appear to enhance DA transmission under stimulated conditions (De Deurwaerdere et al, 2000; Schmidt et al, 1994).

Examinations of the 5-HT2A receptor in the mesolimbic DA system have also focused on the development and expression of psychostimulant sensitization, as well as effects of the 5-HT2A receptor on drug self-administration. 5-HT2A receptor antagonists 1(Z)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(E)-propene (SR 46349B) inhibit the development of sensitization (Filip et al, 2001; Filip et al, 2004; Salomon et al, 2007), while the 5-HT2A receptor agonist DOI has shown no effects on the development of sensitization (Filip et al, 2001; Filip et al, 2004). Other reports have suggested that SR 46349B may also affect the expression of psychostimulant sensitization (Filip et al, 2001). Concurrent with the inhibition of sensitization development by 5-HT2A receptor antagonists, it appears that 5-HT2A receptor antagonists MDL 100, 907 (0.1 µg intra-VTA injection or 0.5 mg/kg s.c.), and ketanserin (1.0-3.0 mg/kg i.p.) block the hyperlocomotive effects, discriminative stimulus effects, and relapse to self-administration of cocaine, and decrease voluntary cocaine consumption (Filip et al, 2001; Fletcher et al, 2002b; McMahon et al, 2001; McMillen et al, 1993). The 5-HT2A receptor is known to downregulate rapidly after
agonist/antagonist exposure, and shows decreased function following chronic cocaine exposure (Filip et al, 2001; Gray et al, 2001). However, supersensitivity of 5-HT2A receptors has also been found during withdrawal from repeated cocaine administration (Baumann et al, 1996; Essman et al, 1994; Levy et al, 1992).

Taken together, published work on the 5-HT2A receptor in acute and chronic psychostimulant exposure appear to reveal a pattern similar to that of the 5-HT1B receptor, although the effects are less well defined and via direct modulation of dendrites in the NAc, rather than the indirect control of GABA release in the VTA seen with the 5-HT1B receptor. It appears that the role of 5-HT2A receptors in regulation of stimulated, phasic DA signaling in the mesolimbic DA system may potentiate play a role in the potentiation of psychostimulant effects both acutely and following repeated exposure.

The 5-HT2C Receptor

The 5-HT2A and 5-HT2C receptor are often studied together in the literature, a phenomenon that results from a lack of truly selective agonists and antagonists for each receptor. The 5-HT2A and 5-HT2C receptors display >50% sequence homology, with >80% homology in the transmembrane regions (Boess et al, 1994; Hoyer et al, 1994), and the 5-HT2C receptor has 458 amino acids in humans, 460 in rat, 459 in mouse (Stam et al, 1994; Yu et al, 1991). 5-HT2A and 5-HT2C receptors are coupled to G_{aq/11} to activate phospholipase C, leading to downstream effects like increased intracellular Ca^{2+}. Both of these receptors also activate phospholipase A2 to release arachidonic acids, modulating synaptic transmission (Conn et al, 1986; Day et al, 2002; Felder et al, 1990; Hoyer et al, 2002; Lucaites et al, 1996; Raymond et al, 2001). In addition, both exhibit secondary
coupling to $G_i/G_o$ to inhibit adenylyl cyclase (Lucaites et al, 1996). However, the 5-HT2C receptor exhibits additional activation of phospholipase D (McGrew et al, 2002).

The 5-HT2C receptor is more abundant than the 5-HT2A receptor, and expressed in more brain areas (Pompeiano et al, 1994; Wright et al, 1995). It can be found in the spinal cord and dorsal root ganglion (Fonseca et al, 2001; Pierce et al, 1996), as well as the choroid plexus, CA3 of the hippocampus, amygdala, and the raphe (Abramowski et al, 1995; Filip et al, 2002; Pompeiano et al, 1994; Serrats et al, 2005), generally on GABA neurons (Pasqualetti et al, 1999; Serrats et al, 2005). In the mesolimbic DA system, the 5-HT2C receptor is expressed on VTA GABA neurons (Bubar et al, 2003; Clemett et al, 2000; Di Giovanni et al, 2001; Di, V et al, 1999; Gobert et al, 2000), where it is postsynaptically located on dendrites (Bubar et al, 2007; Eberle-Wang et al, 1997) and co-localizes to a subset of DA cell dendrites as well (Bubar et al, 2007). As would be expected from its wide expression pattern and concentration in dopaminergic areas, the 5-HT2C receptor has been implicated in analgesia, anxiety, hyperlocomotion, food intake, sleep, and responses to repeated stress (Bagdy, 1998; Bagdy et al, 2001; Chojnacka-Wojcik et al, 1994; Chou-Green et al, 2003a; Chou-Green et al, 2003b; Fone et al, 1998; Frank et al, 2002; Gibson et al, 1994).

**Effects of 5-HT2C receptors on the mesolimbic DA system.** There is abundant expression of the 5-HT2C receptor in the mesolimbic DA system, and the 5-HT2C receptor has been extensively studied with regard to its effects on dopaminergic neurotransmission. The effects appear to be distinct, based on the populations of 5-HT2C receptors. In the VTA, 5-HT2C receptors are present primarily on GABA neurons (Bubar et al, 2003; Clemett et al, 2000; Di Giovanni et al, 2001; Di, V et al, 1999; Gobert et al,
Because 5-HT2C receptor stimulation induces phospholipase C activity and enhancement of intracellular "CA2+", which depolarizes cell membranes to increase neuron firing rate (Stanford et al, 2005), the stimulation of 5-HT2C receptors on GABA neurons in the VTA should increase GABA firing rate, subsequently inhibiting VTA DA neuron firing (Johnson et al, 1992). Stimulation of 5-HT2C receptors in the VTA using the 5-HT2C agonists m-chlorophenylpiperazine (mCPP, 5-320 µg/kg i.v.) and RO 60-0175 (1.0 mg/kg i.p.) result in decreases in DA neuron firing due to increases in GABA activity (Di Giovanni et al, 2001; Di, V et al, 1999; Gobert et al, 2000; Pompeiano et al, 1994). The 5-HT2C receptor has a high level of constitutive activity (Adell et al, 2004; Berg et al, 2005; De Deurwaerdere et al, 1999; De Deurwaerdere et al, 2004; Pozzi et al, 2002), and thus, 5-HT2C receptors in the VTA are inhibitory to DA neuron firing under basal conditions. Reports have shown that stimulation of the 5-HT2C receptor with mCPP (5-320 µg/kg i.v.) decreases VTA DA neuron firing and DA outflow (Di Giovanni et al, 2001; Di, V et al, 2000; Navailles et al, 2006), and that this decrease in DA firing is due to the activation of GABA neurons in the VTA (Alex et al, 2005; Blackburn et al, 2002; Di Giovanni et al, 2001; Gobert et al, 2000). In support of this, the 5-HT2C receptor antagonist SB 242084 (200 µg/kg, i.v. or 1-10 mg/kg i.p.) enhances DA neuron firing and DA outflow, and increases DA in the NAc (De Deurwaerdere et al, 1999; Di, V et al, 1998; Di, V et al, 1999; Navailles et al, 2006). Some studies suggest, however, that intra-VTA infusion of 5-HT2C receptor antagonists change VTA DA outflow, but do not alter levels of DA in the NAc, suggesting an effect on exocytosis without affecting DA neuronal firing (Navailles et al, 2006). Due to the high level of constitutive activity and the robust effects of 5-HT2C receptor agonists and antagonists in this area,
investigators believe that the VTA is the major point of 5-HT2C receptor inhibitory control on the DA system (Navailles et al., 2004).

5-HT2C receptor stimulation appears to have converse effects on DA release when 5-HT2C receptors are studied specifically in the NAc, and some authors suggest that the NAc shell is the primary site of action for the effects of constitutive 5-HT2C receptor activity in the regulation of DA (Navailles et al., 2006) and in the inhibitory control of constitutively active central 5-HT2C receptors (De Deurwaerdere et al., 2004). 5-HT2C receptors exert selective control over DA exocytosis (Porras et al., 2002), and 5-HT2C receptors are stimulatory. Thus, when located on DA neurons, 5-HT2C receptors can increase DA exocytosis (Bubar et al., 2007). Indeed, it appears that the NAc shell 5-HT2C receptor population on the terminals of DA neurons exerts excitatory control over NAc DA release (Cornea-Hebert et al., 1999; Filip et al., 2002; McMahon et al., 2001; Yan, 2000), but that this influence is secondary to the dominant inhibitory influence of 5-HT2C receptors in the VTA.

Overall, it appears that the influence of VTA GABA neuron-localized 5-HT2C receptors on the constitutive activity on DA neuron firing may be the dominant influence in the VTA and NAc. Systemic 5-HT2C receptor activation with agonists such as 6-chloro-2-(1-piperazinyl)pyrazine HCl (MK 212, 2.0 mg/kg i.p.) decreases locomotor activity (Filip et al., 2004; Kennett et al., 1997; Martin et al., 1998), and it is thought that 5-HT dampens locomotor activity mainly through the 5-HT2C receptors in the mesolimbic DA system (Baumann et al., 2008). Neurochemically, systemic 5-HT2C receptor agonist RO 60-0175 (80-320 µg/kg, i.v.) decreases DA in the NAc and VTA (Di, V et al., 1999; Dremencov et al., 2005; Navailles et al., 2006), while systemic administration of 5-HT2C
receptor antagonists such as SB 242084 (160-640 microg/kg, i.v.) increases NAc DA release (Navailles et al, 2006), suggesting a significant influence of 5-HT2C receptor populations on GABA neurons in the VTA. In addition, systemic administration of 5-HT2C receptor antagonists such as mesulergine (200 µg/kg s.c.), SB 206553 (1 and 2.5 mg/kg i.p.), ritanserin (630 microg/kg i.p.), and RP 62203 (2.5 and 4 mg/kg i.p.) enhance DA neuron firing and increase DA in the NAc (De Deurwaerdere et al, 1999; Di, V et al, 1998; Di, V et al, 1999) despite the fact that 5-HT2C receptor antagonists alone do not elevate locomotor activity (Dekeyne et al, 2000; Filip et al, 2002; McMahon et al, 2001; Vickers et al, 2000).

In sum, the effect of 5-HT2C receptors in the mesolimbic DA system is thought to oppose 5-HT1B receptors and inhibit mesolimbic DA (De Deurwaerdere et al, 2004; Di, V et al, 1999; Di, V et al, 2000), exerting a tonic and phasic inhibitory influence on DA transmission and DA-related behaviors (Bubar et al, 2006). While specific NAc population of 5-HT2C receptors must be kept in mind when looking at the effects of local 5-HT2C receptor agonist administration, the overall effects of 5-HT2C receptors in the mesolimbic DA system appear to result from their modulation of GABA neurons in the VTA, and the subsequent generalized inhibitory effect on DA neuron firing when stimulated.

Effects of 5-HT2C receptors on psychostimulant responses. As the VTA is thought to be the primary area of 5-HT2C receptor influence, it is also hypothesized to be the primary location of effects of 5-HT2C receptor agonists and antagonists on the DA-mediated behaviors induced by psychostimulants (Fletcher et al, 2004). The effects of 5-HT2C receptor stimulation on psychostimulant induced behavior and neurochemical
effects appear to be mainly acute. 5-HT2C receptor agonist MK 212 (0.2 mg/kg, i.p.) and antagonists ketanserin (1.0-3.0 mg/kg i.p.) and prazosin (3.0 mg/kg i.p.) had no effect on the development (Filip et al, 2001; Filip et al, 2004), or expression of cocaine sensitization (Filip et al, 2004). In addition, chronic cocaine does not alter the 5-HT2A/2C receptor availability in humans (Wang et al, 1995), suggesting that 5-HT2A/2C receptors are not affected by long term psychostimulant exposure, although there are indications that chronic cocaine may alter 5-HT2A/2C function (Filip et al, 2001). However, it appears that 5-HT2C receptor antagonism or stimulation has the greatest effects on the acute response to psychostimulant exposure.

As discussed above, the 5-HT2C receptor appears to exhibit its greatest effects in the VTA, where stimulation and constitutive activity controls GABA neuronal activity, leading to inhibition of VTA DA neuron firing (Di Giovanni et al, 2001; Di, V et al, 1999; Gobert et al, 2000; Pompeiano et al, 1994). It is believed that the attenuation of psychostimulant-induced locomotor activity 5-HT drugs may be due to the stimulation of 5-HT2C receptors (Fletcher et al, 2002b; Fletcher et al, 2006). Conversely, 5-HT2C receptor antagonists might be expected to have a positive effect on VTA DA firing (De Deurwaerdere et al, 1999; Di, V et al, 1998; Di, V et al, 1999; Navailles et al, 2006), which might increase the dopaminergic effects of psychostimulants. The 5-HT2C receptor antagonists SB 206553 (1.0, 2.0, and 4.0 mg/kg i.p.) and SB242084 (0.5 mg/kg i.p.) enhance the acute locomotor response to MDMA (Bankson et al, 2002); (Fletcher et al, 2006) and cocaine (Filip et al, 2004). Interestingly, low doses of the 5-HT2C receptor agonist MK 212 (0.3 mg/kg i.p.) enhance cocaine-induced locomotor activity, while higher doses (2.0 mg/kg i.p.) inhibited the locomotor stimulation produced by
psychostimulant administration (Filip et al, 2004), an effect that may stem from actions specific to the VTA (Grottick et al, 2000). Further studies have shown that 5-HT2C receptor antagonist SB242084 (0.5 mg/kg i.p.) enhances the discriminative stimulus effects of cocaine and promotes relapse to cocaine self-administration (Fletcher et al, 2002b), while 5-HT2C receptor agonists such as Ro 60-0175 (0.1-3 mg/kg s.c.) reduce responding for cocaine (Grottick et al, 2000)(McCreary et al, 1999b). It appears that these effects of 5-HT2C receptor antagonists on psychostimulant responses are the result of 5-HT2C receptor activation in the VTA, as blockade of 5-HT2C receptors in the VTA enhances MDMA-induced DA release in the NAc (Bankson et al, 2004). Conversely, 5-HT2C receptor agonists reduce cocaine self-administration, possibly by decreasing DA neuron firing in the VTA (Filip et al, 2001).

Some work has also been performed to examine the role of NAc-specific 5-HT2C receptors in psychostimulant responses. 5-HT2C receptor activation in the NAc specifically is known to locally increase DA release (Cornea-Hebert et al, 1999; Filip et al, 2002; McMahon et al, 2001; Yan, 2000). This local activity could be why low doses of 5-HT2C receptor agonist MK 212 (0.1 mg/kg i.p.) enhance cocaine induced locomotor activity, while higher doses inhibit locomotor stimulation, presumably acting in the VTA (Grottick et al, 2000). In addition, stimulation of NAc 5-HT2C receptors specifically enhance cocaine-induced locomotor activity (Filip et al, 2002; Filip et al, 2003). Taken together, these data suggest that the stimulation of 5-HT2C receptors in the NAc may to some extent counteract the effects of 5-HT2C receptor stimulation in the VTA, but that the VTA 5-HT2C receptor population exerts the majority of 5-HT2C receptor-specific control over the mesolimbic DA system.
The 5-HT3 Receptor

The 5-HT3 receptor is the only one of the 5-HT receptors known to be directly coupled to an ion channel via a ligand-gated mechanism, rather than being a seven transmembrane G-protein linked receptor (Derkach et al, 1989; Peters et al, 1989; Yakel et al, 1988). As such, the 5-HT3 receptor is a pentameric structure (Barnes et al, 2009; Barrera et al, 2005; Boess et al, 1995; Green et al, 1995), and when activated, conducts Na+ and K+, resulting in rapid depolarization followed by rapid desensitization (Derkach et al, 1989; Peters et al, 1989; Yakel et al, 1988). Located presynaptically (Laporte et al, 1992), activation of 5-HT3 receptors results in the modulation of GABA, cholecystokinin, acetylcholine, dopamine, norepinephrine, and substance P transmission (Chameau et al, 2006; Fink et al, 2007; Nichols et al, 1996). The 5-HT3 receptor is located both in the PNS and CNS. In the CNS, the highest expression of 5-HT3 receptor is in the caudate and hippocampus (Parker et al, 1996). However, 5-HT3 receptors are also found in the dorsal horn, medulla, brainstem nuclei, entorhinal cortex, cerebral cortex, amygdala, and hypothalamus (Grant, 1995; Laporte et al, 1992; Tecott et al, 1993).

Effects of 5-HT3 receptors on dopaminergic signaling. Given the significant expression of the 5-HT3 receptor in the caudate (Parker et al, 1996), and the modulation of dopamine release by presynaptic 5-HT3 receptors (Campbell et al, 1996; Chameau et al, 2006; Fink et al, 2007; Gillies et al, 1996; Nichols et al, 1996), it is to be expected that 5-HT3 receptors will mediate some of the effects associated with mesolimbic dopaminergic neurotransmission. The 5-HT3 receptor has been known to modulate the
activity of VTA DA neurons (Gillies et al, 1996). Local infusion of the 5-HT3 receptor agonist 1-(m-chlorophenyl)-biguanide (CPBG 10-240 µM) increases the somatodendritic release of DA in the VTA, indicating an increase in spontaneously active cells (Campbell et al, 1996; Liu et al, 2006), while the 5-HT3 receptor antagonist LY 277359 (0.1 or 1.0 mg/kg i.p.) has the opposite effect, reducing the number of spontaneously active DA cells in the VTA (Minabe et al, 1991). In addition, blockade of 5-HT3 receptors with the antagonist ICS 205930 inhibits fluoxetine-induced DA release in the medial PFC (mPFC) (Tanda et al, 1995), suggesting that activation of these receptors would enhance DA release. These results have led some investigators to believe that the 5-HT3 receptor, in conjunction with the 5-HT2A receptor, may mediate the majority of stimulatory actions of 5-HT on DA neurons in the VTA (Adell et al, 2004). The results in the VTA are similar to those seen in the NAc, where 5-HT3 receptors in the NAc core are believed to control depolarization-dependent DA release when DA and 5-HT are increased concurrently (De Deurwaerdere et al, 2005).

**Effects of 5-HT3 receptors on psychostimulant responses.** In light of the stimulatory effects of the 5-HT3 receptor on DA release in the VTA, it is not surprising that 5-HT3 receptors have significant effects on psychostimulant responses. The majority of research on the interaction between 5-HT3 receptor stimulation and psychostimulant responses in the VTA and NAc has been conducted with 5-HT3 receptor antagonists, and so the results of agonists can only be inferred. Administration of the 5-HT3 receptor antagonist ondansetron blocks the development of cocaine-induced locomotor sensitization (King et al, 1997), which suggests that 5-HT3 receptor agonist administration may enhance locomotor sensitization(King et al, 2000). However,
pretreatment with the 5-HT3 receptor antagonists MDL 72222 (0.1 and 3.0 mg/kg i.p.), Y-25130 (0.1-3.0 mg/kg i.p.), or ondansetron (2.0 mg/kg i.p.) does not block the acquisition of cocaine conditioned place preference (Szumlinski et al, 2003), and 5-HT3 receptor knockout mice have blunted cocaine-induced locomotor sensitization with no change in acute response (Hodge et al, 2008). Taken together with the anxiolytic properties of 5-HT3 receptor antagonists (Eison et al, 1994), these data appear to suggest that the acute 5-HT3 receptor stimulation increases somatodendritic DA release in both the NAc and VTA, possibly contributing to the acute effects of psychostimulants and to the development of psychostimulant-induced locomotor sensitization.

The 5-HT6 Receptor

The 5-HT6 receptor is the most recently discovered of the 5-HT receptors with interactions in the mesolimbic DA system, and as such, there have been relatively few published reports addressing the effects of the 5-HT6 receptor on DA system activity. Initial work on the 5-HT6 receptor indicates that it has a CNS-only expression pattern, with highest levels in the olfactory tubercle, striatum, NAc, dentate gyrus, and CA1, CA2, and CA3 layers of the hippocampus (Gerard et al, 1997; Ward et al, 1995; Yoshioka et al, 1998), with lower levels of expression in the cerebellum, diencephalic nuclei, thalamus, superior colliculus, substantia nigra, motor trigeminal nucleus, facial nucleus, and layers 2, 3, 4, and 6 of the cortex (Gerard et al, 1997; Ruat et al, 1993; Ward et al, 1995). 5-HT6 receptor expression appears to be primarily postsynaptic and somatodendritic (Gerard et al, 1997).
The 5-HT6 receptor is another of the seven transmembrane G protein coupled receptors, with 440 amino acids in humans, 436 in rats, and 440 in mice (Kohen et al, 1996; Kohen et al, 2001; Ruat et al, 1993). In contrast to other G protein coupled 5-HT receptors in the mesolimbic DA system such as the 5-HT1A, 1B, 2A, and 2C, the 5-HT6 receptor appears to be coupled primarily to Gs to stimulate adenylyl cyclase, with a resulting increase in cyclic AMP (Boess et al, 1995; Kohen et al, 2001; Ruat et al, 1993; Zhang et al, 2003), and there is evidence for additional coupling to Gq/G11 to stimulate phospholipase C (Zhang et al, 2003).

Studies with the 5-HT6 receptor have indicated that it is involved in the modulation of acetylcholine neurotransmission (Bentley et al, 1999; Bourson et al, 1998; Lacroix et al, 2004) as well as basal GABA and stimulated glutamate transmission (Schechter et al, 2008), and additional results have implicated that the 5-HT6 receptor is involved in learning and memory (Rogers et al, 2001; Woolley et al, 2001). Recently published findings have highlighted the possible role of the 5-HT6 receptor in the treatment of psychiatric disorders such as obsessive compulsive disorder and schizophrenia (Dawson et al, 2001; Dawson et al, 2003; Woolley et al, 2004). Given the involvement of the mesolimbic DA system in these psychiatric disorders, and the ideal positioning of the 5-HT6 receptor in reward-related areas like the striatum and NAc (Gerard et al, 1997; Ward et al, 1995), it is not surprising that work has begun to focus on the role of the 5-HT6 receptor in dopaminergic neurotransmission (Lacroix et al, 2004).

There is evidence that 5-HT6 receptor antagonists may be “pro-cognitive” (Woolley et al, 2004), increasing performance in memory consolidation sets and object discrimination, and so several reports have looked at the effects of 5-HT6 receptor
antagonists on monoamine transmission in areas such as the PFC and hippocampus. 5-HT6 receptor antagonists increase DA, NE, and 5-HT in the mPFC of rats (Lacroix et al, 2004), enhance glutamate neurotransmission (Dawson et al, 2003), and overall enhance excitatory neurotransmission and potentiate DA efflux in the PFC and hippocampus (Dawson et al, 2001; Li, 2007). This suggests that effects of 5-HT6 receptor antagonists may be pro-cognitive as suggested. Further, while 5-HT6 receptor antagonists alone do not affect pre-pulse inhibition, a mouse model of stress (Leng et al, 2003), administration of 5-HT6 receptor antagonists can reverse deficits induced by DA manipulation in both pre-pulse inhibition and fear learning paradigms (Mitchell et al, 2008), which suggest possible protective effects.

Effects of the 5-HT6 receptor on dopaminergic signaling and psychostimulant responses. Due to the relatively recent discovery and characterization of the 5-HT6 receptor, there have been few investigations looking at the effects of 5-HT6 receptor antagonists on dopaminergic signaling and behaviors in the VTA-NAc connection specifically. Even so, studies performed thus far indicate an excitatory role of 5-HT6 receptors on dopaminergic activity, possibly via global serotonergic effects. In a manner similar to studies conducted on the 5-HT2C receptor and psychostimulant interactions, most research has focused on the effects of antagonists, and so the effects of agonists can only be inferred at this date. While acute (i.p.) administration of the 5-HT6 receptor antagonist SB-271046 (10.0 mg/kg) decreased VTA DA neuronal activity, repeated doses of this same antagonist for 21 days had no effect on VTA DA neuron firing, despite a small increase in spontaneously active DA neurons in the substantia nigra (Minabe et al, 2004). With regard to psychostimulant exposure, SB-271046 and another 5-HT6 receptor
antagonist SB 258510A (3.0-10.0 mg/kg) potentiated the effects of amphetamine on DA and 5-HT (Dawson et al, 2003; Frantz et al, 2002), and augmented the locomotor and discriminative stimulus effects of amphetamine (Frantz et al, 2002; Pullagurla et al, 2004). Interestingly, however, this effect does not appear to cross over to cocaine (Frantz et al, 2002; Pullagurla et al, 2004). While artificial increases in 5-HT6 receptor levels caused by viral-mediated gene expression in the NAc block conditioned place preference for cocaine (Ferguson et al, 2008), 5-HT6 receptor antagonists appear to have no effect on locomotor activity or behavioral sensitization. Taken together, it appears that 5-HT6 receptors may play an inhibitory role in the VTA and NAc and responses to psychostimulants, as determined by recent studies with 5-HT6 antagonists.

Summary and Conclusions

While published findings agree that the initial rewarding and reinforcing effects of psychostimulant drugs require activation of the dopaminergic system (Wise, 1987; Wu et al, 2001), the modulatory effects of other neurotransmitter systems such as the 5-HT system should never be discounted. In fact, the six 5-HT receptors that display an extensive presence in the mesolimbic DA system appear to play distinct modulatory roles in dopaminergic neurotransmission, as well as in response to psychostimulant challenge.

From this discussion of the 5-HT1A, 1B, 2A, 2C, 3, and 6 receptors, several patterns seem to emerge. The 5-HT1A receptor appears to have a dual effect on mesolimbic DA neurotransmission. Excitatory influences of 5-HT1A receptors appear to result primarily from stimulation in the raphe, where they inhibit 5-HT cell firing, and thus disinhibit DA neurons in the VTA (Adell et al, 2004). Additionally, there may be a
biphasic effect of 5-HT1A receptor signaling on DA and GABA neurons in the VTA, with low dose agonists exciting DA neurons directly, and high dose agonists exciting GABA neurons preferentially to inhibit DA neuron firing (Lejeune et al, 1998; Lejeune et al, 2000). While the effects of 5-HT1A receptor activation on mesolimbic DA appear to be variable, the effects of 5-HT1B receptor activation are highly consistent. It appears that 5-HT1B receptors, although they do not exhibit any over-riding effects on DA at baseline, inhibit GABA neurons in the VTA when stimulated. This leads to disinhibition of VTA DA neurons and increases in DA cell firing, resulting in the potentiation of psychomotor stimulant effects (Cameron et al, 1994; Hallbus et al, 1997; Iyer et al, 1996; O'Dell et al, 2004; Parsons et al, 1998; Sari, 2004; Yan et al, 2001a; Yan et al, 2004).

The effects of the 5-HT2A receptor appear to be relatively similar to those of the 5-HT1B receptor, occurring through a different mechanism. While the 5-HT1B receptor stimulates DA neuron firing via inhibition of GABA neurons, the 5-HT2A receptor appears to have a phasic excitatory influence over DA release (Adell et al, 2004). This increases DA levels and potentiates the effects of psychostimulants (Cornea-Hebert et al, 1999), in a manner similar to 5-HT1B receptor stimulation (Filip et al, 2004; Ichikawa et al, 1995; Yan, 2000). Although often studied in conjunction with the 5-HT2A receptor due to a lack of pharmacological specificity, the 5-HT2C receptor has strong effects on mesolimbic DA that are distinct from those of the 5-HT2A receptor. The 5-HT2C receptor appears to exert primary modulation of DA neuronal activity via activation of GABA receptors in the VTA, leading to increased inhibitory tone and decreased DA firing (Di Giovanni et al, 2001; Di, V et al, 1999; Gobert et al, 2000; Pompeiano et al, 1994). Thus 5-HT2C receptor activation opposes some of the stimulatory effects of
psychostimulants (Fletcher et al, 2002b), resulting in effects nearly opposite those of the 5-HT1B receptor (Fletcher et al, 2006). In contrast, the ion-channel linked 5-HT3 receptor modulates release at the terminal, leading to increases in DA release as well as in the number of spontaneously active DA neurons (Campbell et al, 1996). Although this receptor is less well studied than its G-protein linked counterparts, stimulation of the 5-HT3 receptor may potentiate the effects of psychostimulants in a manner similar to the 5-HT2A receptor (King et al, 1997; King et al, 2000). Finally, the recently characterized 5-HT6 receptor may increase local DA neuron firing (Minabe et al, 2004), but this receptor appears to have negative modulatory effects on psychostimulant related similar to those of the 5-HT2C receptor (Dawson et al, 2003; Frantz et al, 2002).

Broadly, it may be said that the effects of 5-HT receptor stimulation on mesolimbic DA activity are dominated by 5-HT1B, 5-HT2A, and 5-HT2C receptors, with 5-HT1A, 5-HT3, and 5-HT6 receptor playing secondary roles. The 5-HT1B and 5-HT2A receptors, when stimulated, appear to enhance DA neuron firing and DA release, while the 5-HT2C receptor opposes these effects. The effects of all of these 5-HT receptors appear to depend upon specific stimulation, and it is possible that general psychostimulants may overwhelm the behavioral and neurochemical effects of specific receptor subtypes. However, given the extensive effects of some of these receptor subtypes on psychostimulant responses, specific stimulation could provide an effective avenue for treatment and study into the mechanisms behind psychostimulant addiction, as well as the mechanisms behind the control of the mesolimbic DA system.

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CHAPTER II

CHRONIC METHYLPHENIDATE TREATMENT REVERSES NEGATIVE SEROTONIN EFFECTS OF DRUG REWARD: A ROLE FOR 5-HT1B RECEPTORS

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**Abstract**

Abuse rates of methylphenidate (MPH) are rapidly increasing among adolescents and adults as prescription rates for this drug increase. MPH is a psychostimulant similar to cocaine and amphetamine, except that MPH is a potent inhibitor of DAT and NET with little affinity for the SERT, making it pharmacologically distinct from other psychostimulants. The effects of chronic, high-dose, abuse-like exposure to MPH have not been well defined. In particular, the effects of MPH on 5-HT systems have gone unexplored. We investigated the effects of chronic, high-dose MPH on DA and 5-HT system interactions in mice. Our work reveals that MPH exposure induces 5-HT system changes leading to increased rewarding properties of drugs with serotonergic actions. Following MPH treatment in mice, the 5-HT uptake inhibitor fluoxetine and the 5-HT1B agonist RU 24969 produced locomotor and conditioned place preference (CPP) and increased DA in the nucleus accumbens (NAc), a reversal of the aversive, DA-decreasing
effects seen in saline treated animals. We also found 5-HT neurochemical sensitization
to cocaine and MDMA after MPH treatment, which was correlated with increased
sensitivity to MDMA CPP and increased locomotor activation by both drugs. We
identified the mechanism for these changes as increased 5-HT1B receptor activity in the
ventral tegmental area (VTA), and a significant, 21% increase in 5-HT1B receptor
binding in the ventral midbrain. These results show novel effects of MPH on 5-HT/DA
interactions, with implications for the consequences of MPH abuse on future drug
exposure, and new insights into the serotonergic effects of chronic psychostimulant
exposure.

Introduction

MPH is a common prescription stimulant treatment for attention-
deficit/hyperactivity disorder in patients of all ages (Biederman et al, 2002), and is
increasingly abused in adolescent and adult populations. It is estimated that up to 26% of
adults 18-25 years old have used prescription stimulants non-medically, and abuse rates
are increasing along with prescriptions for these drugs (Barrett et al, 2005; Setlik et al,
2009). MPH abuse is correlated with the use of other stimulants, including cocaine and
3,4-methylenedioxymethamphetamine (MDMA) (McCabe et al, 2005; Yacoubian, Jr.,
2003), and thus evaluations of the neurobiological consequences of MPH abuse are of
critical importance.

It is generally accepted that the initial stimulating and reinforcing effects of
psychostimulants are the result of increases in extracellular DA in brain areas such as the
NAc (Wise, 1987); (Wu et al, 2001), and that dopaminergic adaptations in response to
chronic drug administration play a role in the addictive process. MPH is a
psychostimulant that binds to and inhibits the activity of DA (DAT) and norepinephrine (NE) transporters (NET), leading to increased extracellular levels of these catecholamines (Gatley et al, 1996b; Kuczenski et al, 1997). MPH increases DA levels in a manner similar to cocaine (Volkow et al, 1999), is readily self-administered by animals (Kollins et al, 2001), and can elicit psychomotor sensitization following repeated exposure (Guerriero et al, 2006). Although MPH has fairly high affinity for the DAT and NET (84 nM and 510 nM, respectively), it has low affinity for the serotonin transporter (SERT) (~50,000 nM) (Gatley et al, 1996a). In addition, MPH has been shown to bind as an agonist to the 5-HT1A receptor (Markowitz et al, 2009), making its neurobiological effects different from that of amphetamine or cocaine. Although the effects of chronic MPH on DA and NE systems have been examined, the effects on 5-HT systems remain unclear.

The DA and 5-HT systems have extensive interactions, particularly in the NAc and VTA, brain areas linked with the locomotor stimulating and rewarding effects of many drugs of abuse (Ikemoto, 2002). The VTA in particular receives strong serotonergic projections from 5-HT cell bodies in the raphe nuclei, and 5-HT receptors on DA and GABA cell bodies in this region modulate dopaminergic output to the NAc (14-16). In addition, the DA system provides extensive reciprocal projections to the 5-HT system, where D2 receptors on raphe neurons modulate 5-HT activity (Aman et al, 2007). Thus, although MPH may have no direct effect on 5-HT systems, it is possible for chronic MPH exposure to impact DA/5-HT system interactions.

In the present study, we utilized locomotor activity, radioligand binding, conditioned place preference (CPP), and dual probe in vivo microdialysis to demonstrate
that chronic MPH (20 mg/kg, i.p., once daily for 14 days) can have profound effects on the 5-HT system. Our findings show that chronic, high-dose MPH exposure causes serotonergic drugs to elicit DA release in the NAc, and increases the rewarding properties of psychostimulants and 5-HT selective drugs. Additionally, we have identified the mechanism of this change as supersensitivity of the 5-HT1B receptor in the VTA.

Materials and Methods

Animals. Equal numbers of male and female C57BL/6J mice between 2 and 4 months of age were treated with 20 mg/kg MPH or saline in a volume of 0.1mL i.p. once per day for 14 days prior to testing, which took place 48 hours after the final MPH injection. Animals were group housed with food and water provided ad libitum. Animal care was in accordance with Wake Forest University’s Institutional Animal Care and Use Committee and in compliance with National Institutes of Health guidelines.

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Locomotor Activity. Activity was assessed using Med Associates Open Field Activity Monitors (Med Associated, St. Albans, VT). The open field consisted of a square plexiglass container (27.0 cm X 27.0 cm X 20.3 cm) with three 16-beam I/R arrays. Analyses were conducted during the light phase (9 am - 5 pm). Following a 2-hour period when mice were allowed to habituate to the chambers, animals received either cocaine (10 mg/kg), MDMA (1-20 mg/kg), fluoxetine (1 - 15 mg/kg), RU 24969 (0.3-3.0 mg/kg),
or saline, and data was collected in 5–min bins for a period of two hours following injection. Data was analyzed for distance traveled in centimeters (cm).

**Conditioned Place Preference.** The CPP apparatus consisted of two chambers (13 cm x 13 cm x 20.3 cm, Med Associated, St. Albans, VT) connected with a guillotine door, and tests were conducted in an unbiased design. During the preconditioning phase (day 1), mice were allowed access for 20 min to both chambers to assess possible bias. During the conditioning phase (days 2-4), mice received an i.p. dose of either MDMA (5.0 mg/kg), fluoxetine (5.0 or 15.0 mg/kg), RU 24969 (0.3 or 1.0 mg/kg), or saline in a volume of 0.1 mL. Mice were returned to the home cage for 20 minutes, and then confined to one chamber of the apparatus for 20 min. Mice were then returned to their home cage for 24 h, and given an injection of either drug or saline, whichever they have not yet received, and placed in the opposite chamber. Pairing was randomized across groups. On day 5, mice were placed in the apparatus and the guillotine door was opened. Side of entry was randomized across groups during the testing process. CPP was assessed by the amount of time spent in each chamber over a 20-min observation period.

**Radioligand Binding to the Dopamine Transporter, Serotonin Transporter, D2-Dopamine Receptor, and 5HT1B Receptor.** For determination of dopamine transporter (DAT), serotonin transporter (SERT), and D2-dopamine receptor binding sites, $^3$H-mazindol, $^3$H-paroxetine, and $^3$H-YM-09151-2 were used. Briefly, tissue samples were homogenized in 50mM Tris-HCl containing 150 mM NaCl and 5mM KCl with a glass mortar using a Wheaton motorized tissue grinder and a Teflon pestle. Homogenates were
centrifuged at 48,000 x g for 10 minutes and the supernatant discarded. The pellet was resuspended in the same buffer by homogenization and washed twice more by centrifugation to yield crude membrane preparations.

DAT levels were determined in crude striatal membranes by binding of the specific antagonist $^3$H-mazindol as previously described (Caudle et al., 2005) using a single concentration of 10 nM for 1 hr at 4°C. Non-specific binding was determined by the inclusion of 10µM nomifensine. SERT levels were determined by binding of the specific antagonist $^3$H-paroxetine as previously described (Caudle et al., 2006) using a single concentration of 10 nM for 1 hr at 25°C. Non-specific binding was determined by the inclusion of 10 µM fluoxetine. D2-like dopamine receptor levels were determined by binding of the specific antagonist $^3$H-YM-09151-2 essentially as described by Niznik and co-workers (1985), with modifications to reduce the assay volume to 200 µl. Binding assays were conducted with a single concentration of 1 nM for 1 hr at 25°C. Non-specific binding was determined by the inclusion of 10 µM eticlopride. 5HT1B receptor levels were determined by binding of the specific antagonist $^3$H-GR125743 essentially as described by Scott et al., 2006, with modifications to reduce the assay volume to 200 µl. Binding assays were conducted with a single concentration of 1 nM for 1 hr at 25°C. Non-specific binding was determined by the inclusion of 10 µM 5-HT. For all binding assays, incubations were terminated by rapid vacuum filtration onto GF/B filter plates pre-soaked with 0.1-0.5% polyethylenimine and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the total binding (no unlabeled antagonist included) minus non-specific binding (unlabeled antagonist included), and expressed as fmol/mg protein.
**Microdialysis and HPLC.** 24 h after the final drug or saline injection, mice were anesthetized with ketamine/xylazine (100 mg/kg and 13 mg/kg i.p., respectively), and a microdialysis guide cannula (CMA/7 guide cannula; CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically implanted into the NAc (anterior, +1.4 mm, lateral, +1.0 mm, ventral -4.0 mm, relative to bregma and dura surface). Concentric microdialysis probes (membrane length 2 mm, CMA/7, CMA/Microdialysis AB, Stockholm, Sweden) were implanted while animals were recovering from surgery. Experiments were conducted in freely moving mice 18-24 h following implantation of the probe. Probes were perfused with artificial cerebrospinal fluid (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂, at pH 7.4) at a constant flow rate of 0.8 μl/min. Samples were collected every 20 min and analyzed for DA, 5-HT and metabolites by high-performance liquid chromatography coupled to electrochemical detection. Samples were separated on a Luna 3-μm C18(2) column (50 x 2 mm, Phenomenex, Torrence, CA) using a mobile phase containing 90 mM NaH₂PO₄, 50 mM Citric acid, 1.7 mM 1-cctanesulfonic acid sodium salt (OSA, from Acros, Geel, Belgium), triethylamine, 50 M EDTA, and 10% (±5%) acetonitrile in ultra pure water (mobile phase formula from ESA Analytical, Chelmsford, MA). Microdialysis data were calculated as the percentage change from baseline concentration, with 100% being defined as the average of the last three samples prior to injection of drug. Cocaine (10.0 mg/kg i.p.), MDMA (5.0 mg/kg, i.p.), fluoxetine (15 mg/kg, i.p.), or RU 24969 (1.0 mg/kg, i.p.) was then administered and samples collected every 20 min for 2 h following drug administration.
For experiments involving dual probe microdialysis, surgeries were performed as described above, and microdialysis guide cannulae were stereotaxically implanted into the NAc and VTA (anterior, -3.0 mm, lateral, +0.6 mm, ventral, -4.0 mm, relative to bregma and dura surface). Following collection of a stable baseline, concentrations of 10, 50, and 100 M fluoxetine dissolved in aCSF were infused into the VTA for 80 min each, and samples were collected every 20 min.

**Histology.** Mice were anesthetized with ketamine (100 mg/kg) and xylazine (13 mg/kg) and 0.5% Chicago Blue Dye was perfused through the microdialysis probes for ~5 min. Mice were perfused transcardially with 10 ml of 10 % formalin (pH 7.0; Sigma, St. Louis, MO), and brains were removed and cryoprotected in 30% sucrose solution (in 0.01 M phosphate buffer, pH 7.4). Sections were then sliced (40-µm sections) through the NAc and VTA, to identify microdialysis probe locations.

**Statistical Analyses.** The effect of cocaine, MDMA, fluoxetine, and RU 24969 on extracellular concentrations of DA and 5-HT in the NAc and VTA was assessed by two-way analysis of variance (ANOVA) for repeated measures with a Bonferroni post-hoc analysis where appropriate, with MPH treatment as the between-subject factor and time as the within-subject factor. Receptor binding data were analyzed by two-tailed t-test. Locomotor data were analyzed for distance traveled and vertical rears over 5 min bins. In the case of locomotor dose response curves, data were analyzed by a two-way ANOVA with a Bonferroni post-hoc test where appropriate. For CPP studies, t-tests were performed for analysis of difference between saline and MPH-treated animals, with
further t-tests performed to determine significant difference from unpaired side following conditioning. Values of p<0.05 were considered statistically significant.

Results

MPH treatment decreased DATs and D2 receptors in the striatum, increased 5-HT1B receptors in the midbrain. DAT, SERT, and D2 receptor levels in the striatum of MPH- and saline-treated animals were measured using \(^3\)H-mazindol, \(^3\)H-paroxetine, and \(^3\)H-YM-09151-2 binding, respectively. Measures of radioligand binding in MPH-treated animals showed a significant 50% decrease in DAT levels in the striatum following chronic MPH treatment (p<0.01, Table 1), and a similar significant decrease in D2 receptor binding (p<0.01, Table 1), with no difference in SERT binding detected in the striatum (p>0.05, Table 1). Further studies using \(^3\)H-GR125743 showed that 5-HT1B receptor binding was significantly increased by 20% in the ventral midbrain (p<0.001, Table 1) after chronic MPH.

Locomotor cross-sensitization to cocaine following MPH treatment We used locomotor activity measures to assess behavioral responses to cocaine during the first 48 hours of withdrawal from MPH treatment. Following habituation, animals were given either saline (0.1 mL) or cocaine (10 mg/kg i.p.), and locomotor activity was recorded for two hours. In accordance with previous locomotor studies (Brandon et al, 2001; Guerriero et al, 2006), mice treated with MPH showed no difference from saline-treated mice in locomotor responses to saline, but showed enhanced locomotor activation
following an injection of 10 mg/kg cocaine (p<0.05 effect of MPH treatment, Figure 1A).

**MPH treatment produced an augmented 5-HT response to cocaine, with no change in DA** In order to look at possible neurochemical alterations in response to chronic MPH administration, we conducted single probe *in vivo* microdialysis studies in the NAc of MPH- and saline-treated mice. Following collection of a stable

### Table 1: DAT, D2, SERT, and 5-HT1B Binding

<table>
<thead>
<tr>
<th>Binding (fmol/mg protein)</th>
<th>Saline Mean</th>
<th>SEM</th>
<th>n</th>
<th>MPH Mean</th>
<th>SEM</th>
<th>n</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT (3H-Mazindol)</td>
<td>600</td>
<td>40.42</td>
<td>5</td>
<td>294.5</td>
<td>43.23</td>
<td>4</td>
<td>**p&lt;0.01</td>
</tr>
<tr>
<td>D2 (3H-YM-09151-2)</td>
<td>259.7</td>
<td>14.83</td>
<td>5</td>
<td>152.3</td>
<td>23.11</td>
<td>4</td>
<td>**p&lt;0.01</td>
</tr>
<tr>
<td>SERT (3H-paroxetine)</td>
<td>384.2</td>
<td>17.04</td>
<td>5</td>
<td>369.4</td>
<td>10.48</td>
<td>4</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>5-HT1B (3H-GR125743)</td>
<td>409.7</td>
<td>1.764</td>
<td>3</td>
<td>496.3</td>
<td>6.438</td>
<td>3</td>
<td>***p&lt;0.001</td>
</tr>
</tbody>
</table>
Table 1: Effects of chronic MPH on DAT, D2, and SERT levels in the striatum, and on 5-HT1B receptor levels in the ventral midbrain. **DAT:** Radioligand binding results for DAT levels in the striatum of MPH- and saline-treated mice, expressed as 3H-Mazindol binding (fmol/mg protein). MPH-treated animals showed significant decreases in DAT binding ($t=5.134$, $p<0.01$ effect of MPH treatment on DAT levels, $n=4-5$ per group). **D2:** Radioligand binding results for D2 levels in the striatum of MPH- and saline-treated mice, expressed as 3H-YM-09151-2 binding (fmol/mg protein). MPH-treated animals showed significant decreases in D2 binding ($t=4.076$, $p<0.01$ effect of MPH treatment on D2 binding, $n=4-5$ per group). **SERT:** Radioligand binding results for SERT levels in the striatum of MPH- and saline-treated mice, expressed as 3H-paroxetine binding (fmol/mg protein). No differences between MPH- and saline-treated animals ($t=0.6918$, $p>0.05$, $n=4-5$ per group). **5-HT1B:** Radioligand binding results for 5-HT1B levels in the ventral midbrain of MPH- and saline-treated mice, expressed as 3H-
GR125743 binding (fmol/mg protein. There was a significant (21%, t=12.99, p<0.001) increase in 5-HT1B receptor binding in the ventral midbrain following MPH treatment (n=3 per group).
Figure 1

A

Distance traveled (cm/20 min)

saline

MPH

saline

10 mg/kg Cocaine

***

B

NAc % baseline [DA]

saline

MPH

10 mg/kg cocaine

C

NAc % baseline [5-HT]

10 mg/kg cocaine

**

**
Figure 1: Effects of chronic MPH on behavioral and neurochemical responses to cocaine

A: Locomotor results over the first 30 minutes following i.p. injections of 0.1 mL saline or 10.0 mg/kg cocaine (n=6 per group for 0.1 mL saline, n=24 per group for 10 mg/kg cocaine). There was no significant effect of MPH treatment on locomotor responses to saline (t=0.7688, p>0.05), but MPH treatment produced significant effects on locomotor responses to 10.0 mg/kg cocaine (t=3.500, p<0.01).

B: Single probe in vivo microdialysis measurements of DA in the NAc of MPH and saline-treated animals following 10 mg/kg i.p. cocaine (n=8 per group). There was a significant effect of cocaine injection (F9,135=9.484, p<0.001), but no significant effects of MPH treatment on DA response to cocaine (F1,135=0.3168, p=0.5744).

C: Single probe in vivo microdialysis measurement of 5-HT in the NAc of MPH and saline-treated animals following 10 mg/kg i.p. cocaine (n=6 per group). There was a significant effect of cocaine injection (F9,98=4.179, p<0.001), a significant effect of MPH treatment on 5-HT response (F1,98=17.30, p<0.001), and a significant interaction between MPH treatment and cocaine injection was also observed (F9,98=2.326, p<0.05, **p<0.01).
baseline, animals were injected with 10 mg/kg cocaine, i.p., and samples were collected for two hours. Although both groups showed significant DA responses to i.p. cocaine, MPH-treated animals did not show an augmented DA response (p>0.05, **Figure 1B**). Rather, cocaine-induced 5-HT release in the NAc was significantly enhanced in MPH-treated animals (p<0.001, **Figure 1C**).

**Sensitization of locomotor activity and CPP for MDMA following MPH treatment**

Following work with cocaine-stimulated locomotor activity, we wished to examine the effects of chronic MPH on subsequent exposure to a psychostimulant with a particularly strong serotonergic component. Accordingly, we performed a locomotor dose response curve for the psychostimulant MDMA. As expected, MPH-treated animals showed cross-sensitization to the locomotor stimulating effects of MDMA (p<0.001 effect of MPH treatment, **Figure 2A**). In addition, we measured CPP with a threshold dose of MDMA (5.0 mg/kg, i.p.), which does not produce CPP in control animals (Daza-Losada et al, 2007), to allow us to look for changes in the rewarding properties of the drug. MPH-treated animals showed significant preference for the environment paired with low dose MDMA (p<0.05, **Figure 2B**), while control mice did not.

**MPH treatment produced augmented 5-HT responses to MDMA, with no change in DA**

In results similar to those found with the administration of cocaine, there was no significant difference between the NAc DA response of saline and MPH-treated animals in response to 5.0 mg/kg i.p. MDMA (p>0.05, **Figure 2C**). However, there was a
Figure 2

A. Distance traveled (cm/25-75 min) vs MDMA (mg/kg) with saline and MPH.

B. Time on paired vs unpaired side (s) for Saline and MPH.

C. NAc % baseline [DA] vs time (min) with 5 mg/kg MDMA.

D. NAc % baseline [5-HT] vs time (min) with 5 mg/kg MDMA.
Figure 2: Effects of chronic MPH on behavioral and neurochemical responses to MDMA: A: Full locomotor dose-response curve for i.p. MDMA between 25 and 75 minutes post drug injection is shown (n=12 per group). A significant effect of MDMA dose across all groups (F_{5,127}=239.5, p<0.001), and a significant effect of MPH treatment on locomotor response to MDMA was observed (F_{1,127}=5.354, p<0.05), as well as a significant interaction between MPH treatment and MDMA dose (F_{5,127}=2.711, *p<0.05).

B: CPP results for 5.0 mg/kg MDMA, i.p., shown as difference in time spent in paired chamber compared to bias testing (n=10 saline, 12 MPH-treated animals). Saline-treated animals showed no significant chamber bias following four days of training, while MPH-treated mice showed significant bias for the drug paired chamber (t=2.143, *p<0.05).

C: Single probe *in vivo* microdialysis measurement of DA in the NAc of MPH and saline-treated animals following 5.0 mg/kg i.p. MDMA (n=5 per group). There was a significant effect of MDMA injection (F_{9,79}=12.39, p<0.001), and a significant effect of MPH treatment (F_{1,79}=6.990, p<0.01).

D: Single probe *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline-treated animals following 5.0 mg/kg i.p. MDMA (n=6 saline, 8 MPH-treated animals). There was a significant effect of MDMA injection (F_{9,118}=9.786, p<0.001), and a significant effect of MPH treatment on 5-HT response to MDMA (F_{1,118}=16.03, p<0.001), **p<0.01, *p<0.05 effect of MPH treatment on MDMA response.
significant augmentation in 5-HT response to MDMA in MPH-treated animals (p<0.01 effect of MPH treatment on 5-HT response, Figure 2D).

**MPH treatment produced rewarding effects of an SSRI** In saline-treated animals, increasing doses of fluoxetine resulted in a significant decrease in distance traveled (p<0.05, Figure 3A), while MPH-treated animals showed no decreases in locomotor activity, resulting in greater locomotor activity compared to saline-treated controls (p<0.05, Figure 3A), and indicating alterations in the locomotor effects of fluoxetine in these mice.

We also used CPP to assess whether aversive doses of fluoxetine would become preferred following chronic MPH treatment. MPH-treated animals showed no significant preference or aversion to the highest dose of 15 mg/kg i.p. fluoxetine, while saline-treated animals showed significant aversion as expected (p<0.01, Figure 3B). In response to a lower dose of fluoxetine, 5.0 mg/kg, i.p., MPH-treated mice showed significant CPP, while saline-treated mice showed no preference or aversion (p<0.05 effect of MPH treatment, Figure 3B), implying possible rewarding effects of low-dose fluoxetine in MPH-treated mice.

**Fluoxetine increased NAc DA following MPH treatment** Following work with locomotor activity and CPP, we chose to examine DA responses to 15mg/kg i.p. fluoxetine measured by microdialysis. Although this dose did not result in place preference in MPH treated mice, 15 mg/kg fluoxetine produced clear differences in locomotor and place preference paradigms, and thus might result in important differences
Figure 3

A

B

C

D

Figure 3
Figure 3: Effects of chronic MPH on behavioral and neurochemical measures following administration of an SSRI

A: Locomotor results (n=12 per group) over the first 20 minutes following i.p. fluoxetine injection. There was a significant effect of MPH treatment on overall locomotor responses to fluoxetine (F_{1,108}=5.080, p<0.05).

B: CPP results for 5.0 mg/kg and 15.0 mg/kg fluoxetine (n=12 per group). Saline-treated animals show significant aversion at 15 mg/kg (**p<0.01 compared to pre-conditioning), with no difference from baseline at 5.0 mg/kg. MPH-treated animals show no difference from baseline measures at 15.0 mg/kg (difference from saline-treated control, #p<0.05), and significant increase in time spent in the drug-paired chamber at 5.0 mg/kg (*p<0.05 compared to pre-conditioning, #p<0.05 compared to saline-treated control).

C: In vivo microdialysis in the NAc showing DA response following 15.0 mg/kg fluoxetine i.p. (n=8 per group). There was a significant effect of MPH treatment (F_{1,129}=21.05, p<0.001), and an interaction between MPH treatment and acute fluoxetine (F_{9,129}=2.527, p<0.05). Significant differences between MPH- and saline-treated animals were observed at 20 (t=3.833, p<0.05) and 40 (t=3.496, p<0.05 by Bonferroni post-hoc analysis) minutes post-injection.

D: In vivo microdialysis in the NAc showing 5-HT response following 15.0 mg/kg fluoxetine i.p. (n=9 per group). There was significant effect of fluoxetine observed (F_{9,117}=5.240 effect of fluoxetine, p<0.001), but no difference between saline and MPH-treated animals (F_{1,117}=0.4947, p>0.05).
in neurochemistry. Indeed, in response to 15 mg/kg fluoxetine, saline-treated mice showed a significant decrease in DA from baseline values (p<0.001, Figure 3C), while MPH-treated animals showed no significant effect of i.p. fluoxetine on DA in the NAc (p<0.001 effect of MPH treatment, Figure 3C). Levels of 5-HT in the NAc were also measured in both MPH-treated and saline-treated mice. Though both treatments showed a significant effect of fluoxetine (p<0.001, Figure 3D), there was no difference between saline and MPH treatment.

**Effects of fluoxetine on NAc DA can be localized to the VTA.** The DA and 5-HT systems have many points of interaction, but we hypothesized that 5-HT changes would be localized to the area of the VTA, an area rich in 5-HT innervation, which contains DA cell bodies that project to areas like the NAc. To determine the location of the effects of fluoxetine on DA in MPH-treated mice, we conducted dual probe microdialysis studies in the NAc and VTA. Following collection of a stable baseline, fluoxetine was infused in increasing concentrations (10, 50, and 100uM) into the VTA. While saline-treated animals showed a significant decrease in DA levels in the NAc following infusion of fluoxetine into the VTA, MPH-treated mice exhibited significant increases in DA levels (p<0.001 effect of MPH treatment, Figure 4A). Levels of 5-HT in the NAc were also monitored for changes following infusion of fluoxetine into the VTA. Although both groups of animals showed a significant decrease in 5-HT in the NAc in response to fluoxetine infusion in the VTA (p<0.001, Figure 4B), there was no difference between MPH and saline-treated mice.
Figure 4

A

VTA Infusion: Fluoxetine

NAc % baseline [DA]

time (min)

B

VTA Infusion: Fluoxetine

NAc % baseline [5-HT]

time (min)

C

VTA Infusion: Fluoxetine

VTA % baseline [DA]

time (min)

D

VTA Infusion: Fluoxetine

VTA % baseline [5-HT]

time (min)
Figure 4: Effects of chronic MPH on fluoxetine infused into the VTA

A: Effects of fluoxetine (10, 50, 100μM in aCSF) infused into the VTA on DA measured in the NAc (n=9 per group). There was a significant effect of MPH treatment (F_{1,168}=49.67, p<0.001), and a significant interaction between MPH and fluoxetine infusion (F_{12,168}=2.389, p<0.01). *p<0.05, **p<0.01, ***p<0.001 compared to saline-treated animals.

B: Effects of fluoxetine (10, 50, 100μM in aCSF) infused into the VTA on 5-HT measured in the NAc (n=7 per group). There was a significant effect of fluoxetine on 5-HT levels in the NAc (F_{12,201}=4.910, p<0.001), but no difference between MPH and saline-treated animals.

C: Effects of fluoxetine (10, 50, 100μM in aCSF) infused into the VTA on DA levels measured in the VTA (n=5 saline-treated, 6 MPH-treated). There was a significant effect of fluoxetine infusion on DA levels in the VTA (F_{12,113}=3.992, p<0.001), as well as a significant overall effect of MPH treatment on DA response to fluoxetine infusion (F_{1,113}=4.447, p<0.05).

D: Effects of fluoxetine infused (10, 50, 100μM) into the VTA on 5-HT levels measured in the VTA (n=7 saline-treated, 5 MPH-treated animals). There was a significant effect of fluoxetine infusion on 5-HT levels in the VTA (F_{12,137}=8.628, p<0.001), and a significant effect of MPH treatment on overall 5-HT response to fluoxetine (F_{1,137}=22.67, p<0.001), as well as a significant interaction between MPH treatment and fluoxetine infusion (F_{12,136}=2.789, p<0.01). **p<0.01, *p<0.05 difference from saline-treated control.
To confirm that 5-HT is acting in the VTA to produce the changes seen in the NAc, we also collected and analyzed samples from the VTA. There was a significant overall effect of fluoxetine, and a significant difference between MPH and saline-treated animals. MPH-treated animals showed larger decreases in DA in response to fluoxetine (p<0.05, Figure 4C). Extracellular 5-HT in the VTA showed increases consistent with the infusion of an SSRI, and supersensitive responses in MPH-treated animals, with a significant effect of fluoxetine on 5-HT in the VTA, and an augmented increase in MPH-treated animals (p<0.001 effect of MPH treatment, Figure 4D).

**MPH treatment produced rewarding effects of a 5-HT1B agonist** Following chronic MPH treatment, 5-HT1B receptor density in the ventral midbrain is significantly increased (p<0.01, Table 1), implying a possible increase in the influence of 5-HT1B receptors over mesolimbic DA activity. Accordingly, we conducted a locomotor dose-response curve for 5-HT1B agonist RU 24969. In the first 60 minutes following administration of drug, MPH-treated animals showed a significant augmentation in locomotor activity, specifically at the 1.0 mg/kg dose (p<0.05 effect of MPH treatment, Figure 5A), a possible supersensitivity to the locomotor stimulating effects of RU 24969.

Following the demonstration of CPP for fluoxetine in MPH-treated animals, we conducted CPP studies for the 5-HT1B agonist RU 24969 (Figure 5B). While saline-treated animals showed significant aversion at both the 0.3 and 1.0 mg/kg dose, MPH-treated animals showed no aversion at 1.0 mg/kg, and significant preference at 0.3 mg/kg (p<0.05 Figure 5B).
5-HT1B agonists increased DA in the NAc of MPH-treated animals In previous studies, systemic administration of 5-HT1B agonists has been shown to increase basal and stimulated extracellular DA in the NAc at high doses (Guan et al, 1989). It was our hypothesis that sensitization of 5-HT1B receptors in MPH-treated mice would result in increases in DA in response to a 5-HT1B agonist at doses too low to affect DA in saline-treated animals. Indeed, at 1.0 mg/kg i.p. RU 24969, saline-treated mice showed small decreases in DA in the NAc, while MPH-treated animals showed significant increases, signifying a reversal of drug effect (p<0.001 effect of MPH treatment, Figure 5C).

5-HT in the NAc following i.p. injection of 1.0 mg/kg RU 24969 was also measured. It is known that systemic activation of 5-HT1B receptors will cause decreases in extracellular 5-HT by activating autoreceptors that inhibit 5-HT release (Sharp et al, 1989). Thus, it was our hypothesis that 5-HT1B receptor sensitization might result in an augmented decrease in 5-HT in MPH-treated animals. Accordingly, in vivo microdialysis studies in the NAc showed greater decreases in 5-HT levels in MPH-treated mice following i.p. RU 24969, with no decrease in 5-HT in saline-treated animals (p<0.001 effect of MPH treatment, Figure 5D).

Discussion

The results of this study show that two weeks of high-dose MPH treatment (20 mg/kg, i.p., 14 days) increased sensitivity to the stimulating and rewarding effects of MDMA, and transformed the aversive serotonin agonists, fluoxetine and RU24969, into rewarding drugs. The mechanism underlying this alteration appeared to be an increase in
Figure 5

A

B

C

D

-60 -40 -20 0 20 40 60 80 100 120 140

-60 -40 -20 0 20 40 60 80 100 120 140

-60 -40 -20 0 20 40 60 80 100 120 140

-60 -40 -20 0 20 40 60 80 100 120 140

Figure 5

RU 24969 (mg/kg)

0.3 1.0 3.0 5.0

distance traveled (cm/60min)

0 2000 4000 6000 8000 10000 12000

Distance traveled

0.3 mg/kg 1.0 mg/kg

RU24969 time (min)

Time in paired vs unpaired side (s)

time (min)

NAc % baseline [5-HT]

NAc % baseline [DA]
Figure 5: Effects of chronic MPH on responses to the 5-HT<sub>1B</sub> agonist RU 24969. A: Locomotor activity responses to RU 24969 over 60 minutes following i.p. injection of RU 24969 (0.3-3.0 mg/kg, i.p., n=12 per group). A significant effect of RU 24969 (F<sub>3,96</sub>=12.88, p<0.001), and a significant overall effect of MPH treatment was observed (F<sub>1,96</sub>=4.393, p<0.05). B: CPP results for 0.3 mg/kg and 1.0 mg/kg RU 24969 (n=12 per group). Saline-treated animals showed significant aversion at both the 0.3 and 1.0 mg/kg dose (**p<0.001 , *p<0.01 compared to pre-conditioning). MPH-treated animals show no difference from baseline measures at 1.0 mg/kg (p>0.05), and significant increase in time spent in the drug-paired chamber at 0.3 mg/kg RU 24969 (**p<0.01 compared to pre-conditioning, #p<0.05 compared to saline-treated control). C: Single probe In vivo microdialysis in the NAc showing DA responses following 1.0 mg/kg RU 24969, i.p. (n=7 saline, 8 MPH-treated mice). There was a significant effect of RU 24969 (F<sub>9,128</sub>=2.136, p<0.05), and a significant effect of MPH treatment (F<sub>1,128</sub>=12.28, p<0.001). **p<0.01 at 40 minutes post injection, *p<0.05 at 80 minutes post-injection difference from saline-treated control. D: Single probe in vivo microdialysis in the NAc showing 5-HT responses following 2.0 mg/kg RU 24969, i.p. (n=7 saline-treated, 11 MPH-treated). There was an overall significant effect of MPH treatment (F<sub>9,155</sub>=32.16, p<0.001), as well as a significant interaction between MPH treatment and RU 24969 (F<sub>1,155</sub>=2.849, p<0.05). **p<0.01 at 40 minutes post injection, *p<0.05 at 80 minutes post injection, ***p<0.001 at 100 minutes post injection.
5-HT1B receptors in the VTA, causing increased excitatory 5-HT influence over DA neuron activity.

Chronic psychostimulant exposure has been previously shown to result in decreased DA receptor and transporter number, reflecting adaptations to high levels of DA (Nader et al, 2006; Thanos et al, 2007), and decreases in D2 receptors have been shown to correlate with increased rewarding properties of psychostimulants in animals and to predict the reinforcing effects of stimulants in humans (Volkow et al, 2002). Thus, we hypothesized that chronic MPH treatment might produce changes in DAT and D2 receptors, and that these changes may correlate with alterations in the rewarding properties of drugs. Indeed, we found substantial decreases in DAT and D2 receptor levels in the striatum, reflecting a response to long-term MPH exposure. These changes in transporter and receptor levels might be expected to produce significant alterations in DA-related behaviors and neurochemistry following MPH exposure. With the two psychostimulants tested, cocaine and MDMA, chronic MPH administration produced significant locomotor cross-sensitization, and facilitated place preference for low-dose MDMA. This sensitization to MDMA was correlated with increased 5-HT, but not DA, release in the NAc. These results are consistent with the results seen with cocaine, indicating that stimulants with serotonergic components induce greater 5-HT responses following MPH treatment.

It was our hypothesis, based on results with other psychostimulants and previous work in knockout mice (Mateo et al, 2004), that chronic DAT blockade by MPH causes changes in DA/5-HT interactions such that 5-HT stimulation will increase DA in the NAc. As increases in NAc DA have been linked with changes in locomotor activity
(Christie et al, 1971), we hypothesized that drugs that increase 5-HT will elevate locomotor activity through a secondary DA mechanism in MPH-treated animals. MPH treatment eliminated the normal locomotor attenuation produced by fluoxetine at high doses, and reversed behavioral responses to fluoxetine from aversive to rewarding in the place conditioning paradigm. Systemically administered fluoxetine also had opposite effects on DA levels, increasing DA in the NAc of MPH-treated mice while decreasing DA in controls. There were, however, no differences in NAc 5-HT responses between groups, in contrast to the psychostimulant results. It appears that, in MPH-treated mice, the combined increase in several monoamine neurotransmitters induced by stimulants elicits a greater 5-HT response than 5-HT transporter inhibition alone. These results demonstrate that robust fluoxetine-induced decreases in DA levels and consequent fluoxetine-induced hypoactivity and aversion can be reversed with repeated, intermittent MPH treatment. Our results confirm that this is mediated via an altered 5-HT control of DA systems. It was our prediction that this effect occurred via 5-HT receptor alterations in the VTA, in accordance with similar findings in DAT knockout animals (Mateo et al, 2004). We therefore performed dual probe in vivo microdialysis in the VTA and NAc.

Microdialysis results showed that the locus of action for 5-HT system changes in MPH-treated mice is the VTA, a result predicted from the extensive 5-HT innervation of this area. When we infused fluoxetine into the VTA, MPH-treated mice showed robust increases in NAc DA release, suggesting that 5-HT elevations in the VTA activate receptors that lead to DA neuron excitation and increased DA release in terminal fields. We then sought to determine 5-HT receptor changes that could underlie the altered DA response. The 5-HT1B receptor is highly expressed in the mesolimbic DA system (Pazos
et al, 1985), and 5-HT1B receptor agonists are known to increase basal and stimulated extracellular DA in the NAc (Guan et al, 1989); (O'Dell et al, 2004). The 5-HT1B receptor was also a likely candidate because this receptor enhances the effects of psychostimulant administration (Parsons et al, 1998), is increased in response to chronic cocaine administration (Hoplight et al, 2007), and increases locomotor activity and NAc DA in control animals in a dose-dependent manner (Cervo et al, 2002; Yan et al, 2001). The location of 5-HT1B receptors in the VTA on terminals of GABA neurons, promoting DA cell firing through inhibition of GABA release, places the 5-HT1B receptor in an ideal position to mediate the present altered 5-HT effects (Cameron et al, 1994). We found that locomotor activating responses to the 5-HT1B agonist were augmented following chronic MPH, and MPH-treated animals showed a reversal of hedonic effects of the drug, displaying preference rather than aversion in place conditioning. Additionally, RU 24969 injection increased DA release in the NAc of MPH-treated animals. These behavioral and neurochemical findings were accompanied by a significant increase in 5-HT1B receptor binding levels in the ventral midbrain. It appears that results with the 5-HT1B agonist largely account for the behavioral and neurochemical effects of fluoxetine in MPH-treated animals, suggesting that changes in DA/5-HT interactions following MPH treatment are due to changes in the 5-HT1B receptor.

In light of studies in the literature and the results seen here, we propose a theoretical framework that could explain the effects of chronic MPH on interactions of dopaminergic and serotonergic systems. We hypothesize that the increased extracellular levels of DA induced by chronic MPH treatment will result in desensitization of DA receptors, in particular the D2 receptor. D2 receptors are present on dendrites of 5-HT
neurons in the dorsal raphe, and stimulation of these receptors promotes 5-HT neuron firing (Aman et al, 2007). In addition, MPH may activate 5-HT1A receptors located presynaptically on 5-HT cell bodies (Casanovas et al, 2000; Collu et al, 1997; Yoshimoto et al, 1992). During treatment with chronic MPH, stimulation of the 5-HT1A receptor accompanied by the decrease of D2 receptors found here could decrease 5-HT neuron firing following MPH. This hyposerotonergic state might then lead to a compensatory increase in 5-HT receptors in terminal regions, such as the 5-HT1B receptors seen in MPH-treated mice. In the VTA, 5-HT1B receptors are localized presynaptically on GABA terminals, where their stimulation acts to inhibit GABA release (Cameron et al, 1994; Hallbus et al, 1997; Yan et al, 2001). When these sensitized 5-HT receptors are stimulated, GABA release is inhibited, resulting in greater DA neuron activity in the VTA and increased DA release in the NAc (Cervo et al, 2002; Parsons et al, 1999). These changes would switch the relative balance of all 5-HT receptors in the VTA, inhibitory and excitatory, from DA neuron inhibition to excitation. This hypothetical mechanism would account for the results seen in our studies, with decreases in D2 receptors, increases in 5-HT1B receptors, and reversal of DA responses to fluoxetine and the 5-HT1B agonist RU24969 from aversive/inhibitory to rewarding/excitatory.

It is interesting that administration of psychostimulants with both DA and 5-HT components, such as cocaine and MDMA, produced a robust potentiation of the 5-HT response in MPH-treated mice, while the 5-HT system specific drugs produced only a sensitized dopamine response. With regard to the responses to psychostimulants, dopaminergic sensitization following previous psychostimulant exposure has been shown to be mismatched in time, with locomotor sensitization developing prior to enhanced DA
responses in the NAc (Ito et al, 2002); (Kalivas et al, 1991; Robinson et al, 1988; Robinson et al, 1993); (Murphy et al, 2001). Thus, the lack of DA sensitization seen in response to psychostimulants in MPH treated mice may be time dependent. This would not account, however, for the changes in DA signaling in response to fluoxetine in MPH-treated animals. We hypothesize that, in this case, the auto-inhibitory effects of DA system stimulation due to psychostimulant exposure may override the effects of 5-HT system stimulation in the VTA. Previous studies have shown that psychostimulant administration decreases DA cell firing in the VTA (Chen et al, 1996), and this inhibition could overcome the stimulatory effect of 5-HT on DA cell firing following MPH exposure.

The potentiation of the 5-HT response in MPH-treated mice in response to subsequent psychostimulant exposure is also an interesting phenomenon, as this effect is not accompanied by increases in SERT binding. However, the potentiated 5-HT response to psychostimulants is strongly reflected in the dual probe studies infusing fluoxetine into the VTA, where infusion of fluoxetine produced a significant potentiation of 5-HT response in the VTA, although this effect is not apparently in studies using i.p. injections of fluoxetine. It is possible that the differences between i.p. administration of fluoxetine and VTA infusions are the result of differences in global brain 5-HT elevations versus localized elevations.

It should be noted that infusion of fluoxetine into the VTA decreased extracellular 5-HT levels in both MPH and saline treated mice. This is the first measurement of the effects of VTA-infused fluoxetine on 5-HT levels in the NAc. It would not necessarily be expected, due to the limited region of the infusion, that fluoxetine infused into the
VTA would increase extracellular 5-HT levels in the NAc, so the dose-dependent decrease in extracellular 5-HT in the NAc is somewhat surprising. It is possible that local increases in DA in the NAc may account for the decreases in 5-HT release in this area. While it has been established in previous studies that D2 receptors localized to raphe cell bodies can stimulate 5-HT neuron firing and local 5-HT release (Aman et al, 2007) (Ferre et al, 1993), D2 receptors located on 5-HT terminals appear to have opposite effects. D2 antagonists have been shown to cause local increases in 5-HT release (Nakazato et al, 1998), while local infusions of D2 agonists decrease 5-HT release (Thorre et al, 1998). Thus, it is possible that increases in DA in the NAc, as induced by fluoxetine infusion, could stimulate D2 receptors on 5-HT terminals in this area, reducing 5-HT release in the NAc, though further studies are requires to test this hypothesis.

While the current studies have focused on DA and 5-HT systems, NE is also an important component of MPH actions. MPH is a potent NET inhibitor (Kuczenski et al, 1997), and the effect of MPH on NE is thought to be a major player in the therapeutic effects of MPH on attention in humans (Kuwahata et al, 2002) and animals (Drouin et al, 2006). Further studies will be necessary to determine what changes there are in NE systems following chronic MPH treatment in mice.

Because human abuse rates of prescription psychostimulants are increasing (Barrett et al, 2005; Setlik et al, 2009), we specifically wanted to examine the effects of high doses. The chronically administered dose of MPH (20 mg/kg i.p) was chosen to mimic abused doses in humans, which are between 2 and 10 times the normal prescribed doses (2). The 20 mg/kg dose in mice is known to produce locomotor activation equivalent to that of 2.0 mg/kg i.v., a concentration known to produce a subjective high
in humans (Gatley et al, 1999). Thus, the i.p. route of administration that used in mice results in pharmacokinetic profiles that resemble that observed with intranasal or intravenous self-administration in humans.

The results of this study could have profound implications for individuals engaged in the abuse of MPH, who number in the hundreds of thousands in the US alone, with significant numbers of college and university students affected (Teter et al, 2006). The present results suggest that such abuse may lead to abuse of other drugs, particularly psychostimulants and compounds with 5-HT activity, such as MDMA. Additionally, 5-HT system agonists, such as fluoxetine and other commonly used antidepressants, may show changes in their clinical effects reflecting an excitatory influence on the dopaminergic system, and thus may have different clinical and side-effect profiles in MPH-abusing individuals. In light of our current findings and the growing prevalence of prescription stimulant abuse, more research is needed to define the heretofore unrecognized serotonergic changes associated with MPH abuse.

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References


CHAPTER III

SENSITIZATION OF 5-HT1A and 1B RECEPTORS IN THE VENTRAL TEGMENTAL AREA FOLLOWING TREATMENT WITH CHRONIC METHYLPHENIDATE

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Abstract

Methylphenidate (MPH) is one of the most commonly prescribed stimulants among children and adults, and abuse rates of this drug are increasing. Previous studies have indicated that, following treatment with MPH at doses similar to those abused in humans, mice exhibit alterations in dopamine (DA) and serotonin (5-HT) system interactions, such that 5-HT system agonists elevate DA in the nucleus accumbens (NAc). Changes in the 5-HT control over DA neurons in the ventral tegmental area (VTA) appear to be critically important in this change. We have undertaken a behavioral and neurochemical survey of five 5-HT receptors known to affect DA cell firing in the VTA: 5-HT1A, 1B, 2A, 2C, and 3. MPH treated animals show a trend toward aversion for the 5-HT1A agonist 8-OH-DPAT in trast to previous trends toward preference, and appear to be more sensitive to the DA effects of the 5-HT1A agonist, showing elevations in DA in the NAc in response to i.p. administration, and substantial decreases in NAc DA when 8-OH-DPAT is infused into the VTA. MPH treated mice also showed strong potentiation of dopaminergic responses to 5-HT1B agonists, and a reversal of the rewarding effects of the drug, showing significant place preference for low doses, while saline treated controls showed significant aversion. Finally, we show that the increases in DA induced by fluoxetine challenge in MPH-treated mice can be reversed by co-administration of a 5-HT1B antagonist, indicating that the effects of fluoxetine on DA in MPH-treated mice are primarily mediated by alterations in this receptor. Taken together, these data indicated that the increased serotonergic control over the mesolimbic DA system following chronic MPH can be accounted for by increases in sensitivity of the 5-HT1A and 1B receptors.
Introduction

Methylphenidate (MPH) is one of the most common prescription stimulants on the market in the United States. It is prescribed to children and adults of all ages for treatment of attention-deficit/hyperactivity disorder (Biederman et al, 2002). Abuse rates of MPH and other prescriptions stimulants are increasing as prescription rates increase (Setlik et al, 2009), and these drugs are perceived as less risky than other psychostimulants (Arria et al, 2008). It is thus important to carefully evaluate the effects of chronic MPH exposure at both clinical and abused doses, to determine the behavioral and neurochemical effects of exposure, as well as the effects of MPH treatment on subsequent exposure to psychostimulants and other drugs of abuse.

A great deal of evidence indicates that the initial rewarding and reinforcing effects of psychostimulants such as MPH are the result of increases in extracellular DA in areas such as the nucleus accumbens (NAc) (Wise, 1987). MPH is known to inhibit the DA and norepinephrine transporters (DAT and NET, respectively), increasing extracellular monoamine levels (Gatley et al, 1996; Kuczenski et al, 1997) in a manner similar to that of cocaine (Volkow et al, 1999). While MPH has little affinity for the 5-HT transporter (SERT) (Gatley et al, 1996), the DA and 5-HT systems have extensive interactions allowing 5-HT to influence mesolimbic DA. Very few studies have been conducted to study the effects of MPH on DA/5-HT interactions and how changes in these interactions can affect subsequent drug responses. Previous results from our laboratory have shown that, following chronic MPH, animals showed potentiated locomotor and conditioned place preference responses for psychostimulants such as cocaine and MDMA, a
potentiation that occurred in conjunction with a sensitized 5-HT responses in the NAc following psychostimulant exposure. In addition, MPH treated mice showed conditioned place preference and dopaminergic elevations in the NAc in response to fluoxetine, a selective SERT inhibitor that is aversive, and decreases DA in normal mice (Brookshire, 2010). This reversal of effects seemed to be linked to increases in 5-HT1B levels in MPH-treated mice, coupled with increases in DA in response to 5-HT1B receptor agonists. These results emphasized the strong interactions between DA and 5-HT in the mesolimbic DA system, and suggested that chronic, high DA could result in altered 5-HT modulation of the DA system, which in turn produces altered responses to future drug administration.

Based on the previous results seen in MPH treated mice, we have devised a model that could account for the changes seen following chronic MPH exposure. Briefly, chronic high levels of DA in MPH treated mice could result in desensitization and decreases in D2 receptors, effects we have observed in previous studies (Brookshire, 2010). D2 receptors are known to stimulate 5-HT cell firing in the dorsal raphe (Aman et al, 2007), a region that projects to mesolimbic areas (Jacobs et al, 1992). With a decrease in D2 receptors in the raphe, a decrease in 5-HT cell firing and a hyposerotonergic system would be predicted. This would in turn result in sensitization of 5-HT receptors, such as the 5-HT1B receptor (Brookshire, 2010), which is located presynaptically on GABA neurons in the ventral tegmental area (VTA). When stimulated, 5-HT1B receptors inhibit release of GABA (Cameron et al, 1994; Hallbus et al, 1997; Yan et al, 2001) and disinhibit DA neuron firing (O'Dell et al, 2004). Thus, when these sensitized 5-HT receptors are stimulated following chronic MPH, they would be expected to inhibit
GABA release, resulting in disinhibition of DA neurons in the VTA and increased DA cell firing to areas such as the NAc (Cervo et al, 2002; Parsons et al, 1999) (Brookshire, 2010).

While the 5-HT1B receptor appears to be a promising candidate, it should be noted that there are other 5-HT receptors present in the mesolimbic DA system that could be affected by chronic MPH administration. Therefore, we investigated the 5-HT1A, 5-HT2A, 5-HT2C, and 5-HT3 receptors. The 5-HT1A receptor in particular is a good candidate for changes following MPH administration, as MPH is known to act as an agonist at this receptor (Markowitz et al, 2009). In addition, we performed dual probe microdialysis experiments with 5-HT1A and 5-HT1B receptor agonists to investigate the location of the changes seen in MPH treated animals. Finally, we examined the effects of a systemic 5-HT1B antagonist on the dopaminergic effects of VTA-infused fluoxetine in MPH treated mice, to confirm the involvement of 5-HT1B receptors in the altered DA/5-HT system interactions following MPH.

**Methods**

**Animals:** Equal numbers of male and female C57BL/6J mice between 2 and 4 months of age were treated with 20 mg/kg MPH or saline in a volume of 0.1mL i.p. once per day for 14 days prior to testing, which took place 48 hours after the final MPH injection. Animals were group housed with food and water provided ad libitum. Animal care was in accordance with Wake Forest University’s Institutional Animal Care and Use Committee and in compliance with National Institutes of Health guidelines.
**Locomotor Activity:** Activity was assessed using Med Associates Open Field Activity Monitors (Med Associated, St. Albans, VT). The open field consisted of a square plexiglass container (27.0 cm X 27.0 cm X 20.3 cm) with three 16-beam I/R (define) arrays. Analyses were conducted during the light phase 9 am - 5 pm. Following a 2-hour period when mice were allowed to habituate to the chambers, animals received either the 5-HT1A agonist 8-OH-DPAT (0.5-3.0 mg/kg), the 5-HT1B/1A agonist RU 24969 (0.3-3.0 mg/kg), the 5-HT2A/2C agonist DOI (0.125-2.0 mg/kg), the 5-HT2C agonist MK 212 (1.0-10.0 mg/kg), the 5-HT3 agonist SR 57227 (1.0-4.0 mg/kg), or saline in a 0.1mL injection i.p., and data was collected in 5–min bins for a period of two hours following injection. Data was analyzed for distance traveled in cm and number of vertical rears performed during the active period of the drug.

**Conditioned Place Preference:** The CPP apparatus consisted of two chambers (13 cm x 13 cm x 20.3 cm, Med Associated, St. Albans, VT) connected with a guillotine door, and tests were conducted in an unbiased design. During the preconditioning phase (day 1), mice were allowed access for 20 minutes to both chambers. During the conditioning phase (days 2-4), mice received an i.p. dose of RU 24969 (0.3 or 1.0 mg/kg), 8-OH-DPAT (1.0 mg/kg), or saline in a volume of 0.1 mL. Mice were returned to the home cage for 20 minutes, and then confined to one chamber of the apparatus for 20 minutes. Mice were then returned to their home cage for 24 hours, and then given an injection of either drug or saline, whichever they have not yet received, and placed in the opposite chamber. Pairing was randomized across groups. On day 5, mice were placed in the apparatus and the guillotine door was opened. Side of entry was randomized across
groups for both bias and testing. CPP was assessed by the amount of time spent in each chamber over a 20 min observation period.

**Microdialysis and HPLC:** 24 hours after the final drug or saline injection, mice were anesthetized with Ketamine/Xylazine (100mg/kg and 13 mg/kg i.p., respectively), and a microdialysis guide cannula (CMA/7 Guide Cannula; CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically implanted into the NAc (Anterior, +1.2 mm, Lateral, -0.6 mm, Ventral -4.0 mm, relative to bregma and dura surface). Concentric microdialysis probes (membrane length 2 mm, CMA/7, CMA/Microdialysis AB, Stockholm, Sweden) were implanted while animals were recovering from surgery. Experiments were conducted in freely moving mice 12 hours following implantation of the probe. Probes were perfused with artificial cerebrospinal fluid (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl2, 0.85 mM MgCl2, at pH 7.4) at a constant flow rate of 0.8 µl/min. Samples were collected every 20 min and analyzed for DA, 5-HT and metabolites by high-performance liquid chromatography coupled to electrochemical detection. Samples were separated on a Luna 3um C18(2) column (50 x 2 mm, Phenomenex, Torrence, CA) using a mobile phase containing 75 mM NaH2PO4, 1.7 mM 1-cctanesulfonic acid sodium salt (Acros, Geel, Belgium), triethylamine, 25 uM EDTA, and 10% (±5%) acetonitrile in ultra pure water (ESA Analytical, Chelmsford, MA). Microdialysis data were calculated as the percentage change from baseline concentration, with 100% being defined as the average of the last three samples prior to injection of drug. 8-OH-DPAT (2.0 mg/kg ) or RU 24969 (1.0 mg/kg) was then administered i.p. and samples collected every 20 minutes for two hours following drug administration.
For experiments involving dual probe microdialysis, surgeries were performed as described above, and microdialysis guide cannulae were stereotaxically implanted into the NAc (Anterior, +1.2 mm, Lateral, +1.0 mm, Ventral, -4.0 mm, relative to bregma and dura surface), and VTA (Anterior, -3.0 mm, Lateral, +0.6 mm, Ventral, -4.0 mm, relative to bregma and dura surface). Concentric microdialysis probes (NAc membrane length 2 mm, CMA/7, VTA membrane length 1 mm, CMA/7), were implanted while animals were recovering from surgery. Experiments were conducted in freely moving mice 12 hours following implantation of the probe in the manner described above. Following collection of a stable baseline, concentrations of 10, 30 and 100uM 8-OH-DPAT or 20, 40, and 80uM 5-HT1B agonist CP93129 were infused into the VTA for 80 minutes each, and samples were collected every 20 min and analyzed immediately for DA, 5-HT, and metabolites. In the case of work with the i.p. 5-HT1B antagonist GR 55562, 50uM fluoxetine was infused following collection of a stable baseline, and GR 55562 (5.0 mg/kg) was then administered i.p. concurrent with fluoxetine infusion. Samples were collected for two hours following i.p. administration.

**Histology:** Mice were anesthetized with ketamine (100 mg/kg) and xylazine (13 mg/kg) and 0.5% Chicago Blue Dye (Sigma-Aldrich Inc.) was perfused through the microdialysis probes for ~5 min or microinjected (250 nl) into the VTA. Rats were perfused transcardially with 10 ml of 10% formalin (pH 7.0; Sigma, St. Louis, MO), and brains were removed and cryoprotected in 30% sucrose solution (in 0.01 M phosphate buffer, pH 7.4). Sections were then sliced (40 µm sections) through the NAc and VTA, to identify microdialysis probe locations.
**Statistical Analyses:** The effect of 8-OH-DPAT, RU 24969, DOI, MK 212, and SR 57227, fluoxetine, and GR 55562 on extracellular concentrations of DA and 5-HT in the NAc and VTA was assessed by two-way analysis of variance (ANOVA) for repeated measures, with MPH treatment as the between-subject factor and time as the within-subject factor. Receptor binding data were analyzed by two-tailed t-test. Locomotor data were analyzed for distance traveled and vertical rears over 5 min bins. In the case of locomotor dose response curves, data were analyzed by a two-way ANOVA with a Bonferroni post-hoc test. For CPP studies, t-tests were performed for analysis of difference between saline and MPH-treated animals, with further t-tests performed to determine significant difference from unpaired side following conditioning. Values of p<0.05 were considered statistically significant.

**Chemicals:** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Results**

**MPH treated animals exhibit altered locomotor responding to 5-HT1B agonists.** It was our hypothesis that, following chronic MPH treatment, animals would exhibit altered DA/5-HT system interactions as exhibited previously (Brookshire, 2010), and that specific receptors altered by MPH treatment would produce differential effects on locomotor activity. Thus, we examined five 5-HT receptor subtypes known to be heavily involved in DA/5-HT system interactions: the 5-HT1A, 1B, 2A, 2C, and 3 receptors. MPH and saline treated animals both showed significant decreases in locomotor activity following administration of 5-HT1A agonist 8-OH-DPAT (F4,86=6.995, p<0.001 effect
of 8-OH-DPAT, Figure 1A) as has been previously observed in the literature (Brookshire et al, 2009; Chojnacka-Wojcik, 1992), but no significant effect of MPH treatment on locomotor activity could be observed (F1,86=1.553, p>0.05, Figure 1A).

In contrast, the 5-HT1B agonist RU 24969 produced robust increases in locomotor activity in both MPH and saline treated mice, an effect noted previously (F4,120=7.792, p<0.001, Figure 1B)(Brookshire et al, 2009; Chaoulloff et al, 1999). MPH treated animals showed a significant potentiation of locomotor activity in response to RU 24969 over saline treated mice (F1,120=4.393, p<0.05 effect of MPH treatment, Figure 1B). These results, similar to those observed in the original study in MPH-treated mice (Brookshire, 2010), indicate that MPH treated animals are more sensitive to the locomotor effects of RU 24969.

We additionally investigated other 5-HT receptors known to be present in the mesolimbic DA system that have significant influence over the DA system, in particular the 5-HT2A (Pessia et al, 1994), the 5-HT2C (Martin et al, 1998) and 5-HT3 receptors (Campbell et al, 1996). Both the 5-HT2A/2C agonist DOI and the 5-HT3 agonist SR 7227 increased locomotor activity in saline and MPH-treated animals (F3,39=7.801, p<0.001 effect of DOI, Figure 1C, F3,84=4.404, p<0.01 effect of SR57227, Figure 1E), but no differences were observed due to MPH treatment (F1,39=3.011, p>0.05 effect of MPH treatment on DOI response, Figure 1C, F1,84=0.1850, p>0.05 effect of MPH treatment of SR 57227 response, Figure 1E). Subsequent in vivo microdialysis studies failed to show differences from control in DA measures in the NAc following DOI challenge (data not shown). Similarly, the 5-HT2C agonist MK 212 significantly decreased locomotor activity in MPH and saline-treated animals (F3,74=32.58, p<0.001
Figure 1:

A

B

C

D

E

Figure 1: Distance traveled (cm/20 min) as a function of 8-OH-DPAT (mg/kg) in A and DOI (mg/kg) in C, saline and MPH (black bars) or RU 24969 (mg/kg) in B, saline and DOI (mg/kg) in D, and saline and SR 7227 in E.
Figure 1: Effects of Chronic MPH on Locomotor Responses to 5-HT receptor agonists

A: Locomotor activity counts over the first 20 minutes in response to 8-OH-DPAT (0.5-3.0 mg/kg, i.p.) in MPH- and saline-treated animals (n=9 per group). There was a significant overall effect of 8-OH-DPAT dose, but no significant effect of MPH treatment (p>0.05).

B: Locomotor activity counts over the first 60 minutes for the 5-HT1B agonist RU24969 (0.3-5.0 mg/kg, i.p.) in MPH- and saline-treated animals (n=12 per group). There was a significant effect of RU24969 dose, and a significant effect of MPH treatment (*p<0.05).

C: Locomotor activity counts over the first 60 minutes following injection for 5-HT2A/2C agonist DOI (0.125-2.0 mg/kg, i.p.) in MPH- and saline-treated animals (n=6 per group). There was a significant overall effect of DOI dose on locomotor activity, but no significant effect of MPH treatment.

D: Locomotor activity counts over the first 20 minutes following injection for 5-HT2C agonist MK 212 (1.0-10.0 mg/kg, i.p.) in MPH- and saline-treated animals (n=12 per group). There was a significant effect of MK 212 dose on locomotor activity in both MPH- and saline-treated animals, but no difference between MPH- and saline-treated animals.

E: Locomotor activity counts over the first 20 minutes following injection of 5-HT3 agonist SR 57227 (1.0-4.0 mg/kg, i.p.), in MPH- and saline-treated animals (n=12 per group). There was a significant effect of SR 57227 dose on locomotor activity, but no difference between MPH- and saline-treated animals.
effect of MK 212, Figure 1D), but MPH treatment did not affect the locomotor response observed (F1,74=0.3675, p>0.05 effect of MPH treatment, Figure 1D). Thus it appears that MPH treated animals show increased locomotor sensitivity only to 5-HT1B agonists following treatment.

**MPH treated animals show significant differences in CPP and DA and 5-HT responses to a 5-HT1A agonist.** Although there was no effect of MPH treatment on locomotor responses to the 5-HT1A agonist 8-OH-DPAT, we decided to investigate the 5-HT1A receptor further with conditioned place preference studies and in vivo microdialysis. Previous studies in the DAT knockout (DAT-KO) mouse had indicated that sensitization of the 5-HT1A influence over the DA system was the mechanism behind increases in DA in DAT-KO mice exposed to cocaine (Mateo et al, 2004). In addition, MPH has been shown to act as an agonist at 5-HT1A receptors (Markowitz et al, 2009). Thus, we hypothesized that 5-HT1A might also be altered in MPH treated mice (Brookshire, 2010). We performed a conditioned place preference experiment for the 5-HT1A agonist 8-OH-DPAT in MPH and saline treated mice (Figure 2A). At a moderate dose of 2.0 mg/kg i.p. 8-OH-DPAT, saline treated mice showed neither preference nor aversion for the drug (t=0.4299, p>0.05, Figure 2A), while MPH treated animals showed significant conditioned place aversion (t=2.376, p<0.05 difference from zero, Figure 2A), suggesting that MPH treatment results in increased sensitivity to reward-related behaviors mediated by 8-OH-DPAT.

In addition to behavioral studies, we also looked at neurochemical effects of 8-OH-DPAT following chronic MPH exposure. MPH treated animals showed a small but significant
increase in extracellular DA in the NAc following i.p. 8-OH-DPAT (2.0 mg/kg i.p., F1,139=14.96, p<0.001 effect of MPH treatment, Figure 2B), while saline treated animals showed a small, nonsignificant decrease in extracellular DA. In addition, 8-OH-DPAT produced decreases in extracellular 5-HT in MPH and saline treated mice (F9,107=3.746, p<0.001, Figure 2C), with a significant potentiation of the effect in MPH-treated animals (F1,107=17.97, p<0.001 effect of MPH treatment, Figure 2C).

**MPH-treated animals have differential neurochemical responses to 5-HT1A agonist infusion.** We performed dual probe microdialysis experiments in the VTA and NAc to look at effects of 8-OH-DPAT infused into the VTA on extracellular DA and 5-HT in the NAc. Infusion of 8-OH-DPAT into the VTA resulted in decreases in extracellular DA in the NAc (F12,117=7.884, p<0.001, Figure 3A), with a more sensitive response present in MPH-treated mice (F1,117=15.19, p<0.001, Figure 3A), suggesting that sensitization of 5-HT1A in MPH treated mice may result in increased inhibitory control of DA cell firing. We also examined extracellular 5-HT levels in the NAc following 8-OH-DPAT infusion into the VTA. While saline treated animals showed no dose-dependent decrease in 5-HT in the NAc during 8-OH-DPAT infusion into the VTA, MPH-treated mice showed significant dose-dependent decreases in extracellular 5-HT (Overall effect of 8-OH-DPAT on 5-HT in the NAc F12,75=3.035, p<0.01, effect of MPH treatment F1,75=22.79, p<0.001, Interaction between MPH treatment and 8-Oh-DPAT F12,75=2,241, p<0.05, Figure 3B). In addition to neurotransmitter levels in the NAc, we also examined the effects of 8-OH-DPAT infused into the VTA on VTA DA and 5-HT levels in MPH and saline-treated animals.
Figure 2

A

Time (s)

[drug-paired - saline-paired chamber]

2.0 mg/kg 8-OH-DPAT, i.p.

B

% Baseline [DA]

2.0 mg/kg 8-OH-DPAT, i.p.

C

% Baseline [5-HT]

2.0 mg/kg 8-OH-DPAT, i.p.
Figure 2: Chronic MPH reverses CPP and NAc DA for 5-HT1A agonist 8-OH-DPAT  
A: Effects of 2.0 mg/kg 8-OH-DPAT, i.p. on conditioned place preference measures in MPH- and saline-treated animals (n=12 per group). When paired over a period of four days, on day five MPH treated animals showed significant aversion for the 8-OH-DPAT-paired chamber (*p<0.05 difference from zero). B: Single probe in vivo microdialysis measurement of DA in the NAc of MPH- and saline-treated animals in response to 2.0 mg/kg i.p. 8-OH-DPAT (n=8 per group). There was a significant effect of MPH-treatment on DA response in the NAc, *p<0.05 difference from saline-treated control. C: Single probe in vivo microdialysis measurement of 5-HT in the NAc of MPH- and saline-treated animals in response to 2.0 mg/kg i.p. 8-OH-DPAT (n=6 saline, n=7 MPH-treated mice). There was a significant overall effect of 8-OH-DPAT injection on extracellular 5-HT, and a significant overall effect of MPH treatment. **p<0.01 difference between MPH and saline-treated response.
Although there was a significant decrease in extracellular DA in the VTA over increasing doses of 8-OH-DPAT (F12,114=10.20, p<0.001, Figure 3C), there was no difference between MPH and saline treated mice (F1,114=0.6504, p>0.05, Figure 3C). In contrast, while 8-OH-DPAT infusion failed to alter 5-HT levels in the VTA of saline-treated animals (F12, 90=0.9009, p>0.05, Figure 4D), MPH treated mice showed a significant effect of MPH treatment (F1,90=4.177, p<0.05, Figure 3D) and a small decrease in extracellular 5-HT in the VTA, suggesting an increase in sensitivity to presynaptic 5-HT1A receptor stimulation.

**MPH-treated animals show significant behavioral and neurochemical alterations in response to a 5-HT1B agonist.** We conducted conditioned place preference studies for the 5-HT1B agonist RU 24969 in MPH and saline treated animals. While saline treated animals showed a trend toward aversion at the lower dose of 0.3 mg/kg, i.p. (t=1.934, p=0.0792 difference from zero, Figure 4A), and neither preference nor aversion at the higher dose of 1.0 mg/kg (t=0.6244, p>0.05, Figure 4A), MPH treated animals showed neither preference nor aversion at 0.3 mg/kg i.p. (t=2.217, p<0.05 difference between MPH and saline, Figure 4A), signaling a reversal in the place conditioning properties of this drug at the lower dose (Figure 4A). These results confirm those seen in previous studies (Brookshire, 2010) and suggest that there are altered rewarding properties of 5-HT1B agonists following MPH treatment.

To confirm the changes seen in previous studies showing a dopaminergic and serotonergic response to i.p. administration of a 5-HT1B agonist (Brookshire, 2010). Accordingly, we conducted single probe in vivo microdialysis to evaluate the
Figure 3

A

% Baseline [DA] NAc

-80 -60 -40 -20 0 20 40 60 80 100 120 140 160 180 200

time (min)

10uM 30uM 100uM 8-OH-DPAT (uM)

***

B

% Baseline [5-HT] NAc

-80 -60 -40 -20 0 20 40 60 80 100 120 140 160 180 200

time (min)

10uM 30uM 100uM 8-OH-DPAT (uM)

*** **

C

% Baseline [DA] VTA

-80 -60 -40 -20 0 20 40 60 80 100 120 140 160 180 200

time (min)

10uM 30uM 100uM 8-OH-DPAT (uM)

*

D

% Baseline [5-HT] VTA

-80 -60 -40 -20 0 20 40 60 80 100 120 140 160 180 200

time (min)

10uM 30uM 100uM 8-OH-DPAT (uM)
Figure 3: Infusion of 8-OH-DPAT into the VTA has differential effects in MPH-treated animals. A: Effects of 8-OH-DPAT (10, 30, 100μM in aCSF) infused into the VTA on DA measured in the NAc (n=6 saline, 5 MPH-treated animals). There was a significant effect of 8-OH-DPAT infusion on extracellular DA, and a significant effect of MPH-treatment on 8-OH-DPAT response. B: Effects of 8-OH-DPAT infused into the VTA on 5-HT measured in the NAc (n=4 per group). There was a significant effect of MPH treatment, as well as a significant interaction between MPH treatment and 8-OH-DPAT response. **p<0.01 difference between MPH- and saline-treated animals. C: Effects of 8-OH-DPAT infused into the VTA on DA levels measured in the VTA (n=6 saline-treated, 5 MPH-treated). There was significant effect of 8-OH-DPAT infusion on DA levels in the VTA, but no effect of MPH treatment (p>0.05). D: Effects of 8-OH-DPAT infused into the VTA on 5-HT levels measured in the VTA (n=5 saline-treated, 4 MPH-treated animals). There was no significant effect of 8-OH-DPAT infusion on 5-HT levels in the VTA, but a significant effect of MPH treatment on overall 5-HT response to 8-OH-DPAT (p<0.05) was observed.
dopaminergic and serotonergic responses to i.p. RU 24969. In response to 1.0 mg/kg RU 24969, a dose that produced a reversed conditioned place preference effect in MPH treated mice (Figure 4A), MPH treated mice showed a significant increase in extracellular DA in the NAc (effect of RU 24969 F9,79=3.488, p<0.01, effect of MPH treatment, F1,79=8.896, p<0.01, Figure 4B) while saline treated mice showed no significant response to drug injection (F9,79=3.218, p<0.01 interaction between MPH treatment and RU 24969, Figure 4B).

In addition to extracellular DA levels in the NAc, we also examined 5-HT levels following injection of RU 24969. In accordance with previous studies (Brookshire, 2010), MPH treated mice showed a significant decrease in extracellular 5-HT in the NAc in response to drug injection, while saline treated mice showed no 5-HT response in the NAc at this dose (F1,79=12.09, p<0.001, Figure 4C). These findings confirm those seen previously, and suggest that MPH treated mice are more sensitive to the autoinhibitory effects of 5-HT1B agonists.

**MPH-treated animals show sensitized responses to 5-HT1B agonists in the VTA and NAc.** Previous work has shown that MPH treated mice have an increased excitatory 5-HT influence over DA cell firing in the VTA, and that this increased control may be influenced by 5-HT1B receptor sensitivity. Thus, we hypothesized that infusion of 5-HT1B agonists into the VTA of MPH treated animals would produce a sensitized DA response in the NAc. Accordingly, we
Figure 4

A

Time (s)
[drug-paired - saline-paired chamber]

0.3 mg/kg, i.p.        1.0 mg/kg, i.p.

RU 24969

B

% Baseline [DA]

1.0 mg/kg
RU 24969, i.p.

***

C

% Baseline [5-HT]

1.0 mg/kg
RU 24969, i.p.

**  

*
Figure 4: Chronic MPH reverses CPA and NAc DA for 5-HT1B agonist RU 24969

A: Effects of 1.0 mg/kg RU 24969, i.p. on conditioned place preference measures in MPH- and saline-treated animals (n=11 per group). When paired over a period of four days, on day five saline-treated animals showed a trend toward aversion for the RU 24969-paired chamber (p=0.0792 difference from zero), showing significant difference from MPH-treated animals at 0.3 mg/kg RU24969. At the higher dose of 1.0 mg/kg RU 24969, no difference was observed between MPH- and saline-treated animals. B: Single probe in vivo microdialysis measurement of DA in the NAc of MPH- and saline-treated animals in response to 1.0 mg/kg i.p. RU 24969 (n=5 per group). There was a significant effect of RU 24959 on extracellular DA levels, and a significant effect of MPH-treatment (p<0.01). There was also a significant interaction between MPH-treatment and DA response to RU 24969. **p<0.01 difference from saline-treated control. C: Single probe in vivo microdialysis measurement of 5-HT in the NAc of MPH- and saline-treated animals in response to 2.0 mg/kg i.p. 8-OH-DPAT (n=5 per group). There was a significant overall effect of MPH treatment. *p<0.05 difference between MPH and saline-treated response.
infused the 5-HT1B agonist CP 93,129, a more specific 5-HT1B agonist, into the VTA of MPH- and saline-treated mice. In response to increasing doses of CP 93,129, both MPH and saline treated animals showed increases in extracellular DA in the NAc (F12,114=2.417, p<0.01, Figure 5A), with MPH treated mice showing an augmented increase in extracellular DA levels compared to saline-treated animals (F1,114=17.01, p<0.001, Figure 5A). These results suggest increases in 5-HT1B mediated control over DA cell firing from the VTA to the NAc.

We also measured levels of extracellular 5-HT in the NAc in response to CP 93,129 infusion into the VTA of MPH and saline-treated mice. MPH and saline treated animals showed no significant changes in extracellular 5-HT levels in the NAc following infusion of CP 93, 129 into the VTA (F12,121=1.635, p>0.05, Figure 5B), but a significant difference was observed between MPH and saline treated animals (F1,121=4.047, p<0.05, Figure 5B).

In addition to the effects of infused CP 93,129 on neurotransmitter levels in the NAc, we also looked at DA and 5-HT levels in the VTA. In response to increasing levels of CP 93,129, both MPH and saline treated animals showed increases in extracellular DA in the VTA (F12,108=3.335, p<0.001, Figure 5C), with MPH-treated mice showing a highly enhanced response (F1,108=28,18, p<0.001, Figure 5C), suggesting possible sensitization of the DA response to 5-HT1B stimulation. In addition, MPH-treated animals also showed a significant increase in extracellular 5-HT levels in the VTA in response to CP 93,129 infusion (F1,145=18.74, p<0.001 effect of MPH treatment, Figure 5D), which indicates enhanced feedback mechanisms from the VTA.
Figure 5: Chronic MPH produces sensitization to infusion of 5-HT1B agonist CP93,129 in the VTA. A: Effects of CP 93,129 (20, 40, 80uM in aCSF) infused into the VTA on DA measured in the NAc (n=5 MPH, n=6 saline treated animals). There was a significant effect of CP 93,129 infusion on extracellular DA, and a significant effect of MPH-treatment on CP 93,129 response (p<0.001). B: Effects of CP 93,129 infused into the VTA on 5-HT measured in the NAc (n=6 per group). There was a significant effect of MPH treatment (p<0.05). C: Effects of CP 93,129 infused into the VTA on DA levels measured in the VTA (n=6 MPH, n=5 saline treated animals). There was a significant effect of CP 93,129 infusion on DA levels, a significant effect of MPH treatment, and a significant interaction between MPH treatment and CP 93,129 infusion. *, p<0.05, **p<0.01 difference between MPH and saline-treated response. D: Effects of CP 93,129 into the VTA on 5-HT levels measured in the VTA (n=7 per group). There was a significant effect of MPH treatment on 5-HT response to CP 93, 129 infusion, and a significant interaction between CP 93,129 infusions and MPH treatment. *, p<0.05, **p<0.01 difference between MPH and saline-treated response.
MPH treated animals show altered dopaminergic responses to a 5-HT1B antagonist. In light of the dramatic results seen with infusion of a 5-HT1B agonist into the VTA, we wished to confirm altered responding to 5-HT1B receptor stimulation using an antagonist. Accordingly, we conducted single probe in vivo microdialysis studies in the NAc of MPH and saline treated mice following administration of 5.0 mg/kg GR 55562, i.p. Following administration of GR 55562, MPH-treated mice showed a significant effect of treatment in the dopaminergic response ($F_{1,116}=12.36, \ p<0.001$, Figure 6A), with a small decrease in extracellular DA compared to saline treated animals. Interestingly, extracellular 5-HT levels did not change in MPH or saline-treated mice in response to GR 55562 ($F_{9,84}=0.8546, \ p>0.05$, Figure 6B), providing further evidence that the changes in 5-HT1B receptors sensitivity may be focused on the DA system.

Dopaminergic effects of fluoxetine on MPH-treated mice can be reversed with administration of a 5-HT1B antagonist. It was our hypothesis that sensitization of the 5-HT1B receptor accounted for many of the rewarding and dopaminergic effects of fluoxetine following MPH administration. Thus, we performed dual probe microdialysis studies in which a moderate concentration of fluoxetine was administered into the VTA, and DA levels measured in the NAc, following administration of an i.p. 5-HT1B antagonist. Should 5-HT1B sensitization account for the effects of fluoxetine on DA in the NAc, the effects should be reversed by administration of a 5-HT1B antagonist. Indeed, administration of GR 55562 blocked the dopaminergic effects of fluoxetine administered into the VTA (effect of fluoxetine on MPH treated animals $F_{1,55}=13.80, \ p<0.001$, after GR 55562 administration $F_{4,59}=0.4366$, Figure 7A), while having no effect on the extracellular DA levels of saline treated animals. Administration of GR
Figure 6

A

![Graph showing the effect of saline and MPH on dopamine (DA) levels over time.](image)

B

![Graph showing the effect of saline and MPH on serotonin (5-HT) levels over time.](image)
Figure 6: Effects of chronic MPH on neurochemical responses to 5-HT1B antagonist GR 55562.  

**A:** Effect of 5.0 mg/kg GR 55562 i.p. on DA levels in the NAc of MPH and saline treated animals (n=7 per group). There was a significant effect of MPH treatment on DA response to GR 55562 (**p<0.001**).  

**B:** Effect of GR 55562 on 5-HT levels in the NAc of MPH and saline treated mice (n=5 MPH, n=6 saline treated animals). There was no significant effect of i.p. 5-HT1B antagonist treatment on 5-HT levels in MPH or saline treated animals.
55562 failed to alter 5-HT levels in MPH or saline treated mice in the NAc following fluoxetine infusion (F12,104=1.853, p<0.05 effect of fluoxetine, effect of GR55562, F4,40=1.51, p>0.05, Figure 7B).

We also monitored the effects of GR 55562 and fluoxetine infusion in the VTA on DA and 5-HT levels. There was a general effect of fluoxetine infusion on DA (F6,63=5.117, p<0.001, Figure 7C) and 5-HT levels (F12,143=7.649, p<0.001, Figure 7D), but systemic GR 55562 did not affect the results of fluoxetine infusion on DA or 5-HT levels in the VTA (Effect of GR 55562 on DA F5,52=0.8520, p>0.05, effect of GR 55562 on 5-HT, F4,55=0.1742, p>0.05, Figure 7C-D).

Discussion

The results of this study show that MPH treatment in mice causes alterations in serotonergic control over the mesolimbic DA system, confirming previous findings (Brookshire, 2010). Further, the results shown suggest that sensitized 5-HT1B and, to a lesser extent, 5-HT1A receptor signaling in the VTA mediate these changes. In normal animals, the 5-HT1A receptor produces CPP at low doses, and at higher doses, produces aversion and decreases in extracellular 5-HT through presynaptic inhibition of cell firing. The 5-HT1B receptor plays a modulatory role in normal mice, and when specifically stimulated, will increase DA in the VTA and NAc via presynaptic inhibition of GABA release in the VTA. We have found that, following chronic MPH treatment, MPH treated mice show conditioned place aversion to 5-HT1A agonists, indicating sensitization to the reward-related effects of 5-HT1A stimulation. 5-HT1A agonists also increase DA in the NAc of MPH treated mice and produce augmented decreases in 5-HT, suggesting
Figure 7

A

% Baseline [DA]

50uM Fluoxetine infused VTA

5.0 mg/kg GR55562, i.p.

B

% Baseline [5-HT]

50uM Fluoxetine infused VTA

5.0 mg/kg GR55562, i.p.

C

% Baseline [DA]

50uM Fluoxetine infused VTA

5.0 mg/kg GR55562, i.p.

D

% Baseline [5-HT]

50uM Fluoxetine infused VTA

5.0 mg/kg GR55562, i.p.
Figure 7: 5-HT1B antagonists reverse the effects of fluoxetine infusion in MPH-treated animals. **A:** Effects of 5-HT1B antagonist GR 55562 (5.0 mg/kg, i.p.) on DA in the NAc during fluoxetine infusion (50uM) into the VTA (n=5 per group). There was a significant effect of fluoxetine infusion on DA levels in MPH treated animals. This effect was reversed by administration of i.p. GR 55562. *, p<0.05 difference between MPH and saline-treated response. **B:** Effects of GR 55562 on 5-HT in in the NAc during fluoxetine infusion into the VTA (n=5 per group). There was a significant overall effect of fluoxetine infusion, but no significant response to GR 55562. **C:** Effect of 5-HT1B antagonist GR 55562 on DA in the VTA during fluoxetine infusion (50uM) into the VTA (n=6 MPH, n=5 saline treated animals). There was a significant overall effect of fluoxetine infusion on DA levels in the VTA, but no significant response to GR 55562. **D:** Effect of GR 55562 (5.0 mg/kg, i.p.) on 5-HT in the VTA during fluoxetine infusion (50uM) into the VTA (n=7 MPH, n=6 saline treated animals). There was a significant overall effect of fluoxetine infusion into the VTA on extracellular 5-HT levels, but no significant effect of i.p administration of GR 55562.
sensitization. In contrast, MPH treated mice showed reversals toward preference in response to a 5-HT1B agonist, as well as significant increases in DA in the NAc. Further studies with dual probe microdialysis showed that the VTA is the locus for the altered response to 5-HT1A and 1B agonists. MPH treated mice showed amplified responses to 5-HT1A agonist infusion, producing significant decreases in NAc DA associated with increased inhibition of DA neuron firing. Dual probe studies also confirmed the altered effects of the 5-HT1B receptor, with augmented DA increases in the NAc and VTA of MPH treated mice following 5-HT1B agonist infusion in the VTA. Finally, increases in DA induced by fluoxetine in MPH-treated animals can be blocked by systemic 5-HT1B antagonist administration. Taken together, these data suggest that MPH treated significantly alters DA/5-HT system interactions at the level of the VTA, and that these changes principally manifested as sensitization of the 5-HT1B receptor.

It has been established for some time that the DA and 5-HT systems have many points of interaction, and that the 5-HT system exerts a modulatory influence over reward-related behaviors (Czoty et al, 2005; Howell et al, 1995; Roberts et al, 1994). Although 5-HT specific drugs such as selective serotonin reuptake inhibitors do not have reinforcing efficacy (Locke et al, 1996; Reith et al, 1997; Roberts et al, 1999), activation of specific 5-HT receptors present in the mesolimbic DA system can have both positive (Kelland et al, 1990; Lejeune et al, 1998; Lejeune et al, 2000; O'Dell et al, 2004; Parsons et al, 1999) and negative effects (Di Giovanni et al, 2000; Di, V et al, 2000; Ferguson et al, 2008) on DA cell firing and behaviors related to drug reward. In particular, there are five 5-HT receptors known to have specific effects on DA cell firing in the VTA; the 5-HT1A, 1B, 2A, 2C, and 3 (Di Giovanni et al, 2000; Gillies et al, 1996; Kelland et al,
Previous experiments from our laboratory (Brookshire, 2010) have shown that MPH treated mice show alterations in 5-HT modulation of DA cell firing at the level of the VTA, and thus we chose to examine all of these receptors.

The 5-HT1A receptor is located presynaptically in many areas of the brain, in particular in the dorsal raphe, where stimulation reduces 5-HT cell firing (Collu et al, 1997; Yoshimoto et al, 1992). However, 5-HT1A neurons are also known to be located post-synaptically in the VTA, in particular on DA neurons (Doherty et al, 2000; Doherty et al, 2001), and GABA neurons (Guan et al, 1989). 5-HT1A receptor stimulation in the VTA appears to have a biphasic on DA neuron firing (Kelland et al, 1990). Low doses of the 5-HT1A agonist 8-OH-DPAT are known to increase DA cell firing, elevating DA levels (Chen et al, 1995) and producing place preference (Fletcher et al, 1993; Shippenberg, 1991). Conversely, higher doses are thought to activate GABA neurons in the VTA, increasing the tonic inhibition of DA cell firing and reducing DA outflow (Lejeune et al, 1998) and producing place aversion (Papp et al, 1991). Possibly due to the biphasic activity observed on DA cell firing rates in the VTA, the behavioral effects of 5-HT1A agonists on DA-mediated activity are somewhat varied. 8-OH-DPAT has been shown to decrease cocaine breakpoints in a progressive ratio schedule (Parsons et al, 1998) and can inhibit the expression and development of amphetamine sensitization (Przegalinski et al, 2000), and 5-HT1A agonists produce general depressant effects on behavior (Abe et al, 1996). However, other studies have found that 8-OH-DPAT can enhance the locomotor effects of psychostimulants such as cocaine (Carey et al, 2000;
Carey et al, 2005; De La et al, 2000). Thus it appears that the behavioral effects of 5-HT1A agonists are variable and dose-dependent.

The 5-HT1A receptor was of interest due to previous studies indicating that DAT-KO mice, a model of chronic hyperdopaminergia similar to that induced by chronic MPH, showed effects of 5-HT on DA systems that included altered 5-HT1A receptor activity (Mateo et al, 2004). Specifically, Mateo et al showed that DAT-KO mice exhibit conditioned place preference for fluoxetine, in a manner similar to MPH-treated animals, and that this effect could be blocked with the 5-HT1A antagonist WAY 100635. Accordingly, we hypothesized that if the 5-HT1A receptor showed similar alterations in MPH-treated mice, 5-HT1A agonists might be expected to increase DA in the NAc following MPH treatment and possibly produce place preference. The present studies with in vivo microdialysis showed that there were small DA increases in MPH-treated animals in response to 8-OH-DPAT, results that correlated with lack of locomotor attenuation in locomotor studies. Interestingly, however, the conditioned place preference studies revealed conditioned place aversion in MPH-treated mice, aversion that is usually indicative of higher doses and indicated sensitized responses to 8-OH-DPAT. In addition, while i.p. administration of 8-OH-DPAT produced a small increase in extracellular DA in the NAc, it also produced a significant decrease in synaptic 5-HT in the NAc of MPH-treated mice, which appeared to be a sensitized response. These findings were further complicated by dual probe studies, which showed that 8-OH-DPAT infused into the VTA produced decreases in DA in the NAc of MPH and saline-treated mice, with MPH-treated mice appear more sensitive to the effects. These results suggest that 5-HT1A receptors in MPH-treated mice may be more sensitive in areas like the
VTA, producing augmented decreases in DA and 5-HT in the NAc. The elevations in DA seen in MPH treated mice in response to systemic 5-HT1A agonists, in contrast may be the result of 5-HT1A stimulation in the NAc, which in turn affect the mesolimbic DA system. It is known that 5-HT1A activation can increase DA in the NAc (Campbell et al, 1995; Parsons et al, 1993; Yan, 2000), and the increases seen in MPH-treated mice may be a supersensitive response. Further studies would be required to truly assess the role of 5-HT1A receptors following MPH treatment.

The 5-HT1B receptor also stood out as a possibility for sensitization following MPH treatment. 5-HT1B receptors are present as presynaptic heteroreceptors in the VTA, and in particular are known to inhibit GABA release in the VTA when stimulated (Cameron et al, 1994; Hallbus et al, 1997; Yan et al, 2001). There is also extensive evidence indicating that 5-HT1B agonists can enhance the stimulating properties of psychostimulants (O'Dell et al, 2004; Parsons et al, 1999), at least partly by inhibiting GABA release and thus disinhibiting the DA neurons in the VTA (O'Dell et al, 2004; Sari, 2004). Previous studies from our laboratory have shown sensitized locomotor responses to the 5-HT1B agonist RU 24969 (Brookshire, 2010). In addition, MPH-treated mice showed a significant 20% increase in 5-HT1B expression in the ventral midbrain, indicating that the sensitized responses to 5-HT1B agonists could be due to increases in 5-HT1B expression. In the present studies, we confirmed the locomotor response to RU 24969, and have also shown a reversal in reward-related behaviors, with a lack of aversion. For studies with dual probe microdialysis, we used the specific 5-HT1B agonist CP 93,129, which must be infused as it does not cross the blood-brain barrier. We found a dramatic effect of MPH treatment on the neurochemical responses to
VTA infusion of CP 93,129. MPH-treated mice showed enhanced DA release in the NAc, as well as dramatic increases in extracellular DA and 5-HT in the VTA, suggesting that the primary location of effects seen in i.p. studies was the VTA confirming previous studies with fluoxetine (Brookshire, 2010). These results were further corroborated by results showing that the DA increases induced by fluoxetine infusion in MPH treated animals could be blocked by 5-HT1B antagonist GR 55562. These data, in addition to previous studies, indicate that the 5-HT1B receptor is primarily responsible MPH-induced effects on DA/5-HT system interactions at the level of the VTA.

Other 5-HT receptors in the VTA have also been studied with regard to psychostimulant effects and effects on VTA neuron firing. The 5-HT2A receptor is known to be located in the VTA on dendrites of DA neurons and also on GABA cell bodies (Adell et al, 2004; Cornea-Hebert et al, 1999; Doherty et al, 2000), and 5-HT2A stimulation has been shown to increase DA cell firing in this region (Pessia et al, 1994). Previous studies have suggested that the role of 5-HT2A in the VTA is a phasic, excitatory influence over DA neurons (Adell et al, 2004), and thus we hypothesized that MPH treatment might exert some effects via changes in the sensitivity of 5-HT2A receptors. However, locomotor studies failed to find any difference in behavioral responding to the 5-HT2A/2C agonist DOI, and preliminary microdialysis studies showed no effects of MPH treatment on extracellular DA responses in the NAc to i.p. 5-HT2A agonists. Taken together, this data indicates that MPH treatment may have some effects on the 5-HT2A receptor.

The 5-HT2C receptor also appear to be unaffected by MPH treatment. Stimulation of the 5-HT2C receptor is known to decrease locomotor activity (Kennett et
al, 1997; Martin et al, 1998), and decrease VTA DA neuron firing (Di Giovanni et al, 2000; Di, V et al, 2000). Like the 5-HT1B receptor, the 5-HT2C receptor is located on GABA neurons in the VTA, but unlike the 5-HT1B receptor, it appears to activate these GABA neurons, producing increases in inhibition of DA cell firing (Di Giovanni et al, 2001; Gobert et al, 2000). Thus, it was our hypothesis that, if 5-HT2C receptors in the VTA played a role in the effects of 5-HT in MPH-treated mice, we would see a desensitized response to 5-HT2C agonists, indicated reduced GABA activation in further disinhibition of DA cell firing. However, locomotor results showed no difference from saline treated mice in behavioral responses to the 5-HT2C agonist MK 212, and it appears that these receptors may not altered following MPH treatment.

The 5-HT3 receptor is known to regulate somatodendritic DA release in the VTA (Campbell et al, 1996), and 5HT3 antagonists can reduce the number of spontaneously active DA cell in the VTA (Minabe et al, 1991). In addition, stimulation of 5-HT3 receptors may increase DA release in the NAc (Campbell et al, 1995; Parsons et al, 1993). However, our studies failed to find any difference in locomotor responding to 5-HT3 agonists in MPH treated mice, and it appears that the 5-HT3 may not be involved in the changes in 5-HT effects found following MPH treatment.

It is not surprising that 5-HT receptors may be affected by psychostimulant exposure. Repeated cocaine exposure has been shown to sensitize 5-HT1A receptors (King et al, 1993), and 5-HT1B receptor binding is enhanced during cocaine withdrawal (Przegalinski et al, 2003). However, it should be noted that MPH treatment is significantly different from treatment with other types of psychostimulants such as cocaine, as MPH has extremely low affinity with the SERT (Gatley et al, 1996), and thus
would not be expected to directly increase extracellular 5-HT like cocaine or amphetamine treatment. It therefore appears that any changes in 5-HT receptors that result of MPH treatment must result from changes in DA/5-HT system interactions, rather than via direct stimulation of the 5-HT system. Previous publications from our laboratory (Brookshire, 2010) have shown that chronic MPH treatment produces decreases in D2 receptor binding. D2 receptors are known to stimulate 5-HT cell firing in the dorsal raphe (Aman et al, 2007), and thus reduced D2 receptors could lead to low levels of endogenous 5-HT in areas like the VTA, leading in turn to sensitization of 5-HT receptors that, when stimulated, could exert increased control over DA cell firing. In addition, MPH can act as a 5-HT1A receptor agonist (Markowitz et al, 2009). 5-HT1A receptor stimulation in the raphe produces decreases in raphe neuron firing (Casanovas et al, 2000; Collu et al, 1997; Yoshimoto et al, 1992), and this may contribute to low extracellular VTA in terminal regions like the VTA.

In conclusion, the results of this study expand upon previous efforts in MPH treated mice, examining several 5-HT receptors and providing evidence suggesting that 5-HT1B and 5-HT1A receptors may be sensitized at the level of the VTA following MPH treatment. These findings could have extensive implications for MPH treatment in animals and humans. In particular, the increased control of 5-HT over DA cell firing could produce increased rewarding effects of drugs with strong serotonergic components, such as 3,4 methylenedioxymethamphetamine (MDMA) (Brookshire, 2010). Further, drugs that target 5-HT systems, such as fluoxetine and other 5-HT reuptake inhibitors, could have significantly altered effects following MPH exposure. Finally, it is possible that drugs that target 5-HT1A and 1B receptors in particular, such as buspirone, a partial
5-HT1A agonist used in therapy for schizophrenia (Sumiyoshi et al, 2007) and anxiety (Mathew et al, 2008), and the 5-HT1B agonists sumatriptan, rizatriptan, and zolmatriptan used in acute migraine therapy (Lynch et al, 2009; Mannix, 2008; Ward et al, 2008) could exhibit significant differences in patients exposure to chronic, high dose MPH, suggesting possible differences in therapeutic effects or side effect profiles in these patients, and indicating a need for further research into the effects of MPH treatment on DA/5-HT interactions.

Acknowledgements

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CHAPTER IV

CHRONIC MPH ADMINISTRATION IN MICE PRODUCES DEPRESSIVE-LIKE EFFECTS AND ALTERED BEHAVIORAL RESPONSES TO ANTIDEPRESSANTS

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Abstract

Methylphenidate (MPH) is a psychostimulant that is commonly prescribed among children and adults for treatment of attention-deficit/hyperactivity disorder, and is often abused in young adult populations. While the effects of clinical use on some psychiatric measures have been evaluated, no studies have yet been performed to determine the effects of abused doses of MPH on depressive-like behaviors and antidepressant efficacy. Following chronic, high-dose (20 mg/kg, i.p. for 14 days) MPH treatment, MPH treated mice showed significant decreases in the locomotor attenuating effects of fluoxetine, with no differences in saline response or habituation to the open field. MPH treated mice also showed a decreased latency to the first flotation episode in the forced swim test and, in contrast to saline-treated controls, showed significant anti-depressant effects of acute fluoxetine. The effects of fluoxetine persisted in tail suspension test measures, but no differences could be detected in sucrose drinking behaviors. Taken together, our data indicate that high doses of MPH can produce depressive-like effects in mice and supersensitive behavioral responses to fluoxetine. These results may have implications for individuals abusing MPH and for those with comorbid depressive disorders.
Introduction

Methylphenidate (MPH) is a psychostimulant prescribed to children and adults of all ages (6), and abuse of MPH is increasing as prescription numbers increase (60). MPH is usually prescribed to treat symptoms of attention-deficit/hyperactivity disorder (ADHD) in children (14;65), and psychostimulants such as MPH remain the treatment of choice through adolescence and into adulthood when ADHD symptoms persist (27). While the effects of MPH in adult humans and animal models have been assessed for drug-abuse potential and cross-sensitization to other abused drugs (1;2;8;9;19;27;40;41;44;65), there have been few studies investigating the psychiatric consequences of MPH at abused doses, particularly with regard to psychiatric disorders such as depression. Some case studies in humans have suggested that chronic exposure to MPH may produce depressive symptoms in withdrawal (4;48). In addition, several rodent studies have demonstrated that MPH administration in adolescence produces depressive-like behaviors later in life, including reduced sucrose preference, decreased locomotor responses to novel environments, and increased immobility in the forced swim test (7;12).

This issue is of particular concern given the high comorbidity of psychostimulant abuse and major depressive disorder (15). In addition to comorbidity in human patients, psychostimulant abuse ADHD, and depression are known to involve dysregulation of dopamine (DA) systems. Numerous studies have shown changes in DA systems in ADHD patients (23;25;39;67). The directionality of DA dysfunction in ADHD is still a matter of debate, however, with some studies indicating increases in dopaminergic tone (23;25), and others indicating decreases (67). In addition, psychostimulants such as MPH
are known to treat symptoms of depression in rodents, effects that appear to be separate from their locomotor stimulating effects (5;18). DA agonists such as MPH are also known to increase the antidepressant effects of selective serotonin reuptake inhibitor (55). Thus, the elucidation of the effects of chronic MPH on DA systems and the resulting effects on behaviors related to psychiatric problems such as depression is an important field of study.

Although depression is most commonly associated with 5-HT dysfunction, many studies have also revealed changes in the DA system human depression as well as in animal models (26;54). Inhibitors of DA transporters (DAT), such as MPH, treat the symptoms of depression (28), and DA abnormalities have been seen in several animal models of depression (24;38;71). In addition, anhedonia, one of the core symptoms of human depression, is associated with dysfunction in predominantly dopaminergic reward pathways (22;50;69). It is also well known that DA and 5-HT systems have extensive interactions, particularly at the level of the mesolimbic DA system, an area strongly associated with the initial rewarding and reinforcing effects of drugs of abuse (11;22). Studies have further shown that, while 5-HT can modulate the effects of DA responses to psychostimulants (10;21;34;35;56;58;66), DA can also reciprocally modulate 5-HT associated behaviors (54), making the role of DAT-specific psychostimulants of interest.

To date, the vast majority of studies looking at depressive-like phenotypes following psychostimulant administration have focused on cocaine (4;48). As cocaine and MPH have significant differences in half-life and receptor pharmacology, it is important to study the effects of chronic MPH abuse in a separate paradigm (30). For example, while cocaine has significant affinity for the DAT, norepinephrine (NE, NET),
and serotonin transporter (SERT) (Ki values in humans for cocaine: DAT 0.23 ± 0.03 uM, NET 0.48 ± 0.05 uM, SERT 0.74 ± 0.03 uM. In mouse: DAT 0.49 ± 0.04, NET 0.46 ± 0.06, SERT 0.73 ± 0.12, (33)) MPH is highly preferential for the DAT and NET, with very little affinity for the SERT (Ki values in humans for MPH: DAT 0.06 ± 0.01 uM, NET 0.1 ± 0.01, SERT 132.43 ± 10.71. In mouse: DAT 0.25 ± 0.03, NET 0.17 ± 0.03, SERT 114.37 ± 7.61, (33)). A few studies have been performed using MPH treatment in rats (7;12), and found significant increases in measures of depressive phenotypes using the forced swim test and sucrose drinking tests. However, both of these studies utilized doses of MPH that were more similar to a clinical regimen and administered in adolescents, and thus fail to address the possible consequences and differences resulting from chronic abuse-like exposure to high doses of MPH in adult models.

In light of the increasing rates of MPH abuse, we chose to examine the effects of high dose MPH administration on the development of a depressive-like phenotype in adult mice. We dosed animals once daily for 14 days with 20 mg/kg MPH or saline, i.p., and 48 hours following the final drug administration, animals were tested for various behaviors at baseline and in response to 15 mg/kg fluoxetine using the well-established forced swim and tail suspension tests. Animals were additionally tested for altered hedonic behaviors using sucrose drinking protocols.

**Materials and Methods**

**Animals:** Male and female mice were treated with saline or 20 mg/kg MPH, i.p. in a volume of 0.1mL over 14 days, with each dose given once daily at the start of the dark cycle. All behavioral testing took place 48 hours following the last dose of MPH or
saline. In the case of the sucrose testing protocol, animals were singly-housed for the two weeks of treatment, with food and water provided ad libitum. For all other experiments, animals were group housed with food and water provided ad libitum. Animal care was in accordance with Wake Forest University’s Institutional Animal Care and Use Committee and in compliance with National Institutes of Health guidelines.

**Locomotor Activity:** Activity was assessed using Med Associates Open Field Activity Monitors (Med Associated, St. Albans, VT). The open field consisted of a square plexiglass container (27.0 cm X 27.0 cm X 20.3 cm) with three 16-beam I/R arrays. Analyses were conducted during the light phase (9 am - 5 pm). Following a 2-hour period when mice were allowed to habituate to the chambers, animals received either fluoxetine (1 - 15 mg/kg) or saline, and data was collected in 5-min bins for a period of two hours following injection. Open field habituation was analyzed during the full two hours of testing. Fluoxetine locomotor activity was analyzed for distance traveled in centimeters (cm) over the first 20 minutes following drug administration.

**Forced Swim Test:** The forced swim testing procedures were adapted from Strekalova et al (62). At the beginning of the test, animals were injected with either saline or 15 mg/kg fluoxetine in a 0.1mL volumes, and placed in the home cage for 20 minutes. They were then placed in a transparent pool of water for 6 minutes. The pool consisted of a round glass cylinder (diameter 15cm, height 22cm) partially filled with water (23 degrees C, height 17.5 cm). The room was lit and room temperature maintained to housing colony standards. Latency to the first floating episode, the total time spent floating, and the
number of floating episodes were recorded. Floating episodes were defined as the absence of directed swimming or escape movement persisting for at least 5 seconds.

**Tail Suspension Test:** The tail suspension test procedures were adapted from Crowley et al (20). At the beginning of the test, animals were injected with either saline or 15 mg/kg fluoxetine in a 0.1mL volume and returned to the home cage for 20 minutes. They were then hung by the tail with tape for 5 minutes on a horizontal aluminium bar that was suspended 14cm from the table top. To prevent mice from climbing their own tails during the test, a convex plastic dish was attached to the horizontal bar, with the mouse’s tail threaded through the dish, preventing the mouse from gaining purchase on which to climb. Sessions were 5 minutes in duration. Latency to first hanging episode, the total time spent hanging, and the number of hanging episodes was recorded. Hanging episodes were defined as the absence of any directed struggle or escape movements persisting for at least 5 seconds.

**Sucrose Drinking Test:** The sucrose drinking protocols were adapted from Strekalova et al (63). Beginning 48 hours following the final administration of MPH or saline, animals were given free choice between two bottles of water for 12 hours (between 7pm and 7am) at decreasing concentrations over a three day period. One bottle was filled with tap water, and the other was filled with a sucrose solution (1%, 0.5% or 0.25% sucrose dissolved in tap water). To prevent side bias, the positions of the bottles in the cage were switched daily and randomized across groups. Bottles were filled in advance and kept at the proper angle to minimize leaks and spills, and bottles remained in the housing colony.
room for two hours prior to testing to mitigate possible differences in temperature. During the first 24 hours following the final administration of MPH or saline, animals were permitted to drink a 2.5% sucrose solution in a one-bottle test for 2 hours, to minimize effects of neophobia. Percent sucrose preference and total liquid intake were recorded.

**Statistical Analyses:** The effect of MPH treatment and subsequent administration of fluoxetine open field activity, locomotor responses to fluoxetine, forced swim test, and tail suspension measures was assessed using a repeated measures two-way ANOVA between saline and MPH treated groups with a Bonferroni post-hoc test where appropriate. Sucrose drinking was analyzed for percent sucrose preference and total liquid intake using a Student’s t-test. When appropriate, outliers were detected via Grubb’s test.

**Chemicals:** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Results**

**Effects of chronic MPH on locomotor habituation and locomotor responses to fluoxetine.** Following 14 days of treatment with 20 mg/kg MPH or saline i.p., animals were placed in an open field for 2 hours. MPH treatment had no effect on the time taken to fully habituate to the locomotor chambers (Figure 1A). Similarly, MPH treatment had no effect on locomotor responses to saline challenge (Figure 1B). Interestingly,
however, MPH treatment significantly affected locomotor responses to fluoxetine in the open field. While increasing doses of fluoxetine significantly decreased locomotor activity in saline treated animals, MPH treated animals showed no significant decreases in locomotor activity (Figure 1B), suggesting altered motor responses to fluoxetine that could affect further studies with the drug.

**Effects of chronic MPH on performance in the forced swim test.** Following 14 days of treatment with 20 mg/kg MPH or saline, animals were placed in the forced swim test environment for 6 minutes, and latency to first flotation episode, the total time spent immobile, and the total number of immobility episodes were recorded following a challenge dose of saline or 15 mg/kg i.p. fluoxetine. MPH-treated animals were observed
Figure 1: Locomotor habituation and response to fluoxetine

A

B

distance traveled (cm)

saline

MPH

time (min)

distance traveled (cm/20 min)

saline

MPH

fluoxetine (mg/kg, i.p.)
Figure 1: Effects of chronic MPH on locomotor activity in the open field during habituation and in response to fluoxetine A: Effects of chronic MPH treatment on locomotor habituation over a 2 hour exposure to the open field (n=24 per group). There was no difference between MPH and saline treated animals in the time taken to habituate to the open field, or in the total number of locomotor counts (effect of treatment $F_{(1, 1080)} = 3.345, p>0.05$), though both groups showed a strong effect of time in the open chamber on locomotor activity ($F_{(23, 1080)} = 74.50, p<0.001$). B: Effects of chronic MPH treatment on locomotor responses during the first 20 minutes following i.p. injections of fluoxetine (0-15 mg/kg, i.p., n=12 per group). There was a significant effect of MPH treatment of locomotor responses to fluoxetine (effect of treatment $F_{(1,108)} = 5.080, p<0.05$).
to have significant decreases in the latency to first immobility episode (Figure 1A), a measure that is considered to be a depressive-like phenotype (52). Treatment with 15 mg/kg i.p. fluoxetine increased the latency to first flotation episode in both saline- and MPH-treated animals (Figure 1A), and the effect of fluoxetine in MPH-treated animals was markedly greater.

In measures of total time spent immobile, MPH- and saline-treated animals did not differ in their response to saline, or in their response to 15 mg/kg fluoxetine (Figure 1B). Interestingly, however, MPH-treated animals did show a significant difference in their total immobility time when exposed to fluoxetine, while fluoxetine had no significant effect on total immobility time in saline-treated animals, providing further evidence that the effects of fluoxetine on MPH-treated animals are stronger than in saline-treated controls.

The number of immobility episodes in MPH- and saline-treated animals was also measured in the forced swim test paradigm. There were no significant differences observed between MPH- and saline-treated animals in response to saline or fluoxetine, and the effect of 15 mg/kg fluoxetine on the number of immobility bouts in MPH-treated animals appeared greater but did not reach significance (Figure 1C).

**Effects of chronic MPH on behavioral measures in the tail suspension test.** Following chronic treatment with 20 mg/kg MPH or saline for 14 days, animals were suspended by the tail for a total of 5 minutes, and the latency to first immobility episode, the total time immobile, and the number of immobility episodes were recorded. In contrast to results seen in the forced swim test, MPH treatment did not affect the latency
Figure 2: Forced Swim Test

A

Latency to 1st Floatation Episode (s)

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<th>saline (control)</th>
<th>15 mg/kg Fluoxetine</th>
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B

Total Time Immobile (s)

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<td><strong>p=0.05</strong></td>
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C

Immobility Bouts

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<th></th>
<th>saline (control)</th>
<th>15 mg/kg Fluoxetine</th>
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<td><strong>p=0.14</strong></td>
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Figure 2: Effects of chronic MPH on Forced Swim Test behavior

A: Effects of chronic MPH treatment on the latency to first flotation episode in the forced swim test in response to saline or 15 mg/kg fluoxetine i.p. (n=12 per group). MPH-treated animals showed a significant decrease in latency to first flotation episode compared to saline-treated controls (t=2.755, p<0.05). Both MPH and saline treated animals showed a significant increase in latency to first flotation episode following 15 mg/kg i.p. fluoxetine (t=4.842, p<0.001, t=6.860, p<0.001 respectively), but the latency to first flotation episode following i.p. fluoxetine did not differ between MPH and saline treated animals (t=1.535, p>0.05).

B: Total time spent immobile (s) in the forced swim test in MPH and saline treated mice (n=12 per group) in response to saline or 15 mg/kg i.p. fluoxetine. There was no significant difference in response to saline (t=1.475, p>0.05), and saline-treated animals showed no significant response to fluoxetine in total time spent immobile (t=1.949, p>0.05). MPH-treated animals showed a significant decrease in total time spent immobile following fluoxetine (t=3.181, p<0.01).

C: Total immobility bouts in the forced swim test in MPH and saline treated animals, in response to saline or 15 mg/kg fluoxetine, i.p. (n=12 per group). There were no differences observed in the number of immobility bouts between MPH- and saline-treated mice (t=1.515, p>0.05, t=1.396, p>0.05, respectively), either in response to saline injection (t=0.3227, p>0.05) or in response to 15 mg/kg fluoxetine (t=0.5849, p>0.05).
to the first immobility episode in response to a saline challenge (Figure 2A). 15 mg/kg fluoxetine had no effect on the latency to first immobility episode in saline treated animals, though there was a trend toward increased latency to immobility in MPH-treated mice. There was also no difference between MPH- and saline-treated animals in total time spent immobile in the tail suspension test, either in response to saline or 15 mg/kg fluoxetine injection (Figure 2B).

Although MPH and saline-treated animals showed few differences in the tail suspension test in measures of latency to first immobility episode or total time spent immobile, there were significant effects of MPH treatment on the total number of immobility episodes during the trial (Figure 2C). Saline treated mice showed no difference in the number of immobility episodes in response to saline or 15 mg/kg fluoxetine challenge. In contrast, MPH-treated animals showed a significant increase in the number of immobility episodes in response to 15 mg/kg fluoxetine, partially due to a nonsignificant decrease in the number of immobility bouts at baseline (Figure 2C). These results provide an interesting contrast to results seen with the forced swim test, indicating possible differences between these two anti-depressant efficacy measures.

**Effects of chronic MPH on sucrose preference.** A decrease in the intake of palatable solutions and decreases in preference for these solutions is thought to signal anhedonia, the decreased ability to experience pleasure, which is one of the core symptoms of human depression (36;63). Following 14 days of 20 mg/kg MPH i.p., or an equivalent volume of saline, animals were exposed to a two-bottle choice paradigm of sucrose preference at
Figure 3: Tail Suspension Test

A

Latency to 1st Immobility Episode (s)

Saline MPH

0 25 50 75 100

0 25 50 75 100

saline fluoxetine

p=0.051

B

Total Time Immobile (s)

Saline MPH

0 25 50 75 100 125

0 25 50 75 100 125

saline fluoxetine

injection

*p=0.108

C

Immobility Bouts

Saline MPH

0.0 2.5 5.0 7.5 10.0

0.0 2.5 5.0 7.5 10.0

saline fluoxetine

*p=0.108
Figure 3: Effects of chronic MPH on tail suspension test measures A: Latency to first immobility episode (s) in the tail suspension test in MPH- and saline-treated animals following i.p. saline or 15 mg/kg fluoxetine (n=24 per group). There were no differences observed in the latency to first immobility between MPH- and saline-treated animals in response to saline (t=0.7955, p>0.05) or fluoxetine (t=1.950, p=0.057). Saline treated animals also showed no significant response to fluoxetine injection (t=0.3701, p>0.05), and MPH-treated animals showed only a trend toward a response to fluoxetine injection (t=2.003, p=0.051). B: Total time spent immobile (s) in the tail suspension test in MPH- and saline-treated animals following i.p. saline or 15 mg/kg fluoxetine (n=24 per group). There was no difference between MPH- and saline-treated animals in response to saline (t=1.140, p>0.05) or fluoxetine (t=0.4278, p>0.05), and neither MPH- nor saline-treated animals showed a significant change in total time spent immobile in response to fluoxetine (t=0.3221, p>0.05, t=1.120, p>0.05 respectively). C: Number of immobility bouts in the tail suspension test in MPH- and saline-treated animals following i.p. saline or 15 mg/kg fluoxetine (n=24 per group). There was no difference between MPH- and saline-treated animals in response to saline (t=1.639, p>0.05), or in response to fluoxetine (t=0.4166, p>0.05). MPH-treated animals showed a significant increase in number of immobility bouts in response to fluoxetine (t=2.127, p<0.05), while saline-treated animals showed no effect of fluoxetine injection (t=0.6428, p>0.05).
1.0, 0.5 or 0.25% sucrose for 12 hours during the dark cycle. Total liquid intake did not decrease as sucrose concentration declined (Figure 3A), and there was no significant effect of MPH treatment on sucrose preference (Figure 3B). Thus it appears that chronic MPH treatment does not produce changes that can be detected with the sucrose drinking paradigm, and implies that results in the forced swim and tail suspension tests may not be the result of anhedonia.

Discussion

The results of these studies indicate that chronic, high dose MPH treatment is capable of producing a significant depressive-like phenotype in the forced swim and tail suspension tests, as well as resulting in augmented responses to fluoxetine administration in these tests. MPH-treated mice also show differences in locomotor responses to fluoxetine administration, suggesting decreased sensitivity to the locomotor attenuating effects, with no difference in habituation or saline locomotor responses in the open field. Despite the differences observed in depressive-like phenotype, however, no differences could be detected in the sucrose drinking protocol. These results, found in adult mice, show similarities to results in rats treated during adolescence and tested in adulthood, which showed increased immobility in the FST and reduced sucrose preference in adulthood (7;12), and show that these effects are persistent across species and can be found following adult treatment with MPH. In addition, these findings support the hypothesis that chronic, high-dose MPH produces a depressive-like phenotype in rodents, suggesting that long-term MPH dosing may have significant effects on hedonic responding.
Figure 4: Sucrose Drinking Protocol

A

Intake (mL) vs. % Sucrose

<table>
<thead>
<tr>
<th>% Sucrose</th>
<th>Saline</th>
<th>MPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>4.00</td>
<td>5.00</td>
</tr>
<tr>
<td>0.50</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>0.25</td>
<td>4.00</td>
<td>4.00</td>
</tr>
</tbody>
</table>

B

% Preference vs. % Sucrose

<table>
<thead>
<tr>
<th>% Sucrose</th>
<th>Saline</th>
<th>MPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>0.50</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>0.25</td>
<td>75</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 4: Effects of chronic MPH on sucrose drinking measures A: Percent of sucrose preference at 0.25, 0.5, and 1.0% sucrose in MPH- and saline-treated animals (n=17 per group). There was a significant effect of sucrose dose ($F_{2,98}=20.29$, $p<0.001$), but no effect of MPH-treatment on sucrose preference ($F_{1,98}=0.2667$, $p>0.05$). B: Total liquid intake during sucrose preference testing at 0.25, 0.5, and 1.0% sucrose in MPH- and saline-treated animals (n=12 per group). There was no significant effect of sucrose dose on total liquid intake ($F_{2,98}=1.298$, $p>0.05$), and no effect of MPH-treatment on total liquid intake ($F_{1,98}=0.01565$, $p>0.05$).
Dysfunction of the DA system in MDD

It is well established that dysfunction of the monaminergic neurotransmitter systems correlates with depressive phenotypes in rodents (53;59) and in humans (15). Many studies have focused on the role of 5-HT, and particularly the 5-HT transporter (SERT) in depressive-like phenotypes in both humans and rodents (29;47), and 5-HT receptors have been implicated, in particular the 5-HT1B (64), the 5-HT1A (51), and the 5-HT2C (57). However, there may also be a role for NE and DA systems in depressive disorders (see (49) for review). It is known that monoamine transporter inhibitors such as cocaine produce antidepressant effects in rodents (43;61), and treat major depression in humans (3;28;31). While most monoamine amine transporter inhibitors are relatively nonspecific for DA, 5-HT, and NE, DA in particular has been implicated in several aspects of animal and human depression (26). It is known that DA is involved in anhedonia (50), and DA agonists and DAT inhibitors have effects in the FST apart from effects seen on locomotor activity (5;18). While there is little correlation between forced swim test results and prefrontal 5-HT, there is a strong correlation with DA levels and FST performance (37). In addition, DAT inhibitors in particular can produce clinically effective antidepressant effects (3;31), and DA agonists and DAT inhibitors can increase the anti-depressant effects of selective serotonin reuptake inhibitors, which are often prescribed to treat depression in humans (55). Given the involvement of DA systems in depressive phenotypes, as well as the known depressive effects of psychostimulant withdrawal (4;48), it is thus not surprising that withdrawal from MPH in particular might elicit a depressive-like phenotype.
Possible effects of 5-HT

Several studies have shown that withdrawal from psychostimulants produces anhedonic effects (4;48) and treatment with MPH in adolescence and adulthood has been shown to produce depressive-like effects in the forced swim test (7). This is the first study, however, to look at higher doses of MPH in a chronic abuse-like paradigm and to examine the subsequent depressive-like effects in mice. Although MPH does not have high affinity for the SERT directly (30), it has been shown to act as an agonist at the 5-HT1A receptor (45). This receptor acts as an autoreceptor on 5-HT cell bodies in the raphe (32), and stimulation of 5-HT1A receptors in this area produces dramatic decreases in 5-HT neuron firing (13;17;70). Thus it is possible that stimulation of the 5-HT1A receptor during chronic MPH administration could produce low extracellular levels of 5-HT through decreases in 5-HT neuron firing rate.

The importance of 5-HT systems in depressive phenotypes is well established, due to the clinical efficacy of 5-HT-centric pharmacotherapies in treating depression (16) and indeed treatment with cocaine and other psychostimulants with serotonergic affinity are known to produce changes in 5-HT systems (42;68). Thus, MPH exposure might have been expected to produce differences in the production of depressive phenotypes, given the strong involvement of 5-HT systems in major depressive disorder (16), and the significant inhibitory effect of 5-HT1A receptors on 5-HT neuron firing (13;17;70).

Given the low affinity of MPH for the SERT, the effects on depressive-like behaviors induced by chronic MPH may be results of alterations following chronic 5-HT1A receptor stimulation, as well as alterations in DA and NE systems. It should be noted that MPH treated mice also showed potentiated locomotor responses to the SERT
specific drug fluoxetine. The effects seen with fluoxetine may indicate that changes in depressive-like behaviors arising from MPH treatment have include alterations in the 5-HT system. Further studies will be required to investigate the effects of chronic MPH treatment on 5-HT systems, in particular those arising from stimulation of the 5-HT1A receptor.

It is important to note potential issues associated with studying the behavioral consequences of long-term psychostimulant exposure. The data obtained from the TST does not appear to show as strong an effect as that from the FST, a difference possibly due to different cohorts of mice, or differences in the apparatus. Despite minor differences, however, it is clear that the overall effects of MPH treatment of total time spent immobile and the differences in response to fluoxetine extend across tests and confirm the effects of long-term MPH on depressive phenotypes.

In contrast to the results seen with TST and FST, the sucrose drinking paradigm used to assess anhedonia did not reveal any significant difference between MPH and saline treated mice, though preference did drop below 60% for low dose sucrose, a decrease in preference that other studies have suggested as the cut-off for anhedonia (62). It is possible that, as mice were singly housed throughout the experiment, the effects of single housing on anhedonia (46) eclipsed those of MPH treatment. Further studies would be necessary to elucidate this difference. In addition, it is interesting that fluoxetine had such pronounced effects in MPH treated mice, even though MPH has no affinity for the 5-HT transporter. It might be interesting to differentiate the effects of MPH on DA and 5-HT systems by testing an antidepressant such as buproprion, which is DA/NE as opposed to 5-HT.
Conclusions

In conclusion, the results of this study have shown pronounced effects of chronic MPH treatment in mice on subsequent examinations of depressive phenotypes in the FST and TST. Given the strong affinity of MPH for the DAT and NET, it is possible that these effects arise from the chronic blockade of these systems, and that withdrawal from abused doses of MPH could be the mechanisms for these behavioral effects. However, MPH also has affinity for the 5-HT1A receptor (45), a receptor that reduces 5-HT neuron firing in the raphe, and that could provide a mechanism for decreased levels of 5-HT, producing a depressive phenotype. This study could have implications for the effects of chronic MPH in humans, in particular the effects of abused doses of MPH, incidents of which are becoming more common as prescriptions for MPH increase (60). This study could open up new research avenues into the behavioral effects of chronic MPH exposure beyond that of future psychostimulant abuse, and implicate a role for depression in MPH withdrawal.

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50; 2001.
The following manuscript is in preparation for submission to the journal Psychopharmacology. Stylistic variations are due to the requirements of the journal. Bethany R. Brookshire performed the experiments and prepared the manuscript. Doctor Sara R. Jones acted in an advisory and editorial capacity.
Abstract

Methylphenidate (MPH) is a common prescription psychostimulant in child and adult populations, and abuse of this drug is growing more common as prescription rates increase. MPH, like other psychostimulants, inhibits the dopamine (DA) and norepinephrine transporters, and also has been shown to act as an agonist at 5-HT1A receptors. MPH is very similar in acute effects to psychostimulants such as cocaine, and thus sensitization following chronic, high dose exposure is likely. We investigated the development of locomotor and neurochemical sensitization in response to chronic MPH (20 mg/kg, i.p., 14 days) at both two and ten days following treatment. At two days following MPH exposure, MPH-treated animals showed behavioral sensitization to psychostimulants as well as to the selective serotonin reuptake inhibitor fluoxetine, and microdialysis results in the nucleus accumbens (NAc) indicated a serotonergic component in the sensitized response to these drugs. In contrast, results at ten days following treatment showed behavioral and neurochemical sensitization mediated by DA, as seen with other MPH treatment regimens and as is common of psychostimulant exposure. This study suggests that early behavioral sensitization to MPH is not correlated with dopaminergic sensitization in NAc, but is followed by a later DA mediated sensitization. Our work illuminates some of the mechanisms engaged by the abuse of MPH.
Introduction

Methylphenidate (MPH, Ritalin) is a prescription stimulant used in the treatment of attention-deficit/hyperactivity disorder, and it is commonly prescribed to children and adolescents (Biederman et al, 2002). Ritalin may be taken along with other prescription psychostimulants such as amphetamine. Despite their reputation for safety when taken in low, oral doses, abuse of prescription stimulants has been rising as the prescriptions for these drugs increase (Setlik et al, 2009). In addition, abuse of prescription stimulants such as MPH and amphetamine is correlated with abuse of other psychostimulants such as 3,4-methylenedioxymethamphetamine (MDMA) (McCabe et al, 2005; Yacoubian, Jr., 2003). In light of the increase in prescription stimulant abuse, it is imperative that the consequences of exposure to high doses of MPH be examined, to determine both the short and long-term effects of this drug.

MPH is a psychostimulant that binds to the dopamine (DA) and norepinephrine (NE) transporters (DAT, NET) to increase extracellular levels of these monoamines (Gatley et al, 1996; Kuczenski et al, 1997). Increases in DA in areas such as the nucleus accumbens (NAc) are known to be involved in the initial rewarding and stimulating effects of psychostimulants (Wise, 1987), and at abused doses, MPH shares these effects with other psychostimulants (Kollins et al, 2001; Volkow et al, 1999). MPH is readily self-administered in animals and produces increases in extracellular DA comparable to those observed with cocaine (Kollins et al, 2001; Volkow et al, 1999). In addition, MPH causes robust psychomotor sensitization following repeated exposure (Guerriero et al, 2006).
Until recently, the development of MPH sensitization has not been extensively studied, possibly because it is presumed to produce similar effects to cocaine. However, MPH and cocaine have important pharmacological differences, including a distinct difference in active period (Gatley et al, 1996), and differences in target selectivity. While cocaine inhibits the DAT, NET, and serotonin (5-HT) transporter (SERT) with similar affinity, MPH has high affinity only for the DAT and NET (Gatley et al, 1996), making its pharmacological effects distinct from that of cocaine. In addition, MPH has been shown to act as an agonist at the 5-HT1A receptor (Markowitz et al, 2009). It is therefore possible that the effects of high dose chronic MPH exposure will differ during early and later withdrawal from the effects of similar doses of cocaine.

To probe the effects of chronic MPH administration on early and late time points following treatment, we used locomotor activity measures and in vivo microdialysis in the NAc to look at DA and 5-HT changes following chronic MPH administration in mice. We evaluated the effects of 20 mg/kg i.p. MPH given for 14 days on neurochemical and behavioral response to common psychostimulants and the selective serotonin reuptake inhibitor (SSRIs) fluoxetine, 2 days and ten days following exposure. While fluoxetine is not a drug usually examined for sensitization following psychostimulant treatment, previous work in our laboratory has indicated that chronic DAT inhibition, either through genetic deletion of the transporter or through pharmacologic means, can produce alterations in the locomotor and neurochemical response to fluoxetine (Mateo et al, 2004), and it is important to determine the time course of these effects. Our results show that chronic MPH produced a brief, sensitized serotonin response to psychostimulants, and moderate behavioral and dopaminergic sensitization to fluoxetine during the first 2
days following treatment. These effects were replaced by augmented locomotor and
dopaminergic responses to psychostimulants ten days following MPH exposure, while
responses to fluoxetine challenge returned to normal. These findings shed light upon the
time-dependent effects of abused doses of MPH and possible development of
sensitization that could inform the human abuse paradigm.

**Materials and Methods**

**Animals:** Equal numbers of male and female C57BL/6J mice between 2 and 4 months of
age were treated with 20 mg/kg MPH or saline in a volume of 0.1mL i.p. once per day for
14 days. Groups of animals remained in the home colony until testing at either 2 days or
ten days post-treatment. Animals were group housed with food and water provided ad
libitum. Animal care was in accordance with Wake Forest University’s Institutional
Animal Care and Use Committee and in compliance with National Institutes of Health
guidelines.

**Locomotor Activity:** Activity was assessed using Med Associates Open Field Activity
Monitors (Med Associated, St. Albans, VT). The open field consisted of a square
plexiglass container (27.0 cm X 27.0 cm X 20.3 cm) with three 16-beam I/R (infrared)
arrays. Analyses were conducted during the light phase between 9 am and 5 pm.
Following a 2-hour period when mice were allowed to habituate to the chambers, animals
received either cocaine (10mg/kg), fluoxetine (15 mg/kg), MDMA (5.0 mg/kg), or saline
in a 0.1mL injection i.p., and data was collected in 5–min bins for a period of two hours
following injection. Data was analyzed for distance traveled in centimeters and number of vertical rears performed during the active period of the drug.

**Microdialysis and HPLC:** 24 hours or 9 days after the final drug or saline injection, mice were anesthetized with ketamine/xylazine (100mg/kg and 13 mg/kg i.p., respectively), and a microdialysis guide cannula (CMA/7 Guide Cannula; CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically implanted into the NAc (anterior, +1.2 mm, lateral, -0.6 mm, ventral -4.0 mm, relative to bregma and dura surface). Concentric microdialysis probes (membrane length 2 mm, CMA/7, CMA/Microdialysis AB, Stockholm, Sweden) were implanted while animals were recovering from surgery. Experiments were conducted in freely moving mice 12 hours following implantation of the probe. Probes were perfused with artificial cerebrospinal fluid (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl2, 0.85 mM MgCl2, at pH 7.4) at a constant flow rate of 0.8 µl/min. Samples were collected every 20 min and analyzed for DA, 5-HT and metabolites by high-performance liquid chromatography coupled to electrochemical detection. Samples were separated on a Luna 3um C18(2) column (50 x 2 mm, Phenomenex, Torrence, CA) using a mobile phase containing 75 mM NaH2PO4, 1.7 mM 1-octanesulfonic acid sodium salt (Acros, Geel, Belgium), triethylamine, 25 uM EDTA, and 10% (±5%) acetonitrile in ultra pure water (ESA Analytical, Chelmsford, MA). Microdialysis data were calculated as the percentage change from baseline concentration, with 100% being defined as the average of the last three samples prior to injection of drug. Cocaine (10 mg/kg i.p), MDMA (5.0 mg/kg, i.p.), or fluoxetine (15
mg/kg, i.p.), was then administered and samples collected every 20 minutes for two hours following drug administration.

**Histology:** Mice were anesthetized with ketamine (100 mg/kg) and xylazine (13 mg/kg) and 0.5% Chicago Blue Dye (Sigma-Aldrich Inc.) was perfused through the microdialysis probes for ~5 min. Mice were perfused transcardially with 10 ml of 10% formalin (pH 7.0; Sigma, St. Louis, MO), brains were removed and cryoprotected in 30% sucrose solution (in 0.01 M phosphate buffer, pH 7.4). Sections were then sliced (40 µm sections) through the NAc, to identify microdialysis probe locations.

**Statistical Analyses:** The effect of cocaine, MDMA, and fluoxetine on extracellular concentrations of DA and 5-HT in the NAc was assessed by a mixed design two-way analysis of variance (ANOVA), with MPH treatment as the between-subject factor and time as the within-subject factor. Locomotor data were analyzed for distance traveled and vertical rears over 5 min bins. In the case of locomotor dose response curves, data were analyzed by a two-way ANOVA with a Bonferroni post-hoc test. Values of p<0.05 were considered statistically significant.

**Chemicals:** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.
Figure 1: Locomotor Activity day 2 and day 10

A

Locomotor Activity Day 2

- Saline treated
- MPH treated

p = 0.09
p = 0.10

B

Locomotor Activity Day 10

- Saline treated
- MPH treated

p = 0.13
Figure 1: Effects of chronic MPH on locomotor responses at two and ten days post-treatment A: Locomotor results over the first 30 minutes following i.p. injections of 0.1 mL saline, 15 mg/kg fluoxetine, 10 mg/kg cocaine, or 5.0 mg/kg MDMA (n=24 per group for 0.1 mL saline, n=12 per group for all other drugs), testing performed 48 hours following the final administration of MPH or saline. There was no significant effect of MPH treatment on locomotor responses to saline, but MPH treatment produced significant effects on locomotor responses to 15 mg/kg fluoxetine (p<0.05), and a trend toward locomotor sensitization to 10 mg/kg cocaine (p=0.09) and 5.0 mg/kg MDMA (p=0.1). B: Locomotor results over the first 30 minutes following i.p. injections of 0.1 mL saline, 15 mg/kg fluoxetine, 10 mg/kg cocaine, or 5.0 mg/kg MDMA (n=24 per group for 0.1 mL saline, n=12 per group for all other drugs), testing performed ten days following the final administration of MPH or saline. There was a significant cross-sensitization to 10 mg/kg cocaine in MPH treated animals (p<0.01).
Results

Effects of chronic MPH on locomotor activity responses at two and ten days post-treatment. All animals were habituated and monitored for locomotor activity for two hours prior to drug injection. At both two and ten days post-treatment, there was no difference in baseline/saline-induced locomotor activity between MPH- and saline-treated animals (t=1.115, p>0.05, Figure 1). MPH-treated animals showed a trend toward cross-sensitization to the locomotor stimulating effects of cocaine at day 2 and significant cross-sensitization at ten days post-treatment, (day 2: t=1.733, p=0.09, day 10: t=3.671, p<0.01 Figure 1A, 1B). In addition, MPH-treated animals showed a trend toward cross-sensitization to MDMA, a psychostimulant with a strong serotonergic component, though this effect did not reach significance at two or ten days post-treatment (day 2: t=1.680, p=0.1, day 10: t=1.533, p=0.13, Figure 1). However, in response to the selective serotonin reuptake inhibitor (SSRI) fluoxetine, MPH-treated mice showed a significant difference in locomotor response at two days following treatment. Fluoxetine is a SSRI known to have locomotor attenuating properties in mice (Brookshire et al, 2009). However, MPH-treated mice showed a significant attenuation of the locomotor inhibiting effects of fluoxetine two days post-treatment (t=2.156, p<0.05, Figure 1A). This effect disappeared by ten days post-treatment (t=0.3226, p>0.05, Figure 1B).

Effects of chronic MPH on neurochemical responses to cocaine at two and ten days post treatment. As we observed a trend toward locomotor cross-sensitization to cocaine in MPH treated mice at two days following treatment, we hypothesized that extracellular DA responses would be amplified as well, correlating with the results seen in locomotor
activity measures (Akimoto et al, 1990; Kalivas et al, 1990). Surprisingly, microdialysis for DA in the NAc showed no significant augmentation in extracellular DA response to 10 mg/kg cocaine at two days post-treatment in MPH-treated animals (F1,136=0.1670, p>0.05, Figure 2A) although both MPH and saline treated mice showed a significant effect of cocaine injection on extracellular DA levels (F9,136=12.08, p<0.001, Figure 2A). However, although there was no augmentation in DA response to cocaine at two days post-treatment, there was a significant amplification in 5-HT response in the NAc in MPH-treated mice at this time point (F1,107=7.898, p<0.01, Figure 2B), as well as a significant effect of cocaine challenge (F9,107=6.288, p<0.001, Figure 2B), an effect that has not been measured before in animals exposed to chronic psychostimulants.

Although we observed no augmentation in DA responses to cocaine at two days post-treatment, by ten days withdrawal from MPH treatment, the sensitized DA response to cocaine was robust in MPH-treated animals (F1,97=29.42, p<0.001, Figure 2C), with a significant interaction between cocaine challenge and MPH treatment (F9,97=3.206, p<0.01, Figure 2C). A significant effect of cocaine challenge was observed in both MPH and saline-treated animals (F9,97=12.47, p<0.001, Figure 2C). Interestingly, although there was an augmented 5-HT response at two days post-treatment, by ten days of withdrawal the 5-HT responses of MPH-treated animals were indistinguishable from their saline-treated counterparts (Effect of cocaine injection: F9,99=3.598, p<0.001, Effect of MPH treatment: F1,96=0.3400, p>0.05, Figure 2D).
Figure 2: Cocaine day 2 and day 10

A  Day 2: Extracellular DA response to 10 mg/kg Cocaine, i.p.

B  Day 2: Extracellular 5-HT response to 10 mg/kg Cocaine, i.p.

C  Day 10: Extracellular DA response to 10 mg/kg Cocaine, i.p.

D  Day 10: Extracellular 5-HT response to 10 mg/kg Cocaine, i.p.
Figure 2: Effects of chronic MPH on neurochemical responses to cocaine at two and ten days post-treatment: A: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline treated animals, two days following the final injection of MPH or saline, in response to 10 mg/kg i.p. cocaine (n=8 per group). There was a significant effect of cocaine injection (p<0.001). B: *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline treated animals on day two in response to 10 mg/kg i.p. cocaine (n=8 per group). There was a significant effect of cocaine injection (p<0.001), a significant effect of MPH treatment (p<0.01), and a significant interaction between MPH treatment and extracellular 5-HT response (p<0.01). C: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline-treated animals at day ten in response to 10 mg/kg i.p. cocaine (n=6 per group). There was a significant effect of cocaine on extracellular DA levels (p<0.001), a significant effect of MPH treatment (p<0.001), and a significant interaction between MPH treatment and extracellular DA response (p<0.01). D: Single prove *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline-treated animals at day ten, in response to 10 mg/kg i.p. cocaine (n=6 per group). There was a significant effect of cocaine injection on extracellular 5-HT levels (p<0.001).
Effects of chronic MPH on neurochemical responses to MDMA at two and ten days post treatment. We performed microdialysis in the NAc of MPH- and saline-treated mice at two and ten days post-treatment. At two days post-treatment, MPH- and saline-treated mice showed no difference in their DA response to i.p. MDMA (Effect of MDMA challenge: $F_{9,97}=7.664$, $p<0.001$, Effect of MPH treatment, $F_{1,97}=0.1992$, $p>0.05$, Figure 3A), even though locomotor results indicated a trend toward locomotor cross-sensitization to the drug (Figure 1A). Similar to results seen with cocaine, we observed that 5-HT responses to MDMA at two days post-treatment were significantly augmented in MPH-treated animals ($F_{1,97}=20.82$, $p<0.001$, Figure 3B), although both MPH treated animals and controls showed a significant 5-HT response to MDMA ($F_{9,97}=14.60$, $p<0.001$, Figure 3B).

At ten days post-treatment, DA responses to MDMA remained similar between groups, both in locomotor responses (Figure 1B) and in neurochemical responses in the NAc (Effect of MDMA injection: $F_{9,60}=7.404$, $p<0.001$, Figure 3C), with no significant difference between MPH- and saline-treated animals in extracellular DA response to i.p. MDMA administration ($F_{1,60}=0.6139$, $p>0.05$, Figure 3C). Similar to results seen with cocaine, the augmentation in 5-HT response, which had been so prominent at two days post-treatment, was no longer present following ten days of withdrawal, ($F_{1,80}=1.170$, $p>0.05$, Figure 3D), although a significant effect of MDMA injection on 5-HT levels remained ($F_{1,80}=4.561$, $p<0.001$, Figure 3D).
Figure 3: MDMA day 2 and day 10

A  Day 2: Extracellular DA response to 5 mg/kg MDMA, i.p.

B  Day 2: Extracellular 5-HT response to 15 mg/kg Fluoxetine, i.p.

C  Day 10: Extracellular DA response to 5 mg/kg MDMA, i.p.

D  Day 10: Extracellular 5-HT response to 5 mg/kg MDMA, i.p.
Figure 3: Effects of chronic MPH on neurochemical responses to MDMA at two and ten days post-treatment: A: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline treated animals at day two in response to 5 mg/kg i.p. MDMA (n=6 per group). There was a significant effect of injection (p<0.001). B: *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline treated animals at day two in response to 5 mg/kg i.p. MDMA (n=6 per group). There was a significant effect of MDMA injection (p<0.001), and a significant effect of MPH treatment (p<0.001). C: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline-treated animals at day ten in response to 5 mg/kg i.p. MDMA (n=4 per group). There was a significant effect of MDMA injection (p<0.001). D: Single prove *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline-treated animals and day ten in response to 5 mg/kg i.p. MDMA (n=4 saline, n=6 MPH treated animals). There was a significant effect of MDMA injection on extracellular 5-HT levels (p<0.001).
Effects of chronic MPH on neurochemical responses to fluoxetine at two and ten days post treatment. We conducted in vivo microdialysis in the NAc for DA and 5-HT. In saline treated controls, i.p. fluoxetine decreased extracellular DA in the NAc ($F_{9,128}=3.325, p<0.01$, Figure 2A) (Gobert et al, 1997; Tanda et al, 1995). In contrast, MPH-treated animals showed a small, but significant increase in extracellular DA following fluoxetine administration (effect of MPH treatment, $F_{1,128}=14.15, p<0.001$, interaction between treatment and fluoxetine: $F_{9,128}=2.081, p<0.05$, Figure 4A). Additionally, though MPH-treated animals showed differences in extracellular DA responses to fluoxetine in the NAc, MPH- and saline-treated mice did not differ in their extracellular 5-HT response to fluoxetine administration (Effect of fluoxetine challenge: $F_{9,136}=2.138, p<0.05$, Effect of MPH treatment: $F_{1,136}=1.207$, Figure 4B).

The results seen with microdialysis at ten days post-treatment also mirrored the results seen with locomotor activity measures at the 10-day time point. Measures of DA in the NAc in MPH- and saline-treated mice showed no effects on extracellular DA ($F_{9,98}=0.8678, p>0.05$, Figure 4C), and no difference in response to 15 mg/kg fluoxetine at ten days post-treatment ($F_{1,98}=0.9138, p>0.05$, Figure 4C), and measures of 5-HT in the NAc of MPH- and saline-treated mice also showed no difference between the two groups ($F_{1,96}=0.1601, p>0.05$, Figure 4D), though a significant effect of fluoxetine challenge on extracellular 5-HT remained ($F_{9,96}=3.904, p<0.001$, Figure 4D). These results suggest that the alterations in DA responses to fluoxetine following MPH treatment are temporary, and that the system returns to normal with regard to SSRI responses within ten days following treatment.
Figure 4: Fluoxetine day 2 and day 10

A  Day 2: Extracellular DA response to 15 mg/kg Fluoxetine, i.p.

B  Day 2: Extracellular 5-HT response to 15 mg/kg Fluoxetine, i.p.

C  Day 10: Extracellular DA response to 15 mg/kg Fluoxetine, i.p.

D  Day 10: Extracellular 5-HT response to 15 mg/kg Fluoxetine, i.p.
Figure 4: Effects of chronic MPH on neurochemical responses to fluoxetine at two and ten days post-treatment: A: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline treated animals at day two in response to 15 mg/kg i.p. fluoxetine (n=8 per group). There was a significant effect of fluoxetine injection (p<0.01), a significant effect of MPH treatment (p<0.001), and a significant interaction between MPH treatment and DA response to fluoxetine (p<0.05). B: *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline treated animals at day two in response to 15 mg/kg i.p. fluoxetine (n=8 per group). There was a significant effect of fluoxetine injection on extracellular 5-HT levels (p<0.05). C: *in vivo* microdialysis measurement of DA in the NAc of MPH and saline-treated animals at day ten in response to 15 mg/kg i.p. fluoxetine (n=6 per group). D: Single prove *in vivo* microdialysis measurement of 5-HT in the NAc of MPH and saline-treated animals at day ten in response to 15 mg/kg i.p. fluoxetine (n=6 per group). There was a significant effect of fluoxetine injection on extracellular 5-HT levels (p<0.001).
Discussion

The results of this study reveal marked behavioral and neurochemical differences between early and late time points following MPH exposure. The two day time point showed locomotor sensitization in MPH-treated animals, although no sensitization of the dopaminergic system was present. Instead, there was marked serotonergic sensitization in response to psychostimulant challenge in the NAc. In contrast, results at day ten showed the dopaminergic and locomotor sensitization typical of chronic psychostimulant exposure, in the absence of enhanced 5-HT responses. These results indicate that withdrawal from MPH produces behavioral sensitization within two days following the final dose, but that dopaminergic sensitization is not present until some days after. In addition, these results suggest that there are effects on the 5-HT system during early withdrawal from chronic MPH, in which animals show serotonergic sensitization to psychostimulants, and dopaminergic responses to 5-HT specific drugs. These findings show that the effects of MPH on 5-HT may be similar to results seen with early cocaine withdrawal (Parsons et al, 1993a). Parsons et al has shown that 10 days of cocaine administration produces sensitized 5-HT and DA responses in the NAc following subsequent cocaine challenge (Parsons et al, 1993a). Our results show similar effects of MPH on early 5-HT responses to psychostimulant challenge following treatment. Our results also show two stages following MPH treatment that can be neurochemically characterized.

Chronic MPH and the development of sensitization
The results seen in this study are consistent with the extensive literature documenting the time course of sensitization following chronic cocaine administration. It is well established that repeated, intermittent psychostimulant administration can result in enhanced behavioral responses to subsequent drug challenge (Shuster et al, 1977), and that these enhanced behavioral responses are associated with an enhancement of psychostimulant-evoked DA levels in the NAc (Akimoto et al, 1990; Kalivas et al, 1990). In addition, it is thought that the increased behavioral and neurochemical responses following intermittent drug administration may underlie aspects of developing drug dependence (Wise, 1987). Although the true role of sensitization in subsequent drug administration behavior is still under investigation, prior sensitization is known to facilitate the acquisition of self-administration behaviors (Vezina et al, 2002). In light of these findings with cocaine, it is significant that this study, along with several previous studies (Brandon et al, 2001; Wilson et al, 2006; Yang et al, 2006), have found significant locomotor sensitization to MPH, as well as sensitization of the mesolimbic DA responses to subsequent psychostimulant challenge following chronic MPH exposure. In our studies, in agreement with those listed above, we found MPH-induced behavioral sensitization at both 48 hours and ten days following chronic MPH treatment.

Interestingly, previous studies have shown that there is a clear time-course for cocaine-induced sensitization (Kalivas et al, 1993a; Kalivas et al, 1993b; Koff et al, 1994; Unterwald et al, 1994), and our results indicate a similar time course for MPH. While behavioral sensitization to the locomotor stimulating effects of cocaine may appear as early as 24 hours after the final administration of drug, the sensitized DA response to the drug is not always present, and thus there is a known mismatch between the presence of
sensitized behaviors and a sensitized DA response to psychostimulants (Ito et al, 2002; Kalivas et al, 1991; Murphy et al, 2001; Robinson et al, 1988; Robinson et al, 1993). Psychostimulant studies have shown that the sensitized DA response appears within ten days of drug withdrawal (Meririnne et al, 2001; Shuster et al, 1982), and our work with MPH reinforces these findings, showing that full sensitization of the dopaminergic response to psychostimulant challenge is present ten days following MPH treatment. Additionally, in accordance with some studies performed using cocaine (Kalivas et al, 1993c), we found no differences between MPH- and saline-treated animals in their dopaminergic response to cocaine in the NAc at two days post treatment, while our findings at day 10 show the dopaminergic sensitization that is often present following exposure to other psychostimulants such as cocaine (Kalivas et al, 1993c).

At the two day time point, our studies showed a serotonergic sensitization effect in the NAc in response to cocaine and MDMA, an effect that disappeared at ten days post-treatment, to be replaced with locomotor and DA neurochemical sensitization. Conversely, our results showed the opposite effect when animals were treated with a 5-HT specific drug, fluoxetine, which caused increases in DA in the NAc at two days following treatment in MPH-treated animals, an effect that disappeared by the 10 day time point. These results suggest that the initial locomotor sensitization associated with MPH treatment is not correlated with a sensitized dopaminergic response at early time points, while later locomotor sensitization is correlated with dopaminergic sensitization. Rather, there is an early neurochemical sensitization to the serotonergic effects of psychostimulant challenge, and effect that has been seen in previous studies of cocaine withdrawal (Parsons et al, 1993b), although in the prior studies 5-HT sensitization was
accompanied by dopaminergic sensitization. The lack of early dopaminergic sensitization in MPH treated animals could be in part a result of the distinct pharmacological profile of MPH.

**Differential Effects of Cocaine and MPH**

Effects of chronic MPH may be distinct from effects of other psychostimulants. MPH, unlike cocaine, is not known to have a strong affinity for the SERT (Gatley et al., 1996) and has been shown to act as an agonist at the 5-HT1A receptor (Markowitz et al., 2009). While cocaine inhibits the DAT, NET, and serotonin (5-HT) transporter (SERT) with similar affinity, MPH has high affinity only for the DAT and NET (Gatley et al., 1996). However, MPH does act as an agonist at 5-HT1A receptors (Markowitz et al., 2009). These two factors make the pharmacological effects of MPH potentially distinct from those of cocaine or amphetamine.

Changes following chronic MPH and resulting in serotonergic sensitization may be the result of changes involving circuit-dependent effects. 5-HT1A receptors are located presynaptically on cell bodies in the dorsal raphe, an area that sends 5-HT projections to areas like the ventral tegmental area (VTA) and NAc (Gozlan et al., 1983; Riad et al., 2000). Stimulation of the 5-HT1A receptor results in decreased 5-HT neuron firing to terminal regions. Thus, it is possible that chronic stimulation of the 5-HT1A receptor during MPH treatment could result in receptor desensitization and decreased 5-HT neuron activity (Rossi et al., 2008). In addition, chronic DAT and NET blockade using nomifensine has been shown to desensitize 5-HT1A receptors (Katz, 2009), which suggests that DAT and NET blockade via MPH treatment could contribute to 5-HT1A
receptor desensitization. This receptor desensitization could result in decreased negative feedback following chronic MPH treatment. In a normal system, increases in extracellular 5-HT following SERT blockade would result in stimulation of 5-HT1A autoreceptors and decreases in 5-HT neuron firing, limiting the extracellular increases in 5-HT (Katz et al, 2009). If, however, chronic MPH treatment results in a desensitization of 5-HT1A autoreceptors in the raphe, extracellular increases in 5-HT would not trigger this negative feedback mechanism, and there would be no decrease in 5-HT neuron firing. Increases in extracellular 5-HT would thus be uninhibited by decreases in 5-HT neuron firing, resulting in augmented extracellular 5-HT increases. The 5-HT1A receptor changes may well be temporary and reverse following acute withdrawal, leading to a disappearance of the effect at day 10.

Additionally, our results suggest that early withdrawal from MPH may involve changes in 5-HT function and a sensitized 5-HT control over DA system function. The DA and 5-HT systems have many points of interaction, particularly in the mesolimbic DA system, including areas such as the NAc and ventral tegmental area. In addition, 5-HT receptors, such as the 5-HT1A and 5-HT1B receptors, are known to mediate dopaminergic activity both at baseline and in response to psychostimulant administration (Chen et al, 1995; De La et al, 2000; Gillies et al, 1996; Guan et al, 1989; O'Dell et al, 2004; Parsons et al, 1998; Parsons et al, 1999). Thus it is possible that long-term MPH administration could change DA/5-HT interactions in the mesolimbic DA system. These changes could occur either via changes in extracellular 5-HT levels during or following chronic MPH treatment, or could be occurring via changes in 5-HT receptor expression as a function of stimulation of the 5-HT1A receptor. Increased 5-HT control over DA
system function could explain the effects seen with fluoxetine at the second day following treatment. Fluoxetine administration increased DA in the NAc of MPH treated mice, an effect not present in controls. These effects are similar to those seen in work with the DAT knockout mouse (Mateo et al, 2004), which was found to have increased 5-HT system control over DA function, and that caused increases in NAc DA following fluoxetine challenge. Further, these results replicate those seen previously in our laboratory (Brookshire, 2010), showing that chronic MPH causes increased 5-HT control over DA function, although the present studies suggest that these effects are temporary and replaced with previously seen dopaminergic sensitization to psychostimulants later in withdrawal.

Conclusions

In conclusion, this study examined early and later time points in MPH withdrawal and the effect of psychostimulant and SSRI challenge on DA and 5-HT in the NAc. It appears that chronic MPH results in behavioral sensitization to psychostimulants at both early and later time points following treatment, although the behavioral sensitization at these two time points may be mediated by two different mechanisms. These results suggest that early time points following high-dose, chronic MPH may see changes in DA/5-HT system interactions, which are then ameliorated over time. These findings may have extensive implications for the early treatment of those abusing MPH. While later-stage treatments could be similar to those used for cocaine abuse, it is possible that early-stage treatments for MPH abuse will have to take altered DA/5-HT interactions into account when considering potential pharmacotherapies.
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References


CHAPTER VI

DIRECT AND INDIRECT 5-HT RECEPTOR AGONISTS PRODUCE GENDER-SPECIFIC EFFECTS ON LOCOMOTOR AND VERTICAL ACTIVITY IN C57 BL/6J MICE

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Abstract

It is well established that the dopamine (DA) and serotonin (5-HT) systems have extensive and complex interactions. However, the effects of specific 5-HT receptor agonists on traditionally DA-related behaviors remain unclear. Our goal in these studies was to characterize the effects of 5-HT receptor agonists on measures of locomotor activity and vertical rearing. The SSRIs fluoxetine and citalopram produced significant decreases in locomotor activity and vertical rearing at the highest doses used with females significant more sensitive to citalopram. The 5-HT_{1A} agonist 8-OH-DPAT and the 5-HT_{2C} agonist MK 212 significantly decreased activity in both male and female mice, with females more sensitive to 8-OH-DPAT. In contrast, the 5-HT_{1B} agonist RU 24969 and the 5-HT_{2A} agonist DOI both increased activity, with DOI exhibiting differential effects with regard to sex. Finally, the 5-HT_{3} agonist SR 57227 produced significant locomotor increases only in female mice at the lowest dose. The results of these experiments define locomotor profiles of several 5-HT agonists in male and female C57BL/6J mice, providing a foundation for further explorations of 5-HT receptor effects on activity.
Introduction

It is well established that dopamine (DA) and serotonin (5-HT) systems have extensive and complex interactions, especially within the basal ganglia (1). The activity of the striatum, a sensory-motor-limbic integration region of the basal ganglia that controls locomotor and vertical activity in rodents and includes the caudate-putamen and nucleus accumbens, is modulated by many factors, in particular DA and 5-HT projections arising from the ventral midbrain and brainstem (21). While the effects of direct and indirect DA receptor agonists on locomotor and vertical activity have been well-characterized (4), the effects of 5-HT receptor agonists remain unclear. The ventral midbrain, where DA cell bodies are located, receives the heaviest 5-HT innervations in the brain (25), and 5-HT receptors regulate the activity of DA and GABA neurons in this region (43;40). There is also a strong 5-HT projection to the striatum that provides presynaptic 5-HT regulation of DA release (1). In light of these extensive interactions, the effects of 5-HT receptor agonists on behaviors traditionally associated with activation of the dopaminergic system, such as locomotor and vertical activity (48;15;16), merit further study.

Fourteen 5-HT receptor subtypes have been identified to date, and at least five of these are known to have extensive interactions with the DA system, including the 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{3}$ receptors (1). Many 5-HT receptor agonists are well characterized with regard to anxiety-related, anti-depressant, hallucinogenic and other properties. However, the effects of these compounds on locomotor activity in mice remain relatively unknown. Although some locomotor studies of drugs such as the 5-HT$_{1A}$ agonist, 8-OH-DPAT, have been performed in mice and rats, the doses used are
limited, and studies center almost exclusively on examining the modulatory influences of specific 5-HT receptors on stimulant-induced behaviors. Thus, a study of the effects of 5-HT receptor agonists on mouse locomotor activity could prove useful in further studies of drug-induced behaviors, as well as providing insight into the direct locomotor effects of the 5-HT agonists.

It is known that male and female mice differ in their responses to some psychoactive drugs that activate both the 5-HT and DA systems, such as amphetamine (11;45). Thus, it is possible that 5-HT receptor agonists with actions in the DA system might show sex differences. Additionally, the DA and 5-HT systems are known to have extensive interactions, particularly in the area of the striatum and nucleus accumbens, brain areas linked with the locomotor- and vertical activity-stimulating effects of drugs (29). It is therefore likely that stimulation of specific 5-HT receptor subtypes in this area might have significant effects on locomotor and vertical activity, effects often mediated through the dopaminergic system (30;16;49), but that are not necessarily mediated exclusively by increases in DA (36). Therefore, although locomotor and vertical activation or attenuation may be due to changes in DA via 5-HT receptor stimulation, 5-HT receptor stimulation alone may also alter locomotor and vertical activity.

Although some 5-HT receptors are relatively well-characterized with regard to sex differences in brain distribution and the behavioral effects of stimulation, such as the 5-HT1A, 5-HT2A, and 5-HT2C receptors (5;6;3), others remain poorly characterized, and no studies have examined sex differences in the effects of specific 5-HT receptor agonists on locomotor activity.
Despite what is known about the effects of 5-HT receptor agonists on mesolimbic DA firing rates and control of such behaviors such as drug self-administration (46), the specific function of these receptors in DA-related behaviors, such as locomotor activity in mice, remain unclear. As locomotor and vertical activity are behaviors known to be regulated by DA signaling, our goal in these studies was to characterize the effects of several 5-HT receptor agonists on DA\5-HT interactions in mice. C57BL/6J is the most commonly used mouse strain in neuropharmacological research, and thus we chose to use this strain to characterize the locomotor effects of seven different serotonergic compounds; the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and citalopram, the 5-HT\textsubscript{1A} agonist 8-OH-DPAT, the 5-HT\textsubscript{1B} agonist RU 24969, the 5-HT\textsubscript{2A/2C} agonist DOI, the selective 5-HT\textsubscript{2C} agonist MK 212, and the 5-HT\textsubscript{3} agonist SR 57227. Naïve mice were tested with a variety of drug doses for locomotor distance traveled and measures of vertical rearing activity.

**Methods**

**Animals:** C57Bl/6J mice were obtained from Jackson laboratories (Bar Harbor, ME), and a breeding colony was established. Mice were housed in groups of three or four per cage with food and water *ad libitum* on a 12-hr light-dark cycle with lights on at 7 am. All experiments used both male and female mice that were between 2 and 6 months old, and for each drug, the male and female mice being tested were age matched. Experimental protocols adhered to National Institutes of Health Animal Care Guidelines and were approved by the Wake Forest University Institutional Animal Care and Use Committee.
**Activity Monitoring:** Locomotor activity and vertical rearing were assessed using open field activity monitors (Med Associates, St. Albans, VT). The open field consisted of a square plexiglass container (27.0cm X 27.0cm X 20.3 cm) with three sixteen beam infrared arrays. Two arrays were placed on the periphery of the chamber at floor level for detection of locomotor activity (X and Y planes, measured approximately 0.25” off the floor), while the third array was placed 2” above the X and Y arrays to obtain measures of vertical activity (Z plane, measured 2.25” off the floor). Data was collected using Med Associates Activity Monitoring Software (Med Associates, St. Albans, VT), and distance traveled was measured in cm over a given length of time. Vertical rearings were measured as number of beam breaks in the vertical plane over a given length of time. Behavioral analyses were conducted during the light phase between 9 am - 5 pm. The locomotor chambers contained no bedding, and were cleaned with 70% EtOH and dried thoroughly between testings.

**Drug Administration:** Following a two-hour period where mice were allowed to habituate to the chambers, animals received either saline (0.1 mL injection volume) or drug dissolved in saline (unless specified otherwise), administered in a 0.1 mL volume i.p., by weight, at the doses described below. Separate injections were prepared for male and female mice based on average male and female weight for the cohort. Animals were divided into cohorts, each of which received all doses of a single drug type. Doses were randomized in a Latin-Square design. Data was collected using Med Associates proprietary software (Med Associates, St. Albans, VT) in 5-minute bins for a period of two hours following injection.
Statistics: Data was analyzed for distance traveled in cm and the number of vertical rears performed during the activity profile of the drug. Locomotor activity was grouped in bins for either the first 20 minutes after drug injection (fluoxetine, citalopram, MK 212), the first 30 minutes after drug injection (8-OH-DPAT), or the first 60 minutes after drug injection (RU 24969, DOI, SR 57227), depending on the active period of the drug. Active period of the drug was determined by AUC analysis of activity curves measured over two hours following drug injection (for a series of representative locomotor activity traces over the full two hours of recording following drug injection, see supplemental data). The time course of each drug tested can be seen at representative doses for locomotor and vertical activity in Supplemental figures 1-9. Following summation of data over 20, 30 or 60 minutes, all groups were tested for outliers using the Grubb’s Test for Outliers. Data was then grouped by sex and analyzed by a one-way ANOVA for effect of drug in either male or female mice, with a corrected Bonferroni post-hoc analysis to determine specific effects of dose. For comparisons between sex, activity was compared as percent of saline values, to control for differences in saline response between sex. Data was analyzed for between sex effects and interactions between drug and sex by repeated-measures ANOVA with corrected Bonferroni post-hoc analysis. P<0.05 was considered significant.

Drugs: The SSRIs (±)-N-methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine hydrochloride (fluoxetine) and 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-
Figure 1

A

0.1 mL Saline, i.p.

- distance traveled (cm)
- Distance measured at 20 min, 30 min, and 60 min for male and female groups.

B

- vertical activity
- Activity levels measured at 20 min, 30 min, and 60 min for male and female groups.
Figure 1. Locomotor Responses to 0.1 mL Saline, i.p. A: Effects of 0.1 mL saline injection on locomotor activity across all cohorts of male and female mice for 20, 30 and 60 minutes post-injection (n=67 male, 72 female). *p<0.01 effect of sex at 20 minutes post injection, *p<0.05 effect of sex at all other points. B: Effects of 0.1 mL saline injection on vertical rearing across all cohorts of male and female mice for 20, 30 and 60 minutes post-injection. *p<0.001 effect of sex at 20 minutes post injection, *p<0.05 effect of sex at 30 minutes post injection (n=67 male, 72 female).
dihydro-5-isobenzofurancarbonitrile hydrobromide (citalopram), the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tertraline (8-OH-DPAT), and the 5-HT$_{2A/2C}$ receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2 amino propane ((±) DOI-hydrochloride) were purchased from Sigma (Sigma Aldrich, St. Louis, MO). The 5-HT$_{1B}$ agonist 5-methoxy-n1N-dimethyltryptamine and 5-methoxy-3(1,2,3,6,-tetrahydro-4-pyrindinyl)-1H-indole (RU 24,969), the 5-HT$_{2C}$ agonist 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride (MK 212), and the 5-HT$_{3}$ agonist 1-(6-chloro-2-pyridinyl)-4-piperidinamine hydrochloride (SR 57227) were purchased from Tocris (Ellisville, MO). All drugs were given in a volume of 0.1 mL with concentrations determined by animal weight averages. Fluoxetine HCl was dissolved in ultra-pure water, while all other drugs were dissolved in 0.9% isotonic sterile saline.

**Results**

**Saline injection produces greater locomotor and vertical responses in male mice** All animals were habituated and monitored for locomotor activity for two hours prior to injection of drug. In all cohorts and with all drugs tested, no difference between male and female mice was observed in measures of spontaneous locomotor and vertical activity during the two hours of habituation to the test chamber (see Supplemental Figure 1). Following habituation, animals were injected with 0.1mL saline, and locomotor activity was monitored for two hours (Representative time course of saline injection shown in
Figure 2

A

Distance traveled (cm/20min)

fluoxetine (mg/kg)

B

Vertical activity (20 min)

fluoxetine (mg/kg)
Figure 2. Locomotor and Vertical Responses to the SSRI Fluoxetine: A: Effects of fluoxetine on locomotor activity for 20 min post injection in male and female mice. *p<0.05 effect of fluoxetine at 15 mg/kg in females, *p<0.01 effect of drug at 5.0 mg/kg in males, *p<0.001 effect of fluoxetine at 1.0 mg/kg in males, +p<0.001 effect of sex on locomotor response to fluoxetine (n=12 per group). B: Effects of fluoxetine on vertical rearing for 20 min post injection in male and female mice. *p<0.001 effect of drug, no difference between sexes (n=12 per group).
Supplemental Figure 2). Male mice showed significantly greater locomotor activity compared to female mice during the first 20 (t=3.022, p<0.01), 30 (t=2.513, p<0.05), and 60 minutes following saline injection (t=2.383, p<0.05) (Figure 1A). Similarly, in comparison to females, male mice performed more vertical rears, particularly during the first 20 (t=4.183, p<0.001) and 30 minutes (t=4.391, p<0.05) (Figure 1B). These data suggest that male mice overall may be more sensitive to the locomotor-activating effects of acute injections than female mice.

Fluoxetine decreases locomotor activity in male and female mice It has been generally agreed that fluoxetine produces decreases in locomotor activity at high doses (43;19), but as yet, no studies have been performed analyzing the effect of sex on locomotor activity following fluoxetine administration. Following two hours of monitored habituation to the locomotor chamber, animals were given either saline or fluoxetine (1.0-15.0 mg/kg) (8;34) in a 0.1mL volume by weight. Data was then analyzed for sex differences and overall effect of drug on locomotor activity using a repeated measures two-way ANOVA with corrected Bonferroni post-doc analysis during the first 20 minutes following injection of drug (for full time course of fluoxetine injection at a representative dose, see Supplemental Figure 3A) using percent change from saline control to normalize for differences in saline response. There was no significant effect of sex on overall fluoxetine response (F_{1,109}=1.450, p=0.2311), or significant interaction between sex and fluoxetine response (F_{4,109}=1.610, p=0.1769), although both sexes exhibited a significant decrease in locomotor activity in response to fluoxetine (F_{1,109}=14.14, p<0.001 Figure 2A). When
analyzed for specific effects of dose by sex, results confirmed that males and females showed decreased activity across all doses of drug.

**Fluoxetine decreases vertical activity in male and female mice** Recordings were also conducted for vertical activity in response to fluoxetine. Not surprisingly, there was a significant effect of fluoxetine on vertical activity in all mice (F_{4,108}=13.55, p<0.001). Analyzed for sex differences during the first 20 minutes following injection as percent of saline control, there was no significant effect of sex on vertical activity (F_{1,108}=0.002597, p=0.9595), although a significant interaction between sex and fluoxetine dose (F_{4,108}=2.607, p<0.05, **Figure 2B**) was observed. Additional one-way ANOVA analysis for dose of drug separated by sex confirmed the effects of fluoxetine on vertical activity, and it is possible that the drug by sex interaction is due to differences in saline locomotor response. (For full time course of fluoxetine injection and vertical activity recording at a representative dose, see **Supplemental Figure 3B**) Taken together, our data show that fluoxetine administration produces significant decreases in locomotor and vertical activity, but overall effects do not differ between male and female mice.

**Citalopram decreases locomotor activity at high doses** Although previous studies have shown few effects of citalopram on locomotor activity (7;15), other studies have shown significant effects of citalopram on locomotor activity at higher doses (16). Thus, it would be useful to re-examine the locomotor effects of citalopram, and, if possible, explore the effects of sex on locomotor response. Following two hours of monitored habituation to the locomotor chambers, animals were given either saline or the SSRI citalopram (5.0 -20.0 mg/kg) (34;17). For a full time course of locomotor effects of
Figure 3

A

Distance traveled (cm/20min)

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Vertical activity (20min)

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*p > 0.05
Figure 3. Locomotor and Vertical Responses to the SSRI Citalopram: A: Effects of citalopram on locomotor activity for 20 min post injection in male and female mice. *p<0.05 effect of citalopram at 20 mg/kg. +p<0.05 effect of sex on locomotor response to 10 mg/kg citalopram, +p<0.01 effect of sex on locomotor response (n=12 per group). B: Effects of citalopram on vertical activity for 20 min post injection in male and female mice. *p<0.01 effect of citalopram at 20 mg/kg. +p<0.01 effect of sex on vertical activity at 5.0 and 10.0 mg/kg citalopram (n=12 per group).
citalopram on male and female mice at a representative dose, see Supplemental Figure 4A. When analyzed for sex differences during the first 20 minutes, it was apparent that there was a significant difference between the males and females (effect of sex $F_{1,65}=13.16$, $p<0.001$, analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis), with females showing significant increases in activity at 5.0 and 10.0 mg/kg (Figure 3A), although no significant interaction effect was observed ($F_{2,65}=2.640$, $p=0.079$). When separated and analyzed further for effects of citalopram dose on sex, male and female mice showed overall effects of citalopram, with significant decreases in locomotor activity only at the highest dose (overall effect of citalopram in males $F_{3,46}=6.511$, $p<0.001$, overall effect in females $F_{3,47}=7.544$, $p<0.001$, analyzed by one-way ANOVA for each sex relative to saline control).

Citalopram decreases vertical activity at high doses in male and female mice In measures of vertical activity in response to the SSRI citalopram, analysis revealed a significant effect of sex ($F_{1,65}=13.16$, $p<0.001$, analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis). There was also a significant effect of sex for measures of vertical activity ($F_{2,62}=4.222$, $p<0.05$ analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis), although no interaction between sex and citalopram dose was detected ($F_{3,79}=0.9842$, $p=0.405$). Analyzed separately for effects of citalopram on males and female, citalopram dramatically reduced the number of vertical rears during the first 20 minutes at the highest dose tested in both sexes (males: $F_{3,44}=9.924$, $p<0.001$, females: $F_{3,46}=11.59$,
Figure 4. Locomotor Responses to the 5-HT$_{1A}$ Agonist 8-OH-DPAT. A: Effects of 5-HT$_{1A}$ receptor agonist 8-OH-DPAT on locomotor activity in male and female mice. *p<0.0001 effect of 8-OH-DPAT dose, +p<0.05 effect of sex at 1.0 mg/kg 8-OH-DPAT, +p<0.001 effect of sex at 2.0 mg/kg 8-OH-DPAT (n=9 males, n=17 females). B: Effects of 8-OH-DPAT on vertical rearings for 30 min following drug injection. p<0.001 effect of drug, p<0.001 effect of sex, no interaction. C: Effects of 8-OH-DPAT on vertical rearing for 30 min following drug injection. *p<0.001 effect of 8-OH-DPAT dose (n=9 males, n=17 females).
p<0.001), although females showed increases in vertical activity at 5.0 and 10.0 mg/kg, possibly related to a low initial response to saline injection (Figure 3B). For a full time course of the vertical effects of citalopram at a representative dose, see Supplemental Figure 4B.

In the case of vertical responses to citalopram in females, the effect of 20 mg/kg citalopram was a drastic decrease in vertical activity, compared to significant increases in vertical activity at lower doses. Because the 20 mg/kg dose resulted in vertical activity without sufficient variance to allow for a Bonferroni post-hoc test, we ran a one sample t-test comparing the mean value of the 20 mg/kg dose to saline, and this showed significant effect of drug at 20 mg/kg (t=11.86, df=11, p<0.0001). Overall, it appears that citalopram produced decreases in locomotor and vertical activity at the highest doses used, but that effects at intermediate doses vary with sex of the animal.

The 5-HT₁A agonist 8-OH-DPAT decreases locomotor activity in male mice It has been observed that the 5-HT₁A agonist 8-OH-DPAT decreases locomotor activity in mice (14), but effects with regard to sex differences have yet to be explored. Following two hours of habituation, animals were given either saline or 8-OH-DPAT (0.5-3.0 mg/kg) (5). There was a significant effect of sex ($F_{1,115}=25.92$, $p<0.01$) over the first 30 minutes following drug administration, with a significant interaction between sex and drug dose ($F_{4,115}=2.495$, $p<0.05$ analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis). For a full time course of drug effects at a representative dose, see Supplemental Figure 5A. Interestingly, subsequent analysis revealed that only male mice showed a response to drug, while females showed no significant effects at any
dose tested (males: $F_{4,50}=24.55$, $p<0.001$, females: $F_{4,67}=0.9706$, $p=0.43$, Figure 4A, analyzed by one-way ANOVA for each sex relative to saline control).

**8-OH-DPAT reduces vertical activity in male and female mice**

With regard to vertical activity following injection of 5-HT1A agonist 8-OH-DPAT, no effect of sex ($F_{1,79}=0.05198$, $p=0.82$ analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis), and no interaction between sex and 8-OH-DPAT dose was detected ($F_{1,108}=0.03268$, $p=0.9979$), although there was a significant overall effect of 8-OH-DPAT across all doses ($F_{4,108}=26.20$, $p<0.001$, Figure 4B). For a full time course of drug effects on vertical activity at a representative dose, see **Supplemental Figure 5B**. In analyses for effects of drug dose on gender, both sexes showed significant decreases during the first 30 minutes in the number of vertical rears at all doses of drug, confirming the results of the two-way ANOVA. Overall, it appears that 8-OH-DPAT decreases vertical rearing behavior in both male and female mice, although females may be less sensitive to the effect of this 5-HT1A agonist on locomotor activity.

**The 5-HT$_{1B}$ agonist RU 24969 increases locomotor activity in male and female mice**

5-HT$_{1B}$ agonists have been shown to potentiate stimulant-induced locomotor activity when administered systemically (13), but the effects on vertical activity and the possible effect of sex have not been determined. Following habituation animals were given either saline or RU 24969 (0.3-5.0 mg/kg) (41). For a representative graph of locomotor effects of RU 24969 on male and female mice, see **Supplemental Figure 6A**. Across all mice, there was a significant increase in locomotor activity in response to RU 24969 ($F_{4,78}=2.706$, $p<0.05$), but there was also a significant difference between sexes.
Figure 5. Locomotor Responses to the 5-HT₁B Agonist RU 24969. A: Effect of 5-HT₁B agonist RU 24969 on locomotor activity from 40-70 minutes post administration in male and female mice. *p<0.0001 effect of RU 24969 at 3.0 mg/kg, +p<0.05 effect of sex at 5.0 mg/kg (n=9 per group). B: Effect of RU 24969 on vertical rearing from 40-70 minutes post administration. +p<0.05 effect of sex at 5.0 mg/kg, +p<0.01 effect of sex at 1.0 mg/kg (n=9 per group).
(F_{1,78}=4.604, \ p<0.05 \ analyzed \ by \ two-way \ repeated \ measures \ ANOVA \ with \ corrected \ Bonferroni \ post-hoc \ analysis). \ There \ was \ no \ significant \ interaction \ between \ sex \ and \ dose \ of \ RU \ 24969 \ (F_{4,78}=1.019, \ p=0.4029). \ While \ both \ sexes \ showed \ significant \ increases \ in \ locomotor \ activity \ in \ response \ to \ drug \ administration \ (males: \ F_{4,43}=5.380, \ p<0.01, \ females: \ F_{4,43}=2.619, \ p<0.05, \ Figure \ 5A, \ analyzed \ by \ one-way \ ANOVA \ for \ each \ sex \ relative \ to \ saline \ control), \ males \ showed \ a \ decrease \ in \ locomotor \ responding \ at \ the \ highest \ dose \ compared \ to \ females, \ pointing \ to \ a \ possible \ difference \ between \ sexes \ in \ responses \ to \ RU \ 24969.

5-HT1B agonist RU 24969 produces no effects on vertical activity While male and female mice showed consistent increases in locomotor activity, vertical activity appeared completely unaffected by dose of the 5-HT1B agonist (F_{4,76}=1.060, p=0.3824), and with no effect of sex present (F_{1,76}=0.2187, p=0.6414, Figure 5B, for a representative time course vertical effects of RU 24969, see Supplemental Figure 6B). Overall, it appears that the 5-HT_{1B} agonist RU 24969 has a significant activating effect on locomotor activity in mice, and that females appear to be more sensitive to these effects.

The 5-HT_{2A/2C} agonist DOI increases locomotor activity in females In previous studies, the 5-HT_{2A/2C} agonist DOI alone did not change locomotor activity, although it has been found to potentiate the locomotor effects of psychostimulants such as cocaine (20). However, DOI has been known to exhibit anxiolytic effects, and it is possible that further studies with locomotor activity and vertical activity could reveal differences due to sex (42). Following two hours of habituation to the test chamber, animals were given
Figure 6. Locomotor Responses to the 5-HT_{2A/2C} Agonist DOI. A: Effects of 5-HT_{2A/2C} receptor agonist DOI on locomotor activity in male and female mice. *p<0.01 effect of RU 249696 dose, *p<0.001 effect of RU 24969 dose (n=9 per group). B: Effects of DOI on vertical rearing for 60 min following drug administration. *p<0.05 effect of RU 24969 dose (n=9 per group).
either saline or DOI (0.125 -2.0 mg/kg) (31;42). Although no significant effect of sex (F_{1,63}=1.192, p=0.2790) or interaction between sex and dose of drug (F_{3,63}=0.03913, p=0.7596) was observed in initial analyses (for a time course of locomotor effects of DOI at a representative dose, see Supplemental Figure 7A), further analyses revealed that females showed a significant locomotor effect of DOI (F_{3,34}=4.532, p<0.01 by one-way ANOVA for female responses to drug dose). Thus, while both male and female mice showed overall effects of drug on locomotor activity (F_{3,63}=7.131, p<0.001) only females showed significant increases in activity at specific doses (0.125 and 1.0 mg/kg analyzed by One-way ANOVA, F_{3,35}=4.532, p<0.01, Figure 6A).

**DOI increases vertical activity in males** In contrast to the results with locomotor activity, there was a marked difference between sex in vertical activity responses to the 5-HT_{2A/2C} agonist DOI (F_{1,63}=13.26, p<0.001), although all animals showed a significant response to drug (F_{3,63}=6.342, p<.001). The sexes were subsequently separated to examine effects of DOI by dose. While both male and female mice showed overall effects of DOI on vertical activity (males: F_{3,35}=4.625, p<0.01, females: F_{3,35}=3.407, p<0.05, analyzed by one-way ANOVA for each sex relative to saline control, Figure 6B), only male mice showed significant effects of drug at specific doses (0.125 and 1.0 mg/kg, for a time course of vertical effects on male and female mice at 1.0 mg/kg, see Supplemental Figure 7B). Thus it appears that male and female mice show significant differences in their locomotor and vertical responses to DOI, although both sexes show a significant response to the 5-HT_{2A/2C} agonist.
Figure 7

A

Distance traveled (cm/20 min)

MK 212 (mg/kg)

B

Vertical activity (60 min)

MK 212 (mg/kg)
Figure 7. Locomotor Responses to the 5-HT$_{2C/2B}$ Agonist MK 212. A: Effects of 5-HT$_{2C/2B}$ receptor agonist MK 212 on locomotor activity in male and female mice. *p<0.05 effect of 1.0 mg/kg MK 212, *p<0.001 effect of MK 212 dose (n=12 per group).

B: Effects of MK 212 on vertical rearing for 20 min following drug administration. *p<0.05 effect of 1.0 mg/kg MK 212, *p<0.0001 effect of MK 212 dose (n=12 per group).
5-HT<sub>2C/2B</sub> agonist MK 212 decreases locomotor activity in male and female mice

Following two hours of habituation to the testing conditions, animals were given either saline or the 5-HT<sub>2C/2B</sub> agonist MK 212 (1.0-10.0 mg/kg) (50). Previous studies have shown that preferential activation of the 5-HT<sub>2C</sub> receptor produces significant decreases in locomotor activity (33). Our studies found similar results, with the 5-HT<sub>2C/2B</sub> agonist MK 212 producing drastic decreases in locomotor activity in all animals tested (F<sub>1,88</sub>=18.30, p<0.001), although no differences between the sexes in locomotor response was observed (F<sub>1,88</sub>=1.236, p=0.2694, Figure 7A). Separated by sex, male and female mice showed significant effects of MK 212 at all doses tested (males: F<sub>3,40</sub>=10.65, p<0.001, females: F<sub>3,40</sub>=28.40, p<0.001, analyzed by one-way ANOVA for each sex relative to saline control, for a time course of locomotor activity following a representative dose of MK 212, see Supplemental Figure 8A).

MK 212 decreases vertical activity in male and female mice

MK 212 appears to exert similar effects in both locomotor and vertical activity, with significant effects of drug on vertical rearing (F<sub>3,88</sub>=20.29, p<0.001). No differences between the sexes were observed (F<sub>1,88</sub>=0.2181, p=0.1433 analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis). For a time course of the effects of MK 212 on vertical activity, see Supplemental Figure 8B. When separated by sex, male and female mice both showed significant decreases in vertical activity at all doses of MK 212, confirming the results of the two-way ANOVA, matching results seen in the original two-way analysis. These results complement the work of earlier studies in the literature showing significant locomotor attenuating effects of 5-HT<sub>2C</sub> agonists in mice.
Figure 8. Locomotor Responses to the 5-HT₃ Agonist SR 57227. A: Effects of 5-HT₃ receptor agonist SR 57227 on locomotor activity in male and female mice. *p<0.05 effect of 1.0 mg/kg SR 57227, +p<0.01 effect of sex at 1.0 mg/kg SR 57227 (n=11 per group). B: Effects of SR 57227 on vertical rearing for 60 min following drug administration. No effect of SR 57227 or sex was observed (n=11 per group).
The 5-HT₃ agonist SR 57227 increases locomotor activity in female mice

Very few studies have been performed examining the effects of 5-HT₃ agonists on mouse locomotor behavior, most involving the induction of movement in paraplegic mice (21), with no examination of possible interactions with dopamine-related behaviors. Thus, a thorough examination of the locomotor effects of SR 57227 on male and female mice could provide further insight into the behavioral relevance of this receptor. Following two hours of habituation, animals were given either saline or SR 57227 (1.0-4.0 mg/kg) (23). The 5-HT₃ agonist SR 57227 had a significant effect on locomotor activity when mice were grouped (F₃,₈₂=4.875, p<0.01), but a significant effect of sex was also detected (F₁,₈₂=9.225, p<0.01). Indeed, when sexes were separated and subjected to further analysis, it appeared that SR 57227 produced no locomotor effects in male mice (F₃,₄₃=0.9630, p=0.4196, analyzed by one-way ANOVA for each sex relative to saline control), but produced significant increases in locomotor activity in female mice (F₃,₄₅=4.411, p<0.01, effect of sex F₁,₈₂=9.225, p<0.01 analyzed by two-way repeated measures ANOVA with corrected Bonferroni post-hoc analysis). In female mice, this effect was particularly strong at the lowest dose of 1.0 mg/kg (p<0.05, Figure 8A), although increased activity as an effect of drug was evident at all doses (for a time course of locomotor effects at a selected dose of SR 57227, see Supplemental Figure 9A).

The 5-HT₃ agonist SR 57227 increases vertical activity in female mice only

The effects of SR 57227 were less pronounced in the vertical plane, and although there was a significant overall effect of drug (F₃,₈₄=4.509, p<0.01), no significant overall different in sex was detected (F₁,₈₄=0.1048, p=0.7470), Figure 8B). However, when animals were
separated by sex to look at individual doses of drug, SR 57227 did not show significant
effects on vertical activity in males (males: $F_{3,44}=2.210$, $p=0.1014$, analyzed by one-way
ANOVA for each sex relative to saline control). In contrast, females showed significant
overall effects of locomotor activity, with the strongest effect at the lowest dose (females:
$F_{3,45}=3.091$, $p<0.05$, Figure 8B), which suggests effects of 5-HT$_3$ agonists on locomotor
and vertical activity are weak at best, as intersex differences cannot be effectively drawn
when differences are apparent only at the lowest dose tested (for a time course of the
effects of SR 57227 on vertical activity at a representative dose, see Supplemental
Figure 9B).

Discussion

5-HT receptors have strong modulatory actions on the DA system, influencing
DA neuron firing rates, presynaptic DA release, and postsynaptic DA-responsive cells
(1). In the present studies we documented the effects of systemic administration of
various 5-HT direct and indirect agonists on locomotor activity and vertical rearing, two
behaviors that are known to be DA-related (15;16). We investigated the effects of two
SSRIs, fluoxetine, and the more selective citalopram, as well as receptor agonists for five
receptors known to interact with the DA system: the 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2C}$
and 5-HT$_3$ receptors. Our experiments showed that each receptor agonist can be
distinguished by its locomotor profile, and that significant sex differences exist for many
of the subtype-specific drugs tested.

There is a large literature on sex differences in responsiveness to direct and
indirect DA receptor agonists in rodents. For example, females exhibit more intense
locomotor activation in response to most stimulants, although males show greater DA responses to cocaine (22), and males are known to be more sensitive to the behavioral effects of D₂ agonists (47). Since 5-HT agonists are modulators of dopaminergic activity, underlying sex differences in the DA system could play an important role in the effects of 5-HT receptor agonists on locomotor activity in males and females.

The SSRI fluoxetine dose-responsively decreased activity in the present studies, in contrast to previous reports showing that fluoxetine increases locomotor activity in adult CD1 and NMRI mice (18;8). Our studies also found dose-dependent locomotor decreases. However, it should be noted that in the previous studies showing increases in activity, animals were exposed to a novel environment following drug administration (18), an important difference from the present study. Also, it should be noted that there are differential effects of fluoxetine between strain of mice, as well as between mice and rats. Fluoxetine is known to decrease locomotor activity in young (49) and adult rats (32). Additionally, our study used a specific strain of mouse, C57BL/6J, a strain noted for its insensitivity to antidepressants such as fluoxetine in the forced swim test. The neurochemical effects of fluoxetine that could produce attenuation of locomotor activity are complex because it is an uptake inhibitor, elevating extracellular 5-HT levels and causing activation of potentially diverse receptors in various brain regions at different doses. Also, in addition to being an inhibitor of the 5-HT transporter, fluoxetine is an agonist at 5-HT₂C receptors (38) that are known to decrease locomotor activity (33), and that could have produced some of the effects seen in the current study.

In contrast to fluoxetine, the more selective SSRI citalopram produced decreases in locomotor and vertical activity only at the highest dose used (20.0 mg/kg, i.p.). Given
that citalopram is an indirect agonist, it is possible that increases in extracellular 5-HT produced by this drug will activate diverse subtypes of receptors at different doses, producing mixed effects on activity. In previous studies of habituated rats and mice, citalopram had no effects on locomotor activity, although increases in activity have been shown in unhabituated mice (8). In general, SSRIs have no stimulant properties on their own and do not promote self-administration (51), but acute SSRI administration potentiates the locomotor-activating effects of psychostimulants such as cocaine and amphetamine (9). However, in our hands, acute SSRI administration alone had attenuating effects on locomotor activity. It should be noted, however, that female mice appeared to show a significant locomotor stimulating effect of citalopram at the two lowest doses, although female mice also showed significant locomotor attenuation at the highest dose tested. This augmentation in locomotor activity is surprising, especially considering the significant locomotor attenuating effects of the similar SSRI fluoxetine. However, fluoxetine and citalopram do have significant differences, as fluoxetine is known to be less selective, and in particular, to have activity at the 5-HT2C receptor, which could account for some of the locomotor attenuating effects (32). Additionally, it is possible that the effects seen with citalopram could be due to the extremely low activity exhibited by the female mice in response to saline. However, these mice are the only appropriate control for these locomotor responses. Additionally, because the differences measured are in behavior following drug administration, differences in absorption and distribution of drug in male and female mice should not be discounted.

The 5-HT1A receptor is one of the most-studied 5-HT receptors with regard to sex differences. It appears that, although there are no differences in 5-HT1A receptor binding
levels (53), female rats have increased sensitivity to the anxiolytic effects of 5-HT_{1A} agonists (6). The 5-HT_{1A} agonist 8-OH-DPAT has also been shown to decrease locomotor activity in mice (14), and in the present studies, 8-OH-DPAT significantly attenuated locomotor activity in male mice at all doses tested and attenuated vertical rearing in both male and female mice. In the present studies, female mice were found to be less sensitive to the locomotor attenuating effects of 8-OH-DPAT, and both sexes were affected equally with regard to vertical activity. Our data thus imply that 5-HT_{1A} activation results in locomotor attenuation in male and female mice, although females are less susceptible to the locomotor-attenuating effects. It should be noted that 8-OH-DPAT is also an agonist at 5-HT_{7} receptors (2), and while this may have some effect on locomotor activity, 5-HT_{7} receptors are located in the thalamus, hypothalamus, and amygdala, and thus may not have direct effects on dopamine system interactions resulting in changes in locomotor activity (24). While the effects of 8-OH-DPAT at 5-HT_{7} receptors cannot be ignored and may play a role in the effects, this mechanism seems less likely to directly affect locomotor activity than the actions of 8-OH-DPAT at 5-HT_{1A}.

The 5-HT_{1B} receptor has been relatively well-characterized with regard to locomotor activity, although sex differences have not been extensively explored. It is known that the 5-HT_{1B} agonist RU 24969 enhances locomotor activity (13), and our results are consistent with this, showing that systemic RU 24969 potentiates locomotor activity in both sexes with no effect on vertical activity, although females appear to be more sensitive to the locomotor stimulating effects. It is hypothesized that activation of 5-HT_{1B} receptors on GABA neurons in the VTA produces inhibition of a tonic inhibitory influence on DA neurons, resulting in increased DA neuron firing to regions such as the
NAc (40), where increased DA levels lead to locomotor activation (39). Although RU 24969 is known to also activate 5-HT<sub>1A</sub> receptors, these effects are generally opposite to those seen with 5-HT<sub>1B</sub> agonists, resulting in substantial decreases in locomotor activity. The significant increase in locomotor activity in response to RU 24969 in male and female mice thus seem to indicate the effect of the 5-HT<sub>1B</sub> agonist, although the effects of RU 24969 at 5-HT<sub>1A</sub> receptors should not be discounted. Thus, it appears that 5-HT<sub>1B</sub> receptors have activating effects on the DA system and lead to enhanced locomotion both in isolation (present results, 11) and in combination with stimulants (7;44).

Sex differences in 5-HT<sub>2A</sub> receptors have been documented. For example, juvenile female rats have higher 5-HT<sub>2A</sub> receptor binding levels than their male counterparts (53). In previous studies, 5-HT<sub>2A</sub> stimulation has been shown to increase DA cell firing in the VTA (43). In this study, administration of the 5-HT<sub>2A/2C</sub> agonist DOI increased locomotor activity significantly above baseline in all animals, although only female mice showed significant effects at specific doses. Differences between the sexes were also marked in measures of vertical activity, where males showed significant increases in vertical rearing in response to intermediate doses of DOI. It is interesting that the effects of 5-HT<sub>2A</sub> agonists should reveal such strong sex differences between locomotor and vertical activity measures, and further studies may reveal whether this change is due to differences in receptor densities.

Because DOI is an agonist at both 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors, we also looked at the effects of the 5-HT<sub>2C/2B</sub> agonist MK 212, which activates 5-HT<sub>2C</sub> receptors preferentially (37). 5-HT<sub>2C</sub> receptor activation has been shown to decrease locomotor activity (33), and our findings agree with previous studies in the literature. MK 212
decreased spontaneous locomotor activity at all doses tested in both male and female mice, with no differences between sexes at any dose. Previous literature found that males are more sensitive to 5-HT$_{2C}$ stimulation (3), and it is possible that doses used in this study were not low enough to show such differences. Interestingly, the locomotor profile of MK 212 appeared similar to that of the SSRI fluoxetine, indicating that the locomotor attenuating effects of fluoxetine may be partially due to stimulation of the 5-HT$_{2C}$ receptor.

The ionotropic 5-HT$_3$ receptor remains little studied with regard to sex differences. 5-HT$_3$ agonists increase somatodendritic release of DA in the VTA (12), while antagonists appear to reduce the number of spontaneously active DA cells (35). In this study, the 5-HT$_3$ agonist SR 57227 appeared to have little effect on locomotor activity, increasing activity only in female animals at the lowest dose used. In contrast, SR 57227 significantly elevated vertical activity in both males and females, although no sex difference was observed. This is in accordance with literature on SR 57227, indicating that 5-HT$_3$ agonists have little effect on locomotor activity when administered alone (52), (23).

Although we have attributed the behavioral and sex-specific effects of 5-HT system and receptor agonists to their activity at 5-HT receptors, differences in the absorption, distribution, and metabolism of these drugs cannot be discounted. Unfortunately, few studies have examined the pharmacokinetics of these drugs in mice, and to the best of the authors’ knowledge, no studies have been done on potential sex differences in metabolism in male and female mice. Studies that have been performed with fluoxetine have assumed no difference between male and female mice (27;28).
Studies on the distribution and metabolism of citalopram and fluoxetine in humans have found no differences have been found due to sex (26), although strong effects of age are evident.

In addition, it is best to be careful when making the assumption that the locomotor effects seen in response to 5-HT receptor agonists mediate these effects through interactions with the DA system. Although changes in DA in the area of the striatum and NAc are strongly correlated with changes in locomotor activity (29;15;10), there is also evidence that increases in locomotor activity are not necessarily dependent on increases in DA (36). Thus, it is possible that the effects of the 5-HT receptor agonists tested in this study could be exerting their locomotor effects via both direct and indirect means, either through interactions with the dopaminergic system, or as locomotor agonists in their own right. Unfortunately, the present study cannot address this point, and future studies will be needed to separate the direct and indirect effects of serotonergic agonists.

Taken together, our data indicate that the effects of many 5-HT receptors within the DA system can be detected using a simple locomotor behavior paradigm, and that these effects are characteristic of agonists of specific receptor subtypes. SSRIs and specific 5-HT receptor agonists all exhibited sex differences in their locomotor and vertical activity profiles, indicating that DA/5-HT interactions may differ extensively between sexes. These data could provide an effective measure of differences between male and female mice with regard to DA/5-HT interactions. Given that both the DA and 5-HT systems are intimately involved with many psychiatric disturbances, such as anxiety, depression, and drug abuse, differences in D/-5-HT interactions between males and females could provide insight into sex difference in these disorders, as well as aid in
determining possible sex differences in responses to drugs targeting these receptors in humans. Thus, a convenient behavioral screen for differences in locomotor responses to 5-HT agonists could reveal differences in receptor responses in DA/5-HT interactions. It is hoped that these studies will shed further light on the behavioral effects of DA/5-HT interactions, as well as differences between the sexes, providing a useful template to study DA/5-HT interactions in the context of both normal and neurobiological disease states.

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Reference List


Supplemental Figures

Supplemental Figure 1

A

(distance traveled (cm))

- male
- female

B

(vertical activity)

- male
- female

(time (min))
Supplemental Figure 1. Habituation to Locomotor Chambers in Male and Female C57 Mice

A: Representative time courses of locomotor activity for male and female mice during the first two-hour habituation to the test chamber prior to i.p. administration of drug are shown (n=33 per group). Time courses were randomly selected from all animal cohorts. There was a significant overall effect of time in chamber ($F_{23,1536}=19.06$, $p<0.001$), significant overall effect of sex ($F_{1,1536}=24.86$, $p<0.001$), with no significant interaction between sex and time observed.

B: Representative time courses of vertical activity for male and female mice during the first two hour habituation to the test chamber prior to i.p. administration of drug (n=33 per group). Time courses were randomly selected from all animal cohorts. There was a significant overall effect of time in chamber ($F_{23,1536}=8.050$, $p<0.001$), a significant overall effect of sex ($F_{1,1536}=23.24$), but no significant interaction between sex and time observed.
Supplemental Figure 2

A

distance traveled (cm)

male
female

0.1 mL saline, i.p.

time (min)

B

vertical activity

male
female

0.1 mL saline, i.p.

time (min)
Supplemental Figure 2. Locomotor and Vertical Activity Responses 0.1 mL saline, i.p. A: Representative time courses of locomotor responses for male and female mice to 0.1 mL saline, i.p (n=24 per group) over the full two hours of recording following drug administration. Time courses were randomly selected from all animal cohorts. There were significant effect of saline injection on locomotor activity (F_{28,1332}=11.58, p<0.001), but no significant effect of sex (F_{1,1332}=3.026, p=0.08). B: Representative time courses of vertical activity for male and female mice to 0.1 mL saline, i.p. are shown (n=24). Time courses were randomly selected from all animal cohorts. A significant effect of saline injection on locomotor activity (F_{28,1334}, p<0.001) was found, as well as a significant overall effect of sex (F_{1,1334}=13.82, p<0.001).
Supplemental Fig 3

A

distance traveled (cm)

- male
- female

5.0 mg/kg
Fluoxetine, i.p.

B

vertical activity

- male
- female

5.0 mg/kg
Fluoxetine, i.p.
Supplemental Figure 3. Locomotor and Vertical Activity Responses to 5.0 mg/kg fluoxetine, i.p. A: Shown here are representative time courses for male and female mice in response to 5.0 mg/kg fluoxetine, i.p. (n=12 per group) over the full two hours of recording following drug administration. B: Representative time course of vertical activity responses for male and female mice to 5.0 mg/kg fluoxetine, i.p. are shown (n=12 per group) over the full two hours of recording following drug administration.
Supplemental Figure 4: Locomotor and Vertical Activity Responses responses to 10 mg/kg citalopram, i.p.  

A: Shown here are representative time courses of locomotor activity for male and female mice in response to 5.0 mg/kg fluoxetine, i.p. (n=12 per group) over the full two hours of recording following drug administration.  

B: Representative time courses of vertical activity for male and female mice in response to 5.0 mg/kg fluoxetine, i.p. (n=12 per group) over the full two hours of recording following drug administration are shown.
Supplemental Figure 5. Locomotor and Vertical Activity Responses to 2.0 mg/kg 8-OH-DPAT, i.p. **A:** Presented here are representative time courses of locomotor activity in male and female mice in response to 2.0 mg/kg 8-OH-DPAT, i.p. (n=9 males, n=18 females) over the full two hours of recording following drug administration. **B:** Representative time courses of vertical activity are shown for male and female mice in response to 2.0 mg/kg 8-OH-DPAT, i.p. (n=9 males, n=18 females) over the full two hours of recording following drug administration.
Supplemental Figure 6

A

Distance traveled (cm)

-25 0 25 50 75 100 125

time (min)

1.0 mg/kg
RU 24969, i.p.

B

Vertical activity

-25 0 25 50 75 100 125

time (min)

1.0 mg/kg
RU 24969, i.p.
Supplemental Figure 6. Locomotor and Vertical Activity Responses to 1.0 mg/kg RU 24969, i.p. A: Presented here are representative time courses of locomotor activity in male and female mice in response to 1.0 mg/kg of the 5-HT\textsubscript{1B} agonist RU 24969, i.p. (n=9 per group) over the full two hours of recording following drug administration. B: Shown here are representative time courses of vertical activity for male and female mice in response to response to 1.0 mg/kg RU 24969, i.p. (n=9 per group) over the full two hours of recording following drug administration.
Supplemental Figure 7

A

Distance traveled (cm)

- male
- female

1.0 mg/kg
DCl, i.p.

B

Vertical activity

- male
- female

1.0 mg/kg
DCl, i.p.
Supplemental Figure 7: Locomotor and Vertical Activity Responses to 1.0 mg/kg DOI, i.p. A: Representative time courses of locomotor activity for male and female mice are shown over the full two hours of recording following administration of 1.0 mg/kg 5-HT2A/2C agonist DOI, i.p. (n=9 per group). B: Representative time courses for male and female locomotor activity over the full two hours of recording following administration of 1.0 mg/kg DOI, i.p. (n=9 per group) are shown.
Supplemental Figure 8

A

Distance traveled (cm)

Time (min)

3.2 mg/kg
MK 212, i.p.

B

Vertical activity

Time (min)

3.2 mg/kg
MK 212, i.p.
Supplemental Figure 8: Locomotor and Vertical Activity Responses to 3.2 mg/kg MK 212, i.p.  

**A:** Representative time courses of locomotor activity for male and female mice are shown over the full two hours of recording following administration of 3.2 mg/kg 5-HT2C/2B agonist MK 212, i.p. (n=6 per group).  

**B:** Representative time courses for male and female mouse vertical activity are shown over the full two hours of recording following administration of 3.2 mg/kg MK 212, i.p (n=6 per group).
Supplemental Figure 9

A

distance traveled (cm)

- • male
- ○ female

2.0 mg/kg
SR 57227, i.p.

B

vertical activity

- • male
- ○ female

2.0 mg/kg
SR 57227, i.p.
Supplemental Figure 9: Locomotor and Vertical Activity Responses to 2.0 mg/kg SR 57227, i.p.  

A: Shown here are representative time courses of locomotor activity in male and female mice over the full two hours of recording following administration of 2.0 mg/kg 5-HT3 agonist SR 57227, i.p. (n=11 per group).  

B: Representative time courses for male and female mouse vertical activity are shown over the full two hours of recording following administration of 2.0 mg/kg SR 57227, i.p. (n=11 per group).
CHAPTER VII.
SUMMARY AND DISCUSSION

Summary

The experiments in this dissertation have shown a novel alteration in dopamine (DA)/ serotonin (5-HT) system interactions in the mesoaccumbens pathway following the chronic administration of MPH in mice. Our studies found that chronic MPH treatment produces a temporary sensitization of 5-HT1B and 5-HT1A receptors, resulting in augmented 5-HT system control over mesolimbic DA function when 5-HT levels are increased. We also showed behavioral and neurochemical sensitization to cocaine and MDMA, which persisted for at least 10 days following cessation of MPH treatment. Finally, our studies showed that chronic MPH treatment can produce alterations in behavioral responses in the forced swim and tail suspension tests that indicate depressive-like states. These findings have significant implications for the field of MPH and psychostimulant studies, as well as for the field of DA/5-HT system interactions, suggesting that the chronic, high-dose MPH administration may strongly affect 5-HT system control over DA system function through specific 5-HT receptors,

Major Findings Presented in this Dissertation

Effects of 5-HT receptor agonists on locomotor activity. The chapters in this dissertation were designed with the goal of fully describing the influence of selected 5-HT receptor subtypes over mesolimbic DA in the NAc and VTA, both before and after chronic MPH treatment. It was first necessary to deduce the behavioral effects of various 5-HT
receptor subtype agonists on the DA-correlated measure of locomotor activity, a body of work that has been addressed in Chapter VI. In agreement with previous studies, the general selective serotonin reuptake inhibitors (SSRIs) produced marked decreases in locomotor and vertical activity in the C57Bl/6J mouse. Specific 5-HT receptor agonists produced differential effects, which 5-HT1B, 2A and 3 agonists increasing locomotor and vertical activity, while 5-HT1A and 5-HT2C agonists produced decreases in locomotor and vertical behavior. These data provide behavioral evidence for the involvement of specific 5-HT receptor subtypes in the mesolimbic DA system, and form a baseline from which behavioral alterations in receptor responses can be measured.

Effects of MPH treatment on DA/5-HT System Interactions. The work undertaken in Chapter II examined the effect of chronic MPH at a high dose (20 mg/kg, i.p., 14 days) on measures of subsequent psychostimulant and serotonergic receptor stimulation. Our studies showed that chronic MPH treatment produces increases in the psychomotor effects of stimulants such as cocaine and MDMA, as well as a potential increase in rewarding efficacy of MDMA. We then examined the interplay between DA and 5-HT systems following chronic MPH treatment. MPH treated animals additionally exhibit a lack of locomotor attenuation in response to the SSRI fluoxetine, a reversal of the normal effects of fluoxetine. The results with microdialysis confirmed our behavioral findings. MPH treated animals showed small but significant increases, indicating a reversal of the normal effects of fluoxetine and significant changes in the modulation between 5-HT and DA systems.

While changes in DA and 5-HT had been observed in the NAc of the MPH treated mice, the NAc receives its dopaminergic inputs from the VTA, an area with
extensive 5-HT projections. Accordingly, we infused fluoxetine into the VTA and measured DA and 5-HT levels in the NAc and VTA. MPH treated animals showed significant increases in NAc DA, as well as a highly sensitized 5-HT response in the VTA. The final question of this chapter was, "Which 5-HT receptor subtypes could be mediating the changes seen in MPH treated mice at the level of the VTA?" We performed initial investigations into this receptor using radioligand binding assays, which showed a significant increase in 5-HT1B receptor expression. Locomotor studies with a 5-HT1B receptor agonist showed an augmented increase in locomotor activity in MPH treated mice, and place preference studies showed preference for 5-HT1B agonists resembling that in response to fluoxetine. Finally, i.p. administration of a 5-HT1B agonist produced increases in DA in the NAc of MPH treated mice.

Overall, the work covered in Chapter II provided evidence for sensitization to psychostimulants following chronic MPH, as well as significant evidence for changes in DA/5-HT system interactions at the level of the VTA and NAc in MPH treated mice. Further, these studies provided a potential mechanism for the changes in DA/5-HT interactions, in the form of increased 5-HT1B receptor expression. These studies show that chronic MPH produces drastic changes in DA/5-HT interactions, a novel finding in the effects following chronic psychostimulant treatment.

Involvement of the 5-HT1A and 5-HT1B receptors in the effects of chronic MPH. With the effects of chronic MPH on DA/5-HT interactions confirmed, Chapter III aimed to determine the extent of involvement of other 5-HT receptor subtypes. Preliminary locomotor studies were carried out with agonists for the 5-HT1A, 1B, 2A, 2C, and 3 receptors. Of these, changes in MPH treated mice appeared evident in response to 5-
HT1B agonists. Our studies then continued with an in depth analysis of the changes in 5-HT1A and 5-HT1B control over mesolimbic DA. The 5-HT1A agonist 8-OH-DPAT produced reversed place preference responses and small increases in NAc DA in MPH treated mice. The 5-HT1B agonist RU 24969 produced conditioned place preference and significant increases in NAc DA in MPH treated animals. Results from Chapter II led us to believe that the effects in MPH treated mice causing increases in DA following administration of fluoxetine were the result of increases in DA cell firing in the VTA. While infusion into the VTA of the 5-HT1A agonists did indeed show differences in MPH treated mice, the changes showed substantial decreases in NAc DA, suggesting supersensitive responses in MPH treated animals.

The 5-HT1B receptor seemed a more likely candidate. Indeed, our results showed increases in DA in both the NAc and VTA, indicating that MPH treated animals had augmented increases in DA neuron firing in the VTA, leading to increases in DA in the NAc. We then determined to what extent the 5-HT1B receptor was responsible for the effects seen in Chapter II with fluoxetine. A 5-HT1B antagonist reduced the DA levels induced by fluoxetine, indicating that the 5-HT1B receptor was responsible for the effects seen with fluoxetine in MPH treated mice. These results fully confirmed the results seen in Chapter II, that MPH treated mice showed increased 5-HT system control over DA function, and that these results were mediated primarily by increases in the 5-HT1B receptor.

Effects of MPH treatment on depressive-like behaviors in mice. We hypothesized that the sensitization of the 5-HT1B receptor might be due to decreases in extracellular 5-HT induced by the effects of MPH on 5-HT neuron firing, and possibly also by the effects of
MPH on the 5-HT1A receptor. Low endogenous levels of 5-HT have been linked with depressive-like effects in mice (Gardier et al, 2009). Thus we examined the effects of chronic MPH administration on depressive-like behaviors, including the forced swim test, the tail suspension test, and the sucrose drinking task.

Our results in MPH treated mice showed deficits in the forced swim test, but did not show any difference in response in the tail suspension or sucrose drinking tests. Interestingly, MPH treated mice showed increased responses in the forced swim test and tail suspension test to fluoxetine, an effect that was expected given the altered effects of fluoxetine on DA in MPH treated mice. These data suggest that, if MPH treated do indeed exhibit low levels of endogenous 5-HT, these effects may not be enough on their own to strongly effect depressive behaviors. On the other hand, the dopaminergic effects of fluoxetine showed strong effects in these behavioral tasks, providing evidence for the dopaminergic involvement in these behaviors.

5-HT effects in MPH treated mice are temporary, while DA effects are long-lasting. For the final set of studies covered in Chapter V, we set out to establish the timing of the effects of chronic MPH treatment. We examined the locomotor effects and DA and 5-HT effects in the NAc of MPH treated mice following two and 10 days of withdrawal from MPH treatment. Locomotor studies with fluoxetine indicated that the lack of locomotor attenuation disappeared by 10 days of withdrawal, and microdialysis confirmed that the increase in DA at two days of withdrawal following fluoxetine administration in MPH treated mice is reversed again at day 10. Studies with cocaine and MDMA showed opposite effects. The locomotor sensitization was present at both day 2 and day 10. Microdialysis studies at day 2 replicated previous results, showing increased effects of
cocaine and MDMA on 5-HT levels in the NAc, while DA levels showed no difference from saline-treated controls. These effects appeared reversed at day 10, MPH treated mice showed augmentations in DA responses following acute cocaine challenge, while 5-HT effects following cocaine and MDMA administration were not different from those in saline treated mice. The results of this final chapter suggest that the 5-HT effects seen in previous chapters are temporary at best, while the sensitization to other psychostimulants following MPH exposure increases over time. These findings shed light on a possible withdrawal timecourse characteristic of MPH exposure.

In light of the findings produced in these experiments, as well as previous results from the literature, we have devised a model describing the possible mechanism behind the effect of chronic MPH on DA/5-HT system interactions (Figure 1). Chronic MPH administration produces increased extracellular DA and NE (Volkow et al, 2004), as well as stimulation of the 5-HT1A receptor (Markowitz et al, 2009). In the dorsal raphe, stimulation of D2 receptors results in increased 5-HT neuron firing (Aman, 2007), while stimulation of the 5-HT1A receptor results in decreased 5-HT cell activity (Sprouse, 1987, Sprouse, 1988) and firing to terminal regions such as the VTA. Over time, our results show that chronic MPH produces decreases in D2 receptor binding and increased 5-HT1A sensitivity, which would produce a net result of decreased 5-HT neuron activity. Decreases in 5-HT cell firing to terminal regions would produce supersensitivity of receptors in those regions. In particular, it appears that there is sensitization of the
Figure 1: Proposed model for the effects of chronic MPH on DA/5-HT interactions.

A: The system in the absence of MPH. The raphe receives excitatory inputs from the VTA in the form of dopaminergic projections. The raphe also has large numbers of 5-HT1A receptors that provide inhibitory control over 5-HT cell activity. The raphe projects to many areas, including the VTA, where it activates other 5-HT receptors, including 5-HT1B receptors located on the terminals of GABA neurons projecting from the NAc. The VTA in turn is the location of DA neurons that project to regions such as the NAc. 

B: The system following chronic MPH. Chronic MPH produces long-term increases in extracellular DA, desensitizing D2 and 5-HT1A receptors in the raphe, reducing 5-HT cell activity and creating a hyposerotonergic system. These decreases in extracellular 5-HT result in the upregulation of 5-HT1B receptors on GABA terminals in the VTA. These 5-HT1B receptors, when stimulated, inhibit GABA release in the VTA, disinhibiting VTA DA neuron firing (O’Dell et al, 2004). Thus, following chronic MPH, when these 5-HT1B receptors are stimulated by increases in 5-HT, there is an augmented sensitized response, a decrease in GABA release, and an increase in DA neuron firing to terminal regions, resulting in increased DA in the NAc.
5-HT1B receptor in the VTA, located on GABA terminals. These 5-HT1B receptors, when stimulated, decrease GABA neuron activity, disinhibiting VTA DA neurons and promoting cell firing (O’Dell, 2004). Thus, following chronic MPH treatment, when these 5-HT1B receptors are stimulated with global increases in 5-HT or specific agonists, there is a sensitized response and increase in DA neuron firing, resulting in DA increases in terminal areas like the NAc.

Taken together, the data shown in this thesis reflect an extensive study into the effects of high-dose chronic MPH in mice, showing significant change in the interactions between DA and 5-HT system in mesoaccumbens DA-mediated neurochemistry and behaviors. It appears that chronic MPH treatment produces increases in 5-HT1B receptor expression, changes which deeply influence how the mesolimbic DA system responds to 5-HT system challenge. These changes, seen in the first few days following chronic MPH treatment, could have implications for the abuse of MPH, as will be addressed further.

Applications of this work to the field of psychostimulant abuse and DA/5-HT system interactions.

This work, examining the effects of chronic MPH treatment on DA and 5-HT system interactions in the mesoaccumbens circuit is in some ways a logical extension of other work in the field, and in others represents a new area of investigation. It has been known for some time that 5-HT1B receptors stimulate DA increases and locomotor activity via the mesoaccumbens circuit (Sari, 2004). While these receptors do not appear to be very influential in DA neuron firing under basal conditions, when stimulated they potentiate MPH-induced locomotor activity (Borycz et al, 2008). While the effects of 5-
HT1B agonists on psychomotor activity have been established (O'Dell et al, 2004; Sari, 2004), we have shown the converse, that chronic MPH exposure can have significant effects on 5-HT1A and 5-HT1B receptor sensitivity. Additionally, we have traced the changes in 5-HT1B sensitivity in particular to the VTA, providing a mechanism for the increased 5-HT system control over mesoaccumbens DA function following chronic MPH. While changes in 5-HT1B expression have been seen following cocaine and MDMA (Amato et al, 2007; O'Dell et al, 2006), our studies are the first to show increases in 5-HT1B sensitivity following chronic MPH. We have also examined the altered effects of 5-HT system agonists such as fluoxetine on NAc DA following chronic MPH exposure, and have examined the time course of these effects. The altered effects of 5-HT on NAc DA following MPH exposure are highly novel and provide a new avenue of investigation into the effects of chronic, high-dose MPH, and add to the literature which implicates the 5-HT1B receptor in the adaptations following chronic psychostimulant exposure.

Questions to address in Future Studies

The current studies were performed using non-contingent injections. Could these effects be replicated with MPH self-administration? While the effects are clear in the results of these studies of MPH administered via i.p. injection, it is well know that there are differences in response to drugs of abuse in contingent and non-contingent paradigms. MPH self-administration has been established in rats, and our laboratory has already begun to assess whether the effects seen in mice with 5-HT system agonists following chronic MPH can be replicated in rats.
self-administering MPH, the better to achieve an MPH abuse model. We are already attempting studies utilizing single probe in vivo microdialysis in rats self-administering MPH, who will then receive an acute injection of fluoxetine. Preliminary results suggest that there is a similar effect of fluoxetine in animals following MPH self-administration, with self-administering rats exhibiting small increases in NAc DA following fluoxetine challenge. Given the possible species differences between mice and rats, we would also want to examine the effects of a 5-HT1B receptor agonist in the NAc and VTA, to determine if there are similar alterations in 5-HT/DA system interactions following contingent MPH self-administration. In addition, progressive ratio responding could be used to determine whether MPH administration produces increases the rewarding aspects of other psychostimulants.

Can the effects seen with chronic MPH be replicated using other psychostimulants? Previous work in the DAT-KO mouse (Mateo et al, 2004) indicated that chronic blockade of the DAT, or genetic deletion, produced the DA/5-HT system changes seen in our current studies. Mateo et al (Mateo et al, 2004) observed increases in DA following fluoxetine challenge in DAT-KO mice, as well as in mice given the long-acting DAT inhibitor 2β-propanoyl-3β-(4-tolyl)-tropane (PTT). Studies should also be undertaken to determine whether these effects can be induced with administration of other long-acting psychostimulants such as amphetamine or methamphetamine, both of which are drugs which are commonly abused, and amphetamine in particular is a commonly prescribed drug for treatment of ADHD. Investigation as to whether or not these other psychostimulants induce similar effects could provide more information on the mechanisms behind the plasticity of DA/5-HT system interactions.
Given the effects seen in the DAT-KO mouse, with PTT (Mateo, 2004), and the current findings with chronic MPH, it appears that the alteration in DA/5-HT interactions resulting in increased 5-HT influence over DA neuron firing may require long-term increases in extracellular DA. As hypothesized in Figure 1, high levels of extracellular DA resulting from prolonged DAT blockade could cause desensitization of D2 receptors, a finding seen in our results as well as in other studies (Nader et al, 2006; Thanos et al, 2007), and these decreases could produce decreased 5-HT neuron firing (Aman et al, 2007), resulting in a hyposerotonergic system and subsequent sensitization of 5-HT receptors including the 5-HT1B. Using this model, we would hypothesize that chronic, high extracellular levels of DA might produce these effects, regardless of whether or not DAT blockade is directly involved. This would not only mean that drugs such as amphetamine could cause these effects, but other drugs which produce increases in extracellular DA, such as ethanol or morphine, might have these effects. Additionally, it is possible that behavioral interventions such as stress, which increase extracellular DA, might produce similar effects.

However, the duration and degree of increased extracellular DA might well play a role in whether or not the effects on DA/5-HT interactions are produced, and studies should be performed to determine whether extracellular DA increases are necessary or sufficient for the changes seen in our results. It is possible that smaller increases in extracellular DA may not be enough to achieve the desensitization of the D2 receptors hypothesized in our model. Our laboratory recently conducted studies in adolescent mice using chronic administration of 10 mg/kg i.p. MPH, and did not observe any of the behavioral or neurochemical effects seen in adult mice. While the effects of age are an
extremely important variable in the results of these studies and cannot be discounted, the lower doses used could also be of importance in whether or not the changes in DA/5-HT system interactions took place.

In addition, the duration of the increase in extracellular DA may also play an important role. Preliminary studies attempting to replicate the results seen with MPH in rats self-administering cocaine could not detect any differences in neurochemical or locomotor activity responses to fluoxetine. It is possible that the elevations in extracellular DA produced by cocaine were too transient to produce decreases in D2 receptors at the level of the raphe. Thus, further studies, using shorter acting or more specific DAT inhibitors, as well as different methods to increase extracellular DA, would be necessary to determine the true role of increased extracellular DA in the effects on DA/5-HT interactions.

**Could the effects of chronic MPH render 5-HT system agonists such as fluoxetine and specific receptor agonists reinforcing?** Our studies showed that, following chronic MPH treatment in mice, fluoxetine and 5-HT1B receptor agonists produced conditioned place preference, along with increases in extracellular DA in the NAc. While place preference is often considered to be a measure of reward and reward-related learning, it cannot assess the reinforcing properties of drugs (Tzschenkte, 2007). In addition, it cannot truly assess drug-associated cue discrimination and incentive salience. It would thus be very interesting to examine the effects of chronic MPH in drug discrimination and drug self-administration paradigms, to see if 5-HT1B agonists or SSRIs could substitute for traditional psychostimulants, findings which could have important clinical implications. These studies could easily be performed with MPH self-administering
rats, to examine the possible self-administration of fluoxetine or 5-HT1B agonists. Unfortunately, fluoxetine is a very long-acting agonist, so it is possible that self-administration rates will be low. Shorter acting 5-HT agonists (such as quipazine) or SERT inhibitors (such as paroxetine or sertraline) may have to be obtained or synthesized. Another important examination could also be the 5-HT1B agonist, and studies could examine drug discrimination between the 5-HT1B agonist and psychostimulants, self-administration of a 5-HT1B agonist, and progressive ratio breakpoints for a 5-HT1B agonist following MPH self-administration. We would speculate that chronic MPH self-administration, if it produces alterations in DA/5-HT interactions and 5-HT1B receptors similar to those seen following non-contingent administration, could result in lever-responding for drugs such as the 5-HT1B agonist RU 24969 or intra-VTA infusion of the more specific agonist CP 93,129. We also hypothesize that, while fluoxetine may be too long acting to produce reliable self-administration in MPH-administering animals, other 5-HT drugs such as paroxetine or sertraline may show some reinforcing efficacy following MPH-administration.

Will psychostimulants with varying 5-HT affinities be more rewarding following MPH administration? While it is worthwhile to examine the rewarding and reinforcing effects of pure 5-HT agonists and receptor agonists following chronic MPH, it would also be of interest to look at the effects of psychostimulants. While some studies have shown that sensitizing regimens of MPH can decrease the latency to self-administer cocaine (Schenk et al, 2002), our studies have examined a high dose regimen, and have begun to assess the subsequent rewarding properties of drugs with strong serotonergic components, such as MDMA. We have found that MPH treated animals show a sensitized conditioned place
preference for low doses of MDMA. Given the effects we have found in these studies showing increases in 5-HT control over the mesolimbic DA system, we hypothesize that drugs which strong 5-HT system components might be more rewarding following chronic MPH, as seen in our conditioned place preference studies. MDMA is not generally highly reinforcing compared to other psychostimulants such as methamphetamine (Wang et al, 2007), but chronic MPH exposure could change the reinforcing properties due to changes in DA/5-HT system interactions. These studies are possible in MPH self-administering rats, where we could examine breakpoints for MDMA and for cocaine following MPH self-administration, both to examine sensitization or tolerance to the rewarding effects of cocaine, as well as examining potential increases in the reinforcing value of MDMA. The findings could potentially be of great importance for the consequences of MPH abuse in human populations, making subsequent use of drugs such as MDMA more rewarding and leading to possible increases in abuse risk of these drugs.

Does chronic MPH administration increase the self-administration and preference of ethanol? If chronic MPH self-administration has effects on the subsequent self-administration of psychostimulants, it is highly possible that MPH administration will also influence the subsequent reinforcing properties of ethanol. This is of particular clinical concern due to the high rates of MPH abuse and diversion on college campuses (Teter et al, 2003), and co-administration of alcohol is common (Barrett et al, 2006). These studies could be performed in mice or rats, studying two-bottle choice preference for ethanol following either non-contingent or contingent MPH administration. It would also be possible to look at lick rate and volume consumed in a drinking paradigm in MPH
self-administering rats, and studies in Dr. Weiner’s laboratory are already underway examining the effects of oral, clinically relevant MPH dosing.

What is the mechanism behind the sensitization of the 5-HT1B receptor? It has been our hypothesis that the changes in 5-HT system control over the DA system are the result of circuitry influences during chronic MPH administration. During the administration of chronic MPH, elevated levels of extracellular DA resulting from blockade of the DAT would cause overstimulation of post-synaptic DA receptors. There are post-synaptic D2 receptors on cell bodies in the dorsal raphe, and these receptors stimulate raphe cell firing (Aman et al, 2007). Following chronic stimulation due to MPH administration, D2 receptors would desensitize, and our radioligand binding studies from Chapter II suggest that this is indeed the case. In the case of the raphe, this means that the decreased D2 receptor levels would result in decreased raphe output to regions like the VTA. Under this scenario, the decrease 5-HT output would result in sensitization of other 5-HT receptors such as the 5-HT1B.

However, recent findings (Markowitz et al, 2009) have shown that MPH can bind directly as an agonist to 5-HT1A receptors. 5-HT1A receptors are the primary control over raphe neuronal firing, and stimulation of these receptors could result in direct reductions in raphe cell firing in concert with, or independently from, decreases in D2 receptors. This decrease in extracellular 5-HT would then result in sensitization of 5-HT1B receptors in the VTA.

Several experiments would be required to observe which mechanism is indeed at work in the sensitization of 5-HT1B receptors following chronic MPH. First of all, D2 agonists need to be infused or microinjected into the raphe, and the resulting increases in
5-HT from increased raphe neuron firing measured in the NAc. A similar experiment should also be performed with a 5-HT1A agonist infused or microinjected into the raphe of the MPH and saline treated animals. These experiments would determine the extent of involvement of 5-HT1A receptors and D2 receptors within the raphe. In addition, these experiments would provide potential mechanisms for development of treatment medications for the behavioral consequences following chronic MPH exposure.

Finally, it is of particular important to determine whether chronic MPH treatment produces decreased basal 5-HT levels in the brain hypothesized in Figure 1. We hypothesized that the effects we have seen following chronic MPH are due to stimulation of the 5-HT1A receptors on raphe cell bodies (Markowitz et al, 2009) or to decreases in D2 receptors following increases in extracellular DA. We hypothesize that the net effect of either of these mechanisms is a decrease in 5-HT neuron firing from the raphe resulting in decreased 5-HT signaling, which in turn produces increases in 5-HT receptor sensitivity. If this is the case, decreased 5-HT signaling would show itself in decreased extracellular levels via zero net flux microdialysis. In addition, decreases in extracellular 5-HT seen with zero net flux could provide a mechanism for the depressive-like behavioral effects seen in Chapter IV.

Could the 5-HT effects seen after chronic, high dose MPH be replicated at lower doses applicable to clinical paradigms? While the paradigm used here and the MPH self-administration experiments proposed would replicate a MPH abuse model, there are far more patients currently undergoing treatment with MPH for symptoms of ADHD, narcolepsy, and chronic fatigue (Accardo et al, 2001; Goldman et al, 1998; Harris, 2008; Leonard et al, 2004; Levin et al, 1995; Niederhofer, 2009; Solanto, 1998). While many
studies have been performed on the potential rewarding effects of clinical doses of MPH, and the potential effects on subsequent psychostimulant exposure, no studies have examined possible effects on 5-HT/DA interactions. Our findings have shown that there are potential behavioral consequences of MPH treatment which result in depressive-like effects, and clinical doses of MPH should also be investigated for this consequence and for the mechanisms behind the behavior. We have performed some pilot studies with 10 mg/kg doses in adolescent mice, and were unable to obtain the behavioral or neurochemical alterations seen with fluoxetine at the 20 mg/kg dose. While this could partially be the effect of dose and of treatment length, the adolescent mice used in this study are an important variable. It is possible that a fully developed system is necessary for the changes seen following chronic MPH. Further studies in adult animals using doses as low as 5 mg/kg in mice and 2 mg/kg in rats would be necessary to determine whether the changes in DA/5-HT interactions are present following clinically relevant dosing. It is our hypothesis that higher doses, and thus higher levels of extracellular DA, may be necessary for these effects, given the results seen in our preliminary adolescent studies, and that these changes may be relevant only in abuse-related paradigms.

Can we reverse or prevent the effects of chronic MPH with chronic fluoxetine treatment?

It is known that SSRIs can potentiate the locomotor and discriminative stimulus effects of psychostimulants (Borycz et al, 2008; Bubar et al, 2003; Cunningham et al, 1991; Herges et al, 1998). Some studies have suggested that the mechanism for this is stimulation of 5-HT1B and 5-HT2A receptors in the VTA/NAc circuit, and our studies have found similar effects, with SSRIs and 5-HT1B agonists increasing DA in the NAc independently following MPH treatment. We have also found increased responses of the 5-HT1A and
5-HT1B receptor, as well as increased receptor binding for the 5-HT1B receptor. Thus it would be interesting to examine the effects of chronic fluoxetine treatment following chronic MPH treatment, to determine whether the effects of chronic MPH on DA/5-HT interactions could be reversed, using behavioral and microdialysis measures. In addition, fluoxetine and MPH are being given concurrently in the clinic (Findling, 1996; Kafka et al, 2000; Stoll et al, 1996), and the effects of concomitant administration should be examined, to determine if similar effects on 5-HT receptors are present.

Clinical Implications

MPH is one of the most highly prescribed medications on the market today for ADHD, and is frequently diverted for abuse and purposes of cognitive enhancement (McCabe et al, 2005; Teter et al, 2003). Our studies have focused on the effects of chronic, high-dose MPH administration intended to imitate an abuse paradigm which may increase future psychostimulant abuse (Lambert et al, 1998). The results of our studies provide a detailed look into the effects of chronic MPH treatment on DA/5-HT interactions, showing that, following MPH treatment, other commonly used drugs such as cocaine, MDMA, and fluoxetine, exhibit changes in their rewarding and reinforcing properties. While MPH treated animals developed cross-sensitization for cocaine and MDMA, as well as sensitized conditioned place preference for MDMA, further studies must be performed to determine whether the reinforcing effects of these drugs are increased. However, we also found a reversal in the behavioral effects of fluoxetine. Fluoxetine normally decreases locomotor activity and produced conditioned place aversion, but MPH treated mice showed place preference for low doses of fluoxetine.
This was accompanied by increased levels of DA in the NAc in response to fluoxetine challenge. While it is fairly unlikely that fluoxetine itself would become abused following MPH administration due to its slow pharmacokinetic properties, the changes in the behavioral effects could have important implications for MPH abusers being treated with fluoxetine for anxiety or depressive symptoms. Further, our hypothesis proposed low extracellular 5-HT following chronic MPH administration, and behavioral studies into depressive like effects indicate that chronic MPH may produce depressive effects in rodents. Several studies have already examined the potential for depression following psychostimulant abuse (Barr et al, 2002; Moreau et al, 1995), and it is possible that MPH abuse may produce similar effects. Finally, we have found sensitization of the 5-HT1A and 5-HT1B receptors, and other 5-HT receptors in other brain areas may respond in similar ways to low endogenous 5-HT levels following chronic MPH. Should 5-HT drugs such as MDMA or fluoxetine then be applied in addition to MPH, this may increase the risk of Serotonin Syndrome in MPH abusing patients. There have already been several case studies showing Serotonin Syndrome during concomitant administration of MPH and fluoxetine (Coskun et al, 2008; Ishii et al, 2008; Park et al, 2010), and it is possible that the effects seen in the above studies influence this extra sensitivity.

Given the increased locomotor activating, neurochemical, and reward-associated responses to drugs of abuse following chronic MPH administration, these studies also point to possible changes that should be made in the prescription of MPH at the clinical level. While oral, extended release MPH is not associated with rewarding effects (Fone et al, 2005; Greydanus et al, 2007), immediate release MPH is often diverted for abuse
Given the potential effects of chronic MPH abuse, clinicians might consider decreasing the prescription rates for immediate release MPH in favor of extended release in an effort to diminish diversion of MPH.

Conclusions

In conclusion, the work covered in this thesis examined the effects of chronic, high dose MPH administration on DA/5-HT interactions within the mesoaccumbens circuit. Our findings indicated that there are temporary effects following administration of chronic MPH which produce sensitization of the 5-HT1B receptor, increased 5-HT system control over DA neuron firing, and depressive-like effects. In addition, our work found long term behavioral and neurochemical sensitization associated with withdrawal from chronic MPH. Many questions remain as to the mechanisms behind the changes in DA/5-HT interactions, but our studies suggest that there are extensive neurobiological effects following abuse of MPH, and these effects could strongly influence the way MPH is prescribed in the clinic, encouraging more prescription of long-acting formulas which are less diverted. Overall, chronic MPH has extensive effects on DA/5-HT interactions, effects which influence the subsequent response to various drug classes and have implications for the clinical use of MPH, particularly in the concomitant use of MPH and SSRIs such as fluoxetine.

References


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ABSTRACTS/POSTER PRESENTATIONS:


APPENDIX I

ETHANOL-INDUCED HYPERACTIVITY IS ASSOCIATED WITH HYPODOPAMINERGIA IN THE 22-TNJ ENU-MUTATED MOUSE

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Abstract:

Characterization of neurochemical and behavioral responses to ethanol in phenotypically distinct mouse strains can provide insight into the mechanisms of ethanol stimulant actions. Increases in striatal dopamine (DA) levels have often been linked to ethanol-induced hyperactivity. We examined the functional status of the DA system and behavioral responsiveness to ethanol, cocaine and a DA receptor agonist in an N-ethyl-N-nitrosourea (ENU)-mutagenized mouse strain, 22-TNJ, generated by the Integrative Neuroscience Initiative on Alcoholism Consortium. The 22-TNJ mouse strain exhibited greater locomotor responses to 2.25 g/kg ethanol and 10 mg/kg cocaine, compared to control mice. In vivo microdialysis showed low baseline DA levels and a larger DA increase with both 2.25 g/kg ethanol and 10 mg/kg cocaine. In in vitro voltammetry studies, the 22-TNJ mice displayed increased V_max rates for DA uptake, possibly contributing to the low baseline DA levels found with microdialysis. Finally, 22-TNJ mice showed enhanced in vitro autoreceptor sensitivity to the D2/D3 agonist, quinpirole, and greater locomotor responses to both autoreceptor-selective and postsynaptic receptor-selective doses of apomorphine, compared to controls. Taken together, these results indicate that the dopaminergic system of the 22-TNJ mouse is low-functioning compared to control, with consequent receptor supersensitivity, such that mutant animals exhibit enhanced behavioral responses to DA-activating drugs such as ethanol. Thus, the 22-TNJ mouse represents a model for a relatively hypodopaminergic system, and could provide important insights into the mechanisms of hyperresponsiveness to ethanol’s stimulant actions.
Introduction

In the field of alcohol research, it is apparent that complex genetic and environmental factors contribute to the effects of ethanol, and a variety of animal models have been created, using targeted and random genetic alterations and selective breeding, to examine and understand these effects (e.g., Crabbe and Belknap, 1993; Phillips, 1993; Gorwood et al., 2002; Bergstrom et al., 2003; Hoplight et al., 2007; Bell et al., 2008). Recently, the Integrative Neuroscience Initiative on Alcoholism and Tennesse Mouse Genome Consortia used ENU-mutagenesis to induce random single base-pair mutations in mice and then characterized ethanol-related phenotypes in mutated mouse lines using high-throughput behavioral screens (Kermany et al., 2006; Hamre et al., 2007). In behavioral assays described by Hamre et al (2007), the 22-TNJ mutant mouse strain was noted because it exhibited a hyperactive locomotor response to ethanol compared to 1-TNH controls. Since stimulated locomotor behavior is often associated with activity of the dopamine (DA) system (Imperato and Di Chiara, 1986; Kalivas and Stewart, 1991), the goal of the present studies was to determine if there were alterations in the dopamine system of 22-TNJ mice.

The locomotor stimulant effects of ethanol and other abused drugs are often associated with activation of the ventral tegmental area - nucleus accumbens (NAc) pathway (Amalric and Koob, 1993; Imperato and Di Chiara, 1986) and increased DA release in the NAc (Imperato and Di Chiara, 1986; Kalivas and Stewart, 1991). Many studies show that both systemic injections of alcohol (Imperato and Di Chiara, 1986; Tang et al., 2003; Yim and Gonzales, 2000; Yoshimoto et al., 1992) as well as its voluntary consumption (Doyon et al., 2003; Doyon et al., 2005; Weiss et al., 1993) cause an increase in DA cell activity in the mesolimbic areas of the brain. Additionally, manipulating DA receptors, transporters or other targets within the DA system
leads to alterations in ethanol-induced activity (Cohen et al., 1997; Le et al., 1997; Pastor et al., 2005; Jerlhag, 2008).

Research in rodent models has provided many insights into behavioral and neurochemical phenotypes associated with alcoholism, and their underlying genetic correlates. There are several rodent lines that have been studied with regard to ethanol-induced locomotor activity. The FAST and SLOW mouse lines, selected for locomotor responsiveness to ethanol (Shen et al., 1995), have been postulated to have changes in their dopaminergic system which correlate with changes in locomotor responses to ethanol, as well as responses to psychostimulants acting on the DA system, such as cocaine and methamphetamine (Bergstrom et al., 2003). Additionally, the well-studied alcohol Preferring (P) and Non-Preferring (NP) rats have been found to exhibit enhanced locomotor responses to ethanol which correlate with their enhanced dopaminergic response to the drug (Engleman et al., 2006; McKinzie et al., 2002). Thus, we hypothesized that changes seen in the 22-TNJ mouse with regard to locomotor activity could be the result of changes in the dopaminergic system of these animals. While the FAST and SLOW mouse lines and the P and NP rat lines are selectively bred for ethanol-related phenotypes and may have differences at multiple gene loci, the single-base-pair, random mutagenesis method used to create the 22-TNJ mice may provide a more limited genetic alteration correlating with an ethanol-related phenotype.

In order to better understand the ethanol-induced hyperactivity phenotype of the 22-TNJ mouse strain, we embarked on a neurochemical characterization of their dopaminergic system, specifically focusing on the striatum (including the caudate-putamen (CPu) and NAc), a region linked to the rewarding and stimulating properties of ethanol and known for its abundance of DA. Electrically stimulated DA release, uptake and responses to the D2-type receptor agonist,
quinpirole, were characterized using in vitro fast-scan cyclic voltammetry. Microdialysis was used to evaluate extracellular DA levels and DA responses to ethanol or cocaine in 22-TNJ and control mice. The locomotor stimulating effects of cocaine and two doses of ethanol were also measured to characterize the behavioral phenotype. In addition, apomorphine, a non-selective DA receptor agonist, was used to examine vertical activity in 22-TNJ and control mice. D2-type autoreceptors are known to regulate DA synthesis through inhibition of tyrosine hydroxylase, therefore we measured synthesis rates of L-3,4-dihydroxyphenylalanine (L-DOPA) in the presence of an L-aromatic acid decarboxylase inhibitor and the effects of quinpirole on synthesis. These studies provide a comprehensive picture of DA system function in the 22-TNJ mice and their controls, elucidating neurochemical correlates of ethanol-related behaviors.

Materials and Methods

**Animals:** Male C57BL/6J mice were mutagenized with one 200mg/kg dose of ENU and bred, when fertility returned, to hybrid B6XC3H/RI females that carried the hairy ear (Eh) inversion. The F1 males of this mating were the progenitors strains of each pedigree (or line) of the TNJ experiment that consisted of a total of 30 lines. Thus 22-TNJ was one line of mice generated from this experiment and 1-TNH was the control that received no ENU but underwent the same breeding scheme. The resulting mutations were maintained on a segregating C3H:B6 background, and a mutant with hypersensitivity to the locomotor properties of ethanol was designated as the 22-TNJ strain. Non-mutated background C3H:B6 mice served as controls (1-TNH). Homozygous breeding pairs of 22-TNJ and 1-TNH mice were shipped from the University of Tennessee at Memphis to Wake Forest University Health Sciences, and a breeding colony was established.
Locomotor phenotypes were maintained across all generations used. Animals were housed in groups of three or four per cage with food and water *ad libitum* on a 12-hr light-dark cycle with lights on at 7 am. All experiments used both male and female mice that were between 4 and 16 months old. Experimental protocols adhered to National Institutes of Health Animal Care Guidelines and were approved by the Wake Forest University Institutional Animal Care and Use Committee.

**Locomotor Activity Monitoring:** Initial activity measures were obtained using open field activity monitors (Med Associates, St. Albans, VT) located in a dimly lit room. The monitors consisted of small, square Plexiglas containers (27 × 27 × 20.3 cm) equipped with three 16-beam infrared arrays. All experiments were conducted between 0900 and 1700 hours during the light phase, and locomotor activity following cocaine or ethanol administration was measured as a total distance traveled (cm). Following a two-hour habitation period, mice were injected with either 10 mg/kg cocaine, 1.0 or 2.25 g/kg ethanol, or sterile buffered 0.9% NaCl (saline), given in a volume of 0.1 mL intraperitoneally (i.p.), immediately returned to the activity monitors, and data was collected for 120 minutes in 5-min non-cumulative bins. Locomotor activity in response to apomorphine (0.15, 0.3, 0.6, 1.2, 2.0 or 3.0 mg/kg) administration was measured by number of vertical rears. The effect of cocaine and ethanol on locomotor activity was assessed by two-way analysis of variance (ANOVA). The apomorphine dose-response curve was analyzed using a repeated measures ANOVA with a Bonferroni post-hoc analysis. Values of *P* < 0.05 were considered statistically significant.

**Brain Slices for Cyclic Voltammetry:** Mice were sacrificed by decapitation and the brains rapidly removed and cooled in ice-cold, pre-oxygenated (95% O₂/5% CO₂), modified artificial cerebral spinal fluid (aCSF, Mateo et al., 2004a). The aCSF consisted of (in mM): NaCl (126),
KCl (2.5), NaH$_2$PO$_4$ (1.2), CaCl$_2$ (2.4), MgCl$_2$ (1.2), NaHCO$_3$ (25), glucose (11), HEPES (20), L-ascorbic acid (0.4) and pH was adjusted to 7.4. The tissue was then sectioned into 400 µm-thick coronal slices containing the NAc and CPu with a vibrating tissue slicer (Leica VT1000S, Vashaw Scientific, Norcross, GA). Slices were kept in a reservoir of oxygenated aCSF at room temperature until required. Thirty minutes before each experiment, a brain slice was transferred to a submersion recording chamber, perfused at 1 ml/min with oxygenated aCSF at ~30°C, and allowed to equilibrate.

**Cyclic Voltammetry:** Carbon-fiber microelectrodes were prepared as previously described (Cahill et al., 1996) and reference electrodes were Ag/AgCl wires. For DA recording, the electrodes was scanned linearly from -400 mV to 1200 mV and back to -400 mV vs. Ag/AgCl, at 400 V/s, repeated every 100 ms (Mateo et al., 2004a). Stimulation parameters used to evoke DA release produced consistent, measurable signals and these parameters were chosen based on previous work in mice (John et al., 2006; Mateo et al., 2004b). DA release was evoked by a single, rectangular, electrical pulse (monophasic, 350 µA, 4 ms pulse width) every 5 min from an adjacent bipolar stimulating electrode (Plastics One, Roanoke, VA) placed on the surface of the slice, approximately 150 µm away from the microelectrode. The carbon-fiber microelectrode was placed approximately ~150 µm below the surface of the slice. Each slice served as its own precondition control. Quinpirole was applied to the slice by superfusion for 30 min per concentration, in a cumulative concentration paradigm (Jones et al., 1995b). DA was identified by its characteristic cyclic voltammogram, where the oxidation current for DA is ~600 mV and reductive current is ~-200 mV. Electrodes were calibrated with 1 µM DA to convert the signal from current to concentration.
Data Analysis for Cyclic Voltammetry. DA release and uptake kinetics were analyzed with a Michaelis-Menten based set of kinetic equations (Wightman and Zimmerman, 1990). Electrically stimulated DA release during the rising phase is a balance between the processes of release and uptake, and uptake is the primary process occurring during the decay phase. These parameters were characterized by the following formula:

\[
d [DA] = f [DA]_p - V_{\text{max}} \frac{dt}{(K_m/[DA])+1}
\]

In this equation, [DA] represents the instantaneous extracellular concentration of DA released, \(f\) is the stimulation frequency, [DA]_p is the amount of DA released per stimulus pulse and \(V_{\text{max}}\) and \(K_m\) are Michaelis-Menten uptake rate constants. The following assumptions are made with the above equation: (1) the amount of DA ([DA]_p) released into the extracellular space with each stimulus pulse is consistent, (2) uptake is a saturable process and (3) uptake occurs through the DAT, which is the primary mechanism for clearing DA. Furthermore, a control \(K_m\) value of 0.16 µM for DA was used, and \(V_{\text{max}}\), which is proportional to the number of monoamine transporters present, was determined. These assumptions are best suited for evaluating release and uptake in striatal regions where one-pulse stimulations are used, and where uptake rates are greater than or equal to 1 µM/s (John and Jones, 2007). The curve-fitting algorithm, based on simplex minimization and goodness of fit, was described by a non-linear regression coefficient (\(r^2\)) (Jones et al., 1995a).

The concentration of DA released and \(V_{\text{max}}\) values for uptake were determined from current vs. time curves before and after drug application. When quinpirole was
used, the change in the current vs. time profile was evaluated as a change in [DA]p; inhibition of DA release via D2-type autoreceptors. This change in electrically stimulated DA release was compared to pre-drug values (each animal served as its own control) resulting in a percent change in stimulated DA release. The dose response curve was then plotted as log concentration (M) of quinpirole vs. percent of control DA response, and the data were fit using a non-linear regression curve fit (specifically, sigmoidal dose response curve) to determine EC$_{50}$ concentrations.

All voltammetry statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc. San Diego, CA). In all cases, data are mean ± S.E.M. of at least four brain slices, which were obtained from at least four animals. In vitro voltammetry data provided DA release and uptake data which were compared across mouse lines using Student’s t-tests. Data obtained after administration of quinpirole were subjected to a two-way ANOVA with mouse line and dose of quinpirole as the factors. When significant interactions or main effects were obtained, differences between groups were tested using Bonferroni corrected post hoc tests. In all cases, statistical significance was set at $P < 0.05$.

In vivo Microdialysis: Briefly, mice were anesthetized with Avertin administered in a volume of 16 mL/kg, i.p. (Papaioannou and Fox, 1993). The skin over the skull was shaved, cleaned with alcohol, incised, and the exposed skull was cleaned and dehydrated with 10% H$_2$O$_2$. Mice were placed in a stereotaxic frame equipped with a mouse palate adapter and a burr hole (~1 mm diameter) was drilled in the skull. A guide cannula for a CMA/7 mouse microdialysis probe (CMA/Microdialysis, Chelmsford, MA) was implanted into the CPu using coordinates determined from a mouse atlas (Franklin & Paxinos, 2008) and refined by the Integrative
Neuroscience Initiative on Alcoholism Consortium Neurohistology Core’s (Principle Investigator: Andrea Elberger) histological mouse atlas (CPu: anterior, +0.6; lateral, -1.8; ventral, -2.5 from bregma, NAc: anterior, +1.2; lateral -0.6; ventral -3.3 from bregma). The guide cannula was anchored and the exposed skull sealed with a fast drying two-part epoxy from Loctite (Tak Pak 444, Accelerator 7452 Winona, MN). The microdialysis procedure was as described previously (Bungay et al., 2003, Mateo et al., 2004b), with the following modifications. As mice were recovering from anesthesia, microdialysis probes (1 or 2 mm membrane length (NAc or CPu), 0.24 mm o.d.; Cuprophane, 6 kDa cut-off; CMA-7, CMA/Microdialysis, Chelmsford, MA) were connected to a syringe pump and perfused with aCSF (in mM: 148 NaCl, 2.7 KCl, 1.2 CaCl₂ and 0.85 MgCl₂; pH= 7.4 with NaH₂PO₄) at a flow rate of 0.2 µL/min overnight. Approximately twelve hours later, the flow rate was increased to 0.8 µL/min and allowed to equilibrate for 2 hours before at least four baseline samples were collected at 20 minute intervals and analyzed immediately by high performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytical Instruments, West Lafayette, IN). Following determination of a stable baseline, ethanol (2.25 g/kg) or cocaine (10 mg/kg) was injected i.p. and samples were collected for 2 hours. Immediately after dialysis, mice were sacrificed by inhalation of halothane and cervical dislocation and the brains were removed for histological confirmation of probe placement.

Microdialysis data were calculated as the percentage change from baseline concentration, with 100% being defined as the average of the last three samples prior to drug treatment. 22-TNJ mutant mice were compared to their controls (1-TNH) using Student’s t-tests. The effects of ethanol (2.25 g/kg, i.p.) or cocaine (10 mg/kg, i.p.) on extracellular concentrations of DA in the
NAc were assessed by two-way ANOVA for repeated measures. Values of $P < 0.05$ were considered statistically significant.

**Neurotransmitter Analysis:** HPLC coupled to electrochemical detection was performed using two different methods depending on the sample type. Dialysate samples (10 µl) were injected onto a Luna 50 x 2.0 mm C$_{18}$ 3 µm HPLC column (Phenomenex, Torrance, CA) for separation followed by detection at a glassy carbon electrode (+0.65 V vs Ag/AgCl reference electrode, Bioanalytical Systems, West Lafayette, IN). The mobile phase consisted of (in mM): 19 Na$_2$HPO$_4$, 29 citric acid, 25 sodium acetate, 4.6 1-octane octanesulfonic acid, 0.5 EDTA and 20% acetonitrile (pH ~4). Neurotransmitter peak areas were integrated using Chromgraph software (Bioanalytical Systems, West Lafayette, IN) and quantified against known standards. Concentrations were expressed in nM ± SEM. The limit of detection for DA was 0.5 nM (3 times signal to noise).

**Synthesis:** Tissue samples were assayed for L-DOPA accumulation following inhibition of L-aromatic acid decarboxylase to measure the activity of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis. Mice were injected with the inhibitor 3-(hydrazineomethyl)phenol dihydrochloride (NSD-1015), 100 mg/kg, i.p., and because autoreceptor regulation of DA synthesis was measured, γ-butyrolactone (GBL), 750 mg/kg, was used to inhibit DA neuron firing and reduce extracellular DA levels to a minimum (Jones et al., 1999; Wang et al., 1997). This was done to eliminate autoreceptor activation by endogenous DA tone. In experiments measuring autoreceptor regulation, quinpirole (1 mg/kg) was administered 10 min before NSD-1015 and GBL was administered 5 min before NSD-1015. Mice were sacrificed 40 minutes after NSD-1015 injection and the striatum was dissected and immediately frozen. Tissue samples were stored at -80°C until time of analysis. Tissue samples were removed from the freezer and
homogenized in 0.2 M HClO₄ and 1 mM EDTA containing 250 nM 3,4-dihydroxybenzylamine (DHBA) as an internal standard (Wang et al., 1997). Homogenates were centrifuged for 8 min at 10,000 r.p.m. Separation of L-DOPA was obtained using a Luna 50 x 2.0 mm C₁₈ 3 µm HPLC column followed by detection with an ESA 5011 analytical cell (E₁ = +220 mV, ESA Coulochem III, ESA Inc., Chelmsford, MA). A guard cell (ESA 5020) was placed before the injection loop and set at a potential of +350 mV. The mobile phase consisted of (in mM): 80 citric acid, 10 Na₂HPO₄, 7.8 chloroacetic acid, 1.0 EDTA, 2.4 sodium octanesulfonate and 3 % acetonitrile in a volume of 500 mL (pH =3.0) at a flow rate of 0.36 mL/min. L-DOPA was identified using PowerChrom Software (eDAQ Inc., Colorado Springs, CO) and quantified against known standards. Protein content of 20 µL aliquots was determined with the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Co., Rockford, IL). Analytes were quantified and normalized with respect to mean concentrations measured in control mice.

**Chemicals and Drugs:** Components of HPLC mobile phases, aCSF, Kreb’s buffer and neurotransmitters were of HPLC grade or the highest quality obtainable from Sigma-Aldrich (St. Louis, MO). Sodium octanesulfonate was from Acros Organics (Morris Plane, NJ). The BCA Protein Assay Kit containing all chemicals necessary for the protein assays was purchased from Pierce Co. (Rockford, IL; part #23225).

**Results**

**Locomotor activity responses to cocaine or ethanol challenge.** Following two hours of habituation to the locomotor chambers, mice were given an i.p. dose of 10 mg/kg cocaine, 0.1 mL saline, or 1.0 or 2.25 g/kg ethanol. A 10 mg/kg dose of cocaine was chosen because this dose is known to stimulate locomotor activity in mice (Elliot et al., 2002). The 2.25 g/kg dose of
Figure 1.

A. Cocaine (10 mg/kg)
- 1-TNH
- 22-TNJ

B. Ethanol (1.0 g/kg)

C. Ethanol (2.25 g/kg)
Figure 1. 22-TNJ mice are more sensitive to the stimulating properties of cocaine and ethanol. Locomotor activity measured total distance traveled (cm) after an i.p. injection of cocaine or ethanol at time zero, represented by the arrow. (A) Locomotor activity measured after 10 mg/kg cocaine injection (n = 24 per mouse strain). (B) Locomotor responses of 22-TNJ (n = 14) and 1-TNH (n = 13) mice to 1.0 g/kg ethanol injection. (C) Locomotor activity measured after 2.25 g/kg ethanol injection (n = 17 per mouse strain). Data points represent mean ± S.E.M, *** = p<0.0001 difference from control strain at time point indicated.
ethanol was chosen to be consistent with the high throughput screening analysis used by the Integrative Neuroscience Initiative on Alcoholism and Tennessee Mouse Genome Consortium (Hamre et al., 2007). Interestingly, the 1-TNH control mouse strain showed an augmented response to saline injection relative to the 22-TNJ strain (p<0.05, data not shown). Given this disparity, the subsequent 22-TNJ response to psychostimulants may be considered more pronounced. Cocaine significantly elevated levels of locomotor activity in both 22-TNJ and 1-TNH mice during the 2 hr test session (F_{28,56}=126.1, P < 0.0001), with the activity of the 22-TNJ significantly increased (two-fold) over control (F_{1,58}=115.7, P <0.0001) (Fig. 1A). There was a significant mouse line x time interaction (F_{28,56}=19.8, P < 0.0001) indicating that overall, the different lines of mice responded differently to cocaine with respect to time. In addition, we examined two doses of ethanol, 1.0 and 2.25 g/kg. The lower dose of ethanol caused a significant increase in activity in both 22-TNJ and control mice (F_{10,250}=11.27, P < 0.0001), with the 22-TNJ mice showing a significantly greater enhancement (F_{1,250}= 12.33, P < 0.001). A significant interaction between mouse line x time was found (F_{16,204}=3.958, P < 0.0001). The 2.25 g/kg ethanol dose (Fig. 1C) also caused a significant increase in activity in both 22-TNJ and 1-TNH mice during the 30 min test session (F_{10,320}=14.51, P < 0.0001), with the 22-TNJ exhibiting a significantly greater increase (F_{1,320}=19.65, P < 0.0001). A significant interaction between mouse line and time was also found at the higher dose (F_{10,320}=7.132, P < 0.0001). Thus, the 22-TNJ mutant mice are hyper-responsive to the locomotor stimulating effects of cocaine and ethanol.

**Measures of DA release and reuptake using in vitro voltammetry.** The 22-TNJ mutant was selected for study based on increased locomotor activation following ethanol injection. Since locomotor stimulant activity is often associated with the DA system, we first used voltammetry
Figure 2.

A.

B.

1.0 μM

2 s

1-TNH

22-TNJ
**Figure 2.** Representative traces of electrically-stimulated endogenous DA overflow in the NAc core of 1-TNH (control, left trace) and 22-TNJ mutant (right trace) mice. Dots are data points, sampling extracellular DA levels, plotted every 100 ms. A single electrical pulse (300 µA, 2 ms) was applied where trace begins to rise. Electrically stimulated DA release (DAp) and uptake rates (V\text{max}) were taken from the best fit of the data points to a Michaelis-Menten based kinetic model. Release was not different between the two strains of mice, but DA uptake was significantly faster (* P < 0.05) in 22-TNJ mutant mice compared to the 1-TNH.
to characterize DA release and uptake in controls and mutant 22-TNJ mice. Exemplary traces for the 1-TNH mouse and the 22-TNJ mouse are shown in Fig. 2. $V_{\text{max}}$ for DA uptake was significantly faster (2.8 ± 0.2 μM/s) in the core of the NAc of 22-TNJ mice (n=30) compared to 2.1 ± 0.1 μM/s in the controls (n=31) ($t = 2.64$, $P < 0.05$, data not shown); however, there was no difference in the amount of electrically-stimulated DA release between the control ([DA_p], the concentration of DA released per stimulus pulse = 0.95 ± 0.09 μM) and 22-TNJ ([DA_p] = 1.06 ± 0.10 μM; $t = 0.7852$, $P = 0.4355$, data not shown) mice. Therefore, the 22-TNJ mutant mice have increased maximal DA uptake rates in the core of the NAc.

**Measurement of presynaptic D2 autoreceptors in the NAc.** The activity of DA release-regulating autoreceptors was evaluated in the core of the NAc. Increasing concentrations of the D2-like receptor agonist, quinpirole (0.001 – 1 μM), were added to slices at 25-min intervals. After reaching a plateau effect at each quinpirole concentration, the peak DA efflux was evaluated and expressed as percent of control (Fig 3). The software Graphpad Prism was used to construct a sigmoidal dose-response curve to determine the best fit values for the EC_{50}. Quinpirole significantly reduced DA release in the NAc core ($F_{6,73}=47.08$; $P < 0.001$). The log-concentration of the half-maximal response (log EC_{50}) was -6.9 ± 0.1 (140 nM) for controls (n=6) and -7.3 ± 0.2 (60 nM) for 22-TNJ mice (n=8). There was a significant effect of mouse line on EC_{50} ($F_{1,73}=29.27$; $P < 0.001$), illustrating supersensitivity of D2 autoreceptors in 22-TNJ mice. No interaction between groups was found ($F_{6,73}=0.5563$).

**In vivo microdialysis DA measurements in the CPu and NAc.** Using microdialysis, baseline DA levels were compared in the NAc and CPu of the 22-TNJ and 1-TNH mice. Extracellular DA
Figure 3.
Figure 3. DA release-regulating autoreceptors are supersensitive in the NAc of 22-TNJ mice. Concentration-response relationship of quinpirole and maximal evoked DA release in NAc core in control (n = 6) and 22-TNJ mice (n = 8). 22-TNJ mice showed a supersensitized response of inhibitory D2 autoreceptors on DA release (*** $P < 0.001$). This decrease is greatest at 10 and 100 nM (points -8 and -7, on the graph) between the controls and 22-TNJ mice; * $P < 0.05$ and ** $P < 0.01$ controls vs 22-TNJ.
levels were lower in the CPu of the 22-TNJ mice (1.0 ± 0.4 nM) compared with their controls (3.7 ± 0.8 nM; t= 2.927; P < 0.05, data now shown). Extracellular DA levels in the NAc were not significantly different between the 22-TNJ mice (1.9 ± 0.2 nM) and controls (2.1 ± 0.3 nM) (data not shown).

Microdialysis measurements of ethanol effects on DA levels. Ethanol (2.25 g/kg) was administered i.p. at the end of the last baseline microdialysis sample, and subsequently samples were collected in 20-min intervals for two hours. DA levels in the three samples prior to ethanol administration were averaged to obtain mean baseline values, to determine the effect of ethanol on extracellular DA levels in striatum by calculating the percent change from baseline. After administration of 2.25 g/kg ethanol, DA dialysate levels were measured in the NAc (Fig. 4). A significant increase in DA levels was observed (F_{8,101}=2.00; P < 0.05) in response to ethanol and there was a significant difference between the 22-TNJ and 1-TNH mouse lines (F_{1,96}=25.66; P < 0.0001). Finally, there was a significant interaction between strains over time (F_{8,101} = 2.539; P < 0.05), indicating that the 22-TNJ strain exhibited a significant increase in DA levels over time, above and beyond that of the 1-TNH.

Cocaine-stimulated DA increases in 22-TNJ and 1-TNH mice. Cocaine (10 mg/kg, i.p.) was administered and extracellular DA levels in the CPu and NAc were monitored using in vivo microdialysis. Cocaine elevated extracellular DA concentrations (F_{8,90}=6.746; P < 0.0001) in the CPu (Fig 5A); and there was a significant difference between mouse lines (F_{1,90}=7.358; P < 0.01), indicating a greater increase in the 22-TNJ mice. There was no significant interaction between groups over time (F_{8,90}=1.247; P = 0.2813).
Figure 4.
Figure 4. Effect of ethanol (2.25 g/kg, i.p.) on extracellular concentrations of DA in the NAc of control (□) or 22-TNJ (■) mice. Ethanol significantly elevated extracellular concentration of DA in 22-TNJ ($P = 0.0001$ ; n = 7) and in the control mice (n=7); DA peaked to ~ 30 % above average baseline levels at 20 minutes for the control mice, while the 22-TNJ mice showed a delayed effect and peaked at 100 minutes to ~ 50 % above baseline.
In addition to monitoring extracellular DA levels in CPu, we also evaluated DA in the NAc following cocaine administration (Fig 5B). In these animals, as observed in the CPu, systemic administration of cocaine (10 mg/kg, i.p) elevated extracellular DA concentrations in the NAc in both the 22-TNJ and 1-TNH mouse lines ($F_{8,144}=5.322; P < 0.0001$). The DA response to cocaine between the lines of mice was also significantly different, with the 22-TNJ mice showing an enhanced DA response to cocaine compared to the 1-TNH ($F_{1,144}=5.360; P < 0.05$) and a significant interaction between mouse lines over time was found ($F_{8,144}=2.587; P < 0.05$).

**Behavioral responses to the non-selective DA receptor agonist apomorphine.** Following a two-hour habituation period to the locomotor activity monitoring apparatus, mice were injected with the non-specific DA receptor agonist, apomorphine (0.15, 0.3, 0.6, 1.2, 2.0, or 3.0 mg/kg) or saline in a volume of 0.1 mL, i.p. At low doses, apomorphine is known to suppress vertical rearing in mice, through activation of high-affinity inhibitory presynaptic D2-type autoreceptors. Conversely, at doses over 0.6 mg/kg, apomorphine is known to stimulate vertical activity, through post-synaptic DA receptor actions (Protais et al., 1976). A significant interaction between genotype and dose was found ($F_{6,154}= 3.662, p<0.01$) indicating that, overall, the two genotypes responded differently to both low and high doses of apomorphine. In response to 0.3 mg/kg apomorphine (Fig.6), 22-TNJ mice exhibited a suppression in vertical activity exceeding that of controls ($F_{11,11}= 19.04, p<0.05$). Further, in response to the highest dose of apomorphine given (3.0 mg/kg, Fig. 5), 22-TNJ mice exhibited a significant increase in vertical activity compared to control ($F_{11,11}= 10.89, p<0.05$). Therefore, 22-TNJ mice are supersensitive to both pre- and post-synaptic effects of apomorphine on vertical activity.
Figure 5.

A.

Cognitive Performance (CPu DA dialysate (% of baseline))

Time (min)

-60 -40 -20 0 20 40 60 80 100 120

T NH

22-TNJ

Cocaine

B.

Nucleus Accumbens (NAc DA dialysate (% of baseline))

Time (min)

-60 -40 -20 0 20 40 60 80 100 120

T NH

22-TNJ

Cocaine

**
Figure 5. The effect of cocaine on extracellular DA concentration in the CPu and NAc in control and 22-TNJ mice, as measured by microdialysis. Three baseline samples were collected and then cocaine was administered i.p. during the start of the last baseline sample (arrow). (A) In the CPu cocaine increased DA levels ~ 130 % in control (□) and ~ 315 % 22-TNJ (■) mice. Cocaine significantly elevated extracellular concentration of DA in 22-TNJ mice ($P < 0.01; n = 7$) compared to 1-TNH (n=5) in the CPu. (B) In the NAc cocaine increased DA levels ~ 40 and 70% in control and 22-TNJ mice, respectively. Similarly in the NAc, cocaine significantly elevated extracellular concentration of DA of 22-TNJ ($P < 0.01; n = 8$) compared to 1-TNH (n = 12) mice.
Measurement of D2 autoreceptors controlling synthesis. DA synthesis rates were measured by accumulation of L-DOPA following NSD-1015, an aromatic amino acid decarboxylase inhibitor, and accumulation of L-DOPA was similar in the striatum (containing both the NAc and CPu) of both lines of mice (Fig 7A). The GBL and NSD-1015 treatment combination (Fig 7A) was used to assess the effects of autoreceptors on synthesis. Administration of GBL following NSD-1015 will block cell firing, providing a rate of synthesis in the absence of autoreceptor tone (Walters and Roth, 1976). Mice treated with both GBL and NSD-1015 had significantly elevated L-DOPA accumulation in the CPu compared to NSD treatment alone (F_{1,25} = 14.02; P < 0.001). L-DOPA accumulation increased 4-fold (0.12 ± 0.01 to 0.48 ± 0.06) in control mice (n = 4 and 8) and approximately 3-fold (0.22 ± 0.05 to 0.64 ± 0.15) in 22-TNJ mice (n=8 and 9) following administration of GBL. There were no significant effects of mouse line, or interaction between groups (P>0.05). Since it is known that D2-type autoreceptors in particular can regulate synthesis, the D2-type agonist, quinpirole, was used to inhibit tyrosine hydroxylase and block the accumulation of L-DOPA following administration of NSD-1015 and GBL. Quinpirole significantly (Fig. 6B, F_{1,31}=27.41, P < 0.0001) decreased L-DOPA formation in the striatum in both lines of mice in comparison to NSD and GBL administration alone; however, 22-TNJ mice showed an augmented effect of quinpirole in the striatum compared to 1-TNH mice (F_{1,17}=19.24, P < 0.05).
Figure 6.
Figure 6. Vertical activity as assessed by the non-specific D2 receptor agonist apomorphine. At the highest dose known to inhibit presynaptic receptors apomorphine (0.3 mg/kg) significantly decreased vertical activity in the 22-TNJ mouse strain compared to their controls ($P < 0.05$, $n = 12$ per mouse line). In addition, at the highest dose (3.0 mg/kg) known to predominantly stimulate post-synaptic receptors, apomorphine significantly increased vertical activity in 22-TNJ mouse strain compared to their controls ($P < 0.05$, $n = 12$/mouse line). Results are group means ± S.E.M.
**Discussion**

Overall, we conclude that the striatum of the 22-TNJ mutant mouse is hypodopaminergic compared to wildtype controls. Using a variety of neurochemical techniques such as *in vivo* microdialysis, *in vitro* voltammetry, DA synthesis measures and behavioral testing, we have characterized the striatal DA system of the 22-TNJ mutant mouse and demonstrated that the mutant mice display (1) increased DA uptake rates, (2) reduced extracellular DA levels in the CPu, (3) supersensitive presynaptic D2 DA autoreceptors controlling synthesis and release, (4) supersensitive behavioral responses to the DA receptor agonist apomorphine (5) enhanced DA release in response to cocaine or ethanol and (6) potentiated response to the locomotor stimulating effects of ethanol and cocaine. Taken together, these results show that the 22-TNJ mutant mouse can be considered a model of relative reduced extracellular DA levels and consequent supersensitivity to ethanol, cocaine and DA receptor agonists.

Based on these findings, the 22-TNJ mutant mouse represents a model of supersensitive DA receptor function and concomitantly enhanced response to drugs that elevate extracellular DA levels or activate DA receptors. Because supersensitivity occurs with G-protein coupled receptors in response to reduced agonist stimulation, the enhanced responses seen here are consistent with the documented low tonic DA levels. Our findings show that the 22-TNJ mutant mice displayed supersensitivity to the the locomotor stimulating and dopamine-elevating effects of acute doses of ethanol and cocaine. Although the increases in dopamine, in particular in response to ethanol, appear to be slower than those found in other models, this difference appears to be neurobiological in origin. Blood ethanol concentrations, measured in different groups of animals, do not differ between the two strains of mice, indicating no change in ethanol metabolism following ENU mutagenesis (Hamre et al, personal communication). In addition, *in*
Figure 7.

A.

![Bar graph showing ng/mg L-DOPA levels for 1-TNH and 22-TNJ treatments with NSD1015 and GBL + NSD1015 treatments.]

B.

![Bar graph showing % of GBL treated controls for 1-TNH and 22-TNJ treatments with Quinpirole (1.0 g/kg).]
Figure 7. Tissue levels of L-DOPA accumulation in the striatum of 22-TNJ and control mice measured by HPLC-electrochemical detection. (A) L-DOPA accumulation measured after inhibition of L-aromatic amino acid decarboxylase with NSD-1015 showed no difference between control (0.11 ± 0.01 ng/mg, n=4) and 22-TNJ (0.17 ± 0.02 ng/mg, n=8) mice. Treatment with GBL significantly ($P < 0.01$) increased L-DOPA accumulation in the striatum of both control (0.48 ± 0.06 ng/mg; n =8) and 22-TNJ (0.65 ± 0.14 ng/mg; n =9) mice, with no difference between strains ($P > 0.05$). (B) Modulation of striatal L-DOPA accumulation by quinpirole. Quinpirole (1 mg/kg, i.p.) significantly reduced L-DOPA accumulation in both control and 22-TNJ mice ($P < 0.05$); however, this effect was greater in the 22-TNJ (~ 80 % decrease, n =10) mice compared to their controls (~ 62 % decrease, n = 8). Results are presented as means ± S.E.M.
vivo microdialysis demonstrated that 22-TNJ mutant mice have decreased extracellular DA levels in the CPu, though there was no significant difference in extracellular DA levels in the NAc. In vitro voltammetry data supported the reduced extracellular DA levels found via in vivo microdialysis, providing evidence that the 22-TNJ mutant mice have increased DA uptake rates and supersensitive D2-type autoreceptors controlling DA release in the core of the NAc. Both of these changes could contribute to reduced extracellular DA levels. The findings of no baseline difference in the NAc using microdialysis, but changes in uptake and release regulation measured by voltammetry illustrate the different types of information gathered by the two different techniques. The change in uptake in the NAc might have some influence on the baseline microdialysis data, since with quantitative no-net-flux techniques, it has been shown that extraction fraction is altered by changes in uptake rate.

D2-type autoreceptors located on nerve terminals are also known to inhibit DA synthesis, thus we evaluated the effects of quinpirole on the enzymatic activity of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, and found that autoreceptors regulating synthesis in the 22-TNJ mutant mice were also supersensitive. Finally, apomorphine, a non-selective DA receptor agonist, caused decreased vertical activity responses at an autoreceptor-selective dose (0.3 mg/kg) and caused augmented vertical activity compared to control at the postsynaptic receptor-activating highest dose (3.0 mg/kg) (Becker et al., 1995; Tien et al., 2003), indicating that 22-TNJ mutant mice exhibit supersensitive pre- and post-synaptic D2-type DA receptor function. Taken together, these findings offer sound support for a hypodopaminergic system in the 22-TNJ mouse relative to the 1-TNH control.

The chronically reduced extracellular DA levels and increased DA uptake in the 22-TNJ could be expected to produce supersensitive DA receptors. Indeed, using a variety of methods
including voltammetry, synthesis measurements and behavioral analyses, we determined that pre- and postsynaptic DA receptors were supersensitive. Our voltammetry studies shows that D2-type autoreceptors controlling DA release in the NAc core of 22-TNJ mice were supersensitive to the effects of quinpirole, a D2-type receptor agonist. In addition, quinpirole was more effective in decreasing the rate of L-DOPA synthesis following AADC blockade in the 22-TNJ, providing further evidence for D2 autoreceptor supersensitivity.

Finally, we used apomorphine to assess presynaptic autoreceptor (Becker et al., 1995; Bradbury et al., 1984; Radhakishun and van Ree, 1987) and postsynaptic receptor (Tien et al., 2003) function. Apomorphine is a non-selective DA D1- and D2-type agonist, and thus elicits a U-shaped behavioral dose response curve in which low doses produce behavioral inhibition through presynaptic receptor feedback inhibition, while high doses produce behavioral stimulation due to post-synaptic receptor activation. In our studies, the 22-TNJ mutant mice were more susceptible to both the enhancing (3.0 mg/kg) and the inhibiting (0.3 mg/kg) effects of apomorphine than the 1-TNH mice, indicating supersensitivity of DA receptors in both locations. The 1-TNH mice did not demonstrate significant locomotor stimulation, even at the highest dose of apomorphine given. They appeared less sensitive to the locomotor stimulating effects of apomorphine than other mouse strains such as the C57BL/6J (Cabib and Puglisi-Allegra, 1991; Archer et al., 2003), however the important comparison is between the 1-TNH controls and the 22-TNJ mutants, and the 22-TNJ mice were clearly more sensitive to both locomotor-inhibiting and –stimulating apomorphine effects. Although DA receptor function was measured, we were unable to distinguish between up-regulation, defined as an increase in the number of presynaptic D2 receptors, and individually supersensitive receptors with no change in number. Future studies could include the quantitative analysis of D2-like receptors using autoradiography.
Since the 22-TNJ mutant mice have relatively reduced extracellular DA levels, it may appear paradoxical to call the mutant mice hyper-responsive; however, they are supersensitive to the acute effects of drugs that elevate DA levels or activate DA receptors. Although our data clearly supports compensatory changes in the D2-type receptor system, we have made no attempt to distinguish between D2, D3 and D4 receptors. The D3 and D4 receptors have been difficult to study due to their low abundance and lack of specific agonists. 22-TNJ mutant mice and the genetic knockouts of the DA D3 receptor and D4 receptor mice all show increased sensitivity to ethanol (McQuade et al., 2003; Rubinstein et al., 1997). This finding makes examining D2-type receptor subtypes in the 22-TNJ a potentially interesting avenue to explore.

These data in the 22-TNJ mouse strain provide an interesting complement to other published works of ethanol supersensitivity as a function of genotype. Genotypic differences in locomotor activity as a function of genotype are not unknown (Liljequist and Ossowska, 1994), and some of these differences have been well-characterized with regard to neurotransmitter system alterations. For example, the FAST and SLOW mouse lines have shown supersensitivity to the locomotor stimulating effects of ethanol, and these findings are thought to correlate with changes in their dopaminergic responses (Bergstrom et al., 2003). Additionally, the findings in the 22-TNJ mouse strain appear to bear a marked similarity to recent findings in the VMAT2 heterozygous mouse. VMAT2 heterozygotes show similar locomotor sensitivity to ethanol and psychostimulants, an effect directly related to deficiencies in the storage and release of monoamines (Wang et al., 1997). Finally, P and NP rats are also known to have locomotor changes in response to ethanol with correlate with changes in the dopaminergic system (Engleman et al., 2006; McKinzie et al., 2002). Thus it is not surprising that the locomotor changes associated with the 22-TNJ mouse should be correlated with deficiencies in the
dopaminergic system, especially with the specific changes in monoamine release know to underlie the behavior of such animal models as the VMAT2 heterozygous mouse.

In published works examining ethanol-related behaviors, many differences in DA system parameters have been reported; however, very few have been linked to the overall status of the DA system. When only one, or a few, aspects of the DA system are measured, it is difficult to make conclusions concerning the functional status of the overall system. For example, measured in isolation, a finding of increased numbers of D2 receptors could be interpreted as greater signaling through the DA system, whereas in the case of the 22-TNJ mice, it is more likely an adaptive response to chronically decreased DA. Using a wide variety of methods to measure different aspects of DA neurobiology, we have described here a classic example of a low functioning DA system, producing compensatory hyper-responsiveness to drugs. The results we have obtained could be considered hallmarks of a hypodopaminergic system: supersensitivity to stimulants, D2 receptor supersensitivity, and decreases in synthesis, which culminate in low basal levels of DA and increased uptake by the dopamine transporter. The findings from these studies clearly show hallmarks of a low-functioning DA system, and thus this mouse could serve as a model for future investigations into the consequences of chronically altered dopaminergic tone.

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