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METHYLPHENIDATE AND
ATOMOXETINE TREATMENT DURING
ADOLESCENCE IN THE SPONTANEOUSLY
HYPERTENSIVE RAT: MECHANISMS
UNDERLYING HIGH COCAINE ABUSE
LIABILITY IN ATTENTION DEFICIT/
HYPERACTIVITY DISORDER

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METHYLPHENIDATE AND ATOMOXETINE TREATMENT DURING ADOLESCENCE
IN THE SPONTANEOUSLY HYPERTENSIVE RAT:
MECHANISMS UNDERLYING HIGH COCAINE ABUSE LIABILITY
IN ATTENTION DEFICIT/HYPERACTIVITY DISORDER

DISSERTATION

A dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
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2013

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ABSTRACT OF DISSERTATION

METHYLPHENIDATE AND ATOMOXETINE TREATMENT DURING ADOLESCENCE IN THE SPONTANEOUSLY HYPERTENSIVE RAT: MECHANISMS UNDERLYING HIGH COCAINE ABUSE LIABILITY IN ATTENTION DEFICIT/HYPERACTIVITY DISORDER

Effects of pharmacotherapies for Attention Deficit/Hyperactivity Disorder (ADHD) on cocaine abuse liability in ADHD are not understood. Spontaneously Hypertensive Rats (SHR), an ADHD model, exhibited greater cocaine self-administration than control Wistar-Kyoto and Wistar rats. Methylphenidate, but not atomoxetine during adolescence enhanced cocaine self-administration in adult SHRs compared to controls. The mesocortical dopaminergic system, including medial prefrontal (mPFC) and orbitofrontal (OFC) cortices, is important for ADHD and cocaine addiction. Dopamine and norepinephrine transporter (DAT and NET) are molecular targets for methylphenidate, atomoxetine and cocaine action.

In the current studies, SHR, Wistar-Kyoto and Wistar were administered methylphenidate (1.5 mg/kg/day, p.o.), atomoxetine (0.3 mg/kg/day, i.p.) or vehicle during adolescence (postnatal day 28-55). During adulthood (>77 days), DAT and NET functions in mPFC and OFC were determined as neurochemical mechanisms and locomotor sensitization to cocaine, and impulsivity under differential reinforcement of low rates 30-second (DRL30) schedule were evaluated as behavioral mechanisms associated with greater cocaine self-administration in methylphenidate-treated SHRs.

Maximal velocity of [³H]dopamine uptake (V_{max}) by DAT and DAT cellular distribution in mPFC and OFC did not differ between vehicle-control, adult SHR, Wistar-Kyoto and Wistar. Methylphenidate increased DAT V_{max}, but not cell-surface expression, in SHR mPFC. In contrast, atomoxetine decreased V_{max} and cell-surface expression in SHR OFC. Compared to control strains, norepinephrine uptake by NET in the OFC was increased in vehicle-administered SHR; methylphenidate during adolescence normalized NET function in SHR OFC. Locomotor sensitization was greater in SHR compared to control, and was not altered by methylphenidate. Under DRL30,

methylphenidate increased burst responses in adult SHR compared to vehicle control as well as methylphenidate-treated Wistar-Kyoto and Wistar, indicating increased impulsivity.

Increased OFC NET function, increased impulsivity and cocaine sensitivity may be the neurobehavioral mechanisms associated with the increased cocaine self-administration in SHR. Increased mPFC DAT function may underlie the enhanced impulsivity and cocaine self-administration in SHR administered methylphenidate during adolescence. Decreased OFC DAT function from atomoxetine-treated SHR may explain the reduced cocaine self-administration relative to methylphenidate. Thus, methylphenidate during adolescence in ADHD may increase risk for cocaine abuse, while atomoxetine may represent a therapeutic alternative for at-risk adolescents with ADHD.

KEYWORDS: Attention Deficit/Hyperactivity Disorder, Methylphenidate, Atomoxetine, Dopamine Transporter, Impulsivity

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December 12, 2013

Date

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Dedicated to my late grandmother Mira De,
my mother Rita,
father Sujit,
sister Nivedita
and
life-partner Anupam

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LIST OF ABBREVIATIONS

ADHD	Attention deficit/hyperactivity disorder
SHR	Spontaneously Hypertensive Rats
MPH	methylphenidate
ATO	atomoxetine
WKY	Wistar-Kyoto
WIS	Wistar
PND	postnatal day
DAT	dopamine transporter
NET	norepinephrine transporter
SERT	serotonin transporter
NAC	nucleus accumbens
PFC	prefrontal cortex
mPFC	medial prefrontal cortex
OFC	orbitofrontal cortex
CG	cingulate gyrus
PrL	prelimbic cortex
IL	infralimbic cortex
LO	lateral orbitofrontal cortex
LC	locus coeruleus
DLPFC	dorsolateral prefrontal cortex
vmPFC	ventromedial cortex
DRL	differential reinforcement of low-rate
DRL5LH	differential reinforcement of low-rate 5 second with limited hold
DRL30	differential reinforcement of low-rate 30 sec
OROS	osmotic [controlled] release oral [delivery] system
NE	norepinephrine
DA	dopamine

5-HT	serotonin
GABA	gamma-amino butyric acid
cAMP	cyclic adenosine monophosphate
HCN	hyperpolarization-activated cyclic nucleotide-gated
BOLD	blood oxygenation level dependent
MRI	magnetic resonance imaging
CDER	Center for Drug Evaluation and Research
FDA	Food and Drug Administration
ARCI	Addiction Research Center Inventory
CPP	conditioned place preference
VMAT2	vesicular monoamine transporter type 2
MAO	monoamine oxidase
L-DOPA	L-dihydroxyphenyl alanine
DOPAC	dihydroxyphenyl acetic acid
COMT	catechol-O-methyltransferase
SNAP25	synaptosomal-associated protein 25
TH	tyrosine hydroxylase
D β H	dopamine- β -hydroxylase
GPCR	G-protein coupled receptor
FI-30/EXT	fixed interval 30 sec/extinction
NHE	Naples High Excitability
5-CSRT	5-choice serial reaction time
NCS-R	National Comorbidity Survey Replication
DSM	Diagnostic and Statistical Manual of Mental Disorders
BIS	Barratt Impulsivity Scale
UPPS	urgency, premeditation, perseveration and sensation seeking
WHO	World Health Organization
WMH	World Mental Health
GWAS	genome-wide association studies

SNP	single nucleotide polymorphisms
DTI	diffusion tensor imaging
fMRI	functional magnetic resonance imaging
PET	positron emitted tomography
SPECT	single-photon emission computed tomography

1 CHAPTER ONE: INTRODUCTION

1.1 Attention deficit hyperactivity disorder (ADHD)

Attention deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by inattention and/or hyperactivity-impulsivity that is persistent and is more frequent and more severe than age-matched controls. ADHD was recognized about a century ago, as “hyperactivity” or “hyperkinetic disorder of childhood” that resulted from a biological condition and not from poor parenting (Still, 1902). This disorder was first represented in the Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DSM-III) as Attention Deficit Disorder with or without Hyperactivity (American Psychiatric Association., 1980).

ADHD is a highly debilitating disorder that directly exerts negative impact on the individuals’ social, academic and occupational activities, which if left untreated, may lead to several undesirable outcomes (Meijer *et al*, 2009). A recent study evaluated clinical and functional outcomes of ADHD in adult males three decades after childhood diagnosis (Klein *et al*, 2012). The later study reported that compared to their non-ADHD counterparts, ADHD probands had significantly fewer years of schooling, greater divorce rates, incarcerations, hospitalization and death rates, as well as more frequent diagnosis of psychiatric disorders such as antisocial personality disorders and non-alcohol-related substance disorders. The high rates of criminal conviction, diagnoses of antisocial personality disorders and substance use disorders in adults diagnosed with ADHD during childhood, have been replicated by other independent clinical studies (Barkley *et al*, 2004; Dalsgaard *et al*, 2013; Mannuzza *et al*, 1998; Rasmussen and Gillberg, 2000). Further, results from a WHO World Mental Health (WMH) Survey Initiative indicated that ADHD was associated with a statistically significant 22.1 annual days of lost role performance compared to their non-ADHD counterparts (de Graaf *et al*, 2008). Consequently, ADHD is a major economic burden. The cost of ongoing care for

adolescents with ADHD in the United Kingdom including health, social and educational services is estimated to be 670 million pounds annually (Telford *et al*, 2013). In the United States (US), the estimated societal economic burden associated with ADHD in 2005 was \$36 billion and \$52.4 billion (Pelham *et al*, 2007). Taken together, ADHD has an economic burden on US, as well as the world.

1.1.1 Diagnosis and subtypes

ADHD is diagnosed based on the presence of the hallmark symptoms, including inattention, impulsivity and hyperactivity. The standard for ADHD diagnosis particularly in the US, is detailed by the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition, Text Revision (DSM IV-TR; American Psychiatric Association., 2000). The Fifth Edition of DSM was released recently, and is expected to replace DSM IV-TR (American Psychiatric Association., 2013c). Both the manuals list a series of nine symptoms of inattention and hyperactivity-impulsivity each. Example of inattention includes 'difficulty sustaining attention in tasks or play activities', not listening 'when spoken to directly' and 'difficulty organizing tasks and activities'. Examples of hyperactive-impulsivity includes fidgeting, 'difficulty playing or engaging in leisure activities quietly', 'difficulty awaiting for his/her turn' and frequently 'interrupting or intruding on others'

For an ADHD diagnosis, at least six of the nine symptoms in a category must be presented at a degree that is maladaptive and inconsistent with developmental level, and is persistent for at least 6 months. Furthermore, impairments have to be present in two or more settings (e.g., at work/school and at home), and should clearly impede social, academic, and occupational functioning. Additionally, the impairments should not be better accounted for by another mental disorder (e.g., Pervasive Developmental Disorder, Mood Disorder or Dissociative Disorder). Based on these criteria, ADHD is classified into Predominantly Inattentive Type and Predominantly Hyperactive-Impulsive

Type, depending on whether the criteria for either inattention or hyperactive/impulsive are presented. ADHD, Combined Type is diagnosed if criteria for both inattention and hyperactive/impulsive are presented.

There are two other diagnostic tools for ADHD; Classification of Child and Adolescent Mental Diagnosis in Primary Care: Diagnosis and Statistical Manual for Primary Care (DSM-PC), and the International Classification of Diseases, 10th revision (ICD-10). Unlike the DSM IV-TR, ICD-10 uses the term attention deficit/hyperkinetic disorder and is often used by insurance companies. The DSM-PC is designed for primary care for children and adolescents and details differential diagnoses for commonly coexisting comorbidities (Sabeti *et al*, 2003). However, DSM IV-TR, DSM-PC and ICD-10 all diagnose ADHD based on presentation of certain clinically observable symptoms, and therefore are subjective tools. Currently, there are not objective diagnostic tools for ADHD and this is an area of active research (Sabeti *et al*, 2002).

1.1.2 Prevalence and life-time persistence

Meta-analysis uses a statistical tool called meta-regression to examine the effect of moderator variables, such as sample size, on effect size in a study and combine a set of studies from different sources thereby providing results that more closely resemble the population compared to that obtained from a single study (Paxinos and Watson, 1998). A recent meta-regression analysis of population surveys from North America, South America, Europe, Africa, Asia, Oceania, and the Middle East revealed that the prevalence of ADHD is about 5% in children in most cultures (Polanczyk *et al*, 2007). Also, ADHD was found to be more frequent in males than in females (~ 2:1 ratio in children), with females being diagnosed with the Predominantly Inattentive Type more frequently (Polanczyk *et al*. 2007). Another meta-analytical review reported comparable prevalence rates as the latter study, and indicated that the Predominantly Inattentive

Type is the most prevalent ADHD subtype, but individuals with combined subtype are the most likely to be referred for clinical services (Willcutt, 2012).

In the US, the prevalence rates for ADHD are higher than that seen world-wide. A National Health and Nutrition Examination Survey (Chai *et al*, 2012) reported that the prevalence of ADHD in the US is about 8.7%, with an estimated 2.4 million individuals who meet the DSM-IV criteria (Froehlich *et al*, 2007). Further, the gender ratio of ADHD is the same as that seen world-wide (i.e., 2:1); however boys had an increased likelihood of meeting DSM-IV criteria for all ADHD subtypes (Froehlich *et al*, 2007). Data from the National Ambulatory Medical Care Survey (Yetnikoff and Arvanitogiannis, 2013) for the years 1990-95, found that the a diagnosis of ADHD for children aged 5-18 years increased from 947,208 in 1990, to 2,357,833 in 1995 (i.e., by 232%) calculated using the population-adjusted rate of office-based visits (Robison *et al*, 1999). These increasing trends during early '90s appeared to be related, partially, to the increase in ADHD diagnosis in girls and, partially, to the increasing age of the ADHD patient. Since then, the prevalence rate for ADHD in the US has stabilized, which may be attributable in part to the uniformity in diagnostic and methodological practices subsequent to the release of the DSM-IV in 1996.

Typically, ADHD is identified during childhood (i.e., during elementary school years), which is probably the reason for including the clause that some ADHD symptoms and impairment should be “present before age 7 years” (Criteria B, DSM-IV TR; (American Psychiatric Association., 2000). The early age of diagnosis may be due to high prevalence rates among children of the readily identifiable Predominantly Hyperactive-Impulsive type of ADHD (Froehlich *et al*, 2007). The disorder is relatively stable through early adolescence. Generally, symptoms of hyperactivity become less obvious with increasing age, but problems with restlessness, inattention, poor planning, and impulsivity may persist into adolescence and adulthood (Faraone *et al*, 2006; Turgay *et al*, 2012). Based on the National Comorbidity Survey Replication, the prevalence of adult ADHD in the US was estimated to be about 4.4% with a male:female ratio of ~1.6:1

(Kessler *et al*, 2006). A meta-analytical review found that the pooled prevalence of ADHD in adults worldwide was approximately 2.5% (Simon *et al*, 2009). Controversy exists as to whether the adult ADHD rates are underestimated currently, which is partially, due to the variation in results based on the reporting source (Barkley *et al*, 2002). Specifically, compared to parent-reports, the persistence of ADHD into adulthood is substantially underestimated in studies based on self-reports. Another reason for the supposed underestimation of adult ADHD is the controversy regarding the insensitivity of DSM-IV diagnostic criteria to developmental-changes in ADHD (Simon *et al*, 2009). Furthermore, comorbid conditions such as conduct disorder, antisocial behavior, oppositional defiant disorders (see Table 1) that are expressed during adolescence and adulthood further complicate ADHD diagnosis during adulthood (Biederman *et al*, 1993). All these factors may contribute towards discontinuation of ADHD medications during adolescence and adulthood, and may subsequently exert a negative impact on an individual's social and occupational activities.

1.1.3 Disease mechanisms

1.1.3.1 Genetic mechanisms

ADHD is a heritable disorder; family and twin studies indicate that genetics explains about 76% of the phenotypic variance in ADHD (Biederman and Faraone, 2005a). However, no biological markers have been identified for the ADHD diagnosis; probably because of heterogeneity of phenotypes that are encompassed by this disorder.

Candidate gene studies of ADHD, and meta-analyses thereof, have identified that several genetic factors contribute to the disease etiology, each having a small-to-moderate effect size (Faraone *et al*, 2005). Specifically, functional polymorphisms in genes coding for proteins regulating dopaminergic function, including dopamine receptors D4 (*DRD4*) and D5 (*DRD5*), catechol-O-methyltransferase (*COMT*) and the

dopamine transporter (*DAT1*; or *SLC6A3*), have been implicated in ADHD etiology (Table 1.1). Other candidate genes implicated in ADHD include those coding for synaptosomal-associated proteins of 25 KDa (*SNAP-25*), the noradrenergic receptor $\alpha 2A$ (*ADRA2A*) and monoamine oxidase-B (*MAO-B*) (Faraone *et al*, 2005; Gizer *et al*, 2009). Taken together, these studies indicate that a dysfunctional catecholaminergic system underlies the etiology of ADHD.

Results from candidate gene-based association studies have shown limited overlap with the results from genome-wide association studies (GWAS; Franke *et al*, 2009). Further, a recent meta-analytical review of GWAS studies focusing on single nucleotide polymorphisms (SNPs) within the exonic or intronic regions of genes with an association at $p < 0.0001$ found that about 53% of the top 85 gene candidates for ADHD were involved in neurite outgrowth (Poelmans *et al*, 2011), suggesting that reduced efficiency of neurite outgrowth may underlie ADHD etiology. Furthermore, from the latter pool of 85 candidate genes, only the gene for nitric oxide synthase could be directly placed in the putative ADHD network (Cappellacci *et al*, 2006; Tanda *et al*, 2009). One potential association between the results from candidate gene-based association studies and GWAS, may be that the a dysfunctional catecholaminergic system underlies the reduced efficiency of neurite outgrowth (Poelmans *et al*, 2011); however this putative mechanism has not been validated empirically.

Recent efforts from the Psychiatric Genomics Consortium involved pooling data from several large scale genetics projects for meta-analysis to boost statistical power to identify genetic-phenotypic associations at a genome-wide significance level. Surprisingly, the results revealed no genome-wide significant associations (Neale *et al*, 2010a; Neale *et al*, 2010b). More importantly, of the several specific genes correlated with ADHD, none were either necessary or sufficient to be causally linked with ADHD (Gizer *et al*, 2009). Interestingly, patients with Prader-Willy Syndrome, Turner Syndrome and Fragile-X-Syndrome, despite their divergent neurogenetic etiologies, exhibit ADHD-like symptoms (Lo-Castro *et al*, 2011). Taken together, the genetic abnormalities

associated with the latter disorders may converge on common downstream signaling pathways or neurological circuitry, and thereby express ADHD-like symptoms. A recent meta-analysis examining shared genetic etiology between different psychiatric disorders reported a genetic correlation between major depressive disorder and ADHD (Cross-Disorder Group of the Psychiatric Genomics *et al*, 2013). Specifically, based on weighted prevalence of common SNPs, significant genetic correlation was obtained between ADHD and major depressive disorder. However significant genetic overlap was not found when ADHD was compared to autism spectrum disorder, schizophrenia and bipolar disorder (Cross-Disorder Group of the Psychiatric Genomics *et al*, 2013). Taken together, the most parsimonious explanation for the genetic basis of ADHD etiology is that it involves polygenic interactions with complex transmission patterns to yield genotypic and phenotypic heterogeneity in ADHD (Curatolo *et al*, 2010; Sharp *et al*, 2009).

Table 1.1 Genetics of ADHD

Genetic polymorphisms associated with ADHD etiology and pharmacogenomics; transporters, receptors and other proteins that regulate dopamine (DA) and norepinephrine (NE) neurotransmission. Odds-ratio (OR) was used as the statistical measure for the association of the polymorphism with ADHD.

			Functional effect in ADHD etiology	Association with disease etiology	Pharmacogenetics
Dopaminergic system	Dopamine transporter (DAT, SLC63A)	10-repeat allele at 3'UTR	Increased striatal DAT density (Michelhaugh <i>et al</i> , 2001; VanNess <i>et al</i> , 2005); see also (Martinez <i>et al</i> , 2001)	OR– 1.13 * ^a	10/10 genotypes were less likely to respond to methylphenidate (MPH; Roman <i>et al</i> , 2002; Winsberg and Comings, 1999)
		9 repeat allele		N.A.	9/9 poor responders for MPH (Jooper <i>et al</i> , 2007); 9/10 genotype showed fewer side-effects compared to 9/9 and 10/10 genotypes (Gruber <i>et al</i> , 2009)
	DA receptors	D4 7-repeat allele in exon III	Blunted response to dopamine (Asghari <i>et al</i> , 1995)	OR – 1.45 * ^a Associated with childhood inattention (Rowe <i>et al</i> , 2001)	Excess transmission of the 7-repeat allele compared to the 2- and 4-repeat individuals in MPH responders (Seeger <i>et al</i> , 2001; Tahir <i>et al</i> , 2000)

Noradrenergic system	Receptor α 2A	Promoter region SNP C1291G		ADHD and conduct disorder (Comings <i>et al</i> , 1999); inattention (Roman <i>et al</i> , 2003)	G/G have improved MPH response at reducing inattentive scores (Cheon <i>et al</i> , 2009; da Silva <i>et al</i> , 2008)
	NET, SLC6A2	SNP in exon 9, intron 9 and intron 13	-	Not associated with ADHD (Barr <i>et al</i> , 2002; McEvoy <i>et al</i> , 2002)	No associations met the genome-wide threshold for statistical significance (Kieling <i>et al</i> , 2010)
Catecholaminergic system	Dopamine- β -hydroxylase	TaqI A1 polymorphism in 5'-intron		OR – 1.96* (Smith <i>et al</i> , 2003)	-
	COMT	Val108Met polymorphism	Met-enzyme has a 50-70% reduced enzymatic activity than the Val-enzyme (Lotta <i>et al</i> , 1995)	OR – 1.0 ^a	Pharmacogenetic effects were not strong (Kieling <i>et al</i> , 2010; except Cheon <i>et al</i> , 2008)
	SNAP25	Three markers- In the putative promoter – an SNP -2015 A/T; A microsatellite in intron 1; SNP in intron 7 - 80609 G/A	Reduce calcium dependent dopamine release (Wilson, 2000)	OR – 1.19 ^{*a}	-

* OR was statistically significant for association with ADHD diagnosis; N.A. OR data was not available; ^a as reported in (Faraone *et al*, 2005)

1.1.3.2 Environmental and gene-environment interaction mechanisms

Several groups have proposed that environmental factors as well as complex gene-environment interaction underlies ADHD etiology. Prenatal factors such as maternal life-style during pregnancy have been associated with ADHD. Prenatal exposure to alcohol and smoking have been associated with ADHD symptoms including hyperactivity and impulsivity (D'Onofrio *et al*, 2007; Linnet *et al*, 2003; Milberger *et al*, 1996; Sen and Swaminathan, 2007). Furthermore, prenatal substance exposures are associated with increased risk for autism spectrum disorder which is often comorbid with ADHD (See Table 1.2). These effects of prenatal exposure to abused substances, in part, may be due to the changes in the developmental trajectory of the central catecholaminergic system (Zhu *et al*, 2012), which is developmentally sensitive during gestation (Lee *et al*, 2012).

Recent studies evaluating the combined effects of prenatal substance exposures and DNA polymorphisms in dopamine pathway-related genes, reported that the genetically susceptible children (i.e., with the 7-repeat DRD4 alleles, 9-repeat DAT1 allele, but not the 10-repeat DAT1 allele) had a higher risk for developing ADHD, combined type when prenatally exposed to nicotine (Neuman *et al*, 2007). Another study found that the 10-repeat DAT1 allele was associated with greater hyperactivity-impulsivity (Becker *et al*, 2008). Early deprivation of social environment during the postnatal period also may have significant effects on hyperactivity (McLaughlin *et al*, 2010; Yates *et al*, 2012). Taken together, these studies support that complex gene-environment interactions contributes to the etiological heterogeneity in ADHD.

Table 1.2 Examples of overlapping diagnosis with ADHD and comorbidities with ADHD

Disorder	Similar Features	Differentiating features	References for clinical comorbidity
Tourette's disorder	Developmental disorder, motoric activity	Expressed in bouts of tics, and not general "fidgetiness"	(Melchior <i>et al</i> , 2013)
Oppositional defiant disorder	Aversion to school or work	Resist conforming with other's demands, no sustained attention deficit	(Biederman <i>et al</i> , 1993)
Conduct disorder	Appearance of impulsivity, impaired academic/occupational performance	Antisocial behavior	(Biederman <i>et al</i> , 1993; Hamshere <i>et al</i> , 2013; Willcutt, 2012)
Autism spectrum disorder	Impaired social interaction	Due to indifference to facial and tonal communication cues, instead of peer rejection in ADHD	(Cross-Disorder Group of the Psychiatric Genomics <i>et al</i> , 2013)
	Tantrums	Due to change from their expected course of events, and not impulsivity	
Disruptive mood dysregulation disorder	Impulsivity and disorganized attention	Pervasive irritability and intolerance of frustration	(Biederman <i>et al</i> , 1993)
Personality disorders	Adult ADHD symptoms; social intrusiveness, cognitive dysregulation	ADHD do not necessarily exhibit fear of abandonment, self-injury, extreme ambivalence	(Barkley <i>et al</i> , 2004; Mannuzza <i>et al</i> , 1998)

1.1.3.3 Neurological mechanisms

The neurobiological etiology of ADHD is not understood completely (Tripp and Wickens, 2009). However, converging evidence from structural and functional neuroimaging research indicate a pivotal role for a hypofunctional corticostriatal system in mediating the deficits in higher-order functions, as well as the behavioral and emotional regulation deficits commonly observed in ADHD.

Structural neuroimaging:

ADHD patients have a reduced cerebral volume in the prefrontal cortex (PFC), dorsal anterior cingulate cortex, as well as in subcortical areas including striatum (Aston-Jones and Cohen, 2005; Crunelle *et al*, 2013). The right frontal lobes including PFC (Castellanos *et al*, 1996; Hynd *et al*, 1990) and the caudate nucleus (subregion of dorsal striatum) of children with ADHD were smaller in volume than controls, suggesting a neurodevelopmental lag in the maturation of the associated neuronal pathways and their connectivity (Castellanos *et al*, 1996). Further, the age of attaining peak cortical thickness in the cerebrum was delayed children with ADHD compared to controls (Chandler *et al*, 2013b; Matecka *et al*, 1997b).

A popular hypothesis regarding ADHD etiology is a disrupted connectivity between neuronal networks. Diffusion tensor imaging (DTI) is a structural magnetic resonance imaging (MRI) technique to map white matter tracts for assessing structural abnormalities in neuronal networks (for review; Doughty and Richards, 2002b). DTI studies revealed that compared to controls, individuals with ADHD had reduced white matter tracts in the intra-cortical areas associated with higher-order executive function (Corominas-Roso *et al*, 2013). Further, the frontolimbic white matter tracts, involved with emotional regulation, were reduced in children with ADHD compared to control (Sanchez-Mora *et al*, 2013). Taken together, intra-cortical and frontolimbic networks have structural impairments in children with ADHD that may contribute to reduced speed of neuronal communication compared to age-matched controls.

Functional neuroimaging:

Evidence for functional impairments in the prefrontal cortex (PFC) of individuals with ADHD is provided by functional neuroimaging studies. Functional imaging studies include evaluation of brain activation using changes in blood oxygen level dependent (BOLD) signals monitored in real-time with functional magnetic resonance imaging (fMRI). In the absence of medications, functional deficits in orbitofrontal cortex (OFC), dorsolateral PFC (DLPFC), and medial PFC (mPFC) have been linked to the behavioral deficits observed in individuals with ADHD. For example, compared to control subjects, boys (9-16 years old) with ADHD showed decreased BOLD signal in the OFC during a delayed discounting task that measures impulsivity (Rubia *et al*, 2009a). In male and female adults with ADHD, dysregulated OFC activation was reported during a risky decision making task, suggesting that impairments in OFC function are associated with motivational and emotional challenges faced by ADHD adults (Wilbertz *et al*, 2012). Decreased DLPFC activation was associated with working memory deficits on a Color-Word Stroop test in adults with ADHD (Burgess *et al*, 2010). In the absence of medications, adolescents with ADHD exhibited enhanced attenuation of BOLD signals in the mPFC during the Stroop test with emotional interference, compared to demographically matched control (Posner *et al*, 2011). Taken together, OFC, DLPFC and mPFC functions are associated with the deficits in behavioral and emotional regulation observed in adolescents and adults with ADHD.

Table 1.3 Subregions of the primate frontal cortex and their functions

Subregion	Functionality	Reference
Dorsolateral prefrontal cortex (DLPFC)	Higher order cognition, short and long term memory, impulse control and problem solving	(Petrides, 1994; Vogeley <i>et al</i> , 2004)
Orbitofrontal cortex (OFC)	Processing the value of natural reinforcer, higher order reward and reinforcement, assessment of short- and long-term gain/loss, inhibition, detection of irregularities	(Bechara, 2001) (Aron <i>et al</i> , 2004; Huettel and McCarthy, 2004)
Ventromedial cortex (vmPFC); (includes cingulate gyrus)	Processing decision outcomes, cognitive control, inhibition, suppression of inappropriate behavior	(Elliott and Dolan, 1998; Forstmann <i>et al</i> , 2008; Vogt <i>et al</i> , 1992)

Although the bulk of functional imaging studies use fMRI, the results from these studies do not provide information about the specific neurochemical systems involved in the disease etiology. In this respect, positron emitted tomography (PET) and single-photon emission computed tomography (SPECT) are superior at revealing neurochemical substrates involved in disease etiology (da Silva *et al*, 2011). A significant negative correlation between DAT density (evaluated using SPECT) in striatum and cerebral blood flow in the cingulate gyrus, frontal lobe, temporal lobe of the cortex was found in a group of medication-naïve adolescents with ADHD (da Silva *et al*, 2011). Another PET study revealed that in medication-naïve ADHD adults, striatal DAT and dopamine D2/D3 receptor densities were correlated with motivational deficits evaluated using a personality questionnaire (Goldstein *et al*, 2001); the results were interpreted to suggest that the core symptoms of ADHD such as inattention are related to the broader concept of motivational process. The mesocorticolimbic pathway is involved in reinforcement learning and motivated (also called goal-directed) behavior. The mesocorticolimbic pathway consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc or ventral striatum) and to the PFC (Fig 1.1). Maturation of corticostriatal connectivity is dependent on mesocortical dopaminergic signals (Galinas *et al*, 2009). The nigrostriatal pathway, which projects from substantia nigra to dorsal striatum (Fig 1.1), has been implicated in motor control, and deficits in this pathway are considered to underlie hyperactivity in ADHD (Engert and Pruessner, 2008). The dorsal striatum receives inputs from PFC, which are associated with attentional control (Robbins *et al*, 1998), and hence, may contribute to inattention, a hallmark of ADHD. Taken together, these results of these studies suggest a critical role for deficits in dopaminergic function in the mesocorticolimbic and nigrostriatal pathways in the etiopathology of ADHD.

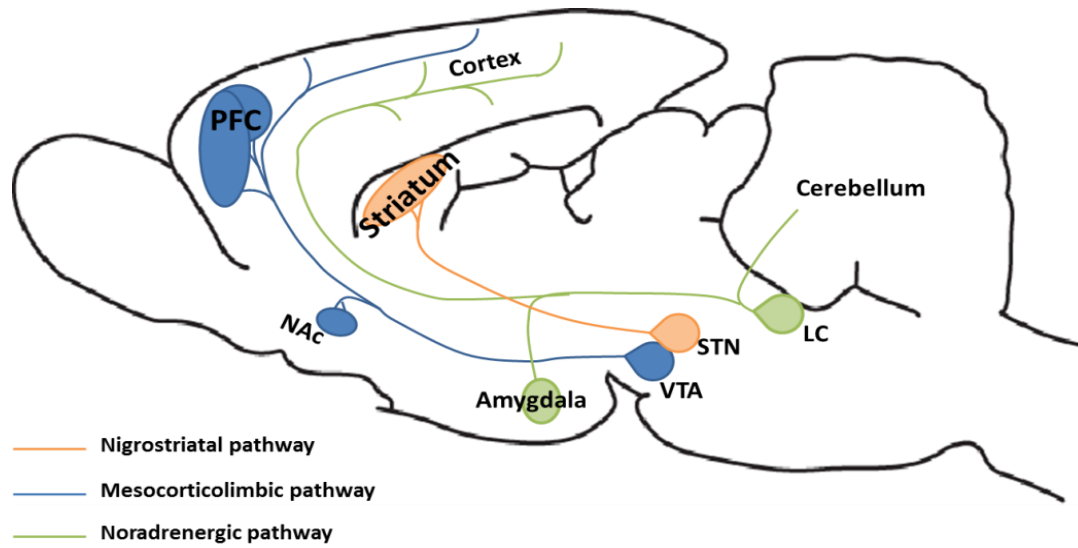


Figure 1.1 Graphical representation of the mesocorticolimbic and nigrostriatal dopaminergic circuitry and of the noradrenergic projections in rat brain

PFC – PFC, NAc – nucleus accumbens, VTA – ventral tegmental area, STN – substantia nigra, LC – locus coeruleus.

In terms of directionality, the elevated striatal DAT expression in ADHD (Gill *et al*, 1997), suggested increased clearance of extracellular dopamine, and thereby lead to the hypodopaminergic hypothesis of ADHD. Further support for the hypodopaminergic hypothesis was provided by genetic polymorphisms in D4, COMT and DAT that potentially lead to reduced dopaminergic signaling have been associated with ADHD (Faraone, Perlis *et al*. 2005). However, a recently published meta-analysis of DAT expression concluded that un-medicated ADHD adults (~30 years old) have lower striatal DAT expression, while previously medicated ADHD adults have higher striatal DAT density compared to non-ADHD individuals (Fusar-Poli *et al*, 2012). These result suggest that the increase in DAT expression is an outcome of treatment and not etiopathology. Thus, ADHD may not be attributable entirely to decreased dopaminergic function.

A seminal review of neuroimaging studies, both clinical and preclinical studies dispelled the hypothesis “suggesting ‘too much’ or ‘too little’ of a single neurotransmitter” underlies ADHD etiology (Pliszka *et al*, 1996). A multistage hypothesis was proposed that suggests a complex interaction of dopamine and norepinephrine and other neurotransmitter systems in the modulation of attention and impulse control, which is disrupted in ADHD. Thus, the most popular hypothesis regarding ADHD etiopathology involves deficits in both dopamine and norepinephrine neurotransmission (Arnsten, 2009; Fernandez-Castillo *et al*, 2013; Levy, 2009).

The primary central noradrenergic pathways project from locus coeruleus (LC) to several regions of brain, including amygdala, ventral striatum and PFC (see Fig 1.1). These projections are involved in arousal and cognitive function (Green *et al*, 2003b; Kalivas and Barnes, 1988). Attentional set-shifting tasks evaluate behavioural flexibility and attention, two functions that are impaired in ADHD (Arnsten, 2009; Viggiano *et al*, 2004a). Reduced noradrenergic function in mPFC is sufficient to produce impaired attentional set-shifting (Newman *et al*, 2008). Further evidence supporting the catecholaminergic hypothesis comes from neuropharmacological studies (described in

section 1.2) and that a large majority of medications efficacious in managing ADHD engage both dopaminergic and noradrenergic systems.

Other neurochemical mechanisms, including cholinergic and glutamatergic pathways, also contribute to ADHD etiopathology (Doughty *et al*, 2002b). For example, ¹H-magnetic resonance spectroscopy revealed reduced glutamate levels in the prefrontal cortex of previously medicated adults with ADHD compared to non-ADHD healthy controls (DeBow and Colbourne, 2003). Furthermore, MPH treatment in ADHD adult was reported to decrease cortical concentration of choline containing compounds (Hebert *et al*, 1999b). Furthermore, cortical dopaminergic function is known to modulate glutamate and cholinergic neurotransmission in the PFC and striatum (Doughty *et al*, 2002b; Hebert and Gerhardt, 1999a). The latter studies contribute to a growing body of literature that suggest that ADHD etiology involves a complex interaction of several neurotransmitter systems.

1.2 ADHD medications

1.2.1 Classifications

Based on overall pharmacology, ADHD medications may be broadly classified into 'stimulants' and 'non-stimulants'. A brief mechanism of action for the currently approved ADHD medications is provided in Table 1.4. Stimulants or psychostimulants are drugs that induce locomotor hyperactivity and promote dopamine release in ventral and/or dorsal striatum (Berridge and Devilbiss, 2011; Wilens, 2006). Examples of stimulant medications for ADHD are amphetamine and its isomers and MPH and its isomers. On the other hand, non-stimulant drugs do not induce striatal dopamine release and do not induce locomotor activation (Swanson *et al*, 2006). Examples of non-stimulant medications are atomoxetine (ATO), guanfacine and clonidine. Further details regarding the mechanism of action and pharmacology of MPH and ATO is provided in section 1.8 and 1.9.

Since the serendipitous discovery of benzedrine for the treatment of “children with learning and behavioral problems” in the 1930s (Bradley, 1950), the psychostimulants, such as amphetamine and MPH have been the drugs of choice for symptomatic treatment of ADHD. Stimulants have been found to be effective at managing ADHD symptoms in 70-80% of ADHD patients (Elia *et al*, 1999). Stimulants are the most widely prescribed ADHD medications; ~3 million prescriptions per quarter are filed for extended release oral MPH preparations alone (Sembower *et al*, 2013). However, doubts have been raised about whether stimulants are safe especially for chronic administration to children and adolescents (Greenhill *et al*, 2001). Specifically, stimulants have established abuse potential that raises concern regarding prescribing stimulants to adolescents with ADHD (Manchikanti, 2007; Sweeney *et al*, 2013). As a consequence, several formulations have been developed with modified pharmacokinetic properties that minimize the abuse liability of these drugs (Spencer *et al*, 2011; Szobot *et al*, 2008b); see Table 1.5 for details).

Over the past six decades, efforts made by both the pharmaceutical industry and academia have led to the development and approval of two non-stimulant ADHD medications, ATO and guanfacine (for review; Heal *et al*, 2012). In particular, for ADHD cases where stimulants are not efficacious or cause unpleasant side effects, non-stimulants have been beneficial. ATO was the first non-stimulant medication approved by the Food and Drug Administration (FDA), for use in children, adolescents, and adults (<http://www.fda.gov/Drugs/DrugSafety>). FDA approved a second non-stimulant drug guanfacine in 2011, for children and teens between ages 6 and 17. Also, the non-stimulant clonidine was approved by FDA in 2009 for use either alone or in combination with a stimulant to enhance its effectiveness. These medications have all shown efficacy in improving attention and impulse control; however the efficacy of these non-stimulants were lower than that for stimulants. Specifically, compared to placebo, efficacy is ~50-60% for ATO in contrast to >70% for stimulants (Garnock-Jones and Keating, 2010; Scahill *et al*, 2001).

Bupropion and modafinil are used off-label and often prescribed for managing ADHD symptoms. Bupropion is a non-stimulant drug, and is a norepinephrine and serotonin uptake inhibitor. Bupropion has been shown to be efficacious compared to placebo in ameliorating ADHD symptoms (efficacy ~20% compared to placebo; Maneeton *et al*, 2011), and has low potential for abuse (Rush *et al*, 1998). On the other hand, modafinil has shown potential as an alternative psychostimulant to amphetamine and MPH for treatment of ADHD (efficacy ~50-60% compared to placebo; Biederman *et al*, 2005b; Heal *et al*, 2012). Unlike amphetamine and MPH, modafinil is not associated with side effects such as abuse potential and locomotor excitability (Deroche-Gamonet *et al*, 2002); however the biochemical mechanisms of action for modafinil is not well understood (Morgan *et al*, 2007).

Cognitive control is defined as the ability to suppress inappropriate behavior in response to contextual and temporal cues and adjust behavior accordingly. Because ADHD includes cognitive deficits that are debilitating, especially under academic settings (Berridge *et al*, 2011; Wallace *et al*, 2011), a number of novel ADHD therapeutics are targeted towards remediating the cognitive dysfunction in ADHD. Moreover, according to one school of thought, impaired cognitive control may be the fundamental disturbance underlying the hallmark features of ADHD (Nigg and Casey, 2005). A review of 40 placebo-controlled clinical studies revealed that MPH improved cognitive flexibility and attention/vigilance in majority of the studies (Pietrzak *et al*, 2006). Interestingly cholinergic drugs, including varenicline and lobeline, that improve attention and working memory by activating the nicotinic acetylcholine receptors ($\alpha 4\beta 2$ and $\alpha 7$ subtypes) have been evaluated for therapeutic efficacy in ADHD (Howe *et al*, 2010; Rollema *et al*, 2009; Wallace *et al*, 2011). Histamine is known also to play a role in cognition, attention and alertness (Leurs *et al*, 2005). MPH (1 mg/kg subcutaneous) and ATO (1 mg/kg subcutaneous) increase extracellular concentrations of histamine in the PFC of awake rats (Horner *et al*, 2007). Thus, an increase in cortical histamine may contribute to the pharmacotherapeutic effects of MPH and ATO that leads to improved cognition and alertness in ADHD. Histamine H_3 receptors are presynaptic autoreceptors

that inhibit histamine release from histaminergic neurons in rat brain (Arrang *et al*, 1983). H₃ antagonist, ciproxifan, improved short-term memory and attention and decreased impulsivity in outbred rats (Day *et al*, 2007; Giovannini *et al*, 1999). Not surprisingly, novel therapeutic strategies for ADHD include antagonists for histamine H₃ receptor that are currently in various phases of clinical trials (Wallace *et al*, 2011).

Table 1.4 Mechanism of action of ADHD drugs

Drug	Pharmacological class	Neurotransmitter target	Site of action	Selectivity	Psychostimulant
D-Amphetamine	Releasing agent	DA, NE, 5HT	Monoamine release	DA >= NE	Yes
			Monoamine uptake inhibition		
			MAO inhibition		
L-Amphetamine	Releasing agent	DA, NE, 5HT	Monoamine release	DA = NE	Yes
			Monoamine uptake inhibition		
			MAO inhibition		
DL-MPH and D-MPH (Ritalin)	Uptake inhibition	DA, NE	Monoamine uptake inhibition	DA = NE	Yes
Bupropion (Wellbutrin, Zyban)	Uptake inhibition	DA (NE)	Monoamine uptake inhibition	DA = NE	No
Atomoxetine (Strattera)	Uptake inhibition	NE (DA in the PFC)	Monoamine uptake inhibition	(DA) = NE	No
Guanfacine (Intuniv)	α 2A-agonist	NE	Adrenoreceptor agonist	NE	No
Modafinil (Provigil)	Undefined	NE, DA, (histamine)			Equivocal

adapted from Heal *et al*, 2012

(DA) – Atomoxetine induced increase in extracellular DA in the PFC is an outcome of NET inhibition.

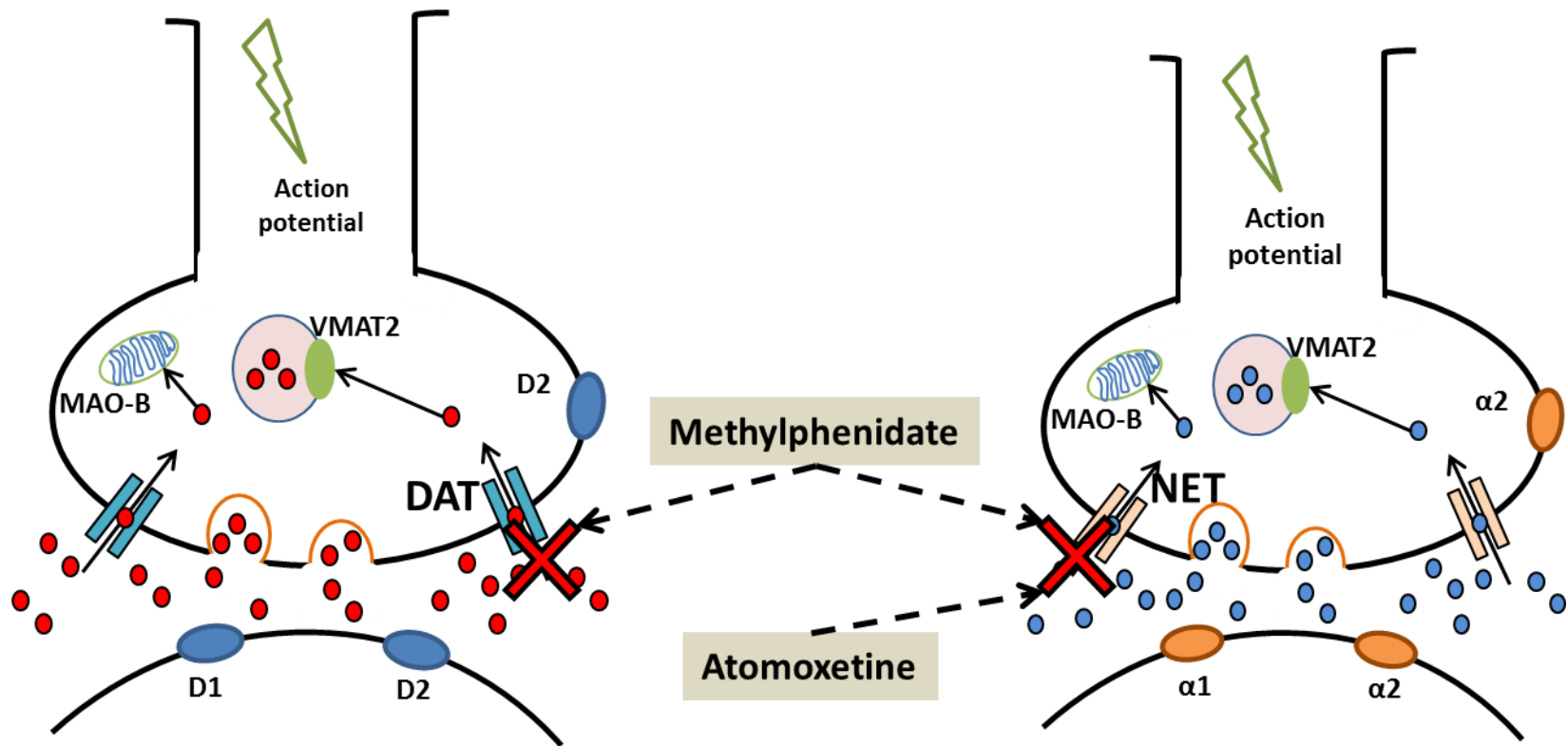


Figure 1.2 Molecular targets of methylphenidate and atomoxetine.

Methylphenidate inhibits dopamine transporters (DAT) and norepinephrine transporter (NET), while atomoxetine is a selective NET inhibitor. VMAT2 – vesicular monoamine transporter type 2, MAO-B – monoamine oxidase B, $\alpha1$ and $\alpha2$ – norepinephrine receptor type 1 and 2, respectively, D1 and D2 – dopamine receptor type 1 and 2, respectively.

Table 1.5 Approved ADHD medications and formulations

Formulations of the currently approved ADHD medications and the efficacies, adapted from (Curatolo *et al*, 2010)

Pharmacotherapy	Formulation	Market name	Efficacy
<i>Stimulant medication</i>			
Methylphenidate	Immediate release	Ritalin, Focalin	0.92 (0.8, 1.05) ^a
	Osmotic release	Concerta	0.90 (0.76, 1.05)
	Extended release	Ritalin LA, Concerta	0.85 (0.65, 1.05)
	Long-acting	Ritalin-SR	0.96 (0.75, 1.16)
	Dexmethylphenidate	Focalin, Attenade	0.76 (0.45, 1.08)
Dextroamphetamine	Immediate release	Dexedrine, Dextrostat	1.24 (0.88, 1.60)
	Extended release	Accurate	1.13 (0.57, 1.69)
	Prodrug	Lisdexamfetamine	1.52 (1.34, 1.71)
Mixed amphetamine salts	Immediate release	Adderall	1.34 (0.95, 1.72)
	Extended release	Adderall XR	0.77 (0.59, 0.94)
<i>Non-stimulant medications</i>			
Atomoxetine	Immediate release	Strattera	0.63 (0.57, 0.69)
Guanfacine	Immediate release	Tenex	
	Extended release	Intuniv	0.8 (0.53, 1.07)

^a Efficacies of the formulation (compared to placebo) and 95% confidence intervals; the values are based on meta-analytical review (Curatolo *et al*, 2010; Kirshenbaum *et al*, 2008)

1.2.2 Prescription rates and relative efficacies

Currently, USA is the world's largest consumer of ADHD medications (Scheffler *et al*, 2007). Stimulants, including MPH and amphetamine, are the first line of pharmacotherapy for ADHD, primarily due to their superior efficacy relative to other approved and non-approved ADHD medications (Table 1.4). The population-adjusted rate of stimulant prescription among ADHD patients between 5 to 18 years of age was increased 2.9-fold from 1990 to 1995 (Robison *et al*, 1999). The prescription rate for MPH increased 2.6-fold in the same period and MPH accounted for 87.6% of all medications prescribed to ADHD individuals (Robison *et al*, 1999). A recent report based in the IMS Health National Disease and Therapeutic Index, reported that between 2005 and 2010, ADHD individuals under 18 years were prescribed stimulants and ATO in 85-90% and 6-13% cases, respectively (Garfield *et al*, 2012). Furthermore, among the stimulants, MPH containing preparations were prescribed in 52-62% of the cases, while amphetamine preparations were prescribed for the rest of the cases (Garfield *et al*, 2012), thus making MPH one of the most widely prescribed ADHD therapeutics for children and adolescents.

Several attempts have been made to evaluate factors underlying the variability in therapeutic efficacy of ADHD medications. Specifically, in neuropsychiatric disorders such as ADHD, there is a paucity of research regarding the predictors of treatment response and side effects (Kieling *et al*, 2010). Pharmacogenomics analyzes how genetic makeup of an individual influences their response to drugs, taking into consideration both the efficacy and side effects of the therapeutic agents. Pharmacogenomics combines the fields of pharmacology and genetics to provide this powerful tool that may expedite the advent of 'personalized medicine'.

Since the efficacious ADHD therapeutics such as MPH and ATO target the catecholaminergic pathways (Table 1.4), the genetic heterogeneity for these molecular targets in the ADHD population may be responsible at least in part for the variability in

response to treatment. Some of the recent pharmacogenetic association studies have been tabulated (Table 1.1) to increase our understanding of why all ADHD individuals are not effectively treated with the current ADHD therapeutics. Again, genetic associations predicting therapeutic efficacy of MPH are not consistent between studies (for review; Kieling *et al*, 2010), which may be due to methodological differences such as heterogeneity in experimental design, differences in baseline scores for ADHD symptoms between studies, non-standardized definition for “clinically significant genetic predictors of treatment effect” (Kieling and Rohde, 2008; Polanczyk *et al*, 2008). Taken together, MPH is one of the most widely prescribed ADHD therapeutic; however several factors including genetics and ADHD subtype and severity contribute to the outcomes (efficacy and side-effects) of MPH treatment.

1.2.3 Mechanisms underlying ADHD etiology: importance of prefrontal cortex

Integration of a wealth of knowledge generated through clinical and preclinical studies employing ADHD medications have culminated in several hypotheses regarding the etiopathology of ADHD. Regulation of behavior and emotion is referred to as “executive functions”. The executive dysfunction hypothesis of ADHD suggests that the core ADHD symptoms arise from primary cognitive/executive impairments (Arnsten, 2009; Sonuga-Barke and Fairchild, 2012). The dual pathway hypothesis posits a combination of executive functional deficits and reward/motivational impairments as responsible for ADHD pathophysiology (Blum *et al*, 2008; Sagvolden *et al*, 1998a; Sonuga-Barke, 2005). A more recent theory in ADHD etiology builds on the dual pathway model by adding the deficit in temporal processing circuitry as an added complexity contributing to the heterogeneity in ADHD (Sonuga-Barke *et al*, 2010). All the above hypotheses converge upon the importance of the prefrontal cortex (PFC) in the regulation of attention, executive function and motivation driven behavior through its projections into the motor and sensory cortex as well as to subcortical areas including the nucleus accumbens (NAc), striatum and amygdala (Arnsten, 2009; Levy,

2008, 2009). The individual processes that are suggested to be dysregulated in ADHD have been described in this section. Further examples have been included that describe the effect of MPH treatment on these individual processes.

Regulation of attention: PFC regulates attention based on relevance to the context/environment (Arnsten, 2009). Specifically, through its projections to the sensory association cortex, the PFC suppresses processing of irrelevant stimuli and thereby enhances processing of relevant stimuli (Knight *et al*, 1995). PFC also helps sustain attention to relevant sources and also shift attention to relevant dimensions through its many intra-cortical networks (Arnsten and Li, 2005). In ADHD children, MPH normalized function in the anterior cingulate cortex and lateral PFC during the suppression of interference during Stroop test (Bush *et al*, 2008; Lee *et al*, 2010).

Reward/motivational deficits in ADHD: Using a multiple schedule of alternating fixed interval 30 sec/extinction, ADHD individuals were found to have a shorter delay of reinforcement gradient compared to non-ADHD control (Sagvolden *et al*, 1998a). Medication naïve ADHD children exhibit increased impulsivity on a delay discounting task (Demurie *et al*, 2012), where impulsivity as increased choice of a smaller immediate reward over a larger delayed reward (Paxinos and Watson, 1986). These results support the reward deficiency hypothesis of ADHD (Blum *et al*, 2008) that explains that hyperactivity, reduced sustained attention, and increased impulsivity in a delay discount task can be explained as reduced motivation for reward. Reward deficiency in ADHD individuals is associated with hypodopaminergic striatum as well as decreased BOLD signal in the OFC and striatum (Rosa-Neto *et al*, 2005; Rubia *et al*, 2009b; Sonuga-Barke, 2005). A [¹¹C]raclopride PET imaging study in adolescents with ADHD revealed that MPH-evoked changes in striatal extracellular dopamine correlate with reductions in inattention and impulsivity using a continuous performance test (Rosa-Neto *et al*, 2005). MPH also normalized OFC activity in medication naïve ADHD children in response to reward during a continuous reinforcement task (Rubia *et al*, 2009b). Taken together,

MPH ameliorates the reward/motivational deficits in ADHD by normalizing striatal dopaminergic function as well as OFC-striatal activation patterns.

Regulation of executive function: PFC regulates behavior and emotion, which are referred to as executive functions. The dlPFC regulates attention-related motor responses through its connections with the basal ganglia and cerebellum (Middleton and Strick, 2000; Robbins, 2007). Projections from mPFC and OFC to amygdala, hypothalamus, NAc and striatum regulates emotion, response inhibition and error detection (Aron, 2007; Aron *et al*, 2004; Price *et al*, 1996; Rubia *et al*, 2003). Go/no-go task measures response inhibition capacity as decreased ability to stop in response to a relatively infrequent stop-cue that is randomly presented in-between the more frequent go-cues (Tannock *et al*, 1989b). The Stroop test evaluates the effect of interference on reaction time to dissociate a complex stimulus (e.g., identify the color of the following word, **BLUE**), thereby evaluating both attention and cognitive flexibility (Paxinos and Watson, 2005). Inattention and response inhibition deficits in ADHD children was associated with an attenuated decrease in BOLD signals in the PFC regions including anterior cingulate cortex compared to age-matched control (Schwarz *et al*, 2006; Stephens *et al*, 2010; Stephens *et al*, 2011). MPH has been found to normalize the decrease in PFC BOLD signal in ADHD children during go/no-go tasks (Liddle *et al*, 2011), and normalize decreased BOLD signal in anterior cingulate cortex and dlPFC during the suppression of interference during the Stroop test (Bush *et al*, 2008; Lee *et al*, 2010). Taken together, MPH treatment normalizes PFC function to improve response inhibition and sustained attention in children with ADHD. Comparing results from clinical studies and preclinical rat models, it is important to note that the DLPFC in primates is considered to be functionally analogous to the Fr2 and the anterior cingulate cortex subregions of mPFC in rodents, and the OFC in humans corresponds to OFC in rodents (Uylings *et al*, 2003). Thus, based on results from human studies, the prefrontal cortical subregions, mPFC and OFC, are regions of interest in rodent studies.

Further support for the role of frontal cortex in ADHD behaviors include that lesions of the frontal lobes result in a breakdown of goal directed activity, executive function, attention and produce hyperactivity (Benson and Stuss, 1982; Petrides and Milner, 1982; Stuss *et al*, 1982). Predominantly glutamatergic pyramidal neurons project from the PFC to the subcortical regions to modulate goal directed activity and executive function (Goldman-Rakic, 1995). The dendritic spines on the pyramidal neurons respond to small changes in the catecholamine via their noradrenergic α 2A receptors and dopamine D1 receptors (Sawaguchi and Goldman-Rakic, 1994; Smiley *et al*, 1994; Wang *et al*, 2007). The catecholaminergic inputs to these neurons arise from the basal ganglia and the arousal systems in the brain stem (for review; Engert *et al*, 2008) and as depicted in Fig 1.1. Thus, increasing catecholaminergic neurotransmission in cortical areas may be involved in the efficacy of psychostimulants and ATO in ADHD, which further lends support to the catecholaminergic hypothesis of ADHD etiopathology.

With a few exceptions, clinically efficacious ADHD medications potently inhibit DAT and/or NET function (see Table 1.4, and Fig 1.2). PET studies reveal that at clinically-effective doses, MPH inhibits DAT and NET (Volkow *et al*, 2002). Intracranial microdialysis studies in outbred rats report that at clinically or pharmacologically relevant doses, ADHD medications elevate extracellular concentrations of dopamine and norepinephrine in the PFC (Berridge *et al*, 2006; Bymaster *et al*, 2002). ADHD medications also increase neuronal firing in PFC (Devilbiss and Berridge, 2008). The MPH induced cortical excitability was decreased by α 2 adrenergic antagonist (Andrews and Lavin, 2006), suggesting the effects of MPH are mediated partially via α 2 receptor activation. Inhibition of D1 receptors in the PFC of rhesus monkeys decreased working memory in oculomotor delayed response tasks (Sawaguchi *et al*, 1994). In outbred rats, the cognitive enhancing effects of therapeutically relevant doses of MPH are inhibited by α 2 and D1 receptor antagonists intracranially applied to PFC (Arnsten *et al*, 2005). Taken together, these results suggest that the molecular mechanism by which MPH exerts its therapeutic effects includes increased cortical α 2 and D1 receptor activation via increased extracellular norepinephrine and dopamine (Arnsten, 2009). The functions

of individual receptor and transporter involved in ADHD etiology and therapeutics have been detailed in section 1.6 and 1.7 for dopaminergic and noradrenergic systems, respectively.

1.3 Animal models of ADHD

For the validation of an animal model for a human disorder, specific criteria have been suggested (Willner, 1986).

- 1) Face validity, i.e., mimicking the symptoms of the human disorder
- 2) Construct validity, i.e., sharing common etiology with the human disorder
- 3) Predictive validity, i.e., a model conforming to the first two conditions predicts the outcomes in the human patient population.

Since the precise etiology of ADHD is still unclear, the face-validity becomes the yard-stick for establishing an ADHD model. Such ADHD models may be viewed as experimental preparations developed for the purpose of studying ADHD-related phenotypes observed in humans (Koob, 2012). This approach has limitations related to extrapolating results to predicting clinically verifiable outcomes regarding both therapeutics and etiology.

A common obstacle for preclinical researchers is the inherent difficulty in operationalizing clinical descriptors of a psychiatric disorder (e.g., the symptoms of ADHD as described in DSM-IV) into behavioral tasks for rodent models. The second challenge is associated with providing a validation for modeling every aspect of a complex psychiatric disorders such as ADHD or addiction (Koob, 2012). For ADHD, the phenotypic traits of inattention, impulsivity and hyperactivity have been used to validate an animal model for this disorder. Also, individuals with ADHD are not hyperactive in a novel environment, but rather express hyperactivity in familiar

environments (Sagvolden and Sergeant, 1998b). Ideally, the deficits in these core symptoms should be ameliorated by stimulant and non-stimulant medications. Taking these primary criteria into consideration, several animal models of ADHD have been employed.

1.3.1 Spontaneously hypertensive rats (SHR)

The most widely accepted animal model of ADHD are the spontaneously hypertensive rats (SHR), a genetic rat model generated from the inbred Wistar-Kyoto (WKY) progenitor strain by inbreeding for hypertension. Historically, SHRs were characterized against the WKY control to define the ADHD phenotype. SHRs have been studied and characterized extensively to ascertain validity as a model for this complicated neuropsychological disorder.

SHR exhibit several dopaminergic deficits that contribute to its popularity as a model of ADHD (see Table 1.6). Specifically, the gene sequence for DAT differs from WKY; a 160 basepair insertion is present upstream of exon 3 of DAT in SHR (Mill *et al*, 2005). Compared to WKY, striatal DAT expression is decreased in juvenile SHR, and is increased in adult SHR (Paxinos and Watson, 2007; Paxinos *et al*, 1980). Electrically evoked [³H]dopamine release is decreased in the dorsal striatum and PFC of adult SHR (Russell *et al*, 1995). Adult SHR also exhibit greater extracellular dopamine in NAc, attenuated K⁺-evoked release compared to WKY; accumbal dopamine release in adult SHR is increased with MPH (Carboni *et al*, 2003). Taken together, SHR exhibit corticostriatal dopaminergic deficits compared to WKY which may contribute to the increased hyperactivity, impulsivity and inattention (Sagvolden *et al*, 2005b). SHR also exhibit other neurobehavioral deficits (Table 1.6) that further increase their face-validity as a model of ADHD.

Table 1.6 Validation of Spontaneously Hypertensive Rats (SHR) as a model for ADHD

ADHD Phenotype	Findings in SHR	References
Inattention	Enhanced responding during extinction trials (responding in the absence of cues) in a multiple fixed interval 30 sec/extinction (FI-30/EXT) schedule	(Sagvolden <i>et al</i> , 2005b)
Impulsivity	Increased premature responses during the FI-30/EXT schedule	(Sagvolden <i>et al</i> , 2005b)
	Deficits in withholding a prepotent response	(Sanabria and Killeen, 2008)
	Increased choice of a small immediate reward over a delayed large reward	(Wooters and Bardo, 2011)
Hyperactivity	Operant hyperactivity	(Hill <i>et al</i> , 2012b)
	Increased open-field activity	(van den Bergh <i>et al</i> , 2006)
Working memory	Increased number of errors on an radial maze version of win-shift task and win-stay tasks	(Kantak <i>et al</i> , 2008)
Behavioral flexibility	Increased sessions to criterion on radial maze version of attentional set-shift task as well as an operant strategy set-shifting task	(Harvey <i>et al</i> , 2013; Kantak <i>et al</i> , 2008)
Dopaminergic dysfunction in striatum	Decreased electrically evoked [³ H]dopamine release from caudate putamen slices	(Russell <i>et al</i> , 1995)
	Greater extracellular dopamine in NAc, attenuated K ⁺ -evoked release, but enhanced extracellular dopamine release with MPH	(Carboni <i>et al</i> , 2003)
Dopaminergic dysfunction in PFC	Enhanced electrically evoked [³ H]dopamine release from PFC slices	(Russell <i>et al</i> , 1995)
	Greater extracellular dopamine in PFC of juvenile SHR	(Viggiano <i>et al</i> , 2004b)
	Greater DAT function and expression levels in frontal cortex	(Pandolfo <i>et al</i> , 2012; Roessner <i>et al</i> , 2010)
Genetics	Differ from WKY rats in DAT-1 gene sequence	(Mill <i>et al</i> , 2005)

In summary, SHR display the core characteristics of ADHD, i.e., inattention, impulsivity and hyperactivity, and also display dopamine dysfunction in striatum and PFC. Further, MPH improves attention and behavioral flexibility and decreases impulsivity and hyperactivity in SHRs (Harvey *et al*, 2013; Kantak *et al*, 2008; Roessner *et al*, 2010; Russell *et al*, 1998; Sagvolden, 2011). Taken together, SHR display the hallmark features as well as other behavioral deficits associated with ADHD thereby proving to be a heuristically useful model of ADHD.

Valuable insights into mechanisms of ADHD pharmacotherapeutics as well as etiology may be obtained using an animal model of ADHD. However, appropriate interpretation of results obtained from ADHD rat models with strong face validity, such as the SHR, requires cautious selection of suitable reference control groups. WKY, being another inbred and by definition a genetically homogeneous strain of rats, were later found to display several behavioral and neurochemical deficits and as a result is currently used as a model of depression (De La Garza and Mahoney, 2004). For example, hyperactivity in SHR appears to have been overestimated when the only control employed was WKY, because WKY are hypoactive relative to outbred control rats (van den Bergh *et al*, 2006). WKY show altered dopaminergic function compared to outbred Wistar (WIS) rats such as decreased striatal DA D1 and D2 receptors (Novick *et al*, 2008; Yaroslavsky *et al*, 2006) and decreased accumbal DAT expression (Jiao *et al*, 2003). Several of these deficits may exaggerated the observed ADHD phenotype that SHR display, and consequently, studies that employ WKY as the only reference control (Alsop, 2007b) have been criticized. Therefore, the current studies include both the more commonly used WKY control and the WIS control as comparators to SHR. The rationale for using two controls was to disambiguate differences between SHR and WKY, and thereby provide a more complete understanding of the behavioral and neurochemical mechanisms underlying ADHD pathology.

1.3.2 Other models

Naples High Excitability rats: Naples High Excitability (NHE) rats are either inbred rats from outbred Sprague-Dawley rats selected based on behavioral arousal to a novel environment (Sadile *et al*, 1993; Viggiano *et al*, 2002). These animals are considered to model the hyperdopaminergic and hyperactive mesocortical system activity that may underlie the etiology of the inattentive and hyperactive phenotype in a subset of the ADHD population (Viggiano *et al*, 2004b). However, there are no studies demonstrating impulsivity in these rats. These rats have several neurochemical and behavioral similarities with the SHR model, but are not as widely accepted (Oades *et al*, 2005).

Poor 5-CSRT task performers: In this model, outbred Wistar rats were segregated into normal and poor-performers based on insufficient stimulus control in a 5-choice serial reaction time task (5-CSRT). The 5-CSRT task is based on the continuous performance task used for evaluating attention in clinical laboratories (Robbins, 2002). In a typical 5-CSRT schedule, a 5 sec delay or inter-trial interval (ITI) is followed by illumination of the cue-light in one of the 5 nose-poke holes. Responding on the illuminated nose-poke hole is considered a correct response and is reinforced. Responding during the 5-sec delay is considered premature responding. A poor 5-CSRT performer, as defined using an accuracy of less than 64% on this schedule, models the inattention aspect of ADHD. Low therapeutically relevant doses of MPH improve accuracy in the poor-performers, but not normal-performers (Puumala *et al*, 1996). Impulsivity, evaluated as % of premature responding was not different between the groups. Also, MPH increased impulsivity in normal-performers, but not in poor-performers. Another study using a similar 5-CSRT schedule but different selection criterion for accuracy, found poor-responders to be more impulsive than control (Barbelivien *et al*, 2001). This study did not test the effect of an ADHD medication on behavior, although the uptake of [C^{14}]deoxyglucose in the anterior cingulate and OFC was lower in the poor-performers. Poor 5-CSRT task performers model is considered to

resemble the predominantly inattentive subtype of ADHD (Koffarnus and Katz, 2011; Puumala *et al*, 1996).

Transgenic models: These models utilized a top-down approach, where proteins with associated genetic polymorphisms in the clinical population of ADHD were over-expressed or under-expressed to evaluate their role in attention, impulsivity or hyperactivity.

DAT knockout and knockdown mice: Several transgenic DAT mutants have been evaluated for ADHD phenotype, the oldest being the DAT knockout mice (Gainetdinov, 2008). For example, homozygous DAT knockout mice were ~6-times more active and required ~100-times longer to clear extracellular dopamine compared to heterozygous and wild-type mice (Giros *et al*, 1996). Also, hyperactivity in the DAT knockout mice is reduced by administration of amphetamine and dopamine receptor agonist (Gainetdinov and Caron, 2000; Zhuang *et al*, 2001). However, there are no published studies showing impulsivity or inattention in these mutants. The importance of this model is that it shows that DAT deficiency is neither necessary, nor sufficient, for inducing ADHD, lending further credence to the complicated multistep catecholaminergic interaction hypothesis of ADHD etiopathology.

Coloboma mice with SNAP-25 deficient mutation: Heterozygous mutant mice expressing coloboma mutation on chromosome 2 (region coding for *SNAP25*) have been proposed as an ADHD model. The SNAP25 coloboma mice exhibit spontaneous locomotor hyperactivity. These mutant mice also display a learning deficiency in achieving complex neonatal motor abilities as a consequence of reduced calcium-dependent dopamine release in dorsal striatum (Wilson, 2000). Compared to wild-type agouti mice, the coloboma mutant mice display greater sensitivity for delay in a delay discounting task as well as impaired attention evaluated as attenuated latent inhibition for acquiring conditioned taste aversion (Bruno *et al*, 2007). However, the effect of a stimulant medication on these behaviors has not been tested. Overall, the results with this mouse model together with the genetic association studies in subjects with ADHD

(Faraone *et al*, 2005) suggest that functional deficiency of SNAP-25 may contribute to ADHD.

Chemically-induced model, neonatal 6-hydroxydopamine lesioned rats have been proposed as a useful model of the hyperactive aspects of ADHD; however they do not display impulsivity (Luthman *et al*, 1989a; Luthman *et al*, 1989b; Shaywitz *et al*, 1978). These studies demonstrate that the dopaminergic, but not noradrenergic depletion was associated with hyperactivity in adult rats.

Environmental models: Environmental effects including, maternal lifestyle, stress and nicotine exposure during gestation have been associated with cognitive deficits including those associated with ADHD (Cornelius and Day, 2009; Linnet *et al*, 2003). A mouse model for prenatal nicotine exposure displayed hyperactivity, reduced cortical volume and dopaminergic deficits in frontal cortex (Zhu *et al*, 2012). A rat model of variable prenatal stress expressed working memory deficits, impulsivity and inattention under certain behavioral paradigms (Wilson *et al*, 2012). Rats raised in environmentally impoverished conditions also express impulsivity, hyperactivity and altered cortical dopaminergic function (Yates *et al*, 2012). These models, while limited in terms of face-validity and construct validity, are important preparations for parsing out environmental effects in ADHD etiology and may be valuable predictive tools for developing treatment strategies in individuals in whom such an environmental basis for ADHD is anticipated.

1.4 Cocaine abuse liability in the ADHD population

The mesocorticolimbic dopaminergic pathway dysfunction implicated in ADHD is also the target of drugs of abuse including cocaine (Ernst and Fudge, 2009; Koob and Volkow, 2010). Not surprisingly, adults with ADHD have a higher risk of developing substance use disorders compared to non-ADHD individuals, and cocaine abuse is often

comorbid with ADHD (Biederman *et al*, 1998; Wilens *et al*, 1998a). The mechanistic basis for this observation is not understood completely.

In this section, a brief review of the clinical literature on ADHD and cocaine addiction comorbidity will be discussed, followed by a brief description of the preclinical preparations available for evaluating factors influencing abuse liability under carefully controlled experimental conditions. The last section will delineate the possible behavioral, neurochemical and neurobiological mechanisms in the overlap between ADHD and cocaine abuse.

1.4.1 Clinical literature on liability for substance abuse

ADHD is usually overrepresented among individuals with substance use disorder (Carroll and Rounsaville, 1993; Szobot and Bukstein, 2008a; Szobot *et al*, 2007). Individuals with ADHD have 35% higher incidence of cocaine abuse compared to the general population (Carroll *et al*, 1993; Levin *et al*, 1999). ADHD is present much earlier than the usual age for initiation of experimentation with drugs of abuse; ADHD is identified during childhood (i.e., during elementary school years), or at least impairment are “present before age 7 years” (Criteria B, DSM-IV TR, American Psychiatric Association., 2000). Taken together, ADHD has been suggested to be a risk factor for developing substance use disorders.

A report derived from 18–44 year old respondents of the National Comorbidity Survey Replication (NCS-R) suggested that compared to non-ADHD, adults with ADHD (~4.4% of respondents) had substantial role impairment (e.g., high divorce rates, and current unemployment status) as well as high comorbid with other NCS-R/DSM-IV disorders, including but not limited to, substance use disorder (Kessler *et al*, 2006). Thus, the association between ADHD and risk of cocaine abuse may be complicated by the presence of other comorbid conditions and social factors.

ADHD is associated with social impairments such as peer rejection and academic problems through childhood and adolescence (Elia *et al*, 1999; Greene *et al*, 2001). These social impairments may contribute to the high substance abuse liability in ADHD, particularly during adolescence (Greene *et al*, 1997; Tarter, 2002). ADHD treatments reduce the risk of developing these social problems, and could be expected to reduce substance abuse liability. However, the prevalence of prescription of MPH and ATO for ADHD individuals between ages 15 to 21 has declined more rapidly than the previously reported age-related decreases in ADHD symptoms (McCarthy *et al*, 2009), suggesting that treatment may be prematurely discontinued in some young adults. Further, the clinical literature is divided on the correlation between cocaine abuse and previous treatment of ADHD (Greene *et al*, 1999; Kollins, 2008a, b). This issue is discussed further in sections 1.8.4 and 1.9.4.

ADHD is highly comorbid with other psychiatric conditions such as oppositional defiant disorder and conduct disorder (Biederman *et al*, 1991; Reinhardt and Reinhardt, 2013), which may be present at childhood (see Table 1 for comorbid conditions) or may develop later during adulthood (Miller *et al*, 2007). These comorbid conditions have been implicated in substance abuse liability even in individuals without ADHD (Molina and Pelham, 2003). Additional evidence that complicates the disentanglement of causality is that compared to age-matched controls, children with ADHD have a higher likelihood of developing conduct disorder during adolescence and antisocial personality disorder during adulthood (Mannuzza *et al*, 1998). Thus, elevated substance abuse liability (as well as the development of other risky behaviors, including criminality) in ADHD may be mediated by the development of comorbid conduct disorder, antisocial personality disorder or oppositional defiant disorder (Dalsgaard *et al*, 2013; Klein *et al*, 2012). Indeed a longitudinal study found that ADHD is associated with a greater incidence of substance abuse liability in adolescents only in the presence of oppositional defiant disorder or conduct disorder (August *et al*, 2006). In contrast, another longitudinal study found that ADHD is associated independently with substance use disorder (Gau *et al*, 2007). Taken together, comorbid conditions may increase the

vulnerability of substance use disorder, but do not necessarily mediate substance abuse in individuals with ADHD.

Indeed, the hallmark symptoms of ADHD have been established as predictors for the development of substance use disorder. Specifically, a longitudinal study in non-ADHD individuals found that lower baseline attention and executive function scores significantly predicted substance use and dependence 8 years later (Tapert *et al*, 2002). Further, impairments in behavioral inhibition in childhood (10-12 years of age) predicted substance use frequency 4-6 years later and substance use disorders 7-9 years later (Tarter *et al*, 2003). High impulsivity has also been established as a predictor for substance use disorders, and specifically, cocaine abuse liability (Bechara and Martin, 2004; Vonmoos *et al*, 2013). Interestingly, cocaine abuse may exacerbate ADHD symptoms (Vonmoos *et al*, 2013). ADHD patients with cocaine dependence were more impulsive compared to ADHD individuals without comorbid cocaine dependence as well as compared to healthy controls (Crunelle *et al*, 2013).

1.4.2 Brain imaging studies that provide insights into mechanism underlying ADHD and comorbid drug abuse

A working hypothesis regarding ADHD-cocaine abuse comorbidity is that executive function deficits in individuals with ADHD impair evaluation of risks and negative consequences associated with drug use, and interferes with inhibition of drug taking behavior despite negative consequences (Goldstein *et al*, 2009; Nigg *et al*, 2005; Szobot *et al*, 2008a). An alternate hypothesis is that altered reward and reinforcement mechanisms, a putative basis of ADHD, also mediate high substance abuse liability (Kalivas and Volkow, 2005b; Sagvolden *et al*, 1998a). Thus, the ADHD phenotype may enhance the behavioral response to the initial cocaine experience (Lambert *et al*, 2006); which is likely because the neuronal circuitry involved in ADHD (Fig 1.1) is involved in cocaine addiction (Adinoff *et al*, 2003; Bolla *et al*, 2003; Kaufman *et al*, 2003).

The mesocorticolimbic dopaminergic pathway (Fig 1.1) is also a part of the brain reward circuitry that is hijacked by drugs of abuse including cocaine (Ikemoto, 2007; Koob *et al*, 2010). DLPFC, OFC and the vmPFC mediate executive functions (Table 1.6) that are relevant for drug abuse and relapse (Aron and Paulus, 2007). Functional changes in these three areas of the frontal cortex are expected to underlie the deficits in executive function such as compulsive cocaine seeking, cocaine use, atypical pattern of evaluating reward expectancy and decision making observed in cocaine users (Bechara *et al*, 2004; Goldstein *et al*, 2009). Not surprising, cocaine users display “hypofrontality” or decreased functioning in these prefrontal cortical areas, as demonstrated by lower rates of glucose utilization measured using PET and [F^{18}]-fluorodeoxyglucose (Goldstein and Volkow, 2002).

As seen with ADHD, persistent decreases in striatal D2 receptor availability has been observed also in cocaine abusers compared to control (Volkow *et al*, 1993; Volkow *et al*, 2007b). Compared to age-matched controls, cocaine abusers displayed reduced glucose metabolism in frontal cortical areas such as OFC and cingulate gyrus compared to control (Volkow *et al*, 1993). The connectivity between the cingulate gyrus, OFC and ventral striatum is thought to underlie the increased reactivity to cocaine cues in cocaine-dependent subjects relative to naturally appetitive stimuli (Wilcox *et al*, 2011). Specifically, compared to age- and education-matched healthy control individuals, cocaine-dependent subjects showed increased activation (BOLD signals) in OFC and DLPFC and decreased activation of cingulate gyrus following cocaine cues relative. Imaging using fMRI during a decision making task (Iowa Gambling task) revealed greater activation of OFC and decreased activation of DLPFC and medial prefrontal cortex (mPFC) in cocaine abusers compared to control (Bolla *et al*, 2003). This compromised decision making is thought to contribute to the development of addiction and undermine efforts for abstinence. Go/No-Go task is commonly used to evaluate impulsivity which requires the subject to emit a simple motor response to a ‘Go’ stimulus and inhibit that response in the presence of a ‘No-Go’ stimulus (Wooters *et al*, 2006). Compared to cocaine-naïve controls, chronic cocaine users show reduced

activation of cingulate and insula during successful inhibition of responding during a no-go trial (i.e. correct no-go responses) and during an incorrect no-go response (i.e., commission errors) in a Go/No-Go task (Kaufman *et al*, 2003). Taken together, these results suggest that the brain structures required for higher-order cognitive control of behavior are disrupted in individuals using cocaine. As discussed previously (section 1.2.3), impulse control and decision making functions are impaired in individuals with ADHD, and thus may be the neurological vulnerability that underlies the high cocaine abuse liability in ADHD.

1.4.3 Animal preparations for evaluating cocaine abuse liability

Animal models are useful for testing intoxication, withdrawal and preoccupation or craving stages of addiction described in the DSM IV (American Psychiatric and American Psychiatric Association. Task Force on, 2000). Abuse liability and abuse potential of drugs are typically evaluated using several behavioral paradigms (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM198650.pdf>), which primarily model binge/intoxication and craving stages of the addiction cycle (Koob, 2012).

Self-administration studies assess the reinforcing properties of a drug under a non-dependent state. If animals work actively in an operant task to receive a dose of the drug, this result indicates that the drug will likely exert reinforcing properties in humans (Collins *et al*, 1984; O'Connor *et al*, 2011). Thus, if a certain disease condition is clinically associated greater risk for substance abuse, the animal model of the said disorder is expected to exhibit greater cocaine self-administration compared to control. Modifications of the schedule of reinforcement reveal useful information about the reinforcing properties of the drug and may be linked to different addiction symptoms described in DSM IV (American Psychiatric Association., 2000). For example, a shift in the dose-response functions under a fixed-ratio of responding (i.e., number of

responses for single reinforcement is constant during the schedule) for a drug of abuse indicates a difference in the efficacy of reinforcement of the drug. Further, increased responding during a progressive-ratio schedule, where the number of responses to obtain a single reinforcement is progressively increased, has been linked with drug craving described as spending a “great deal of time” obtaining substance of abuse (American Psychiatric Association., 2000; Arnold and Roberts, 1997). Second order schedules of reinforcement are used to evaluate differential reactivity for cues that predict availability of a drug (Di Ciano and Everitt, 2005; Schindler *et al*, 2002). Under the second order schedules, animals learn a complex sequence of responding; each step in the sequence is reinforced by a conditioned reinforcer and completion of the sequence results in delivery of the primary reinforcer (Cain *et al*, 2004). The later procedure is especially important model for drug addiction vulnerability because of the strong association between responding for drug-cues and context and likelihood of relapse (Childress *et al*, 1988; McLellan *et al*, 1986).

Conditioned place preference (CPP) is used to evaluate rewarding effect of a drug of abuse in the absence of the drug itself (Bardo and Bevins, 2000). The animal is exposed to two distinct environments, one of which is paired with the drug, and the other with saline through several training sessions. Subsequently, the animal is given the choice to spend time in either of the two environments in the absence of the drug. Spending more time in the drug-paired chamber indicates greater rewarding properties of the drug. However, negative results do not necessarily suggest the absence of abuse potential, given that “psychedelic” drugs such as 5-HT₂ agonist hallucinogens and cannabinoids do not produce CPP in rodent models (Vlachou *et al*, 2007). Another disadvantage of the CPP procedure is that unlike self-administration, it often does not exhibit a clear dose-response relationship (Bardo *et al*, 2000).

Drug discrimination evaluates interoceptive cues produced by a test compound, i.e. it is a method in which the animal indicates whether a test drug produces physical or

psychological perceptions similar to those produced by a known drug of abuse (Ator and Griffiths, 2003; Preston, 1991). In this paradigm, rats are trained to produce a particular response (e.g., press right lever in an operant chamber) in the presence of the drug to obtain a food reinforcer and produce a different response (e.g., press left lever) under placebo condition to obtain the same reinforcer. Subsequently, the response produced by different doses of a test compound reveals whether the rat perceives the test compound as drug-like or placebo-like with respect to the interoceptive cue properties of the training drug (Koob, 2012). This schedule may also be used to evaluate if a test compound can enhance or inhibit the interoceptive cues associated with the drug of abuse (Borta and Schwarting, 2004).

Psychomotor tests assess the ability of drugs to enhance motor functioning in rats, as has been demonstrated by well-characterized drugs of abuse including cocaine and amphetamines (Eisener-Dorman *et al*, 2011; Nordquist *et al*, 2008). Repeated administration of drugs of abuse, including cocaine, is known to enhance the psychomotor response elicited by the drug via a phenomenon termed psychomotor sensitization (Castner and Williams, 2007). Sensitization has been suggested to enhance the probability of relapse (evaluated using preparations like reinstatement of self-administration), long after the discontinuation of drug use (Robinson and Berridge, 2001). The neuropsychological basis for this association has been offered through the concept of “incentive sensitization” (Robinson and Berridge, 1993, 2000). In the words of the authors,

- i. ‘Potentially addictive drugs share the ability to produce long-lasting changes in brain organization.
- ii. The brain systems that are changed include those normally involved in the process of incentive motivation and reward.
- iii. The critical neuroadaptations for addiction render these brain reward systems hypersensitive (“sensitized”) to drugs and drug-associated stimuli.

- iv. The brain systems that are sensitized do not mediate the pleasurable or euphoric effects of drugs (drug “liking”), but instead they mediate a subcomponent of reward we have termed incentive salience or “wanting”.

Further, “incentive salience” has been suggested as the factor responsible for the “instrumental responding for the drug (i.e. drug-taking and drug-seeking)”. However, this concept has been a source of controversy in the field of drug addiction, where some research supports this contention (Lambert *et al*, 2006; LeBlanc *et al*, 2013), while others argue against it (Small *et al*, 2009).

1.4.4 Preclinical studies evaluating factors mediating cocaine abuse in ADHD

Preclinical studies also support the idea that the comorbidity of ADHD and cocaine abuse may be the result of commonalities in neuronal substrates. The mPFC in rodents is functionally analogous to DLPFC and portions of vmPFC in primates (Uylings *et al*, 2003). With respect to rodents, behavioral flexibility, working memory and sustained attention are regulated by the mPFC and OFC (Floresco *et al*, 2009; Ragozzino, 2007).

Both primates and rodents displaying cocaine addiction-like behavior show impaired cognitive control and executive function, suggesting functional impairments of mPFC and OFC (Beveridge *et al*, 2008; Harvey *et al*, 2009; Kantak *et al*, 2009). Importantly, mPFC and OFC also regulate cocaine-seeking behavior in outbred rats self-administering cocaine (Di Pietro *et al*, 2006; Grakalic *et al*, 2010; Kantak *et al*, 2013; Mashhoon *et al*, 2010). Cellular adaptations in the glutamatergic projections from the PFC to the NAc, including decreased D2 and increased D1 signaling in the PFC and decreased glutamate release in accumbens observed in cocaine withdrawn rats (for review, have been suggested to underlie addiction-like behavior (Kalivas *et al*, 2005a; Volkow *et al*, 2005b). By extrapolation, the latter adaptations in the cortico-striatal glutamatergic neurons are proposed to mediate the motivation to obtain drugs of abuse

following the presentation of drug-associated stimuli, which is hallmark diagnostic criteria for drug addiction (American Psychiatric Association., 2000).

The dorsal striatum mediates procedural learning and habit formation (Ikemoto, 2007; Squire *et al*, 1993). Cocaine self-administration studies in primates have shown that although the initial adaptations following cocaine use is predominantly in the accumbens (decreased DAT expression and glucose utilization), chronic self-administration is associated with adaptations in the dorsal striatum, including increased DAT expression and decreased glucose utilization in the caudate and putamen (Letchworth *et al*, 2001; Porrino *et al*, 2004). Striatum plays a role in motor control and compulsive or habitual drug seeking (Goldstein *et al*, 2009; Katak *et al*, 2005). Taken together, these results suggest that cocaine abuse is associated with functional adaptations, particularly in the dopaminergic transmission, in the PFC and striatum. Further, ADHD medications may alter cocaine abuse liability by altering dopaminergic function in PFC and striatum.

1.5 Impulsivity

1.5.1 Definition and classifications

Impulsivity is a complex multidimensional phenotype encompassing a wide variety of maladaptive behavior, including but not limited to, actions that are poorly conceived, prematurely executed, unnecessarily risky, inappropriate, and often with undesirable outcomes (Evenden, 1999b). Impulsivity includes several distinct and discreet components, each of which may have different neurological and genetic underpinnings. However, the high levels of impulsivity in individuals with ADHD are considered to predispose these individuals for future drug abuse vulnerability (Groman *et al*, 2009).

For decades, efforts have been made to fractionate the broad construct of impulsivity. From the psychological perspective, three separate and potentially independent ways have been identified in which impulsivity can modify behavior: in preparation for action, in execution of behavior patterns and in assessment of the consequences of an action (Evenden, 1999b). The majority of the clinical research in impulsivity has relied upon self-reported questionnaires that were developed based on different personality theories, which categorizes impulsivity in a relatively-overlapping manner (Patton *et al*, 1995; Whiteside and Lynam, 2001). As a result, impulsivity suffered from “jingle” fallacy, where two different constructs have equivalent label, as well as from “jangle’ fallacy, where a similar construct has two different labels (Block, 1995). One conceptually founded and well accepted method for deconstructing impulsivity is the UPPS Impulsive Behavior Scale (UPPS; Whiteside *et al*, 2001)) which is based on the Five Factor Model of personality (Merritt and Bachtell, 2013; Russell, 2003). UPPS scale measures four pathways to measure impulsivity: the urgency, (lack of) premeditation, (lack of) perseveration and sensation seeking (Whiteside *et al*, 2001). Urgency refers to the tendency to act impulsively under a positive or negative affect. Lack of Premeditation refers to a tendency to act without sufficient forethought. Lack of Perseverance refers to an inability to focus or follow through on mundane or challenging tasks. Sensation Seeking refers to a tendency to pursue activities that are exciting and often dangerous (Whiteside *et al*, 2005). The UPPS Impulsive Behavior Scale successfully differentiated the ADHD subtypes, ADHD-combined, ADHD-predominantly inattentive and ADHD with comorbid oppositional defiant disorder (Carter and Griffiths, 2009). The latter study also identified the impulsivity factors that differentiated the ADHD subtypes. Given the superiority of the UPPS scale for evaluating impulsivity clinical subjects, efforts to operationalize the impulsivity factors for preclinical studies are currently underway.

Another approach to simplify the construct of impulsivity is by defining endophenotypes of impulsivity; endophenotypes are a part of the biological pathway that links genes with a complex clinical phenotype (Bearden *et al*, 2004; Gottesman and

Gould, 2003). Endophenotypes help establish specific gene-behavior linkages and therefore may assist in identifying the molecular basis of impulsivity in ADHD (Almasy and Blangero, 2001; Crosbie *et al*, 2008). The defining criteria for an endophenotype are sensitivity and specificity to disease (Crosbie *et al*, 2008; Goos *et al*, 2009). For example, an ADHD-related impulsivity endophenotype must be present in ADHD individuals (sensitivity) and uncommon among the non-ADHD population (specificity). Furthermore, the endophenotype must be present in the genetic relatives of the individuals with ADHD even though they do not have ADHD themselves (Almasy *et al*, 2001). Endophenotypes, once established, can be trait markers for susceptibility for disease (Doyle *et al*, 2005b; Goos *et al*, 2009). Also endophenotypes may allow the categorization of ADHD into genetically homogeneous subgroups with distinct etiologies (Doyle *et al*, 2005b; Goos *et al*, 2009). However, given the complexity of executive function deficits in ADHD (Doyle *et al*, 2005a), evaluating endophenotypes of impulsivity, ADHD and cocaine addiction comorbidity is expected to be challenging.

1.5.2 Role in ADHD and cocaine abuse comorbidity

Genetic polymorphisms observed in patients with ADHD, such as DAT and D4 polymorphisms, are thought also to be the genetic basis of impulsivity (Congdon and Canli, 2008). As discussed previously, elevated impulsivity is a hallmark of ADHD (section 1.1.1). ADHD medications such as d-amphetamine, MPH and ATO have been shown to reduce impulsivity in clinical studies (Newman *et al*, 2008; Rubia *et al*, 2009a) and also have been shown to have beneficial effects in other neuropsychological disorders such as substance use disorders for managing impulsivity.

Clinical research on ADHD has employed a number of behavioral tasks for empirically evaluating impulsivity, many of which have strong face-validity and are easily translated to preclinical research (Evenden, 1999a; Evenden, 1999b). Two such tasks, the Choice-Delay Task, measuring preference for a larger, but delayed reward over an

immediate small reward, and the Stop Signal task, measuring the ability to inhibit a prepotent action, form very specific and sensitive predictors of ADHD (Solanto *et al*, 2001). However, there is very little correlation between performance on the latter tasks (Solanto *et al*, 2001), probably because Choice-Delay Task and the Stop Signal task measure two different forms of impulsivity that engage different, but overlapping neural substrates. The behavioral tasks evaluating impulsivity have been broadly categorized into those measuring 'impulsive action or motor-impulsivity' and those evaluating 'impulsive choice or cognitive/non-planning impulsivity' (Winstanley *et al*, 2010a). Motor impulsivity is described as inability to withhold from making a response, while impulsive choice is described as making decisions that are not beneficial for the future (Evenden 1999; Winstanley, Eagle *et al*. 2006). While each of these methods of classification has their own advantages and disadvantages, there is a need to standardize the rules for categorization to facilitate translational research between clinical and preclinical studies (Wickens *et al*, 2011; Winstanley *et al*, 2006a).

Impulsive action has been associated with initiation and acquisition of drug-taking behavior, while impulsive choice has been associated drug-seeking and reinstatement of self-administration (Diergaarde *et al*, 2008; Winstanley *et al*, 2010a). Compared to age-matched controls, individuals with ADHD select a less-likely but larger reward over a certain small reward (Drechsler *et al*, 2010a; Ernst *et al*, 2006). Conceptually, the latter risky decisions making is independent of delay aversion evaluated using Choice-Delay Task, but the two constructs share common neural substrates (Cardinal, 2006; Drechsler *et al*, 2010b). Both risky decision making and delay aversion stem from deficit processing of negative feedback, and may impair judgment and realistic assessment of the risks associated with actions (Cardinal, 2006; Drechsler *et al*, 2010b). Enhanced risk-taking behavior is associated with current and future drug use and abuse (Doremus-Fitzwater *et al*, 2010).

Chronic drug taking has been shown also to increase various facets of impulsivity (Cardinal, 2006). The working hypothesis in the field is that psychostimulants, such as

cocaine, enhance impulsivity as a feed-forward mechanism (Winstanley *et al*, 2010a). Specifically, the enhanced impulsivity impairing attempts to quit using drugs and exacerbating psychological effects of withdrawal, thereby leading to continuation and escalation of drug abuse (Koob *et al*, 2010; Winstanley *et al*, 2010a). Taken together, both ADHD and cocaine abuse are associated with increased impulsivity. However, the long-term effect of chronic administration of the stimulant ADHD medication, MPH, in individuals with ADHD on impulsivity later in life has not been empirically evaluated.

1.5.3 Brain regions/Neurotransmitters involved

Dopaminergic transmission in PFC and striatum are associated with impulsivity, risk-taking behavior and drug abuse (Cardinal, 2006; Koob *et al*, 2010; Perry *et al*, 2011; St Onge and Floresco, 2009, 2010). Inactivation of dopamine terminals in NAc leads to a shift in choice preference from a larger less-likely reward to smaller certain rewards and blocks acquisition of cocaine self-administration (Cardinal and Howes, 2005; McGregor and Roberts, 1993), thus demonstrating that a functional NAc dopaminergic system is necessary for impulsive choice as well as cocaine seeking.

OFC lesions increase impulsive action and impair learning about the relative value of larger reward, under probabilistic as well as delayed delivery conditions (Eagle and Baunez, 2010; Mobini *et al*, 2002). mPFC mediates goal-directed behavior and attention (Dalley *et al*, 2004), and regulates ability of rats to use within-session cues to update their choice according to recent reward contingencies (St Onge *et al*, 2010). Thus, OFC and mPFC functions critically mediate impulsive decision making as well as motor impulsivity.

Dopamine plays an important role in determining the rewarding and reinforcing value of stimuli. Changes in the phasic firing rates of dopamine neurons are thought to code for reward prediction errors and carry information about reward uncertainty (Tobler *et al*, 2005). Stimulants at low doses increase synaptic dopamine levels and

decrease impulsive behavior in humans as well as rodents (Richards *et al*, 1999). Systemic administration of D1-type receptor antagonists does not affect delay discounting tasks while D2-type receptor antagonists increase impulsive choice (Wade *et al*, 2000). Thus, dopaminergic signaling in mesocorticolimbic system may be a link between enhanced impulsivity and cocaine abuse liability in ADHD.

Other neurotransmitter systems implicated in impulsivity are serotonin and norepinephrine. Disruption of serotonergic function by intra-raphe administration of 5,7-dihydroxytryptamine in rats increases preference for the smaller immediate reward over larger delayed reward (Mobini *et al*, 2000). While SSRIs have been useful in managing impulse control disorders such as pathological gambling, these drugs have not been effective at managing ADHD symptoms, suggesting that altered serotonin transmission is not central to ADHD etiology (Winstanley *et al*, 2006a).

On the other hand, inhibition of norepinephrine transporters and activation of noradrenergic α 2A receptors have been shown to reduce ADHD symptoms including impulsivity (Fernando *et al*, 2012; Robinson *et al*, 2008a). Based on differences in sensitivity to delay in a delay discounting task, one study divided SHR into impulsive and non-impulsive subtypes (Adriani *et al*, 2003). The latter study found that the impulsive SHR sub-population had lower extracellular norepinephrine in the cingulate gyrus and mPFC. However, the role of norepinephrine neurotransmission in impulsivity has been relatively underexplored. Of note, local infusion of α 1 and α 2 receptor agonist into the mPFC and OFC did not alter impulsive decision making (Pardey *et al*, 2013). In terms of impulsive action using 5-CSRT task, systemic administration of α 2 receptor antagonist increased premature responding (Onali *et al*, 1988), and β -adrenergic antagonist decreased MPH-mediated increased premature responding (Milstein *et al*, 2010). Taken together these results suggest that noradrenergic transmission modulates impulsivity, however, further studies are needed to evaluate whether PFC noradrenergic receptors are involved.

1.6 Dopamine system

1.6.1 Dopamine biochemistry and neurotransmission

Dopamine is synthesized in the presynaptic neuron from amino acid tyrosine. Tyrosine hydroxylase converts tyrosine to L-dihydroxyphenyl alanine (L-DOPA), and this step is rate-limiting in the biosynthesis of dopamine. L-DOPA is further decarboxylated by DOPA decarboxylase to dopamine. Dopamine is packaged via vesicular monoamine transporter-2 (VMAT2) into presynaptic vesicles, where it is stored for release. In response to an action potential, the synaptic vesicles fuse with the presynaptic membrane and release dopamine into the synaptic space.

The basal striatal extracellular dopamine concentration is ~4 nM, which transiently rises ~60-fold to 250 nM following dopamine release during a standard nerve impulse (Tannock *et al*, 1989a). Dopamine released into the synaptic space binds to postsynaptic D1-like receptors and D2-like receptors to produce their downstream signaling cascade. The extracellular concentration of dopamine returns to ~4 nM within milliseconds, primarily by diffusion of the transmitter into the extracellular space and assisted by uptake by DAT which translocates the dopamine back into the presynaptic terminal.

The low basal level of extracellular dopamine concentration is thought to maintain presynaptic D2 autoreceptors at a steady-state partial activation. D2 autoreceptors detect changes in extracellular dopamine and counter regulate it by modulating tyrosine hydroxylase activity, tonic release of dopamine and DAT mediated uptake. This phenomenon is called tonic dopamine regulation (Costa *et al*, 1990; Dalley and Kelleher, 1966; Siegler *et al*, 1990). Dopamine taken up by DAT into the presynaptic neuron is either metabolized by monoamine oxidase (MAO) into dihydroxyphenyl acetic acid (DOPAC) or repackaged and stored into synaptic vesicles. Extracellular dopamine is metabolized by catechol-O-methyltransferase (COMT) to 3-methoxytyramine. DOPAC

and 3-methoxytyramine are further metabolized into homovanillic acid by COMT and MAO, respectively.

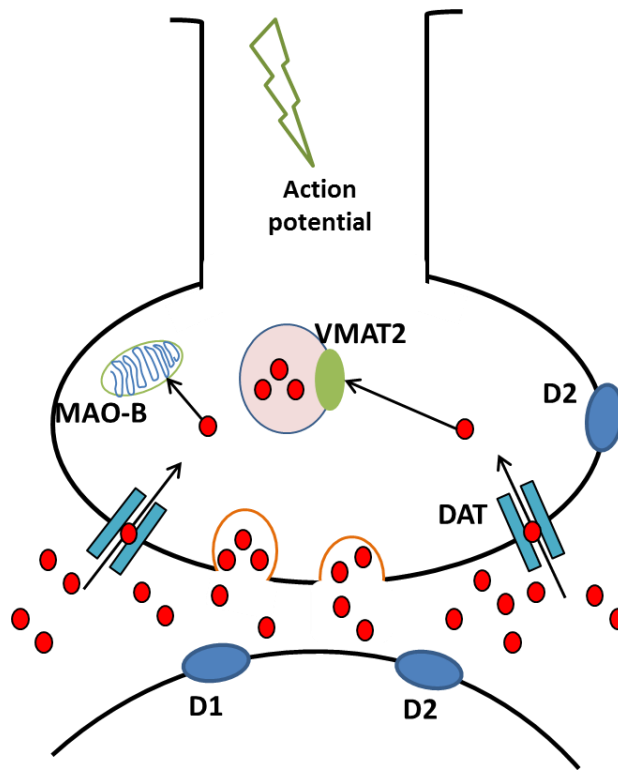


Figure 1.3 Dopaminergic neurotransmission at an ordinary synaptosome

VMAT2 – vesicular monoamine transporter type 2, MAO-B – monoamine oxidase B, D1 and D2 – dopamine receptor type 1 and 2, respectively, and Dopamine transporter – DAT

1.6.1.1 Role in ADHD and cocaine abuse

When DAT is blocked by therapeutically relevant doses of MPH, extracellular dopamine in the striatum is increased by ~6-fold. This elevated resting state extracellular concentration of dopamine increases D2 autoreceptor activation and reduces action potential evoked dopamine release in striatum (Tannock *et al*, 1989a; Trobst *et al*, 2000). The decreased background firing of striatal neurons results in strengthening of corticostriatal signal via the glutamatergic prefrontal cortical afferents (McCrae *et al*, 2008). Taken together, the increased change in signal-to-noise ratio in the striatum is thought to mediate the increased attention and reduced distractibility following MPH treatment. Further support for the role of striatal D2-receptors in ADHD etiology and cocaine addiction comes from imaging studies. Specifically, striatal D2/D3 receptor availability is lower in ADHD compared to non-ADHD individuals (Volkow *et al*, 2007b) and in cocaine abusers (Volkow *et al*, 1993) compared to control. Cocaine is thought to exert its primary rewarding and reinforcing properties by increasing extracellular dopamine in NAc, while the striatum, mPFC and OFC are involved in the development and expression of drug addiction (Koob *et al*, 2010), also see section 1.4.3).

As discussed previously (Table 2), genetic studies revealed that hypofunctional D4 receptor polymorphisms are associated with ADHD etiology. Unlike other D2-like receptors, D4 does not have autoreceptor function. D4 receptors are expressed on neuronal presynaptic terminals that do not co-express tyrosine hydroxylase (Rivera *et al*, 2008). This suggests that the primary function of D4 signaling is modulation of release of non-dopamine neurotransmitters, i.e. D4 functions as a heteroreceptor (Svingos *et al*, 2000). Dopamine and norepinephrine are equal affinity agonists for D4 receptors (Newman-Tancredi *et al*, 1997). In the dorsal striatum and the NAc, D4 is expressed on medium spiny neurons and modulates glutamatergic signaling (Mrzljak *et al*, 1996; Thomas *et al*, 2009). Taken together, D4 receptors appear to be important modulators

of striatal dopaminergic signaling, and hence, play a critical role in ADHD and cocaine addiction.

D2-like receptors in PFC also are important for ADHD etiology and therapeutics. D2 receptors have a significant presynaptic autoreceptor role in the mPFC (Cubeddu *et al*, 1990). Specifically, the D2 agonist quinpirole reduced electrically-evoked [³H]dopamine release from mPFC slice preparation (Cubeddu *et al*, 1990). D2 receptor stimulation in PFC modulates response-related firing of PFC neurons (Wang *et al*, 2004). Further, activation of D2 on pyramidal glutamate neurons modulates cocaine-induced behavior by exerting inhibitory control over cocaine-induced dopamine release in NAc (Beyer and Steketee, 2000, 2001; Liu and Steketee, 2011). D4 postsynaptic receptors are responsible for suppression of GABA-containing inhibitory interneurons (Rivera *et al*, 2008; Wang *et al*, 2002). Thus, hypoactivation of D4 may result in excessive inhibition of firing of PFC networks.

D1 receptors are the most abundant dopaminergic receptor in the PFC (Lidow *et al*, 1991). Stimulation of D1 receptors by cortical dopamine weakens firing of inappropriate connections (Arnsten *et al*, 2005). Thus, insufficient D1 receptor activation impairs PFC function by ineffectively blocking “noise”. Moderate levels of D1 stimulation suppresses irrelevant inputs or “noise” to layer IV and V cortical neurons and thereby, improve PFC function, including attention and working memory (Gamo *et al*, 2010; Vijayraghavan *et al*, 2007; Yang and Seamans, 1996). However over-stimulation of D1 receptors by high concentrations of stimulants (observed during abuse of drugs such as cocaine), weakens PFC function by suppressing too many network connections (Vijayraghavan *et al*, 2007).

1.6.1.2 Ontological changes in dopaminergic transmission, emphasis on adolescence

During early adolescence (~PND 28-35 in rats), a transient generalized increase in D1 receptor density and arborization of glutamatergic pyramidal neurons in PFC results

in decreased cortical control over reward related behavior, which results in higher impulsivity and risk taking (Casey and Jones, 2010; Ernst *et al*, 2009; Somerville and Casey, 2010a; Somerville *et al*, 2011). Fine-tuning of cortical control is achieved with synaptic pruning and selective down regulation of D1 receptors (Brenhouse *et al*, 2008; Doremus-Fitzwater *et al*, 2010) thus, contributing to an age-dependent decrease in impulsivity (Andrzejewski *et al*, 2011; Laviola *et al*, 2003). D1 receptor function in cortex is a therapeutic target for MPH action (Gamo *et al*, 2010). D2 and D4 receptors in PFC also are upregulated between postnatal day 7 (PND7) to PND28, and subsequently, downregulated between PND35-60 (Tarazi and Baldessarini, 2000). Currently, no reports are available that evaluate age-dependent changes in DAT expression or function in cortical areas.

In contrast to the PFC, ontogenic changes in dopaminergic reward circuitry follow a different developmental trajectory. In the rodent NAc, density of dopamine D1, D2 and D4 receptor peaks by 4 weeks of age and is pruned subsequently during early adulthood (PND60) (Tarazi *et al*, 2000). In contrast, DAT density in NAc and dorsal striatum peaks by PND60 and is not down regulated in adult rats (Tarazi *et al*, 1998).

1.6.2 Dopamine transporter (DAT)

1.6.2.1 Involvement in ADHD and cocaine abuse comorbidity

DAT has been associated with several neuropsychiatric disorders ever since the discovery of a 40-basepair variable number of tandem repeats (VNTR) polymorphism in the 3'-untranslated region of the *DAT1* gene (Vandenbergh *et al*, 1992a; Vandenbergh *et al*, 1992b). As discussed in the previous sections, DAT play a critical role in ADHD etiology and therapeutics as well cocaine abuse and addiction. DAT inhibition is one of the primary mechanisms of action by which MPH exerts its therapeutic effects in ADHD and by which cocaine exerts its reinforcing properties. Genetic variations of DAT in ADHD etiology and therapeutics have been described previously (see Table 2).

Striatal DAT density evaluated using SPECT with [¹²³I]altropane revealed a 70% elevated DAT density in ADHD individuals compared to non-ADHD proband (Dougherty *et al*, 1999). A recent meta-analytical review showed that previously medicated ADHD individuals have higher striatal DAT density compared to non-ADHD individuals (Fusar-Poli *et al*, 2012). However, cortical DAT expression in ADHD relative to control has not been reported.

Molecular mechanisms and pharmacological properties of ADHD medication, including MPH, are comparable to cocaine (Volkow *et al*, 1995); the Ki of cocaine and MPH for inhibition of DAT is 640 nM and 390 nM, respectively, and intravenous administration produces comparable DAT occupancy and subjective effects in humans. Striatal DAT function is elevated also in cocaine users (Mash *et al*, 2002). However, cortical DAT expression in cocaine users has not been reported.

1.6.2.2 Structure, Function and regulation of trafficking

Neuronal DAT is responsible for ensuring that dopaminergic signals are restricted, temporally and spatially (Melikian, 2004). DAT is coded by the *DAT1* gene (*SLC6A3*) on chromosome 5 (Vandenberg *et al*, 1992b). DAT is a member of the high affinity, sodium- and chloride-dependent solute transporter SLC6 gene family. DAT is a transmembrane protein the crystal structure of which is not available currently. Information about DAT structure is derived from *in silico* modeling that is currently based on X-ray structure of the homologous bacterial leucine transporter, LeuT (Manepalli *et al*, 2012).

DAT, like the other members of the SLC6 family, are thought to have 12 membrane-spanning domains with intracellular amino- and carboxy-terminals and a large, glycosylated extracellular loop between transmembrane domains 3 and 4 (Melikian, 2004). DAT is thought to have two substrate binding sites, one lodged in the interior of the transmembrane domains, called S1 and the second on the “extracellular

vestibule”, called S2 (Manepalli *et al*, 2012). DAT inhibitors including cocaine and MPH are expected to dock at the S2 site and impair dopamine transport by blocking access to the S1 site (Huang *et al*, 2009).

At the presynaptic surface, DAT functions as regulators of extracellular dopamine concentration. A considerable pool of DAT is present in the intracellular portion of vesicular structures (Nirenberg *et al*, 1997). Immunocytochemical studies suggest that DAT is present on the presynaptic nerve terminal and in the cell bodies of the dopaminergic neurons (Hersch *et al*, 1997). Several stimuli regulate DAT function through trafficking dependent mechanisms that ultimately change the expression of DAT at the cell surface (Melikian, 2004). Specific examples of such mechanism include inhibition of DAT recycling by inhibition of phosphoinositol-3-kinase, increasing DAT endocytosis by PKC-mediated phosphorylation and several protein-protein interactions (Melikian, 2004; Sager and Torres, 2011). Importantly, increases in D2 receptor activation and excess concentrations of transporter substrate downregulates surface DAT expression (Gulley and Zahniser, 2003). Thus, chronic increases in extracellular dopamine by inhibition of DAT and NET in the PFC and by inhibition of DAT in the striatum by drugs including MPH, ATO and cocaine may lead to lasting adaptations in the cellular distribution of DAT.

1.7 Norepinephrine system

Norepinephrine is structurally similar to dopamine. The most important noradrenergic system neurons originate from locus coeruleus (LC) located in the dorsal pons. Axons of these neurons project to several areas of the brain, including but not limited to cortex, thalamus, hypothalamus, cingulate gyrus, hippocampus, and amygdala (see Fig 1.1). ADHD medications as well as cocaine are known to modulate firing of noradrenergic LC neurons (Gamo *et al*, 2010).

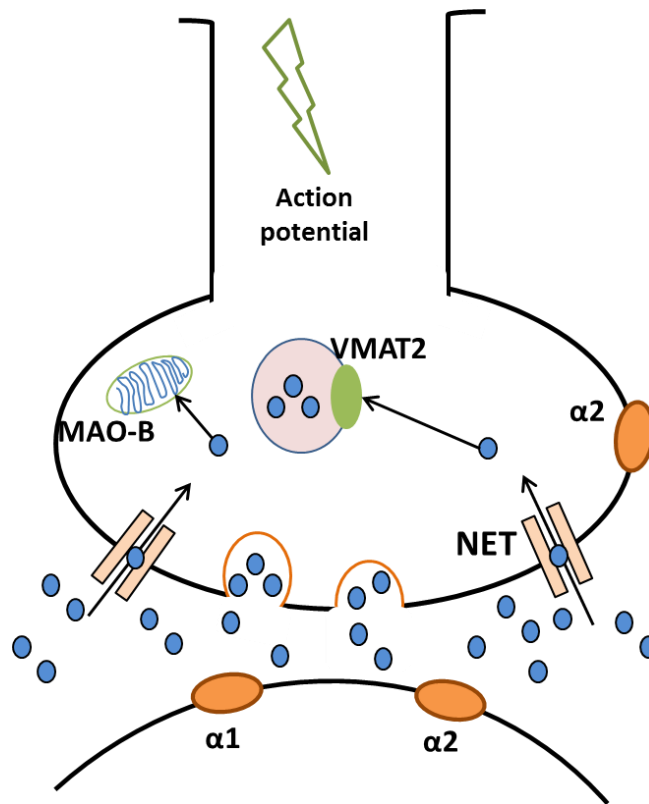


Figure 1.4 Noradrenergic neurotransmission at an ordinary synaptosome

VMAT2 – vesicular monoamine transporter type 2, MAO-B – monoamine oxidase B,
 α_1 and α_2 – norepinephrine receptor type 1 and 2, respectively, NET – norepinephrine
 transporter

1.7.1 Norepinephrine biochemistry and neurotransmission

Norepinephrine is synthesized from dopamine in synaptic vesicles. Dopamine is hydroxylated by dopamine- β -hydroxylase (D β H), simultaneous with the reduction of an ascorbic acid molecule. Norepinephrine is released following an action potential from the presynaptic terminal and is subsequently cleared from the synaptic space by uptake by norepinephrine transporters (NET) and by metabolism by the extracellular enzyme COMT. Norepinephrine in the presynaptic terminal is either repackaged into synaptic vesicles, or metabolized by MAO. Norepinephrine is converted to normetanephrine and dihydroxymandelic acid by COMT and MAO, respectively. These metabolites are converted subsequently to vanillylmandelic acid by the complementary enzyme from the previous biochemical step in the metabolic pathway.

1.7.1.1 Role of noradrenergic transmission in ADHD in cocaine abuse

Clinical studies have found that polymorphisms in NET, COMT, D4 and D β H enzymes are associated with ADHD (see Table 2). NET clears both dopamine and norepinephrine (Moron *et al*, 2002), and is especially important for dopamine clearance in the cortical areas (regions of importance to ADHD etiology). D β H is the neuronal enzyme responsible for the conversion of dopamine to norepinephrine. COMT metabolizes extracellular dopamine and norepinephrine. Dopamine D4 receptors share equal affinity dopamine and norepinephrine, although not expressed on D β H expressing neurons in PFC (Rivera *et al*, 2008).

ADHD medications such as d-amphetamine, MPH, and ATO increase norepinephrine levels in cortical areas. Further, guanfacine is a α 2A adrenergic agonist. All effective ADHD medications increase norepinephrine neurotransmission. Moreover MPH and d-amphetamine at clinically relevant doses increase dopamine and norepinephrine levels in PFC, with the % increase in norepinephrine concentrations being greater than the % increase in dopamine concentration. Extracellular concentration of norepinephrine in PFC is \sim 0.9 nM (Bymaster *et al*, 2002). MPH at

0.5mg/kg, i.p. increases extracellular norepinephrine in PFC by 280% above baseline (Berridge *et al*, 2006). In contrast, in the subcortical region like medial septal area, MPH at 0.5 mg/kg i.p. increased extracellular norepinephrine to 122% of baseline of baseline (Berridge *et al*, 2006).

ADHD is thought to be the result of reduced norepinephrine neurotransmission in PFC. Inhibition of $\alpha 2$ receptors (A, B and C subtypes) with intra-cortical administration of yohimbine has been shown to induce an ADHD profile in primates (Li and Mei, 1994; Ma *et al*, 2005; Ma *et al*, 2003). Inattention, impulsivity and emotional dysregulation may be due to decreased norepinephrine mediated PFC regulation of function of sensory and motor cortices, and dorsal and ventral striatum.

Increasing cortical norepinephrine signal via increasing norepinephrine or activation of $\alpha 2A$ receptors (by both stimulants and non-stimulants) is thought to improved PFC-mediated functions including attention and impulsivity (Arnsten *et al*, 2005; Bari *et al*, 2011). At moderate levels extracellular norepinephrine activates $\alpha 2A$ receptors, decreases intracellular cAMP, and consequently enhances the strength and duration of firing of cortical pyramidal neurons (Wang *et al*, 2007).

Extracellular concentrations norepinephrine produce an inverted-U shaped dose response curve for modulation of PFC function; higher cortical norepinephrine engage the low-affinity $\alpha 1$ -adrenoreceptors, increase intraneuronal cAMP, and protein kinase C activity and suppress firing of PFC cortical neurons (Birnbaum *et al*, 2004). Thus, stimulants at higher doses (comparable to that seen during drug abuse) are detrimental to cortical function.

Cocaine is an inhibitor of monoamine transporters DAT, NET and serotonin transporter, and cocaine administration leads to increases in norepinephrine concentrations in brain (Amara and Sonders, 1998). Systemic administration of $\alpha 1$ -agonist, prazosin, has been shown to increase motivation for cocaine self-administration (Wee *et al*, 2008). However, DAT inhibition, and not NET inhibition by cocaine is thought

to mediate the reinforcing properties of cocaine (Dewit and Wise, 1977; Ritz *et al*, 1987; Wilson and Schuster, 1974). Norepinephrine neurotransmission is engaged by aversive and conditioned appetitive stimuli (Feenstra *et al*, 1999; Mingote *et al*, 2004). Norepinephrine depletion in mPFC has been shown to block cocaine-induced increase in dopamine in NAc and conditioned place preference in mice (Ventura *et al*, 2007). In contrast, inhibition of $\alpha 1$ receptors in mPFC or in VTA did not alter cocaine self-administration (Ecke *et al*, 2012). Taken together, these results suggest that NET in cortical areas is involved in ADHD etiology and therapeutics but may not be directly related to cocaine abuse liability.

1.7.1.2 Ontological changes in noradrenergic transmission, emphasis on adolescence

Cortical norepinephrine concentration in rhesus monkeys exhibited a steady increase from birth to adulthood, and during adulthood, cortical norepinephrine far exceeded dopamine and serotonin concentrations (Goldman-Rakic and Brown, 1982). In the rodent LC, NET expression was high during the early postnatal period, and significantly decreases from adolescence to early adulthood (Sanders *et al*, 2005; Zhu *et al*, 2005b). Downregulation of D β H in the LC occurs more gradually and is not significantly lower until 2 years of age (Zhu *et al*, 2005b). In contrast, in the forebrain, NET expression increased from birth to PND 15, and these levels were typically maintained through adulthood (Sanders *et al*, 2005). The latter age-dependent changes in NET expression are consistent with developmental changes in cortical NET function (Coyle and Axelrod, 1971). $\alpha 2$ adrenergic receptors followed a similar developmental trajectory in rat cortex (Happe *et al*, 2004). Taken together, these studies suggest that cortical noradrenergic systems are not as plastic as the dopaminergic system during adolescence. Thus, the cortical noradrenergic system is not developmentally vulnerable during adolescence, and is not expected to be altered by mild pharmacological manipulations, such as treatment with therapeutically relevant low doses of MPH and ATO.

1.7.2 NE transporter (NET)

The basal dopamine and norepinephrine extracellular concentrations in PFC are 0.4 nM and 0.9 nM, respectively (Bymaster *et al*, 2002). ATO, a selective NET inhibitor, at 0.3 mg/kg, i.p. increases PFC extracellular norepinephrine and dopamine by 170% and 50%, respectively, of baseline (Bymaster *et al*, 2002).

1.7.2.1 Structure, Function and regulation of NET

Neuronal NET is responsible for ensuring that noradrenergic signals are restricted, temporally and spatially (Melikian, 2004). NET is coded by the *NET1* gene (*SLC6A2*) on chromosome 16 (Pacholczyk *et al*, 1991); NET is a member of the high affinity, sodium- and chloride-dependent solute transporter SLC6 gene family. The polypeptide sequence for NET shares 67% homology with DAT, and as a consequence most of the information about NET structure is derived from DAT models (Manepalli *et al*, 2012).

NET, like the other members of the SLC6 family, is thought to have 12 membrane-spanning domains with intracellular amino- and carboxy-terminals (Melikian, 2004). Like DAT, NET too has two substrate binding sites, one lodged in the interior of the transmembrane domains, called S1 and the second on the “extracellular vestibule”, called S2 (Manepalli *et al*, 2012). NET inhibitors including cocaine, as well as specific NET inhibitors including desipramine are expected to dock on the S2 site and impair dopamine transport by blocking access to the S1 site (Hill *et al*, 2011).

NET at the synaptic surface functions as regulators of extracellular norepinephrine; surface NET in the cortex regulates extracellular dopamine concentrations (Bymaster *et al*, 2002). Unlike striatal and accumbal DAT, during the resting stage, NET is predominantly located in the intracellular compartment in the

prefrontal cortex (Miner *et al*, 2003) and is redistributed to the cell surface when conditions, such as stress, increase extracellular norepinephrine (Miner *et al*, 2006). Several proteins regulate trafficking of NET to and from the cell surface, including but not limited to proteinphosphatase2A, syntaxin1A, tyrosine kinase and mitogen-activated protein kinase (Apparsundaram *et al*, 2001; Gulley *et al*, 2003; Sager *et al*, 2011).

1.8 Methylphenidate

MPH is a piperidine-derived chemical moiety with two chiral centers (Fig 1.5). Of the 4 stereoisomers, d-threo MPH is the pharmacologically active stereoisomer (Challman and Lipsky, 2000; Heal *et al*, 2008). The chemical name for MPH is (\pm)-methyl α -phenyl- α -(2-piperidyl)acetate hydrochloride. The mechanism of action and pharmacology of MPH will be described in the subsequent sections.

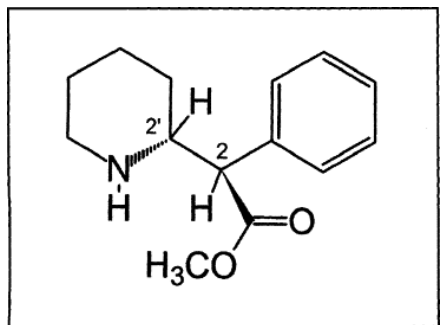


Figure 1.5 Methylphenidate

The structure of d-threo (2R, 2'R)-methylphenidate, the pharmacologically active stereoisomer (Challman *et al*, 2000).

MPH, following its synthesis in 1944, was used originally as an analeptic to reverse barbiturate induced coma (Wax, 1997). MPH is approved by FDA for treatment of ADHD, narcolepsy and postural orthostatic tachycardia syndrome. Off-label clinical uses of MPH include treatment of lethargy, bipolar depression, major depressive disorder and obesity. MPH is the first line of pharmacological treatment for ADHD in

children, adolescents, and adults (Dodson, 2005; Rosler *et al*, 2010; 2009), partially because of its superior efficacy (~70% cases) compared to placebo (Castells *et al*, 2011; Koesters *et al*, 2009), as well as its relative safety and mild side-effects (Godfrey, 2009; Merkel, 2010; Merkel and Kuchibhatla, 2009). However, MPH is not approved for use in children under 6 years of age (Vitiello, 2001) since the long-term effects of MPH on the developing brain are not understood clearly. Currently, several formulations of MPH are available in the US and world market (See table 4) each of which has its own set of advantages and disadvantages.

1.8.1 Mechanism of action

MPH is an inhibitor of DAT and NET, with K_i for inhibition of uptake of [³H]dopamine and [³H]norepinephrine about 160 nM and 40 nM, respectively (Richelson and Pfenning, 1984). MPH acts by enhancing extracellular concentrations of DA and NE in cortical areas (see Fig 1.2). Stimulation of postsynaptic α_2A -receptors by NE is thought to strengthen appropriate network connections by extending the duration of the “signal”. On the other hand stimulation of D1 receptors is thought to weaken inappropriate connections and thereby weaken “noise” (Arnsten *et al*, 2005).

1.8.2 Pharmacology

Following oral administration of the immediate release formulations, maximal plasma concentrations of MPH are observed within 1-3 hours (T_{max}) and a half-life ($T_{1/2}$) of 1.5 to 2.5 hours has been reported (Faraj *et al*, 1974). For sustained release formulations, the T_{max} and $T_{1/2}$ for MPH was 3-4 hrs and 4 hrs, respectively (Birmaher *et al*, 1989). MPH is primarily metabolized to ritalinic acid by enzymatic de-esterification; alternate metabolic degradation pathways include trans-esterification to

ethylphenidate, hydroxylation of the phenyl-ring or oxidation of the piperidine-ring to form lactams. Less than 1% of MPH is excreted unchanged (Markowitz *et al*, 2003).

Clinically, MPH exerts its therapeutic actions at low plasma concentrations of 8-40 ng/mL by occupying >50% DAT in brain, and SPECT studies show that the highest MPH concentrations occur in striatum (Ding *et al*, 1997; Volkow *et al*, 1998a). Striatal DAT inhibition and subsequent increases in extracellular dopamine mediate the therapeutic actions of MPH (Volkow *et al*, 2012). The latter study used changes in [¹¹C]raclopride binding in the striatum to infer changes in extracellular dopamine concentration and reported that MPH-induced (0.5 mg/kg, i.v.) increases in extracellular striatal dopamine correlated with reductions in inattention ratings in ADHD individuals (Volkow *et al*, 2012).

MPH has been shown to normalize deficits in cortical function in ADHD individuals. Specifically, during an emotional Stroop task, the activation and deactivation patterns in the mPFC following the presentation of a positive- and negative-valence distraction, respectively, were enhanced in adolescents with ADHD compared to control subjects and MPH normalized these aberrant activation patterns in individuals with ADHD (Posner *et al*, 2011). In another study, MPH improved reaction time during a working memory task in adolescents with ADHD and simultaneously strengthened connectivity in the frontoparietal regions of cortex (McCrae *et al*, 1986b). In another study, MPH normalized the elevated rates of omission errors, but not commission errors, during a continuous performance task in medication naïve children with ADHD, suggesting that acute MPH ameliorates inattention, but not impulsivity in children with ADHD (Rubia *et al*, 2009b). Behavioral effects in the latter study were associated with activation of the dysfunctional mPFC-striato-cerebellar attentional networks and a deactivation of the hypersensitive OFC activation for reward processing. Taken together, the above studies provide further evidence that the therapeutic efficacy of MPH is mediated, in part, by altering the function of neural networks including those in striatum, mPFC and OFC.

Clinically relevant MPH doses produce peak plasma concentration of 8-40 ng/mL (Wargin *et al*, 1983). Comparable plasma concentrations of MPH are observed in rodents following intraperitoneal injection of 0.25 - 1.0 mg/kg or oral administration of 0.75 - 3.0 mg/kg (Berridge *et al*, 2006; Kuczenski and Segal, 2002). Low oral doses of MPH (2.0 mg/kg, p.o) preferentially increased dopamine in PFC (125% above baseline) rather than in NAc (50% above baseline; Berridge *et al*, 2006). The latter study also found that therapeutically relevant doses of MPH (0.5 mg/kg, i.p.) increased extracellular norepinephrine preferentially in PFC (280% above baseline) compared to the subcortical medial septal area (122% above baseline). Further, within the PFC, the magnitude of increase in extracellular norepinephrine was greater than dopamine (280% versus 130% above baseline) following 0.5 mg/kg, intraperitoneally. MPH improved working memory by increasing activation of cortical α 2A adrenergic and D1 dopaminergic receptors in non-human primates and rats (Arnsten, 2009; Gamo *et al*, 2010). MRI studies in outbred rats revealed that MPH (2.0 mg/kg, i.p.) produced positive BOLD signals in NAc, substantial nigra and OFC, and produced negative BOLD signals in motor and somatosensory cortices, caudate putamen, globus pallidus and bed nucleus of stria terminalis (Easton *et al*, 2009). This pattern of changing brain activation was observed also with ATO, suggesting some similarities in the outcome of ADHD medications (Easton *et al*, 2007). Taken together, these results from animal studies show that the pharmacological actions of therapeutically relevant doses of MPH, in part, involve enhanced catecholamine signaling in PFC and the modulation of mPFC, OFC and striatal activation.

1.8.2.1 Cardiovascular effects

Concerns have been raised about potential cardiovascular side effects including myocardial infarction, stroke and sudden death, associated with use of prescription stimulants such as MPH. The reasons behind such concerns regarding prescription stimulants are that prescription stimulants:

1. increase blood pressure and heart rate (Findling *et al*, 2001; Wilens *et al*, 2005)
2. increase circulating levels of catecholamines that can induce vasospasm (Bromberg-Marín *et al*, 2007; Rumbaugh *et al*, 1971; Thompson and Thompson, 2010)
3. induce formation of proinflammatory immunoactive glycation end products that can cause vasculitis (Rumbaugh *et al*, 1971; Rumbaugh *et al*, 1976; Schteinschnaider *et al*, 2000; Treweek *et al*, 2009)
4. prolong cardiac QT interval that may increase the risk for torsades de pointes (Reimherr *et al*, 2007; Yap and Camm, 2003).

The concern for adverse cardiovascular events is higher in the adult population compared to children and adolescents primarily due to comorbid conditions, higher dose of stimulants administered, slower rates of systemic elimination and higher background rate of cardiovascular events (Castle *et al*, 2007; Olfson *et al*, 2008). A recent review of the clinical literature found that MPH and ATO treatments in children and adolescents with ADHD were not associated with increased risk of adverse cardiovascular events (Westover and Halm, 2012). However, caution must be exercised while interpreting these results; since the incidence of cardiovascular events in this age-group is low, the possibility of type-II error (false negative outcome) is high. In contrast to the effects in adolescents, results from studies evaluating cardiovascular events in adults were mixed, such that some indicated that stimulant treatments were safe (Habel *et al*, 2011) while others found an increased risk for adverse cardiovascular events (Holick *et al*, 2009; Schelleman *et al*, 2012). The later reports indicated an increased risk of events, such as transient ischemic attack among individuals prescribed ATO (Holick *et al*, 2009), and increased risk for ventricular arrhythmia and sudden death among those prescribed MPH (Schelleman *et al*, 2012). However, there is a paucity of clinical studies evaluating predefined clinical endpoints such as, myocardial infarction, stroke and death, as primary outcomes as opposed to soft endpoints such as stimulant induced increased blood-pressure or heart-rate. The former more definitive endpoints are particularly important because there isn't a well-established causative relation of the latter soft-endpoints with adverse cardiovascular events (Psaty *et al*, 2001). Therefore,

further studies are required to ascertain whether chronic treatment with MPH and ATO during childhood and adolescence alters the risk of cardiovascular events during adulthood (Westover *et al*, 2012).

1.8.3 Abuse liability

Although MPH has been approved for medical use, it is classified as a schedule II drug by the US Controlled Substance Act (CSA; 21 U.S.C. 811(b), 811(c)). Schedule II controlled substances have high abuse potential (<http://www.deadiversion.usdoj.gov/schedules/index.html>). The Center for Drug Evaluation and Research (CDER) of the Food and Drug Administration (FDA) describes abuse liability as “used in nonmedical situations, repeatedly or even sporadically, for the positive psychoactive effects it produces.” (<http://www.fda.gov/cder/guidance/index.htm>). A survey revealed that children and adolescents prescribed MPH are often approached to sell or trade their medications (Musser *et al*, 1998). The nonprescription use of MPH increased from 1.2% to 2% between 2000-2006, with a doubling of non-prescription use in the college-age students (18-25 yrs old) in this period (Bogle and Smith, 2009). The mechanism of action of MPH is comparable to cocaine, a drug of abuse (Kollins *et al*, 2001). Specifically, both MPH and cocaine inhibit DAT and increase extracellular dopamine in nucleus accumbens, the reward center of the brain (Ritz *et al*, 1987). The brain distribution of MPH and its *in vivo* potency at inhibiting striatal DAT in humans is comparable to that for cocaine (Volkow *et al*, 1995; Volkow *et al*, 1998b). Humans have been shown to self-administer MPH, which produced significant positive effects and drug-liking scores compared to placebo on the Addiction Research Center Inventory (ARCI) subject-rated drug-effect questionnaire (Jasinski *et al*, 2008). Taken together, these results establish that MPH has significant abuse liability, and as a consequence is diverted for non-medical use, including to individuals without ADHD (United States. Substance Abuse and Mental Health Services Administration. Office of Applied Studies., 2006).

An internet-based survey revealed that ~7% of adults report using MPH for non-medical reasons at least once in their life-time (Novak *et al*, 2007). However, the prevalence of nonmedical use was more for short-acting formulations. These results indicate that different mechanisms underlie the therapeutic and reinforcing effects of MPH. As described above (section 1.4.4), stimulants produce have addictive properties by increasing dopamine release in striatum. The therapeutic actions of MPH also depend, at least in part, upon an increase in extracellular dopamine in striatum (section 1.2.3).

One of the factors that differentiate the reinforcing and therapeutic effects of MPH is dose. Low, therapeutically relevant doses of MPH do not act as a stimulant; i.e., do not increase locomotor activity or increase accumbal and striatal extracellular dopamine concentrations (Berridge *et al*, 2006). Rather, low doses of MPH reduce home-cage activity in rodents, and preferentially increase cortical dopamine and norepinephrine concentrations (Berridge *et al*, 2006; Kuczenski *et al*, 2002) thereby resulting in therapeutic efficacy.

Pharmacokinetic profile associated with the route of administration of MPH contributes to the reinforcing effect. Compared to cocaine, intravenously administered MPH resulted in comparable T_{max} and temporal profile of subjective self-reports of “high”; however the half-life of [^{11}C]MPH was longer than that of [^{11}C]cocaine (90 min vs 20 min; Volkow *et al*, 1995). The therapeutically relevant oral route of administration of MPH resulted in a longer T_{max} , and as a consequence, did not produce the subjective self-reported “high” despite comparable increases in extracellular dopamine in striatum found with intravenous administration (Volkow and Swanson, 2003). These results suggest that formulations that result in rapid increase in plasma concentration of MPH have a greater abuse potential compared to formulations that have a low rate of systemic absorption.

A third factor to consider is individual differences; i.e., some individuals are more sensitive to the rewarding and reinforcing effects of MPH than others (Volkow *et al*,

2003). For example, preexisting conditions including history of drug abuse, impulsivity, and ADHD (section 1.4.1) may confer higher abuse liability. Taken together, dose, route of administration and individual differences contribute to the abuse liability of MPH and must be taken into consideration while evaluating the risks of MPH treatment in children and adolescents with ADHD.

1.8.4 Impact of methylphenidate treatment on ADHD/cocaine abuse comorbidity

The high comorbidity of cocaine abuse in individuals with ADHD is well documented in the clinical literature (see section 1.4). Individuals with ADHD have 35% higher incidence of cocaine abuse compared to the general population (Carroll *et al*, 1993; Levin *et al*, 1999). However, the effects of MPH on this comorbidity are highly debated and controversial. Clinical reports suggest that MPH treatment initiated in childhood may be protective against cocaine addiction (Fischer and Barkley, 2003; Wilens *et al*, 2003), or not modify cocaine abuse liability in individuals with ADHD (Molina *et al*, 2013). Another study reported that MPH treated adolescents and adults with ADHD showed higher abuse and dependence on stimulants, including cocaine and tobacco, compared to untreated and age-matched non-ADHD subjects (Lambert and Hartsough, 1998). The latter study has been criticized. The authors did not control for comorbid conduct disorder, which is critical given the strong association of conduct disorder with drug abuse liability (Burke *et al*, 2001). Another study that controlled for lifetime severity of conduct disorder found that MPH treatment >1 year during childhood was associated with greater likelihood of ever using cocaine, but only in the presence of comorbid conduct disorder (Barkley *et al*, 2003). In contrast, a study that excluded children with conduct disorder found a positive correlation between age of initiation of MPH treatment and cocaine abuse during adulthood (Mannuzza *et al*, 2008), such that lifetime rates of cocaine abuse were higher when treatment was initiated in early adolescence. Thus, the age of initiation of MPH treatment may be a critical factor modulating cocaine abuse liability in individuals with ADHD.

Adolescence is an vulnerable developmental period, and treatment with stimulant medication at this age may lead to several lasting detrimental outcomes (Spear, 2000b); also see section 1.5.5). ADHD is typically diagnosed during childhood, especially given the diagnostic criteria that ADHD symptoms and impairment should be “present before age 7 years” (American Psychiatric Association., 2000). However, several factors, including ADHD subtype and socioeconomic status, may delay the age of ADHD diagnosis (Froehlich *et al*, 2007), and consequently, delay the age of initiation of MPH treatment. Also, older children (12-15 years) have a greater likelihood of initiating treatment compared to younger children (Froehlich *et al*, 2007). Another study reported that although the typical age of ADHD diagnosis was <8 years, ~12% of newly diagnosed ADHD individuals were in the 12-17 years age range (Chen *et al*, 2011). Further, later initiation of MPH treatment in this cohort was associated with increased discontinuation of treatment. A recent review documented high rates of non-adherence to ADHD medications and peak rates of discontinuation of MPH after 5 years of treatment initiation in children and adolescents (Adler and Nierenberg, 2010). Taken together, these studies suggest that initiation of MPH treatment during adolescence as well as discontinuations of MPH treatment are common clinical observations, and its impact on drug abuse liability needs to be evaluated.

Another question raised by the clinical studies is why did specifically cocaine abuse liability stand out in these studies with MPH treatment in ADHD subjects? The commonalities in the mode of action of MPH and cocaine may underlie, in part, the increased cocaine abuse liability following MPH treatment. Both drugs are psychostimulants that block DAT and NET and increase extracellular dopamine concentration in brain reward circuitry (see section above, 1.8.3). Thus, MPH treatment may lead to enhanced sensitivity for later stimulant exposure, which is reported to be a predictor of cocaine dependence and life-time use (Lambert *et al*, 2006). However, whether enhanced sensitization for cocaine preceded cocaine abuse in MPH-treated individuals is difficult to address in clinical studies; preclinical studies with carefully

controlled experimental conditions are advantageous for systematically addressing these questions.

1.8.5 Relevance of adolescence

The developing brain is more vulnerable to pharmacological insult than the fully developed brain, and drugs at doses well below the toxic concentrations are capable of producing long-lasting alterations in a developmentally malleable system. Adolescence represents a developmentally critical period for the neurocircuitry regulating motivation and decision-making (Chambers *et al*, 2003). Because of reduced impulse control, adolescence is associated with enhanced sensation seeking and risk-taking behaviors (Adriani and Laviola, 2004; Arnett, 1992). Furthermore, first use of psychoactive substances during early adolescence has been associated with higher life-time rates of drug-use, rapid development of dependence and higher rates of psychopathological comorbidities (Clark *et al*, 1998). However, these effects are a consequence of a combination of biological and social factors, and as such, animal models provide a better control to evaluate the contribution individual factors associated with enhanced drug abuse vulnerability during adolescence (Adriani *et al*, 2004).

An MRI study revealed age-related differences in neuronal activation patterns following acute MPH in adult and adolescent outbred rats (Canese *et al*, 2009). Specifically, in adult rats, a high dose MPH (4 mg/kg, i.p.) increased BOLD signals in NAc and PFC. In contrast, in adolescent rats, the same MPH dose reduced BOLD signal in NAc and PFC, suggesting that neurological effects of pharmacological manipulations differ between adolescent and adult rats.

Development of higher cognitive function, including inhibitory control occurs throughout adolescence concurrently with the maturation of PFC (Casey *et al*, 2005). Specifically, maturation of cognitive capacity coincides with extensive synaptic pruning of glutamatergic pyramidal neurons in PFC and simultaneous strengthening of the

remaining synaptic connections. For example, during early adolescence, a transient increase in D1 receptor density suppresses cortico-amygdalar emotional control and simultaneously, increases cortical drive controlling reward-related behavior. (Casey *et al*, 2010; Ernst *et al*, 2009; Somerville *et al*, 2010a; Somerville *et al*, 2011). Fine-tuning of cortical drive is achieved with synaptic pruning and selective down regulation of D1 receptors (Brenhouse *et al*, 2008; Doremus-Fitzwater *et al*, 2010), leading to a decrease in impulsivity by adulthood. Expression of D2 and D4 receptors in PFC are also upregulated between PND7-28, and subsequently, downregulated between PND35-60 (Tarazi *et al*, 2000). DAT expression in the cortical areas is sparse; limitations of detection of the currently available methods, such as autoradiographic binding, do not provide information about the developmental changes in the cortical areas during adolescence (Moll *et al*, 2000). Chronic treatment with MPH during adolescence may alter the developmental trajectory of cortical dopaminergic system.

During adolescence, the reward circuitry also undergoes changes; in the rodent NAc, dopamine D1, D2 and D4 receptor density peaks by 4 weeks of age and is subsequently pruned in early adulthood (PND60; Tarazi *et al*, 2000). In contrast, DAT density in the NAc and dorsal striatum peaks by PND60 and does not get down regulated in outbred rats (Tarazi *et al*, 1998). Since the nigrostriatal pathway and the mesolimbic pathways are implicated in motor activity and altered responsiveness to reward-related learning in ADHD (see section 1.2.3), MPH treatment also alters function in these areas. Taken together, the development-related neuronal plasticity confers additional vulnerability, such that MPH at doses well below stimulant concentrations may permanently enhance sensitivity for future drug abuse vulnerability.

Chronic treatment with low doses of MPH during adolescence was associated with decreased cocaine CPP, and decreased cocaine self-administration in adult outbred rats (Carlezon *et al*, 2003; Thanos *et al*, 2007). In contrast, another study reported that a low dose of MPH during early adolescence did not alter psychomotor response to cocaine, but increased cocaine self-administration evaluated during late adolescence

(Brandon *et al*, 2001). Furthermore, MPH treatment in juvenile primates followed by treatment discontinuation did not enhance cocaine self-administration during adulthood (Gill *et al*, 2012). A limitation of these studies was that the subjects involved did not have an ADHD phenotype. Chronic treatment with MPH during adolescence may produce different effects in subjects with an already compromised dopaminergic system, as found in patients with ADHD. Thus, subjects with the ADHD-like phenotype are needed to evaluate the effect chronic MPH treatment during adolescence on future cocaine abuse liability.

Studies using the SHR model for ADHD found several interesting results with MPH treatment. Chronic administration of MPH (0.6 mg/kg, i.p.) during adolescence did not cause sensitization in adolescent rats, but led to sensitization in adult rats (Barron *et al*, 2009). In contrast, MPH treatment (2.5 mg/kg, i.p.) during adolescence (PND35-45) led to decreased CPP for cocaine in adult SHRs (Augustyniak *et al*, 2006). Another study found that MPH (1 mg/kg, i.p.) treatment during peri-adolescence (PND 21-35) decreased CPP for MPH in adolescent SHR (>PND 45) and increased CPP in adolescent Wistar rats (dela Pena *et al*, 2012a). CPP evaluates rewarding effects of drugs; however, self-administration is the strongest predictor of abuse liability of drug (McCrae and Costa, 1995). In adult rats, MPH pretreatment (2 mg/kg, i.p.; 14 days) did not alter MPH self-administration in SHR, but increased MPH self-administration in adult Wistars (dela Pena *et al*, 2012b). In contrast, when MPH (1.5 mg/kg, p.o.) was administered during adolescence (PND 28-55), SHRs showed increased cocaine self-administration during adulthood (PND >77) (Harvey *et al*, 2011). Specifically, compared to WKY and WIS controls, SHR treated with MPH orally (1.5 mg/kg) during adolescence exhibited more rapid acquisition of cocaine self-administration, greater responding across a range of cocaine doses, and higher progressive ratio breakpoints (refer Fig 1.6). The increase in cocaine self-administration was associated with decreased DAT function in whole prefrontal cortex, which includes both mPFC and OFC. mPFC and OFC are distinct based on cytoarchitecture and behavioral function, but are also highly interconnected (Gilbert and Burgess, 2008; Lawrence *et al*, 2009). Taken together, MPH treatment during

adolescence increased later vulnerability to cocaine addiction and altered cortical DAT function may underlie these effects. Furthermore, these reports emphasize that a rat strain exhibiting an ADHD phenotype is required for addressing a key controversial question regarding the impact of MPH treatment during adolescence on later cocaine addiction vulnerability. Further studies are required for evaluating behavioral and neurochemical mechanisms mediating the increased cocaine self-administration in SHR to inform therapeutic strategies for adolescents with ADHD and to protect against future cocaine abuse.

Methylphenidate

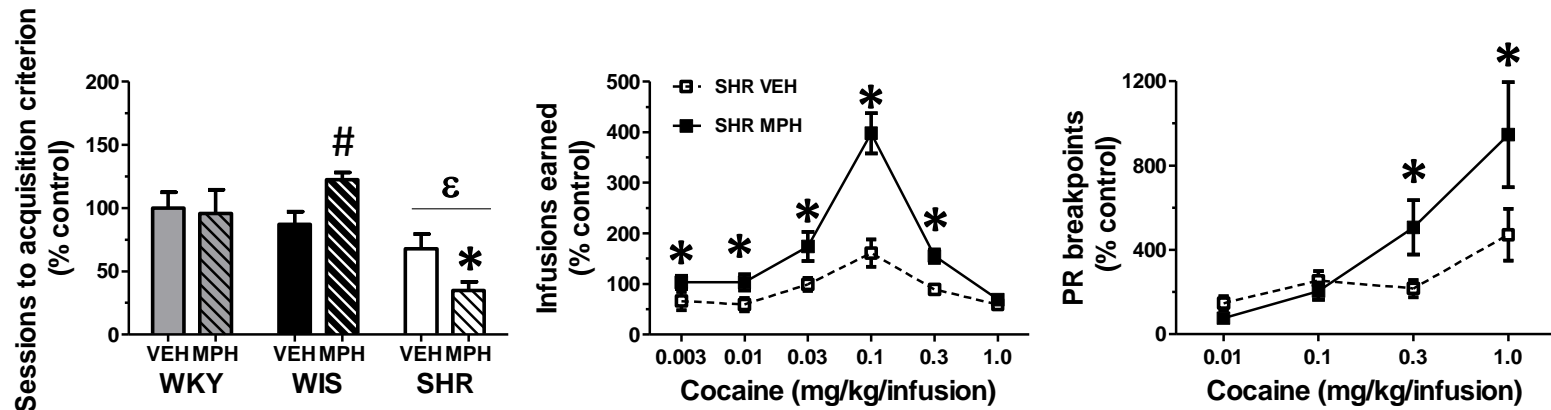


Fig 1.6. Cocaine self-administration: In adult SHR, discontinuation of methylphenidate (MPH) treatment after adolescence expedites acquisition (left) of cocaine self-administration, and leads to upward shifts in the cocaine dose-response under Fixed-Ratio 1 (center) and Progressive Ratio (PR) breakpoint (right) schedules, indicating that cocaine is a more efficacious reinforcer in SHR. In adult WIS, discontinuation of MPH treatment after adolescence increases the number of sessions to acquisition criterion. Data are presented as percentage of WKY-VEH control (left) and as a percentage of WKY-VEH receiving cocaine 0.3 mg/kg/infusion as control (center and right). * $p < 0.05$ compared SHR-VEH, WIS-MPH and WKY-MPH; # $p < 0.05$ compared to respective vehicle control, $\epsilon p < 0.05$ main effect of strain, compared to WKY and WIS. Figure adapted from Harvey et al, 2011.

1.9 Atomoxetine

Stimulants such as MPH and amphetamine have been the first line of pharmacotherapy for ADHD in children, adolescents and adults (Dodson, 2005; Rosler *et al*, 2010; 2009). Stimulants are efficacious controlling ADHD symptoms in ~70% patients (Spencer *et al*, 1996). However, about 10-30% of the individuals with ADHD do not benefit from either MPH or amphetamine treatments as they are non-responders or intolerant to psychostimulant therapy (Barkley, 1977; Elia *et al*, 1991). Psychostimulants are controlled substances with documented abuse liability (Holman, 1994). As a consequence, there is a need to develop pharmacotherapies with different mechanism of action that may not have abuse liability.

Increase in extracellular dopamine and norepinephrine in the PFC which is associated with the therapeutic efficacy of stimulants, while increase in dopamine in the nucleus accumbens contributes to the reinforcing effects of stimulants (Gamo *et al*, 2010; Heal *et al*, 2008). Selective inhibitors of NET increase extracellular dopamine and norepinephrine in the PFC, but not in the nucleus accumbens (Bymaster *et al*, 2002; Moron *et al*, 2002). Thus compared to stimulants, NET inhibitors exhibit a neurochemical profile that mitigates ADHD symptoms without the risk of abuse and diversion.

Tricyclic antidepressants such as desipramine and nortriptyline, which have a higher affinity for NET compared to DAT and serotonin transporters (Wong *et al*, 1995) emerged as “off-label” therapeutics for ADHD (Biederman *et al*, 1989; Spencer *et al*, 1996; Wilens *et al*, 1996). However, tricyclic antidepressants are associated with persistent side effects such as cognitive impairments, sedation, dry mouth, weight-gain and cardiovascular events due to their affinity for α 1-adrenergic receptors, cholinergic receptors and histaminergic receptors (Cookson, 1993; Walsh *et al*, 1994; Wong *et al*, 1995). Subsequent efforts to identify an ADHD therapeutic with improved selectivity for NET led to ATO (Spencer *et al*, 1998).

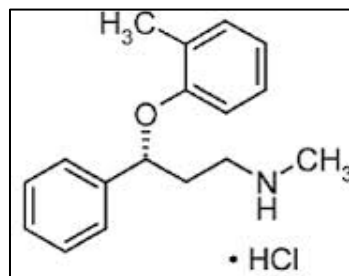


Figure 1.7 Atomoxetine

Alternate names: tomoxetine, LY139603. IUPAC (3*R*)-*N*-methyl-3-(2-methylphenoxy)-3-phenylpropan-1-amine; other chemical names - (*R*)-*N*-methyl-3-phenyl-3-(*o*-tolylloxy)propan-1-amine; (+/-)-*N*-methyl-gamma-(2-methylphenoxy) phenylpropylamine hydrochloride.

ATO was efficacious in the treatment of ADHD over placebo in adults (Spencer *et al*, 1998). Also, ATO reduced core symptoms of ADHD in children and adolescence, produced a graded dose-response, and was found to be well tolerated in this age group (Michelson *et al*, 2001; Spencer *et al*, 2001). The efficacy of ATO was found to be comparable to the immediate release formulations of MPH, but was less than that of extended-release formulation OROS MPH (Garnock-Jones and Keating, 2009).

1.9.1 Mechanism of action

ATO is a selective, potent competitive inhibitor of NET with a K_i of 0.1 nM inhibiting norepinephrine uptake into rat hypothalamic synaptosomes (Bolden-Watson and Richelson, 1993; Wong *et al*, 1982). The latter studies also demonstrated that ATO was highly selective for NET. Specifically, ATO has very low affinity for DAT and serotonin transporters, with K_i 's of ~1600 nM and ~750 nM, respectively, for inhibiting monoamine uptake. With respect to noradrenergic receptors, the IC_{50} for inhibition of binding of [3H]WB4101 to α_1 -adrenergic receptors and binding of [3H]clonidine to α_2 -

adrenergic receptors on membranes isolated from crude synaptosomal fractions of rat cerebral cortex were 10 and 56 μM , respectively. IC_{50} for inhibition of [^3H]pyrilamine to histamine H_1 receptor, and of [^3H]3-quincylindinyl benzilate to muscarinic receptors was 6.4 and 21 μM , respectively. ATO at 10 μM failed to inhibit binding of [^3H]serotonin into rat PFC. Furthermore, ATO produced less than 10% inhibition in binding of [^3H]dihydroalprenolol, flunitrazepam and GABA in membranes from calf cerebellum, suggesting that ATO has little to no affinity for β -adrenergic receptors, GABA_A and other GABAergic receptors (Wong *et al*, 1982).

ATO inhibited radioligand binding in membrane preparations from heterologous expression systems transfected with human NET, SERT and DAT with K_i values of 5, 77 and ~ 1450 nM, respectively (Bymaster *et al*, 2002), suggesting that the mechanism of action of ATO is comparable between rats and humans. Using *in vivo* microdialysis in freely moving rats, ATO (0.3 to 3 mg/kg i.p.) was reported to increase extracellular norepinephrine in PFC to $\sim 300\%$ of basal NE concentrations (Bymaster *et al*, 2002). ATO also increased cortical extracellular dopamine concentrations to $\sim 300\%$ of basal DA concentrations (Bymaster *et al*, 2002). The extracellular 5-HT concentration in PFC was not significantly increased by ATO at doses up to 3 mg/kg i.p. (Bymaster *et al*, 2002). Further, ATO (3 to 10 mg/kg i.p.) did not increase extracellular dopamine in the nucleus accumbens or striatum of freely moving rats (Bymaster *et al*, 2002). Taken together, these results suggest that ATO increased extracellular dopamine in PFC by inhibiting uptake of dopamine into noradrenergic terminals via NET (Moron *et al*, 2002).

1.9.2 Pharmacology

NET inhibition by ATO increases extracellular dopamine and norepinephrine concentration in the PFC. ATO exerts its therapeutic action via enhanced catecholamine neurotransmission in PFC (Bymaster *et al*, 2002; Gamo *et al*, 2010). ATO reduces impulsivity in outbred rats evidenced by reduced premature responding using 5-CSRTT

and increased preference for a delayed larger reward in a delay-discounting task (Robinson *et al*, 2008a). ATO also improved behavioral flexibility in rats with noradrenergic-lesions in the mPFC (Newman *et al*, 2008). ATO also decreased locomotor activity of SHRs, but not WKY, in open- field chambers (Umehara *et al*, 2013a). Taken together, these results from preclinical studies suggest that ATO ameliorates ADHD related symptoms.

Increased extracellular dopamine concentration in PFC activates D1 receptors and enhances working memory (Sawaguchi *et al*, 1994). Further, activation of cortical areas impacts neuronal projections to subcortical areas such as striatum, VTA and NAc (Taber *et al*, 1995; Taber and Fibiger, 1993). Thus, deficits in subcortical function associated with ADHD, such as altered processing of reward and reinforcement (Sagvolden *et al*, 1998a; Williams *et al*, 2009b) are ameliorated, partially, by activation of D1 receptors in the PFC.

Increased cortical norepinephrine by ATO leads to activation of α 2A receptors (Gamo *et al*, 2010), which in turn mediates improvement in PFC function (Arnsten *et al*, 2005). Activation of α 2A receptors decreases intracellular cAMP, disinhibits cAMP-HCN channels and thereby result in enhanced strength and duration of firing of cortical pyramidal neurons (Wang *et al*, 2007). The latter mechanism of action is thought to underlie the efficacy of ADHD medications for increasing attention and reducing impulsivity and working memory deficits (Sagvolden, 2006; Wang *et al*, 2007).

A MRI study using outbred Sprague Dawley rats found negative BOLD response in the caudate putamen (dorsal striatum), which may indicate decreased neural activity in the striatum (Allison *et al*, 2000; Easton *et al*, 2007). Further, negative BOLD response in the brain areas associated with the cortico-basal thalamic loop circuit and positive BOLD response in the OFC was obtained following acute ATO administration (Easton *et al*, 2007). These results, although observed in anesthetized outbred rats, may indicate the pharmacological mechanisms by which ATO reduces hyperactivity and enhance behavioral control in ADHD.

Alternate mechanisms which may contribute to enhanced attention and working memory following ATO (1 mg/kg, i.p.) treatment are increased extracellular acetylcholine and histamine in the mPFC (Horner *et al*, 2007; Tzavara *et al*, 2006). The ATO-induced increase in cortical acetylcholine was reversed by SCH23390, a D1 antagonist and by prazosin, an α 2A antagonist (Tzavara *et al*, 2006). Thus, ATO induced increased cortical catecholamines may mediate the increased extracellular acetylcholine. Taken together, these results suggest that ATO treatment recruits several neurological circuits to produce its therapeutic efficacy, and that increased cortical dopamine and norepinephrine is central to the mechanism of action of ATO.

1.9.3 Abuse liability

As a screening strategy for abuse liability, CDER recommends several binding studies to evaluate the interaction of new drugs with neurotransmitter systems associated with abuse potential (<http://www.fda.gov/cder/guidance/index.htm>), including dopamine, norepinephrine, serotonin, GABA, acetylcholine, opioid, NMDA and cannabinoids. Based on the results from the recommended binding studies (Bymaster *et al*, 2002), ATO was approved by the US FDA as an uncontrolled, non-stimulant treatment for pediatric adolescent and adult ADHD.

In terms of direct effects on the reward circuitry, ATO does not increase extracellular dopamine concentrations in NAc or striatum (Bymaster *et al*, 2002). These microdialysis results have a high translational validity for predicting abuse liability since increases in extracellular dopamine concentrations and DAT occupancy in striatum are closely related to reinforcing effects (Murnane and Howell, 2011). In contrast to the stimulant MPH, ATO increased the number of Fos-positive cells in PFC, but not in striatum or nucleus accumbens (Bymaster *et al*, 2002). Taken together, these results support the interpretation that ATO exerts its pharmacotherapeutic action via

modulation of catecholaminergic neurotransmission locally in cortex, and not via activation of the mesocorticolimbic dopaminergic system.

Preclinical behavioral studies including self-administration and drug-discrimination paradigms are used typically for evaluating abuse potential. Using drug discrimination preparations in rats and rhesus monkeys, ATO generalized to cocaine only under conditions employing low training doses of cocaine, but not when cocaine doses were increased to levels that produce psychomotor stimulation (Terry *et al*, 1994). Also, high doses of ATO substituted for methamphetamine in squirrel monkeys; however, these doses produced substantial decreases in response rates (Tidey and Bergman, 1998), suggesting a non-specific behavioral suppression at high doses. In contrast to ATO, the stimulant MPH produced dose-related cocaine-appropriate responding with complete substitution for cocaine in drug discrimination studies (McCrae and Costa, 1986a). In self-administration studies, ATO did not support self-administration, whereas MPH served as positive reinforcer at doses of 0.03 mg/kg, i.v. (Gasior *et al*, 2005). Taken together, these results also suggest limited abuse liability of ATO.

In a cohort of non-dependent, light drug users, the subjective effects of ATO (20, 45 and 90 mg) were not different from placebo, except for a significantly higher “bad” and “sick” score at the highest dose (Heil *et al*, 2002). In another cohort of stimulant-preferring individuals with a history of drug abuse, significant “liking” scores were reported with MPH (90 mg dose), but not with ATO (45, 90 and 180 mg dose) (Jasinski *et al*, 2008). Further, abrupt discontinuation of ATO in children and adults with ADHD was not associated with drug withdrawal syndrome (Wernicke *et al*, 2004), suggesting that ATO treatment does not lead to drug dependence. To date, there are no published studies evaluating abuse potential of ATO in individuals with ADHD. Taken together, converging evidence from *in vitro* and *in vivo* animal studies and from human studies supports that the low-abuse liability of ATO, indicating that this medication is a valuable alternative for patients who choose not to be treated with a controlled substance.

1.9.4 Impact on ADHD/cocaine abuse comorbidity

ATO does not increase dopamine in NAc or the striatum (Bymaster *et al*, 2002). In a clinical study evaluating the discriminative and subjective effects of ATO in subjects with a substance abuse history, ATO at a 90 mg dose significantly increased drug-appropriate responding for MPH relative to placebo. This partial substitution of ATO for MPH may suggest also that ATO is a useful candidate for a replacement therapy for stimulant abuse (Lile *et al*, 2006). In cocaine-dependent volunteers, ATO was well tolerated, did not alter cocaine pharmacokinetics, improved working memory and sustained attention, and decreased the subjective effects of cocaine (Cantilena *et al*, 2012), further supporting the utility of ATO as an agonist replacement therapy for cocaine abuse.

ATO is effective as an ADHD therapeutic and has favorable safety profile and negligible risk of abuse or misuse in clinic (Garnock-Jones *et al*, 2010). ATO reduced symptoms in adult ADHD patients with comorbid ethanol abuse/dependence (Wilens *et al*, 2008). In the same cohort, reductions in ADHD symptoms were not altered despite relapse to alcohol abuse (Wilens *et al*, 2011). In a small cohort of cocaine dependent subjects comorbid for ADHD, ATO reduced ADHD symptoms, but the high drop-out rate from the study of cocaine-dependent subjects and the lack of effect on cocaine use through the trial, indicates that the utility of ATO in this patient population may be limited (Levin *et al*, 2009). Thus far, no clinical studies have been reported that determine whether ATO treatment of individuals with ADHD prospectively alters liability for future cocaine abuse liability. Using a heuristically useful animal model of ADHD, chronic ATO treatment during adolescence was not found to alter cocaine self-administration during adulthood compared to vehicle control (Somkuwar *et al*, 2013b); Fig 1.8). Taken together, these results suggest that ATO may be a valuable alternative for ADHD patients at risk for substance abuse or who choose not to be administered a controlled substance.

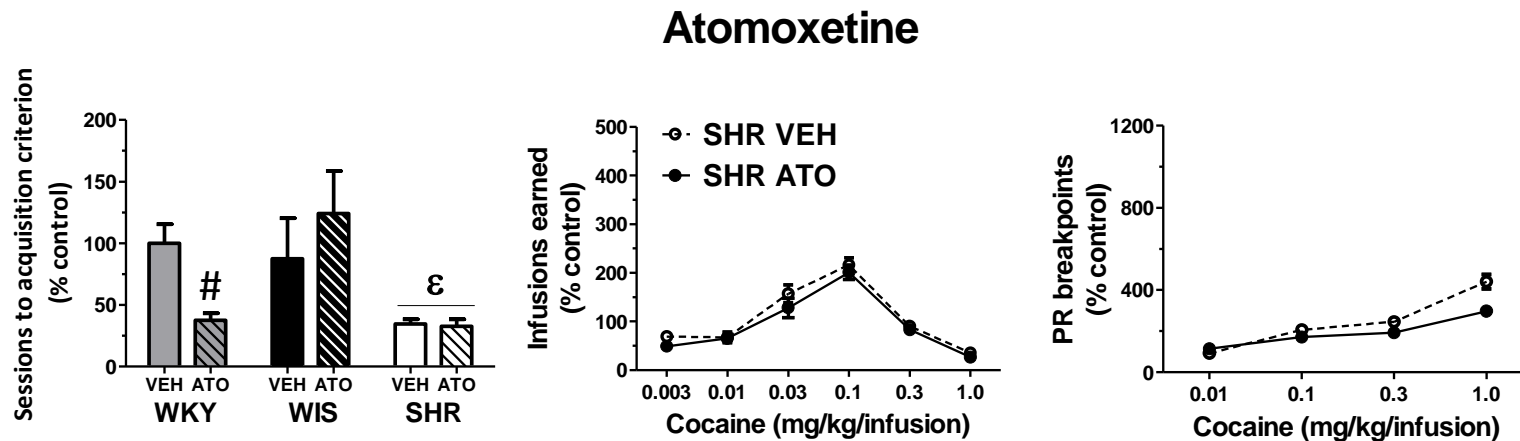


Figure 1.6 Cocaine self-administration in adult rats following atomoxetine treatment during adolescence

In adult SHR, discontinuation of atomoxetine (ATO) treatment after adolescence does not alter acquisition (left) of cocaine self-administration, dose-response of cocaine under fixed-ratio 1 (center) and progressive ration (PR; right) schedules. SHR required fewer sessions to acquire cocaine self-administration, and showed upward shifted dose-response for cocaine under fixed-ratio 1 and PR schedules compared to WKY and WIS (data not shown). In adult WKY, discontinuation of ATO treatment after adolescence decreased the number of sessions to acquisition of cocaine self-administration. Data are presented as percentage of WKY-VEH control (left) and as a percentage of WKY-VEH receiving cocaine 0.3 mg/kg/infusion as control (center and right). * $p < 0.05$ compared SHR-VEH, WIS-MPH and WKY-MPH; # $p < 0.05$ compared to respective vehicle control, $\epsilon p < 0.05$ main effect of strain, compared to WKY and WIS. Figure adapted from Somkuwar et al., 2013.

1.10 Overall hypothesis and specific aims

Mechanisms underlying the high cocaine abuse liability in ADHD are not well understood. Clinical studies are ambiguous about whether MPH treatment in children and adolescents with ADHD increase or decrease cocaine abuse liability. Thus far, no clinical studies have been reported that show that ATO treatment in individuals with ADHD alters liability for future cocaine abuse. Our previous studies using the Spontaneously Hypertensive Rats (SHR), a validated animal model of ADHD, also model the high cocaine abuse liability reported in ADHD (Harvey *et al*, 2011). Specifically, SHRs acquired cocaine self-administration faster and showed greater motivation for lower doses of cocaine compared to control strains Wistar-Kyoto and Wistar. Treatment of adolescent rats (PND28-55) with a therapeutically relevant dose of MPH (1.5 mg/kg/day, p.o.), followed by discontinuation of treatment after adolescence led to an enhanced cocaine self-administration during adulthood (>77 days old) in the MPH-treated SHR compared to vehicle-administered SHR as well as MPH-treated Wistar-Kyoto and Wistar rats (Harvey *et al*, 2011) see Fig 6). Specifically, MPH treatment reduced the number of sessions to acquire cocaine self-administration in SHR, with upward shifts in cocaine dose-response curve under fixed-ratio and progressive-ratio schedules. Our study also found that MPH treatment decreased DAT function in PFC of adult SHR, but did not alter DAT function in striatum. Using an identical experimental design, we showed that ATO treatment (0.3 mg/kg/day, i.p.) did not increase cocaine self-administration in adult SHR (Somkuwar *et al*, 2013b), see Fig 8). The purpose of this dissertation research is to identify neurochemical and behavioral mechanisms associated with the increased cocaine self-administration in MPH-treated SHRs, and determine the protective effects of ATO treatment from further exacerbating cocaine self-administration in SHR.

The overall hypothesis of this dissertation research is that in SHR, MPH treatment during adolescence increases cocaine self-administration in SHR which is associated with

- increased DAT function and cell surface expression in the mPFC and OFC,
- altered NET function in the mPFC and OFC
- increased sensitivity to the psychomotor effects of cocaine
- increased impulsivity under differential reinforcement of low rate schedule

ATO treatment, on the other hand, will not increase cocaine-self administration in SHR and will not increase DAT function in mPFC and OFC.

Hypothesis 1: MPH treatment during adolescence, followed by treatment discontinuation, will increase DAT function and cell surface distribution in the mPFC and OFC of adult SHR, while ATO will not.

Specific Aim 1 (Chapter 2) Determine the effects of MPH during adolescence on DAT function and cellular distribution in mPFC, OFC and striatum during adulthood. DAT function was evaluated using [³H]dopamine uptake and synaptosomal preparation, DAT cellular distribution was evaluated using biotinylation of synaptosomal preparations followed by western blotting analyses.

Specific Aim 2 (Chapter 3) Determine the effects of ATO during adolescence on DAT function and cellular distribution in mPFC, OFC and striatum during adulthood. DAT function was evaluated using [³H]dopamine uptake and synaptosomal preparation, DAT cellular distribution was evaluated using biotinylation of synaptosomal preparations followed by western blotting analyses.

Hypothesis 2: MPH treatment during adolescence, followed by treatment discontinuation, will alter NET function in the mPFC and OFC of adult SHR.

Specific Aim 3 (Chapter 4) Determine the effects of MPH during adolescence on NET function in mPFC, OFC and striatum during adulthood.

- a. Optimize an *in vivo* voltammetry protocol for evaluating clearance of exogenously applied norepinephrine from mPFC and OFC of anesthetized rats
- b. Determine NET function in the mPFC and OFC of adult rats that were treated with MPH or vehicle during adolescence.

Hypothesis 3: MPH treatment during adolescence, followed by treatment discontinuation, will increase sensitivity to the psychomotor effects of cocaine in adult SHR

Specific Aim 4 (Chapter 5) Determine the effects of MPH treatment during adolescence on

- a. sensitivity to cocaine-induced locomotor activation
- b. development of sensitization to repeated cocaine administration
- c. expression of sensitization to the locomotor effects of cocaine

Hypothesis 4: MPH treatment during adolescence, followed by treatment discontinuation, will increase impulsivity in adult SHR

Specific Aim 5 Determine the effects of MPH during adolescence on impulsivity during adulthood using differential reinforcement of low-rate (DRL) schedules.

Chapter 6 describes a set of experiments were conducted to optimize the assay and analysis to identify effects of strain and treatment on behavior;

- a. Determine strain differences between SHR, Wistar-Kyoto and Wistar using two DRL schedules and optimize the analysis of impulsivity endophenotypes using mathematical modeling approach

- b. Determine the effects of MPH treatment in adolescent rats on impulsivity endophenotypes

Chapter 7 describes experiments that were designed to address hypothesis;

- c. Determine the effects of chronic MPH treatment in adolescent rats on impulsivity during adulthood
- d. Determine the effects of chronic MPH treatment in adult rats on impulsivity during adulthood

2 CHAPTER TWO

Adolescence methylphenidate treatment in a rodent model of attention deficit/hyperactivity disorder: Dopamine transporter function and cell surface distribution in adulthood

Portions of this chapter have been published in the manuscript:

Somkuwar S.S., Katak K.M., Dwoskin L.P. Adolescence methylphenidate treatment in a rodent model of attention deficit/hyperactivity disorder: Dopamine transporter function and cellular distribution in adulthood. *Biochemical Pharmacology*, 2013 Jul; 86(2):309-16.

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2.1 Introduction

Attention deficit/hyperactivity disorder (ADHD) is a highly debilitating, heterogeneous disorder typically diagnosed in childhood, and characterized by age-inappropriate levels of inattention, impulsivity and hyperactivity. The neurobiological etiology of this disease is not understood completely (Tripp *et al*, 2009). ADHD is ascribed partially to dopaminergic deficits in prefrontal cortex (Arnsten, 2009; Levy, 2009; Sonuga-Barke, 2005). Further, ADHD is associated with increased dopamine transporter (DAT) expression in striatum and with specific polymorphisms in the DAT gene (Faraone *et al*, 2005; Mill *et al*, 2002).

The Spontaneously Hypertensive Rat (SHR) is the most widely accepted rodent model of ADHD and displays all of the behavioral diagnostic characteristics of ADHD (Katak *et al*, 2008; Sagvolden *et al*, 2005b). SHR display diminished dopamine (DA) release from prefrontal cortical and striatal slices *in vitro* (Russell *et al*, 1995), but greater DA release from nucleus accumbens *in vivo* (Heal *et al*, 2008). Also, DAT

function and expression levels in frontal cortex and striatum were greater in SHR than in control rats (Pandolfo *et al*, 2012; Roessner *et al*, 2010). Taken together, these previous findings strengthen the predictive value of the SHR for evaluating the consequences of long-term pharmacotherapeutic treatment of ADHD on DA neurochemistry and related behaviors.

Methylphenidate (MPH; Ritalin[®]) is a gold standard treatment for ADHD, providing successful relief from ADHD symptoms. In SHR, MPH improves attention and decreases hyperactivity (Kantak *et al*, 2008; Sagvolden *et al*, 2005b). In terms of underlying neurochemical mechanisms of action, MPH acts as an inhibitor of striatal DAT function and prefrontal cortical DAT and norepinephrine transporter (NET) function, increasing extracellular DA concentrations and DA receptor occupancy (Andersen, 1989; Engert *et al*, 2008; Richelson *et al*, 1984; Volkow *et al*, 2005a). Furthermore, MPH decreases basal firing rates of striatal neurons (Engert *et al*, 2008), which has been suggested to strengthen corticostriatal signals, thus contributing to its pharmacological effects. Based on these findings, DAT is critically involved in the dopaminergic dysfunction associated with ADHD and serves as an important molecular target for the treatment of ADHD.

Adults with ADHD have been reported to have a higher risk of developing substance use disorders compared to individuals without ADHD (Wilens *et al*, 1998b). In comparison to the general population, those with ADHD have a 35% higher incidence of cocaine abuse (Levin *et al*, 1999). However, the impact of prior treatment with MPH on cocaine abuse liability in this population is controversial. While some studies suggest MPH treatment is protective against cocaine abuse (Fischer *et al*, 2003; Wilens *et al*, 2003; Winters *et al*, 2011), others indicate that MPH exposure during adolescence may increase cocaine abuse liability (Barkley *et al*, 2003; Lambert *et al*, 1998; Mannuzza *et al*, 2008). Also, the mechanisms underlying the high comorbidity between ADHD and cocaine abuse are not well understood. Since cocaine competitively inhibits DAT, which leads to a compensatory increase in DAT cell surface expression and function (Daws *et*

al, 2002; Huang *et al*, 2009; Mandt and Zahniser, 2010), one explanation for the comorbidity of ADHD and cocaine abuse may be a greater DAT expression and function in these individuals.

Another explanation for the comorbidity of ADHD and cocaine abuse may be the preexisting impairments in cortically-controlled executive function, including increased impulsivity and risk-taking behavior in this population (Groman *et al*, 2009; Winstanley *et al*, 2010a). Compared to control subjects, boys with ADHD showed decreased orbitofrontal cortex (OFC) activation using fMRI during a delayed discounting task that measures impulsivity (Rubia *et al*, 2009a). Also, decreased dorsolateral prefrontal cortex (DLPFC) activation was associated with working memory deficits in ADHD individuals compared to controls (Burgess *et al*, 2010). Unmedicated ADHD individuals also showed increased deactivation of fMRI signals in the medial prefrontal cortex (mPFC) assessed during the Stroop test with emotional interference, compared to medicated ADHD and control individuals (Posner *et al*, 2011). With respect to understanding molecular mechanisms in cortical areas involved in ADHD, it is important to note that the DLPFC in primates is considered to be functionally analogous to the subregions of mPFC in rodents, including the Fr2 and the anterior cingulate cortex (Uylings *et al*, 2003). Thus, functional impairments in these cortical regions (OFC, DLPFC and mPFC) are strongly associated with behavioral deficits in ADHD; however, few studies have evaluated molecular mechanisms in the OFC and mPFC of SHR.

Of importance, cocaine abuse is associated with impulsive behavior and with lasting neurochemical changes in these same cortical regions (Beveridge *et al*, 2008). Individuals abusing cocaine display reduced response inhibition in the Stroop test and increased glucose metabolism in OFC, compared with demographically-matched controls (Goldstein *et al*, 2001). In cocaine users, reduced inhibitory control in the Go-No Go tasks was associated with decreased mPFC activation using fMRI (Kaufman *et al*, 2003). In contrast, increased activation of DLPFC was found in cocaine-dependent individuals during the Stroop test and in response to cocaine-related cues (Brewer *et al*,

2008; Maas *et al*, 1998). With respect to animal models, outbred rats self-administering cocaine showed decreased cocaine-seeking and taking behavior following inactivation of OFC and mPFC (Di Pietro *et al*, 2006; Grakalic *et al*, 2010; Kantak *et al*, 2013; Mashhoon *et al*, 2010), thereby supporting the critical involvement of OFC and mPFC function in cocaine abuse. Thus, both impulsivity and functional impairments of the OFC, DLPFC and mPFC are associated with ADHD and cocaine abuse, and these commonalities may in part underlie their comorbidity.

Valuable insights into the mechanisms underlying comorbidity of ADHD and cocaine abuse may be obtained using an animal model of ADHD that exhibits high cocaine self-administration behavior. However, appropriate interpretation of results obtained from ADHD rat models with strong face validity, such as SHRs, requires cautious selection of suitable reference control groups (Sagvolden *et al*, 2009). Studies that employ the SHR-progenitor strain, Wistar-Kyoto (WKY) as the only reference control (Sagvolden *et al*, 2005b) have been criticized. For example, hyperactivity in SHR appears to have been overestimated when the only control employed was WKY, because WKY are hypoactive relative to outbred control rats (Alsop, 2007a; van den Bergh *et al*, 2006). Neurochemical studies also reveal differences between WKY and outbred control rat strains. WKY show altered dopaminergic function compared to outbred Wistar (WIS) rats (e.g., decreased striatal DA D1 and D2 receptors and increased striatal D3 receptors (Novick *et al*, 2008; Yaroslavsky *et al*, 2006); decreased accumbal DAT expression (Jiao *et al*, 2003); and decreased mPFC DA content (De La Garza *et al*, 2004)). Thus, inclusion of both the more commonly used WKY control and the WIS control as comparators to SHR provides a more complete understanding of the behavioral and neurochemical mechanisms underlying ADHD pathology and of the effects of long term ADHD pharmacotherapeutics.

Using this optimized experimental design, our previous results showed that SHR treated with a therapeutically relevant oral dose of MPH during adolescence exhibit increased cocaine self-administration in adulthood compared to vehicle-administered

SHR and MPH-administered WKY and WIS controls (Harvey *et al*, 2011). The increase in cocaine self-administration was associated with decreased DAT function in whole prefrontal cortex, which includes both OFC and mPFC. OFC and mPFC are distinct based on cytoarchitecture and behavioral function, but are also highly interconnected (Gilbert *et al*, 2008; Lawrence *et al*, 2009). Thus, the current study determined DAT function and expression in OFC and mPFC in adult SHR following a therapeutically relevant oral dose of MPH administered during adolescence.

2.2 Materials and methods:

2.2.1 Materials

(±)-MPH hydrochloride, desipramine hydrochloride, paroxetine hydrochloride, nomifensine maleate, pargyline, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), sucrose, β-mercaptoethanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-hydroxytyramine (DA), sodium chloride and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO). The current experiments used racemic MPH, which also is administered to ADHD patients under the trade name Ritalin[®]. α-D-Glucose, L-ascorbic acid, and monobasic potassium phosphate were purchased from Aldrich Chemical Co. (Milwaukee, WI), AnalaR-BHD Ltd. (Poole, UK) and Mallinckrodt (St. Louis, MO), respectively. [³H]DA (dihydroxyphenylethylamine,3,4-[7-³H]; specific activity, 30.3 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences Inc. (Boston, MA). All other chemicals in the uptake assay buffers were purchased from Fisher Scientific Co. (Pittsburgh, PA).

For the cell surface localization assays, antibodies recognizing rat DAT (C-20; goat polyclonal antibody), demethylated protein phosphatase 2A-C (PP2A-C; 4B7; mouse monoclonal antibody), anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP; sc2954 chicken polyclonal antibody) and anti-goat IgG-HRP (sc2020; donkey polyclonal antibody) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz,

CA). Antibodies against alpha-1 Na⁺/K⁺ ATPase type-1 (mouse monoclonal antibody) were obtained from Abcam (Cambridge, MA). Antibodies recognizing β-actin (A 5441, mouse monoclonal antibody) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfosuccinimidobiotin (sulfo-NHS-biotin), d-biotin and immunoPure immobilized monomeric avidin gel were purchased from Pierce Chemical (Rockford, IL). Complete protease inhibitor cocktail tablets were obtained from Roche diagnostics (Indianapolis, IN). Immunobilon-P PVDF membranes were procured from Millipore. HyGLO Quickspray Chemiluminescent HRP Antibody Detection Reagent and HyblotCL autoradiography films were purchased from Denville Scientific Inc. (Metuchen, NJ). All other chemicals in the buffers for cell surface localization assays were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

2.2.2 Animals and Treatments

Male SHR and WKY (inbred control) rats at postnatal day 25 (P25) were obtained from Charles River Laboratories (Kingston, NY), and male WIS (outbred control) rats at P25 were obtained from Charles River Laboratories (Raleigh, NC). Rats were individually housed with free access to food and water in a colony room maintained on a 12-h light:dark cycle (lights on 07:00 h) in the Division of Laboratory Animal Resources (University of Kentucky, Lexington, KY). From P28 to P55, rats were administered MPH (1.5 mg/kg, p.o. in oyster crackers, Monday - Friday) or vehicle (1 ml/kg, tap water in oyster crackers) to mimic the clinically relevant plasma concentrations, route of administration and weekly pattern of dosing (1996; Berridge *et al*, 2006; Kantak *et al*, 2008; Kuczenski *et al*, 2002). Specifically, a dose of 1.5 mg/kg delivered orally produces peak plasma levels of MPH in rats comparable to plasma levels (8-40 μg/ml) obtained in the ADHD population (Berridge *et al*, 2006; Kuczenski *et al*, 2002). Oyster crackers containing MPH or water were placed in the individual home cage and consumption was monitored to ensure reproducible dosing between days and between animals. Rats consumed the crackers within 3 min of presentation. P28 to P55 includes a time period from early adolescence to late adolescence (Doremus-Fitzwater *et al*, 2010; Spear,

2000a), the typical age during which MPH is administered clinically. Rats were also maintained under mild food restriction (90% of their free-feeding body weight) to mimic the conditions of our previously published studies (Harvey *et al*, 2011) to allow for comparison of current neurochemical findings with those previously reported. Further, all rats consumed the entire daily allotment of food, and body weight did not differ between the MPH- and vehicle-treated groups (data not shown). After P55, MPH treatment ended and *ad libitum* access to food was reinstated. The number of days varied between the last day of treatment (P55) and the day (P77-P84) that the neurochemical assay was conducted. Separate cohorts of rats were used for the DA uptake assays and the DAT cellular distribution assays. Rat handling procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and were performed in accordance with the 1996 version of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

2.2.3 DA Uptake Assay

DAT function was assessed using kinetic analysis of [³H]DA uptake into OFC, mPFC and striatal synaptosomes using a previously published procedure (Marusich *et al*, 2011) with minor modifications. For each experiment, OFC, mPFC and striatum from both hemispheres of one MPH-treated and of one vehicle-treated rat of the same strain and age (P77-P84) were homogenized in separate glass homogenizers, each containing 20 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM sodium bicarbonate, pH 7.4) with 16-20 passes of a Teflon pestle. Synaptosomal suspensions were subjected to two centrifugation steps (2,000g, 10 min, 4 °C followed by 20,000g, 17 min, 4 °C) using an Avanti-J30I centrifuge (Beckman Coulter, Brea, CA). Resulting pellets were resuspended in 2.2 ml (OFC and mPFC synaptosomes) or 2.4 ml (striatal synaptosomes) ice-cold uptake buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO₄, 1.25 mM CaCl₂, 1.5 mM KH₂PO₄, 10 mM glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM L-ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). OFC and mPFC samples

(100 μ l aliquots of the 2.2 ml synaptosomal suspensions) and striatal samples (30 μ l aliquot of the 2.4 ml synaptosomal suspension) were incubated for 5 min in a metabolic shaker (Dubnoff incubator; Precision Scientific, Winchester, VA) at 34 °C in a saturated 95% O₂/5% CO₂ atmosphere in the absence or presence of 10 μ M nomifensine. Nomifensine, a DAT inhibitor, was used to obtain nonspecific [³H]DA uptake. OFC and mPFC suspensions also contained 5 nM each of paroxetine and desipramine to prevent [³H]DA uptake by serotonin transporters and NETs, respectively. Subsequently, 1 of 7 final concentrations (0.01-1.0 μ M) of [³H]DA was added to the buffer, and incubation of the mPFC, OFC and striatal synaptosomal suspensions continued for 5, 5 and 10 min, respectively. [³H]DA uptake was terminated by addition of 3 ml of ice-cold assay buffer, immediately followed by filtration through Whatman GF/B glass fiber filters (presoaked with 1 mM pyrocatechol for 3 hr at 4 °C) using a cell harvester (Biochemical Research and Development Laboratories, Gaithersburg, MD). Values for total and nonspecific [³H]DA uptake were obtained from the amount of radioactivity retained on the filters as determined by liquid scintillation spectrometry (model B1600TR; PerkinElmer Life and Analytical Sciences, Downers Grove, IL). Protein concentrations were determined with bovine serum albumin standards using the Bradford method (Bradford, 1976). Specific [³H]DA uptake was obtained by subtracting nonspecific uptake from total uptake, and the values were used to determine kinetic parameters (V_{max} and K_m) by employing the commercially-available GraphPad Prism 5.0 program (GraphPad Software Inc., San Diego, CA).

2.2.4 DAT cellular distribution assay

Synaptosomal pellets of OFC, mPFC and striatum were resuspended in 1.25 ml (OFC and mPFC) or in 3 ml (striatum) of ice-cold sucrose solution. Synaptosomal protein concentrations were determined as previously described. Biotinylation and Western blotting assays were performed using a previously published method (Zhu *et al*, 2005a). Briefly, synaptosomal suspensions contained about 1 mg protein (OFC and mPFC) or 500

μg protein (striatum). Suspensions were incubated with shaking for 1 hr at 4 °C in 500 μl of 1.5 mg/ml sulfo-NHS biotin in PBS/Ca/Mg buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 9.6 mM Na_2HPO_4 , 1 mM MgCl_2 , 0.1 mM CaCl_2 , pH 7.4), which labels all surface proteins with biotin. Free sulfo-NHS biotin was removed by centrifugation (8000g, 4 min, 4 °C) using a model 5417R centrifuge (Eppendorf, Hauppauge, NY) followed by washing with 1 ml ice-cold 100 mM glycine in PBS/Ca/Mg buffer, and these steps were repeated twice. Then, samples were centrifuged using the same conditions and washed with 1 ml ice-cold PBS/Ca/Mg buffer, and these steps were repeated twice. Subsequently, OFC, mPFC and striatal synaptosomes were lysed by sonication for 2-4 s followed by incubation, with continuous shaking for 20 min at 4 °C in Triton X-100 buffer (150, 150 and 300 μl , respectively; 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 μM pepstatin, 250 μM phenylmethanesulfonyl fluoride, pH 7.4). Lysates underwent centrifugation (21,000g, 20 min, 4 °C). Supernatants constituted the total protein fraction. To obtain the cell surface and intracellular fractions, 2/3 of the supernatant was incubated with Avidin beads with shaking for 1 hr at room temperature, and samples centrifuged (17,000g, 4 min, 4 °C). Supernatants constituted the non-biotinylated fraction (intracellular fraction). Pellets contained the Avidin-conjugated biotinylated proteins (cell surface fraction). Pellets were washed three times with 1% Triton-X-100 and incubated with Lamelli buffer containing 5% v/v β -mercaptoethanol to free the cell surface proteins from the Avidin-biotin complex. Total, non-biotinylated and biotinylated fractions were frozen at -20 °C until Western blot analysis.

The total, non-biotinylated and biotinylated fractions were thawed and subjected to gel electrophoresis and Western blotting as described previously (Salvatore *et al*, 2003) using a gel running apparatus and Trans-Blot[®] SD Semi-Dry Transfer Cell, respectively (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were incubated (overnight, 4 °C) with primary antibody for DAT, followed by secondary antibody (1 hr, room temperature). DAT protein was detected using enhanced chemiluminescence and HyblotCL autoradiography films (Denville Scientific Inc.). Blots were probed

simultaneously for detection of Na⁺/K⁺ ATPase (a plasma-membrane enriched protein), PP2A (an intracellular protein) for determining efficiency of biotinylation of surface proteins, and β-actin (a cytoskeletal protein) to control for loading of proteins. Band densities, expressed as relative optical density (arbitrary units), were determined for DAT and β-actin in total, non-biotinylated and biotinylated fractions using ImageJ software (<http://imagej.nih.gov/ij>).

2.2.5 Statistical Analysis

Data analyses were conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). Data are reported as mean ± S.E.M. and n represents the number of rats per group. To determine if there was a between group difference in the number of days between the cessation of treatment and the conduct of the neurochemical assay, post-treatment periods were analyzed by two-way ANOVAs, with strain and treatment as between-subject factors. Km values for DA were expressed as μM and were log transformed to adjust for the skewed distribution, and then analyzed by two-way ANOVA, with strain and treatment as between-subject factors. Vmax values were expressed as pmol/mg/min. Vmax values for the individuals in the MPH-treated group were expressed as a percent of the mean Vmax value for the vehicle-treated control group and analyzed using one-way ANOVAs followed by Tukey's *post-hoc* test to compare between MPH-treated strains. Within each strain, Vmax values from MPH-treated rats were compared to the vehicle-control (100%) using Student's one-sample *t*-test for matched subjects (Vadum and Rankin, 1998). For cellular distribution assays, total, non-biotinylated and biotinylated fractions were analyzed using mixed-model ANOVAs with strain and treatment as between-subject factors. Day of experiment was used as a covariate to account for day-to-day variations in experimental conditions (Verbeke and Molenberghs, 2000). Outliers were eliminated using the Grubbs test (GraphPad Software; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>). When

appropriate, Tukey's *post-hoc* analyses were used to determine between group differences.

2.3 Results

2.3.1 Maximum Velocity (Vmax) of DA Uptake by DAT

No between-group differences and no interaction of MPH x strain were found for the number of days between the cessation of treatment and the DA uptake assays (interaction terms: OFC $F[2, 38] = 0.51$; mPFC $F[2, 45] = 0.08$; striatum $F[2, 30] = 0.10$; $ps > 0.05$). In the vehicle-control groups, no strain differences in Vmax of [³H]DA uptake for either OFC ($F[2, 17] = 0.34$, $p > 0.05$), mPFC ($F[2, 24] = 0.70$, $p > 0.05$) or striatum ($F[2, 16] = 0.30$, $p > 0.05$; Table 1) were found. Figure 1 shows the strain comparisons after MPH treatment. MPH treatment during adolescence increased ($164 \pm 21.6\%$ of control) Vmax in the mPFC of adult SHR compared to SHR vehicle-control ($t[6] = 2.98$, $p < 0.05$; Figure 1). Also, Vmax in mPFC was greater in MPH-treated SHR compared to MPH-treated WKY and WIS ($F[2, 18] = 4.36$, $p < 0.05$; Figure 1b). Vmax was decreased ($74.0 \pm 5.12\%$ of control) in OFC of the MPH-treated WKY group compared with the WKY vehicle-control ($t[7] = 5.09$, $p < 0.005$), but was not different from the MPH-treated WIS and SHR ($F[2, 20] = 2.55$, $p > 0.05$; Figure 1). MPH treatment during adolescence did not alter DAT function in adult striatum ($F[2,14] = 0.92$, $p > 0.05$; Figure 1).

2.3.2 Affinity (Km) for DA at DAT

In mPFC and striatum, no MPH x strain interaction for the Km for [³H]DA at DAT was found (mPFC, $F[2, 45] = 1.08$; striatum, $F[2, 29] = 0.20$; $ps > 0.05$; Figure 2). In OFC, a MPH x strain interaction ($F[2, 38] = 6.14$, $p < 0.005$) was found, but main effects of strain ($F[2, 38] = 0.18$, $p > 0.05$) and treatment ($F[1, 38] = 2.33$, $p > 0.05$) were not obtained (Figure 2). Post-hoc evaluation of the interaction term revealed that MPH

treatment during adolescence decreased the K_m value by 50% in WIS compared to the WIS vehicle-control group ($p < 0.01$).

2.3.3 DAT cell surface distribution

No between-group differences and no MPH X strain interaction was found for the number of days between the cessation of treatment and the DAT cell surface distribution assays ($F[2, 36] = 0.0, p > 0.05$). DAT expression and distribution did not differ between treatment groups or between strains for either the total, non-biotinylated or biotinylated fractions in OFC (Figure 3), mPFC (Figure 4) or striatum (Figure 5). Results from the statistical analyses of the strain x treatment interaction, and the main effects of treatment and strain are provided in Table 2.

2.4 Discussion

In the current study, effects of MPH treatment during adolescence on DAT function and expression in adulthood were determined in the SHR model of ADHD. WKY and WIS rats served as inbred and outbred control groups, respectively. Surprisingly, treatment of the WIS and WKY during adolescence with a therapeutically relevant dose of MPH, and then cessation of MPH treatment in adulthood, resulted in lasting changes in DAT function in OFC. Specifically, in OFC from WIS, an increase in affinity of DAT for DA was found, with no alteration in maximal uptake of DA. In contrast, in OFC from WKY, the MPH treatment paradigm resulted in no change in affinity for DA, and a decreased maximal DA uptake, which was trafficking independent. These results suggest that a misdiagnosis of ADHD, and subsequent treatment with MPH during adolescence, could result in lasting alterations in OFC DAT function. Thus, with respect to the current data, the outbred control (Wistar) has greater value than the inbred control (WKY) for comparisons to SHR, due to the greater stability of DAT function despite MPH treatment

in the outbred control. Moreover, in the SHR model of ADHD, the MPH treatment regimen produced persistent increases in mPFC DAT function that were trafficking independent. Thus, the increase in mPFC DAT function was a long term consequence of MPH treatment during adolescence, was specific to the SHR strain, and may be responsible for the treatment-induced alterations in behavior. Furthermore, the current results emphasize the importance of the ADHD animal model, and the appropriate control strains, for investigations of neurochemical mechanisms underlying long-term effects of ADHD pharmacotherapeutics.

The current study found that DAT function and cellular distribution in mPFC, OFC and striatum did not differ between SHR, WKY and WIS vehicle-control groups. With regard to striatum, the current results support previous findings showing no difference in total DAT expression and function between SHR and WKY (Harvey *et al*, 2011; Li *et al*, 2007; Miller *et al*, 2012; Womersley *et al*, 2011). The current results further extend these previous findings by showing that cell surface DAT distribution similarly is not different between these inbred strains. In contrast, others have reported greater DAT function and greater total DAT expression in SHR compared to WKY (Pandolfo *et al*, 2012; Roessner *et al*, 2010). Inconsistencies in the results may be explained in that greater DAT function in adult SHR was observed following exposure of striatal synaptosomes to a single concentration (0.022 μM) of [^3H]DA (Pandolfo *et al*, 2012) whereas, the current results were obtained from a comprehensive analysis of DAT kinetic parameters (K_m and V_{max}) using a wide [^3H]DA concentration range (0.01-1.0 μM). No differences in the kinetic parameters for DAT were found between SHR, WKY and WIS. Furthermore, inconsistencies in the levels of striatal DAT expression may be explained by the use of [^3H]GBR 12935 to assess expression, since this radioligand binds to both intracellular and cell surface DAT protein (Roessner *et al*, 2010). However, an explanation for the discrepancy in the analysis of striatal DAT protein by Western blot is not apparent currently. Close inspection of the clinical literature also reveals inconsistencies regarding striatal DAT levels in ADHD. While greater striatal DAT expression has been observed (Faraone *et al*, 2005) others report that DAT levels are

lower in left caudate in ADHD individuals, and not different from control in other subregions of striatum (Volkow *et al*, 2007a). A recent meta analysis reported that striatal DAT levels in ADHD individuals may depend on previous stimulant treatment, with lower DAT density in medication naïve and higher DAT density in previously medicated ADHD individuals (Fusar-Poli *et al*, 2012). Thus, ADHD is a complicated condition that cannot be confined to an explanation regarding alterations in DAT function and/or expression in striatum.

Compared to WKY and WIS controls, SHR treated with MPH during adolescence exhibited improvements in neurocognitive function in adolescence, as demonstrated by performance on maze- and operant-based visual discrimination learning tasks, and increased cocaine self-administration in adulthood (Harvey *et al*, 2011). Specifically, the increase in cocaine self-administration was characterized as a more rapid acquisition of cocaine self-administration, greater responding across a range of cocaine doses, and higher progressive ratio breakpoints. Further, MPH dose and treatment during adolescence resulted in a decrease in DAT function in whole prefrontal cortex in SHR and an increase in DAT function in this brain region in WKY and WIS, compared to the respective vehicle controls (Harvey *et al*, 2011). In contrast, the identical MPH treatment regimen in the current study resulted in an increased DAT function in SHR mPFC, whereas no changes were observed in OFC, compared to vehicle control. These subregions of prefrontal cortex have distinct cytoarchitecture, connectivity and differentially contribute to behavioral processes including decision making and impulsivity (Gilbert *et al*, 2008; Perry *et al*, 2011). As such, the apparent discrepancy with respect to DAT function between the previous and current results may be due to MPH effects in specific cortical subregions. Evidence that mPFC and OFC are critically involved in cocaine self-administration comes from studies in which local inactivation with lidocaine decreased cocaine-seeking and taking behavior (Di Pietro *et al*, 2006; Grakalic *et al*, 2010; Katak *et al*, 2013; Mashhoon *et al*, 2010). Following MPH treatment during adolescence, the increase in mPFC DAT function would be expected to decrease extracellular DA concentration in mPFC. Consistent with these findings, mPFC

DA depletion resulted in an increased motivation for and increased sensitivity to cocaine (McGregor *et al*, 1996; Schenk *et al*, 1991). Thus, mPFC may be at the intersection of MPH effects on DAT function and the increased cocaine self-administration observed following adolescent MPH treatment.

A limitation of the current study was that a single dose of MPH was tested. Nevertheless, this dose of MPH is therapeutically-relevant (Berridge *et al*, 2006; Kuczenski *et al*, 2002) and has been shown to ameliorate behavioral deficits in the adult and adolescent SHR in working memory and behavioral flexibility tasks (Harvey *et al*, 2013; Kantak *et al*, 2008). The increased DAT function in SHR mPFC in the current study did not correspond with a concomitant increase in cell surface localization of DAT. Thus, the MPH-induced long-term changes in DAT function in mPFC were not dependent on trafficking of DAT to the plasma membrane. Although the biotinylation method of evaluating protein trafficking differentiates intracellular and cell surface proteins (Sorkina *et al*, 2005; Zahniser and Sorkin, 2009), this method does not differentiate between cholesterol-rich lipid raft and cholesterol-deficient non-raft regions of the cell surface membrane (Adkins *et al*, 2007; Foster *et al*, 2008). DAT expressed in the lipid raft membrane compartment is more sensitive to protein kinase C mediated phosphorylation (Foster *et al*, 2008). Increased phosphorylation of DAT results in reduced DA uptake, and conversely, decreased DAT phosphorylation results in increased DAT function (Ramamoorthy *et al*, 2010; Samuvel *et al*, 2008; Zhu *et al*, 1997). Furthermore, phosphorylation at serine7 in DAT transitions the protein into a low affinity state (Moritz *et al*, 2013). Taken together, the MPH-induced increase in DAT function in mPFC in SHR rats observed in the current study may be explained by localized DAT expression in the cholesterol-deficient non-raft regions of the cell surface membrane in mPFC, reducing DAT phosphorylation and increasing function.

In conclusion, the present study demonstrates that MPH treatment during adolescence increases DAT function during adulthood in the mPFC of SHR, the ADHD

model, and not in the control strains. The increased DAT function in mPFC may underlie the increased cocaine self-administration observed in MPH-treated SHR.

2.5 Tables

Table 2.1 Maximal velocity of [3H]DA uptake by DAT in OFC, mPFC and striatum in vehicle-treated adult SWKY, SHR and WIS rats

Brain regions ^a	WKY	SHR	WIS
OFC	4.3 ± 0.8	3.0 ± 0.8	2.8 ± 0.7
mPFC	4.0 ± 0.5	3.3 ± 0.6	3.0 ± 0.6
striatum	19.1 ± 2.5	17.1 ± 2.5	21.0 ± 3.4

^a Values are mean ± S.E.M. in pmol/mg/min. n = 6/group for striatal samples, and n = 8-10/group for OFC and mPFC samples

Table 2.2 Statistics comparing DAT levels in total, non-biotinylated (Non-biot) and biotinylated (Biot) fractions of mPFC, OFC and striatum from methylphenidate- and vehicle-treated adult SHR, WKY and WIS rats

F-statistics		Treatment main effect (F_t)	Strain main effect (F_s)	Treatment x Strain interaction (F_i)
OFC	Total	$F_t[1, 17] = 0.65$	$F_s[2, 17] = 0.15$	$F_i[2, 17] = 0.13$
	Non-biot	$F_t[1, 17] = 0.00$	$F_s[2, 17] = 0.54$	$F_i[2, 17] = 1.13$
	Biot	$F_t[1, 16] = 0.06$	$F_s[2, 16] = 2.12$	$F_i[2, 16] = 0.97$
mPFC	Total	$F_t[1, 18] = 1.74$	$F_s[2, 18] = 0.46$	$F_i[2, 18] = 0.82$
	Non-biot	$F_t[1, 18] = 0.06$	$F_s[2, 18] = 3.11$	$F_i[2, 18] = 0.62$
	Biot	$F_t[1, 18] = 0.04$	$F_s[2, 18] = 1.36$	$F_i[2, 17] = 0.37$
striatum	Total	$F_t[1, 14] = 0.75$	$F_s[2, 14] = 1.03$	$F_i[2,14] = 0.66$
	Non-biot	$F_t[1, 14] = 2.50$	$F_s[2, 14] = 0.75$	$F_i[2,14] = 1.22$
	Biot	$F_t[1, 13] = 0.09$	$F_s[2, 13] = 0.41$	$F_i[2,13] = 1.64$

The main effect of treatment (F_t), strain (F_s) and treatment x strain interaction (F_i) have been reported individually; $ps > 0.05$.

2.6 Figures

Figure 2.1 Effect of MPH on the Vmax of DAT in OFC, mPFC and striatum

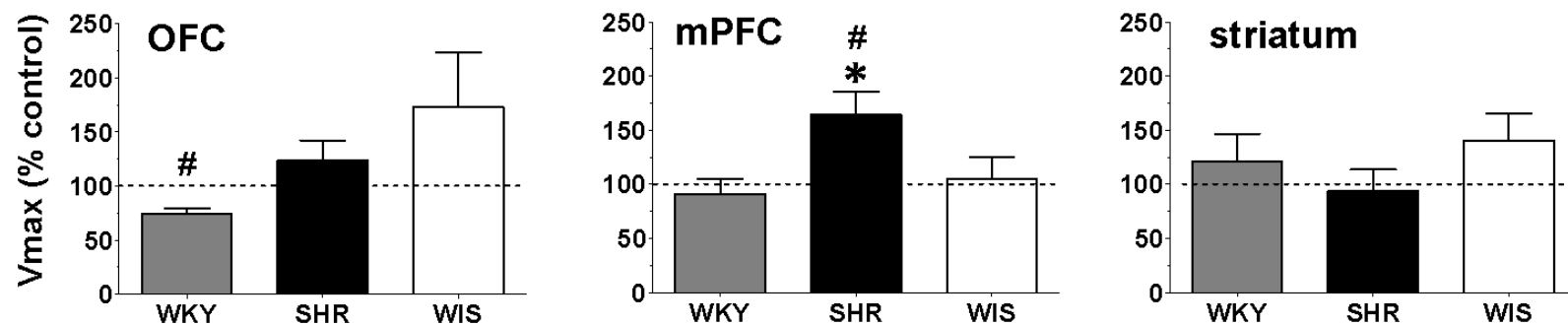


Fig. 2.1. Vmax for DAT in OFC, mPFC and striatum from methylphenidate-treated WKY (grey bars), SHR (black bars) and WIS (open bars) rats expressed as pmol/mg/min as a percentage of vehicle control (dotted line). Values are mean \pm S.E.M. # Different from the vehicle control, $p < 0.05$. * Different from WKY and WIS, $p < 0.05$. $n = 8-10$ /group for OFC and mPFC; $n = 6$ /group for striatum.

Figure 2.2 Effects of MPH treatment on Km of DAT in OFC, mPFC and striatum

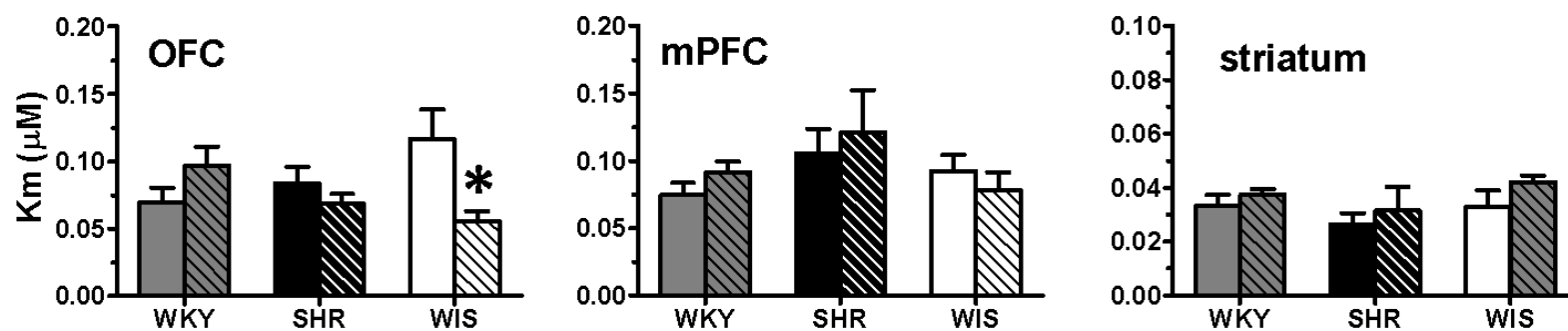


Fig. 2.2. Km for DAT in OFC, mPFC and striatum from methylphenidate-treated (striped) and vehicle-treated (plain) WKY (grey bars), SHR (black bars) and WIS (open bars) rats. Values are expressed in μM as mean \pm S.E.M. # Different from vehicle, $p < 0.05$. $n = 8-10/\text{group}$ for OFC and mPFC; $n = 6/\text{group}$ for striatum.

Figure 2.3 Effects of MPH treatment on cellular distribution of DAT in the OFC

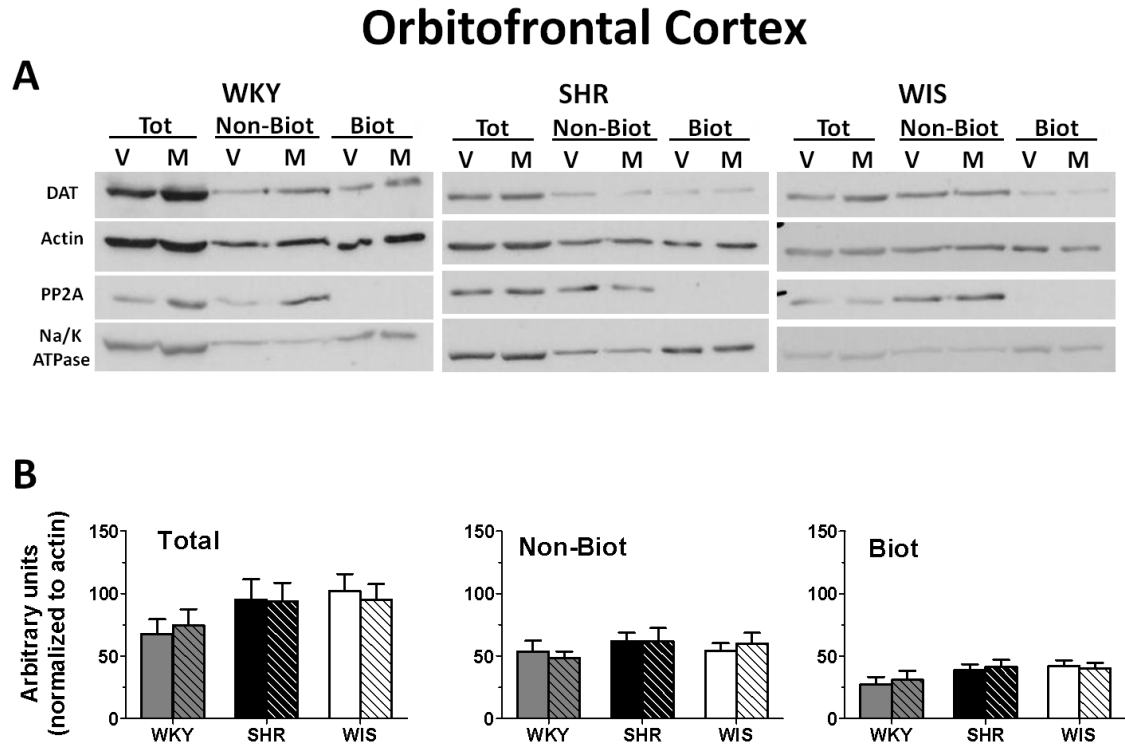


Fig. 2.3 (A) Representative blots for cellular distribution of DAT in OFC synaptosomes from methylphenidate-treated (M) and vehicle-treated (V) WKY, SHR and WIS rats. Actin was used to monitor uniform protein-loading while Na^+/K^+ ATPase and PP2A served to ascertain the efficiency of biotinylation of surface proteins. (B) Distribution of DAT between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions of OFC synaptosomes from methylphenidate-treated (striped) and vehicle-treated (plain) WKY (grey bars), SHR (black bars) and WIS (open bars) rats. Values are mean \pm S.E.M in arbitrary units. $n = 6-7/\text{group}$.

Figure 2.4 Effects of MPH treatment on cellular distribution of DAT in the mPFC

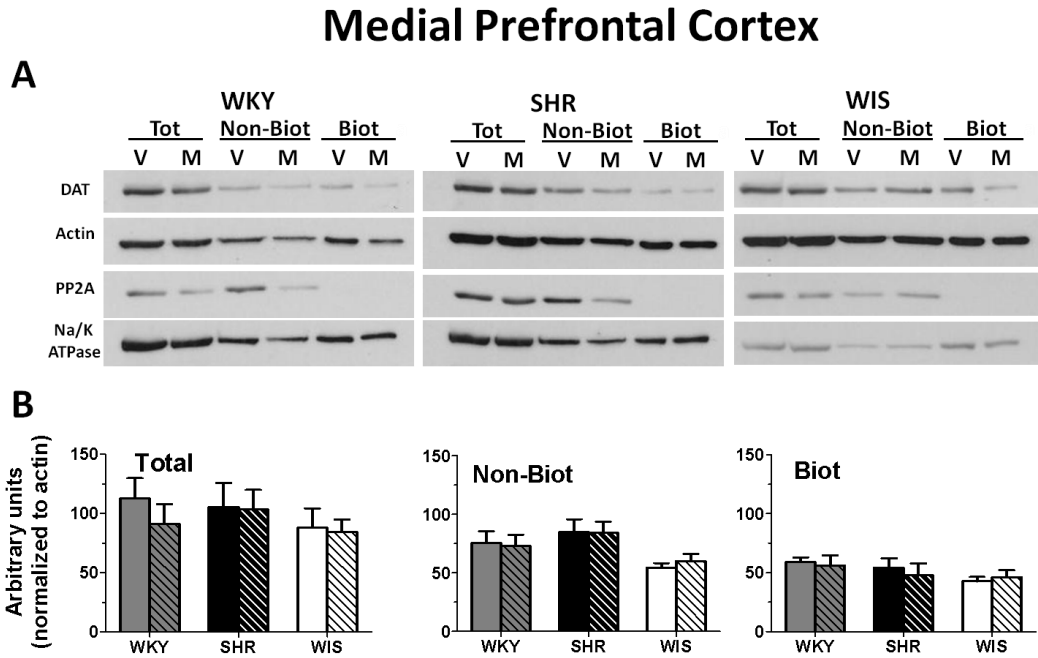


Fig 2.4 (A) Representative blots for cellular distribution of DAT in mPFC synaptosomes from methylphenidate-treated (M) and vehicle-treated (V) WKY, SHR and WIS rats. Actin was used to monitor uniform protein-loading while Na^+/K^+ ATPase and PP2A served to ascertain the efficiency of biotinylation of surface proteins. (B) Distribution of DAT between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions of mPFC synaptosomes from methylphenidate-treated (striped) and vehicle-treated (plain) WKY (grey bars), SHR (black bars) and WIS (open bars) rats. Values are mean \pm S.E.M in arbitrary units. n = 6-7/group.

Figure 2.5 Effects of MPH treatment on cellular distribution of DAT in the striatum

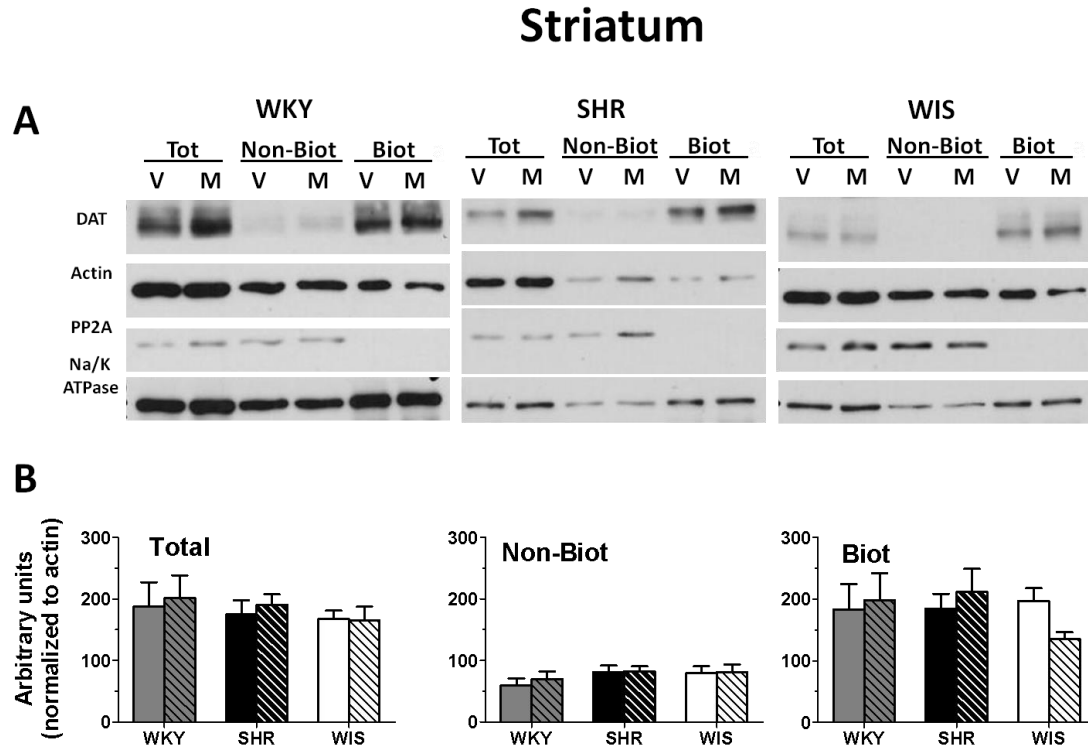


Fig. 2.5 (A) Representative blots for cellular distribution of DAT in striatal synaptosomes from methylphenidate-treated (M) and vehicle-treated (V) WKY, SHR and WIS rats. Actin was used to monitor uniform protein-loading while Na⁺/K⁺ ATPase and PP2A served to ascertain the efficiency of biotinylation of surface proteins. (B) Distribution of DAT between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions of striatal synaptosomes from methylphenidate-treated (striped) and vehicle-treated (plain) WKY (grey bars), SHR (black bars) and WIS (open bars) rats. Values are mean ± S.E.M in arbitrary units. n = 5-6/group.

3 CHAPTER THREE

Adolescent atomoxetine treatment in a rodent model of ADHD: Effects on cocaine self-administration and dopamine transporters in frontostriatal regions

Portions of this chapter have been published in the manuscript:

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3.1 Introduction

Attention deficit/hyperactivity disorder (ADHD) affects 8-12% of children, and up to 5% of adults, making it one of the most prevalent disorders (Biederman *et al*, 2010). Adults with ADHD have a higher risk of developing substance use disorders compared to individuals without ADHD (Wilens *et al*, 1998). In particular, individuals with ADHD have a 35% higher incidence of cocaine abuse compared to the general population (Levin *et al*, 1999), and children with ADHD are twice as likely to use cocaine during adulthood (Lee *et al*, 2011).

One explanation for comorbid ADHD and cocaine abuse may be commonalities in neuronal substrates. Hypoactivation of the orbitofrontal cortex (OFC) and dorsolateral prefrontal cortex (DLPFC) is evident in both disorders and leads to deficits in working memory, decision-making, and response inhibition (Adinoff *et al*, 2003; Bolla *et al*, 2003;

Burgess *et al*, 2010; Cubillo *et al*, 2011; Wilcox *et al*, 2011). With respect to rodents, behavioral flexibility, working memory and sustained attention are regulated by both the medial prefrontal cortex (mPFC) and OFC (Floresco *et al*, 2009). Moreover, mPFC and OFC also regulate cocaine-seeking behavior in outbred rats self-administering cocaine (Di Pietro *et al*, 2006; Kantak *et al*, 2013). As parts of the mPFC in rodents is functionally analogous to DLPFC in primates (Uylings *et al*, 2003), these findings suggest that mPFC and OFC function is critical for understanding the mechanisms underlying comorbid ADHD and cocaine abuse.

There is a paucity of information concerning the effects of ADHD medications on the comorbidity of ADHD and cocaine abuse. Primarily, two categories of medications are used to treat ADHD: stimulants (e.g., methylphenidate; MPH) and non-stimulants (e.g., atomoxetine; ATO). MPH is a dopamine and norepinephrine transporter (DAT and NET, respectively) inhibitor (Richelson and Pfenning, 1984), whereas ATO is a selective NET inhibitor (Bolden-Watson and Richelson, 1993). While MPH is the first-line treatment for ADHD, ATO has efficacy nearly comparable to MPH in reducing ADHD symptoms (Garnock-Jones and Keating, 2009).

Clinical reports suggest that MPH treatment initiated in childhood may be protective against cocaine addiction (Wilens *et al*, 2003), or alternatively, not modify cocaine abuse liability in ADHD individuals (Molina *et al*, 2013). However, a positive correlation was reported between age of initiation of MPH treatment and cocaine abuse during adulthood (Mannuzza *et al*, 2008), such that lifetime rates of cocaine abuse were higher when treatment was initiated in early adolescence. Preclinical models employing carefully controlled experimental conditions may be valuable for evaluating mechanisms underlying these controversial clinical results. Previous work using spontaneously hypertensive rats (SHR), a well established model of ADHD (Kantak *et al*, 2008; Sagvolden *et al*, 2005), found that MPH treatment during adolescence increased cocaine self-administration in adult SHR compared to untreated SHR and compared to MPH-

treated Wistar-Kyoto (WKY) or Wistar (WIS) control rats (Harvey *et al*, 2011). In the latter report, repeated administration of MPH during adolescence increased the self-administration efficacy for a range of doses of cocaine, without altering sensitivity for cocaine in SHR. Increased vulnerability to cocaine self-administration in SHR may be due to MPH-induced increases in DAT function in mPFC relative to untreated SHR and MPH-treated WKY or WIS (Somkuwar *et al*, 2013). These findings from the SHR model are complimentary with observations in humans, such that previously medicated ADHD individuals have higher striatal DAT density compared to non-ADHD individuals (Fusar-Poli *et al*, 2012). Striatal DAT function is elevated also in cocaine users (Mash *et al*, 2002). However, cortical DAT expression in ADHD and in cocaine users has not been reported.

ATO, unlike MPH, has very low affinity for DAT (Heal *et al*, 2008). However, selective inhibition of NET by ATO increases extracellular concentrations of both norepinephrine and dopamine in PFC (Arnsten, 2009), because NET is responsible primarily for dopamine clearance in this brain region (Moron *et al*, 2002). In outbred rats, ATO reduces cocaine seeking and cocaine cue-induced reinstatement (Economidou *et al*, 2011; Janak *et al*, 2012). However, effects of ATO on cocaine self-administration and on DAT function and cell surface expression in SHR have not been determined. The current study tests the hypothesis that, in contrast to the effects of MPH treatment, treatment with a pharmacologically relevant dose of ATO (Bymaster *et al*, 2002) during adolescence does not increase vulnerability for cocaine self-administration in adult SHR after the ATO treatment has been discontinued. To identify long-term changes in dopaminergic systems, DAT function and expression in mPFC, OFC, and striatum in adult SHRs were evaluated following the administration of a pharmacologically relevant dose of ATO during adolescence. Further, to identify changes in DAT function that may influence cocaine self-administration, neurochemical studies were conducted in rats at the same age at which cocaine self-administration was initiated.

3.2 Materials and Methods

3.2.1 Subjects

Male Wistar (WIS)/Cr, Wistar-Kyoto (WKY)/Cr and Spontaneously Hypertensive (SHR)/Cr rats (Charles River Laboratories, Wilmington, MA, Kingston, NY or Raleigh, NC) arrived on postnatal day 25 (P25). SHR served as an animal model of ADHD; and WKY and WIS as inbred and outbred comparator strains, respectively. Experiments 1, 2 and 3 employed the same group of rats. Experiment 4 employed a separate group of rats. Experiment 5 again employed a separate group of rats. Housing has been described previously (Harvey *et al*, 2011) and in the Supplementary Materials. From P28 through P55, rats received ATO or vehicle (see below) Monday through Friday to mimic the weekend “medication holiday” often recommended for individuals with ADHD (American Academy of Pediatrics, 1996). For rats used in the behavioral studies, an intravenous catheter was implanted on P67 (see Supplementary Materials for details). Protocols were approved by the Institutional Animal Care and Use Committee at Boston University and at the University of Kentucky, and were performed in accordance with the 1996 version of the *NIH Guide for the Care and Use of Laboratory Animals*.

3.2.2 Drugs

Atomoxetine ((R)-N-methyl-γ-(2-methylphenoxy)-benzenepropanamine hydrochloride; Tocris Biosciences, Ellisville, MO; 0.3 mg/ml) was dissolved in 0.9% sterile physiological saline and injected i.p. at a dose of 0.3 mg/kg. This relatively low dose was chosen to increase extracellular norepinephrine concentrations in PFC, although this dose also has

been shown to increase extracellular dopamine concentrations in PFC through inhibition of NET (Bymaster *et al*, 2002). An i.p. route was utilized due to poor oral bioavailability of ATO in rats (Mattiuz *et al*, 2003). For intravenous self-administration studies, cocaine hydrochloride (NIDA, Bethesda MD) was dissolved in sterile 0.9% physiological saline containing 3 IU of heparin/ml. A cocaine unit dose of 0.3 mg/kg was used for training, and doses from 0.003 to 1.0 mg/kg were used to evaluate dose-response functions.

3.2.3 Experiment 1: Acquisition of Cocaine Self-administration (Fixed Ratio).

Experiment 1 determined the speed at which WIS, WKY, and SHR acquired cocaine self-administration, and evaluated strain-dependent effects of adolescent ATO treatment on acquisition. Sessions (2 hr) were conducted daily Monday – Friday beginning on P77 in lighted chambers described in Supplementary Materials. Rats were allowed to press the active lever (left or right, counterbalanced across rats) for a 0.3 mg/kg cocaine infusion under a fixed ratio 1 (FR1) schedule of reinforcement. Responses on the inactive lever were recorded, but had no consequences. Animals received no external inducements to respond on either lever (spontaneous acquisition). A stimulus light located above the active lever was illuminated upon receipt of a cocaine infusion and remained illuminated during a 20 sec timeout period during which additional infusions could not be earned, but lever responses were counted. The house light was extinguished during the timeout. Acquisition of cocaine self-administration was defined as earning ≥ 20 infusions in a 2-hr session for two consecutive sessions, and discriminating the active from inactive lever by a factor of 2 or greater (Harvey *et al*, 2011).

3.2.4 Experiment 2: Cocaine Dose-Response Functions (Fixed-Ratio).

Experiment 2 evaluated the efficacy of cocaine reinforcement in WIS, WKY, and SHR, and identified strain-dependent effects of adolescent ATO treatment on responding maintained by a range of cocaine doses under an FR1 schedule. Following achievement of acquisition criterion, rats continued under an FR1 schedule of 0.3 mg/kg cocaine delivery until active lever responses and infusions varied less than 10% across 5 consecutive sessions. A range of cocaine unit doses (0.003, 0.01, 0.03, 0.1, and 1.0 mg/kg/infusion) was then substituted in random order twice each week (Tuesdays and Fridays). The 0.3 mg/kg training dose was available on intervening days. Following determination of FR1 cocaine dose-response functions, baseline responding was reestablished for the 0.3 mg/kg dose for 2-3 days prior to beginning Experiment 3.

3.2.5 Experiment 3: Cocaine Dose-Response Functions (Progressive Ratio).

Experiment 3 assessed the motivating influence of cocaine reinforcement in WIS, WKY, and SHR, and identified strain-dependent effects of ATO treatment during adolescence on progressive ratio (PR) breakpoints across a range of cocaine unit doses. The PR schedule of Loh & Roberts (1990) was implemented, such that response requirements on the active lever increased exponentially for each subsequent cocaine infusion. Self-administration sessions terminated when rats failed to meet the response requirement within 1 hr. The last FR completed was defined as the PR breakpoint. Baseline responding under the PR was established for the 0.3 mg/kg dose for 5 consecutive sessions, after which test doses (1.0, 0.1, and 0.01 mg/kg/infusion) were substituted in descending order.

3.2.6 Experiment 4: Dopamine Uptake Assay.

Experiment 4 assessed DAT function in mPFC, OFC and striatum of WIS, WKY, and SHR treated with ATO or vehicle. Kinetic analysis of [³H]dopamine uptake into mPFC, OFC and striatal synaptosomes was conducted using a previously published procedure (Marusich *et al*, 2011) with minor modifications. Purified synaptosomal suspensions from mPFC, OFC and striatum from one ATO- and one vehicle-treated rat of the same strain and age (P77-P84) were prepared as detailed in Supplementary Materials. Briefly, mPFC, OFC and striatal samples were incubated for 5 min in the absence or presence of an excess concentration of nomifensine, a DAT inhibitor, to determine nonspecific [³H]dopamine uptake, and paroxetine and desipramine to prevent [³H]dopamine uptake by serotonin and norepinephrine transporters, respectively. Subsequently, 1 of 7 final concentrations (0.01-1.0 μM) of [³H]dopamine was added to the assay buffer and incubations continued for mPFC (5 min), OFC (5 min) and striatal (10 min) synaptosomal suspensions. Specific [³H]dopamine uptake was obtained by subtracting nonspecific uptake from total uptake; these values were used to determine kinetic parameters (V_{max} and K_m) using the commercially available GraphPad Prism 5.0 program (GraphPad Software Inc., San Diego, CA).

3.2.7 Experiment 5: DAT Cellular Distribution Assay.

Experiment 5 assessed DAT cellular distribution in mPFC, OFC and striatum of WIS, WKY, and SHR treated with ATO or vehicle. Synaptosomal pellets of mPFC, OFC and striatum were resuspended in 1.25 ml (mPFC and OFC) or in 3 ml (striatum) of ice-cold sucrose solution. Biotinylation and Western blotting assays were performed using a previously published method (Somkuwar *et al*, 2013) detailed in Supplementary Materials. Briefly, synaptosomal suspensions were incubated with sulfo-NHS biotin to label all surface

proteins. Synaptosomes were lysed by sonication and incubation in Triton X-100 buffer. Total protein fractions were obtained by centrifugation. Two-thirds of the total protein fractions were incubated with Avidin beads to separate non-biotinylated (supernatant) from Avidin-conjugated biotinylated fractions (pellet). Total, non-biotinylated and biotinylated fractions were subjected to gel electrophoresis and Western blotting and subsequently probed for DAT protein, Na⁺/K⁺ ATPase (plasma-membrane enriched protein) and PP2A (intracellular protein) for determining efficiency of biotinylation, and β -actin (cytoskeletal protein, loading control) to ascertain protein loading. Band density, expressed as relative optical density, was determined for DAT and β -actin using ImageJ software (<http://imagej.nih.gov/ij>).

3.2.8 Data Analyses

Dependent measures for self-administration experiments included sessions to reach acquisition criterion (square root transformed prior to analysis), cocaine infusions earned, active and inactive lever responses, and progressive ratio breakpoints. Dependent measures for DAT functional assays included K_m (log transformed prior to analysis) and V_{max} (pmol/mg/min; ATO-treated rats were normalized as percent of vehicle-treated control of the same strain). For the cellular distribution assay, DAT from each fraction (total, intracellular and surface) for each brain region was normalized to β -actin levels in the same sample. Dependent measures were analyzed by 1-factor (strain), 2-factor (strain X treatment, drug dose X treatment, or drug dose X strain) or 3-factor (drug dose X strain X treatment) ANOVAs, with repeated measures for dose. Post-hoc Tukey's tests were used in behavioral studies and Tukey's or one-sample t-tests (compared to a hypothetical value of 100) for matched subjects in neurochemical studies. Outliers in neurochemical studies were removed prior to analysis using the Grubbs test (GraphPad Software; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>).

3.3 Results

3.3.1 Experiment 1: Acquisition of Cocaine Self-Administration (Fixed-Ratio).

Sessions to reach the acquisition criterion for the 0.3 mg/kg dose is shown in Figure 1. Strains differed significantly ($F[2, 47] = 7.0, p \leq 0.002$), and there was a trend for a strain X treatment interaction ($F[2, 47] = 2.7, p \leq 0.07$). Overall, SHR acquired cocaine self-administration faster than WKY and WIS ($p \leq 0.04$ and 0.002 , respectively). Treatment comparisons within each strain revealed that in WKY, acquisition of cocaine self-administration was faster after ATO than vehicle ($p \leq 0.03$). ATO did not alter acquisition speed in SHR or WIS. Strain comparisons within each treatment revealed that in vehicle-treated rats, SHR acquired cocaine self-administration faster than WKY ($p \leq 0.02$). In ATO-treated rats, both SHR and WKY acquired cocaine self-administration faster than WIS ($p \leq 0.01$ and 0.05 , respectively).

Analyses of active and inactive responses as well as infusions earned at criterion also were performed (Table S1 and Figure S1). These analyses confirm strain-level differences, with SHR emitting more active lever responses and earning more cocaine infusions than WKY or WIS, overall. These analyses also revealed that at criterion, ATO treatment increased active lever responses exclusively in SHR. Moreover, numbers of inactive lever responses emitted were not different between strains and between treatment conditions, and rats discriminated the active from inactive lever by a factor of 2 or greater (Table S1).

3.3.2 Experiment 2: Cocaine Dose-Response Functions (Fixed-Ratio).

Cocaine dose-response functions based on number of infusions earned under FR1 are shown in Figure 2A. For the 3-way ANOVA, strain ($F[2, 43] = 18.4, p \leq 0.001$) and dose ($F[5, 215] = 185.5, p \leq 0.001$) differed, and there was a strain X dose interaction ($F[10, 215] = 9.3, p \leq 0.001$). The treatment factor and its interactions with strain and/or dose were not significant. Overall, SHR earned more cocaine infusions than WKY and WIS ($p \leq 0.001$). Further testing of the strain X dose interaction indicated that SHR earned more infusions than WKY for cocaine doses ranging from 0.003 to 0.3 mg/kg ($p \leq 0.04$), and more infusions than WIS for cocaine doses ranging from 0.003 to 0.1 mg/kg ($p \leq 0.01$ except at 0.01 mg/kg, where $p \leq 0.08$). In addition, WIS earned more infusions than WKY at 0.1 mg/kg ($p \leq 0.001$). No strain differences were observed at 1.0 mg/kg. Analyses of the cocaine dose-response functions based on number of active lever responses were similar to the above number of infusions earned (Figure S2).

3.3.3 Experiment 3: Cocaine Dose-Response Functions (Progressive Ratio).

Cocaine dose-response functions based on the last FR completed under the PR schedule are shown in Figure 2B. For the 3-way ANOVA, strain ($F[2, 42] = 10.1, p \leq 0.001$) and dose ($F[3, 126] = 53.3, p \leq 0.001$) differed. The treatment factor and its interactions with strain and/or dose were not significant. Overall, SHR had higher breakpoints than WKY and WIS ($p \leq 0.001$ and 0.01, respectively). Further analysis of the dose factor revealed that animals maintained the highest breakpoints at 1.0 mg/kg, which differed from all other doses, and maintained the lowest breakpoints at 0.01 mg/kg, which also differed from all other doses ($p \leq 0.001$ to 0.03). Breakpoints maintained by 0.3 mg/kg and 0.1 mg/kg did not differ from each other. PR breakpoints based on infusions earned and active

lever responses also were analyzed and results were similar to the last FR completed measure (Figures S3 and S4).

3.3.4 Experiment 4: Dopamine Uptake.

K_m values for [^3H]dopamine uptake in mPFC, OFC and striatum did not differ among strain or treatment groups (Table S2). Also, the V_{\max} for [^3H]dopamine uptake by DAT in mPFC, OFC and striatum did not differ between strains treated with vehicle (Figures 3A-5A). ATO treatment did not alter V_{\max} in mPFC in any strain when compared to the corresponding vehicle control. Also, there were no differences in V_{\max} in mPFC between strains treated with ATO (Figure 3A). Conversely, with respect to the OFC, ATO decreased V_{\max} for [^3H]dopamine uptake in SHR ($t[7] = 2.42, p \leq 0.05$) and WIS ($t[5] = 5.67, p \leq 0.005$) by 25% and 51% of vehicle control, respectively (Figure 4A). Furthermore, there were strain differences in OFC in the ATO treated groups. Specifically, V_{\max} was lower for ATO-treated SHR and WIS (14% and 55%, respectively) compared to ATO-treated WKY ($F[2, 21] = 8.22, p \leq 0.005$). ATO also decreased V_{\max} in SHR striatum ($t[6] = 2.74, p \leq 0.05$) by 18% of vehicle control (Figure 5A). However, V_{\max} in striatum did not differ among the ATO-treated groups.

3.3.5 Experiment 5: DAT Cellular Distribution Assay.

In the vehicle-treated groups, no between-strain differences in DAT cellular distribution were found in mPFC, OFC and striatum (Table S3). ATO treatment did not alter DAT cellular distribution in mPFC (Figures 3B and 3C). However in OFC, ATO treatment significantly decreased (22% of vehicle control) DAT expression in the surface fraction

only in SHR (Figures 4B and 4C; $t[7] = 2.50$, $p \leq 0.05$). DAT cellular distribution in OFC in the ATO-treated groups was not different between the strains (Figures 4B and 4C). In striatum, ATO treatment during adolescence increased total DAT expression in SHR (18% of vehicle control; $t[7] = 2.76$, $p \leq 0.05$) and WIS (12% of vehicle control; $t[6] = 4.35$, $p \leq 0.05$; Figures 5B and 5C). Also, surface DAT in striatum of WKY was increased (13% of vehicle control; $t[7] = 2.88$, $p \leq 0.05$; Figures 5B and 5C). DAT cellular distribution in striatum did not differ among ATO-treated groups (Figures 5B and 5C).

3.4 Discussion

3.4.1 Strain Differences in Behavior and DAT Neurochemistry

Compared to inbred WKY and outbred WIS comparator strains, SHR acquired cocaine self-administration faster, and showed greater intake and higher breakpoints across a range of cocaine unit doses under FR1 and PR schedules. These results suggest that SHR exhibit a vulnerable cocaine self-administration phenotype, characterized by faster acquisition, higher efficacy and a greater motivating influence of cocaine reinforcement. Vulnerability is reflected by vertical shifts in FR and PR dose-response functions (Piazza *et al*, 2000). Thus, the current findings further verify the utility of the SHR for modeling comorbid cocaine abuse and ADHD (Harvey *et al*, 2011).

Comparator strains did not differ, except that WIS had greater cocaine intake and made more active lever responses than WKY for 0.1 mg/kg cocaine under FR1. This dose produced peak rates of responding in all strains. Cocaine doses commonly abused in people and those associated with peak rates of responding in rhesus monkeys produce similar levels of striatal DAT occupancy (Wilcox *et al*, 2002). This suggests that strain differences in cocaine self-administration may reflect strain differences in DAT

function or expression. Compared to WIS, WKY have decreased DAT density in nucleus accumbens (Jiao *et al*, 2003). In the present study, however, there were no strain differences in DAT function in mPFC, OFC or striatum under vehicle conditions. Further, no differences were found in DAT cellular distribution in mPFC, OFC and striatum among SHR, WKY and WIS, which is in agreement with some previous findings (Jiao *et al*, 2003; Li *et al*, 2007), but not others (Pandolfo *et al*, 2012; Roessner *et al*, 2010).

Inconsistencies in striatal DAT expression may be explained by use of [³H]GBR 12935 to assess expression, since this radioligand binds to both intracellular and cell surface DAT protein (Roessner *et al*, 2010). Further, prior history of the subjects, including participation in behavioral assays, may explain some inconsistencies between studies in striatal DAT function and expression (Pandolfo *et al*, 2012). With respect to striatal cell surface DAT expression, there was a trend suggesting WKY had lower DAT expression than SHR and WIS, although this was not associated with differences in striatal DAT function (Harvey *et al*, 2011; Womersley *et al*, 2011). Thus, cocaine may be a more efficacious reinforcer in SHR and WIS than WKY, due to greater DAT cell surface expression within the reward circuit in SHR and WIS.

3.4.2 Effects of Atomoxetine Treatment

Following discontinuation of MPH treatment in adolescent SHR, an increase in cocaine self-administration was observed; and importantly, this was not observed in the control WKY and Wistar rats (Harvey *et al*, 2011). These findings appear to differ from those of other investigators reporting no increase in cocaine self-administration after discontinuing MPH treatment (Gill *et al*, 2012; Thanos *et al*, 2007); however, rats or monkeys that did not have an ADHD phenotype were employed in these studies. Thus, our results with the control strains (not expressing the ADHD phenotype) are in agreement with the latter findings. Further, our results employing SHR, which display

the ADHD phenotype, extend the literature, and moreover, are in agreement with clinical reports that teens with ADHD treated with stimulant medication have greater liability for cocaine abuse (Harvey *et al*, 2011; Mannuzza *et al*, 2008). Interestingly, when the ADHD stimulant treatment was initiated during childhood, a decreased drug abuse liability was found in adulthood (Wilens *et al*, 2003). Taken together, the current approach using an animal model of ADHD is appropriate and clinically relevant for determining the effects of alternate ADHD medications such as ATO.

Adolescent ATO treatment did not alter cocaine self-administration behavior in SHR, with one exception. ATO-treated SHR made more active lever responses at the acquisition criterion under FR1, without having greater cocaine intake. This indicates that SHR made more responses during the 20 sec timeout/cue light presentation period following each cocaine infusion. Acute ATO pretreatment has cognitive enhancing effects via increased noradrenergic transmission (Gamo *et al*, 2010; Janak and Corbit, 2011). Thus, it is possible that chronic ATO treatment increased the salience of cocaine-paired cues in SHR during acquisition of cocaine self-administration via inhibition of norepinephrine uptake. As chronic ATO treatment during adolescence selectively increases NET mRNA in the OFC during adulthood (Sun *et al*, 2012), the OFC may be an important site of action for ATO-induced changes in cocaine cue salience.

The results of the current report suggest that alterations in DAT function and expression in OFC after ATO treatment may also be of importance to cocaine self-administration behavior. Adolescent ATO treatment increased the speed of acquisition of cocaine self-administration in adult WKY, but not SHR and WIS, and correspondingly decreased DAT function in OFC of SHR and WIS, but not WKY. In our previous work, ATO treatment during adolescence was shown to differentially influence performance on a strategy set shifting task as well (Harvey *et al*, 2013). Specifically, learning speed during the initial discrimination phase was decreased in SHR and increased in WKY and WIS after ATO. During the set shift phase, ATO improved learning accuracy exclusively in

SHR, suggesting strain-dependent effects of ATO on prefrontal cortical functions (Floresco *et al*, 2009). In addition to decreases in V_{max} of dopamine uptake at DAT in OFC, ATO decreased cell surface expression of DAT in OFC in SHR, revealing an underlying trafficking-dependent mechanism for the observed decrease in OFC DAT function. It has been shown that repeated application of dopamine decreases DAT function and expression in striatum *in vivo* and in a heterologous expression system *in vitro* (Gulley *et al*, 2002). Since dopamine is cleared by NET in mPFC and OFC, chronic ATO treatment will increase extracellular dopamine concentrations in these brain regions, which may lead to decreased DAT surface expression, and subsequently to decreased DAT function as observed in the SHR OFC. In WIS, the identical ATO treatment decreased DAT function in OFC, but did not decrease DAT cell surface expression, indicating a trafficking-independent mechanism. Taken together, these results suggest ATO may be protective against further enhancement of vulnerability to cocaine self-administration in SHR, via a long-lived reduction in DAT function and/or surface expression in OFC. Another variable that may confer this protection in SHR is the failure of adolescent ATO treatment in SHR to increase DAT function in mPFC during adulthood. In our previous studies, adolescent MPH treatment increased mPFC DAT function in SHR (Somkuwar *et al*, 2013) and further enhanced vulnerability to cocaine self-administration in SHR (Harvey *et al*, 2011) during adulthood.

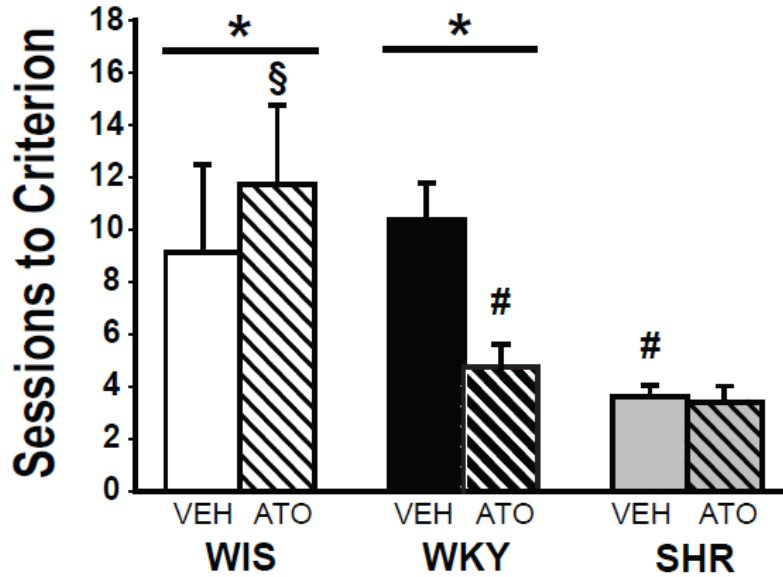
ATO unexpectedly decreased DAT function and increased total DAT expression in SHR striatum. The increase in total striatal DAT expression may be a compensatory response to the decrease in DAT function. Also, ATO increased total DAT expression in WIS and surface expression in WKY. Thus, ATO alterations in striatal DAT expression were not specific to the strain with the ADHD phenotype. These results may reveal neuronal cross talk between dopaminergic neurons in striatum and noradrenergic neurons in cortex, where ATO has a direct effect at NET (Swanson *et al*, 2006).

3.4.3 Conclusions

The present study demonstrates that the SHR phenotype models comorbid cocaine abuse and ADHD. Contrary to previous findings with MPH, ATO treatment did not further enhance vulnerability to cocaine self-administration in SHR. Though additional work is needed to confirm mechanisms and signaling pathways involved, our work suggests that the protection by ATO against further enhanced cocaine abuse vulnerability in SHR may occur through decreased DAT function and decreased DAT cell surface localization in OFC, and sustained DAT function in mPFC. Taken together, the behavioral and neurochemical results suggest that ATO may be a suitable alternative to stimulant treatment in ADHD teens in whom the risk of drug abuse may be a concern. Moreover, while these studies do not raise critical concerns about the safety of ATO, they do emphasize the importance of accurate diagnosis of ADHD. In the WKY control, ATO speeded acquisition of cocaine self-administration and did not alter OFC DAT. Misdiagnosis of ADHD, and subsequent ATO treatment in teens could result in a more rapid development of abuse of cocaine.

3.5 Figures

Figure 3.1 Number of sessions to reach criterion for acquisition of cocaine self-administration



Experiments were performed using adult Wistar (WIS; white bars), Wistar-Kyoto (WKY; black bars), and Spontaneously Hyperactive (SHR; grey bars) rats after discontinuation of adolescent treatment with atomoxetine (ATO; striped bars) or vehicle (VEH; solid bars). Values are mean \pm S.E.M. (n=8-11/strain and treatment). * $p \leq 0.05$ compared to SHR (denoted by horizontal line under the symbol). # $p \leq 0.05$ compared to VEH-treated WKY. § $p \leq 0.05$ compared to ATO-treated WKY and SHR.

3.2 Cocaine dose-response functions under fixed-ratio and progressive-ratio schedules

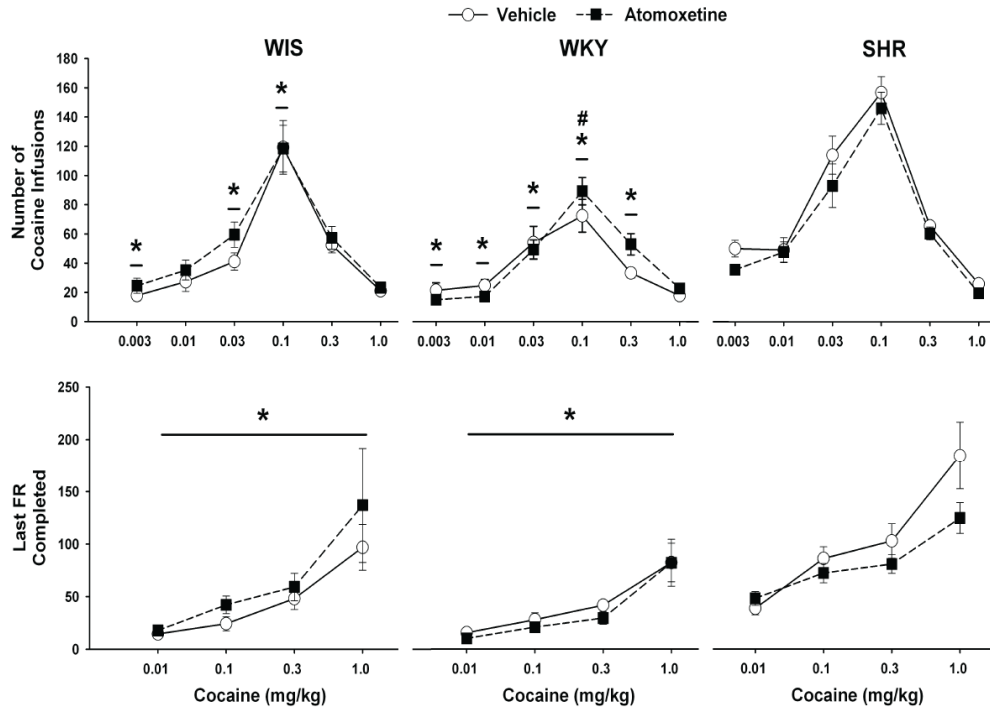


Figure 2. (A) Cocaine dose-response functions based on number of infusions earned under an FR1 schedule ($n=8-9$ /strain and treatment). (B) Progressive ratio breakpoints based on the last FR completed ($n=8$ /strain and treatment). Experiments were performed using adult Wistar (WIS), Wistar-Kyoto (WKY), and Spontaneously Hypertensive (SHR) rats following discontinuation of adolescent treatment with atomoxetine (ATO; squares, dashed lines) or vehicle (VEH; circles, solid lines). Values are mean \pm S.E.M. * $p \leq 0.05$ compared to SHR overall (denoted by horizontal line under the symbol). # $p \leq 0.05$ compared to the same dose in WIS.

3.3 Effects of atomoxetine on DAT function and cellular distribution in mPFC

Medial Prefrontal Cortex

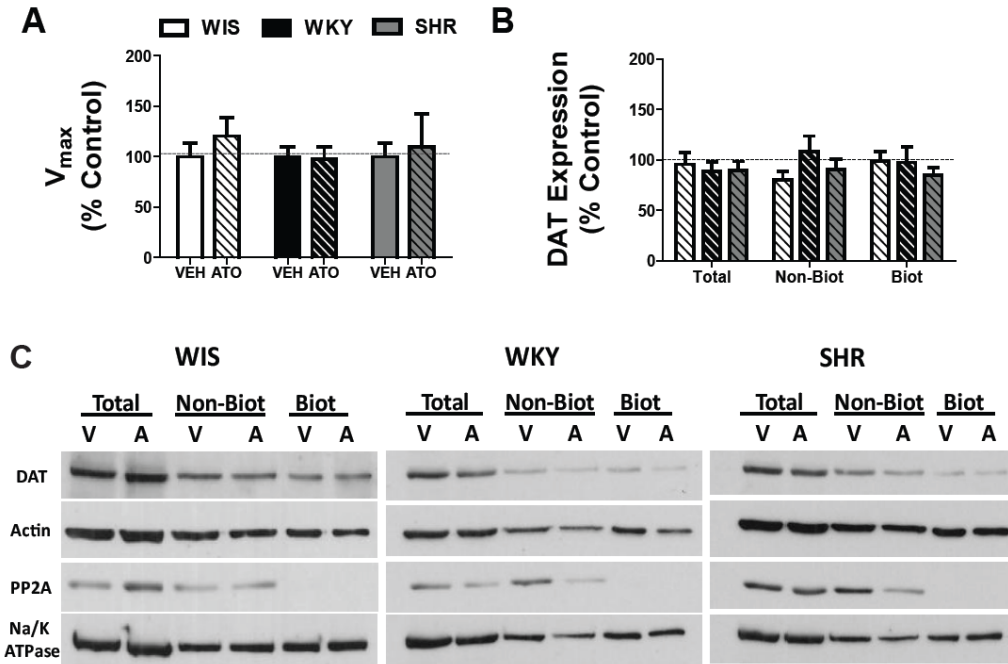


Figure 3. Dopamine transporter V_{max} and cell surface distribution in the medial prefrontal cortex (mPFC) of adult Wistar (WIS), Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) following discontinuation of adolescent treatment with atomoxetine (ATO) or vehicle (VEH). (A) V_{max} values are mean \pm S.E.M. pmol/mg/min expressed as a percent of vehicle control; (n = 6-10/strain and treatment). V_{max} values for WIS, WKY and SHR vehicle control groups were 3.0 ± 0.4 , 2.4 ± 0.3 and 3.9 ± 0.7 pmol/mg/min, respectively, and were not different from each other. (B) DAT expression values are mean \pm S.E.M. arbitrary units for DAT density following ATO treatment expressed as a percentage of vehicle control (dotted line, see Supplementary Materials for vehicle control values). (C) Representative blots for DAT cellular distribution between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions in mPFC synaptosomes from ATO-treated (A) and vehicle-treated (V) WIS, WKY and SHRs. Actin was used to normalize DAT expression for each individual sample while Na^+/K^+ ATPase and PP2A served to ascertain efficiency of biotinylation of the surface proteins; (n = 6-7/strain and treatment).

Figure 3.4 Effects of atomoxetine on DAT function and cellular distribution in OFC

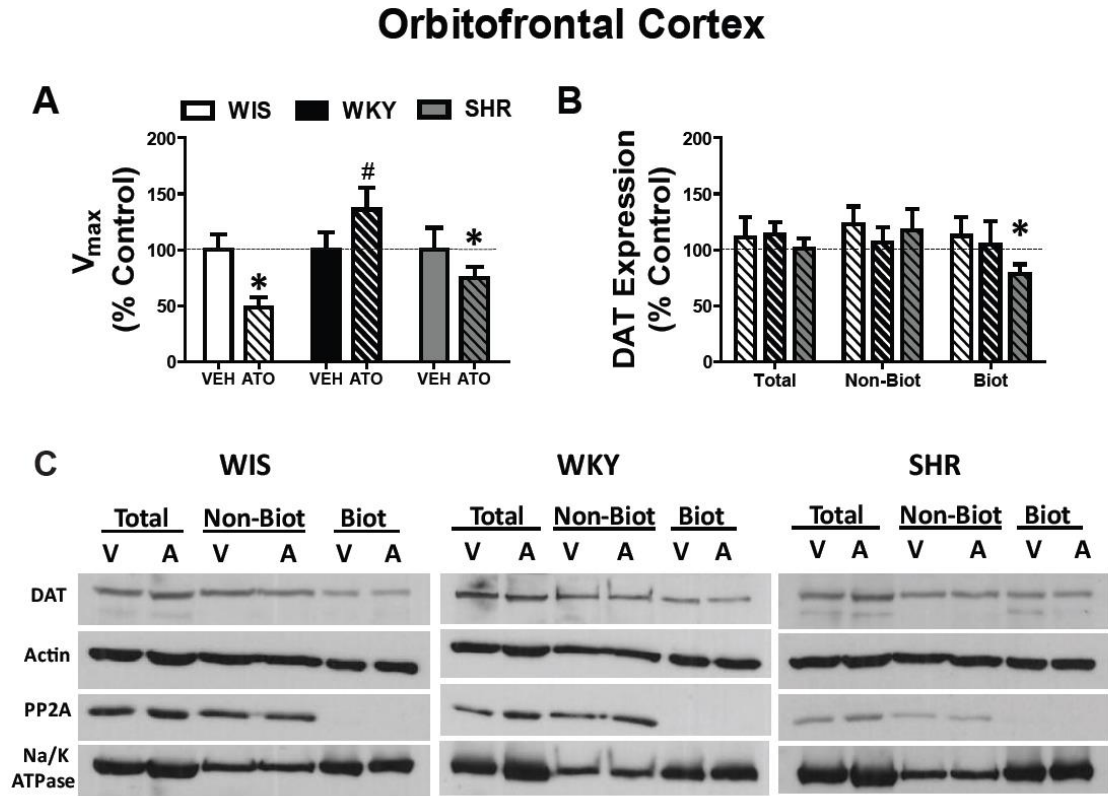


Figure 4. Dopamine transporter V_{max} and cell surface distribution in the orbitofrontal cortex (OFC) of adult Wistar (WIS), Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) following discontinuation of adolescent treatment with atomoxetine (ATO) or vehicle (VEH). (A) DAT V_{max} values are mean \pm S.E.M. pmol/mg/min expressed as a percent of vehicle control; (n = 6-10/strain and treatment). V_{max} values for WIS, WKY and SHR vehicle control groups were 3.5 ± 0.4 , 2.8 ± 0.5 and 4.3 ± 0.9 pmol/mg/min, respectively, and were not different from each other. (B) DAT expression values are mean \pm S.E.M. arbitrary units for DAT density following ATO treatment expressed as a percentage of vehicle control (dotted line, see Supplementary Materials for vehicle control values). (C) Representative blots for distribution of DAT between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions in OFC synaptosomes from ATO-treated (A) and vehicle-treated (V) WIS, WKY and SHRs. Actin was used to normalize DAT expression for each individual sample, while Na^+/K^+ ATPase and PP2A served to ascertain the efficiency of biotinylation of surface proteins; (n = 6-7/strain and treatment). * $p \leq 0.05$ compared to the vehicle control value of 100%, # $p \leq 0.05$ compared to ATO-treated WIS and SHR.

Figure 3.5 Effects of atomoxetine on DAT function and cellular distribution in striatum

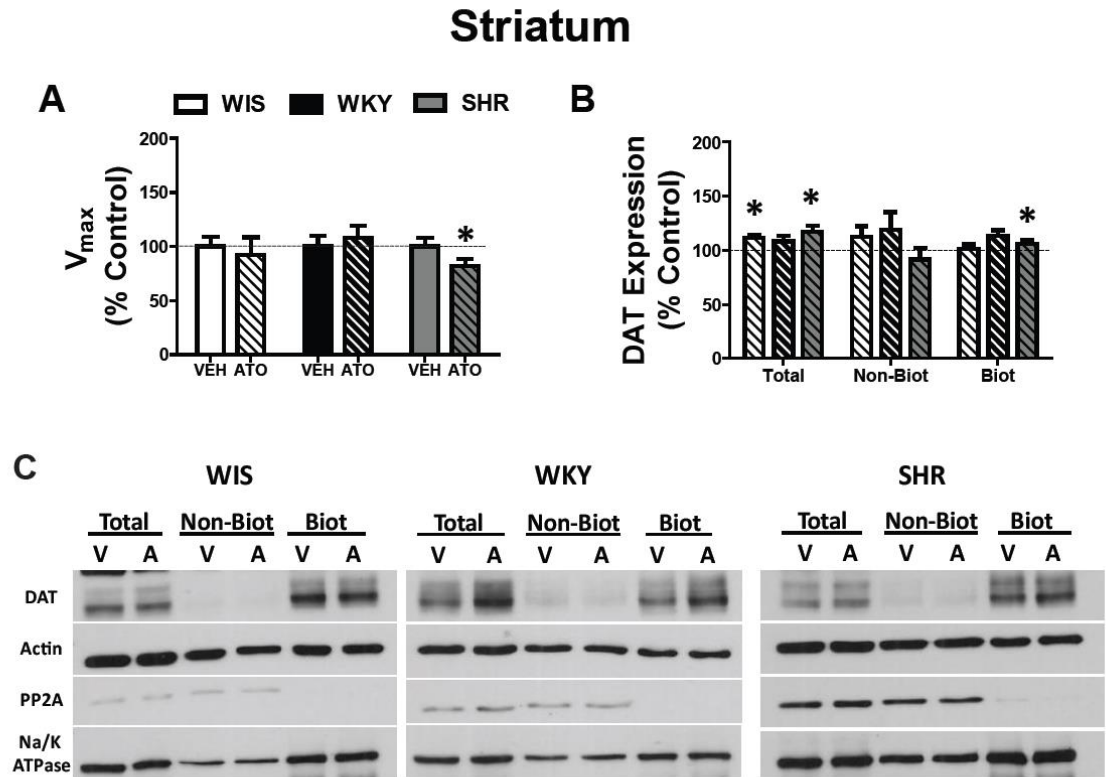


Figure 5. Dopamine transporter V_{max} and cell surface distribution in the striatum of adult Wistar (WIS), Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs) following discontinuation of adolescent treatment with atomoxetine (ATO) or vehicle (VEH). (A) DAT V_{max} values are mean \pm S.E.M. pmol/mg/min expressed as a percent of vehicle control; (n = 5-6/strain and treatment). V_{max} values for WIS, WKY and SHR vehicle control groups were 19.0 ± 1.7 , 15.4 ± 2.3 and 17.3 ± 1.8 pmol/mg/min, respectively, and were not different from each other. (B) DAT expression values are mean \pm S.E.M. arbitrary units for DAT density following ATO treatment expressed as a percentage of vehicle control (dotted line, see Supplementary Materials for vehicle control values). (C) Representative blots for distribution of DAT between total, non-biotinylated (Non-Biot; intracellular) and biotinylated (Biot; cell surface) fractions in striatal synaptosomes from ATO-treated (A) and vehicle-treated (V) WIS, WKY and SHRs. Actin was used to normalize DAT expression for each individual sample while Na^+/K^+ ATPase and PP2A served to ascertain the efficiency of biotinylation of surface proteins; (n = 5-6/strain and treatment). * $p \leq 0.05$ compared to the vehicle control value of 100%.

3.6 Supplementary Materials and Methods

Housing conditions and treatment: Male Wistar (WIS)/Cr, Wistar-Kyoto (WKY)/Cr and Spontaneously Hypertensive (SHR) rats (Charles River Laboratories, Wilmington, MA, Kingston, NY or Raleigh, NC) arrived on postnatal day 25 (P25). The rats were housed individually. Rats were not given environmental enrichment because past research has demonstrated that environmental enrichment reduces ADHD-like symptoms in the SHR strain (Pamplona *et al*, 2009). For the rats used in the cocaine self-administration assays, rats received ATO (0.3 mg/kg, i.p., dissolved in 0.9% sterile physiological saline) or saline (2.0 ml/kg, i.p.) from P28 until P55. For the rats used in dopamine transporter function and cellular distribution assays, rats received ATO (0.3 mg/kg, i.p., dissolved in 0.9% sterile physiological saline) or saline (2.0 ml/kg, i.p.) from P28 until P55. Double volume was used to enable complete dissolution of ATO. Administration was from Monday through Friday to mimic the weekend “medication holiday” often recommended for individuals with ADHD (1996). In addition, rats were mildly food restricted (85-90% of their free-feeding body weight) to enable comparison with our previously published results (Harvey *et al*, 2011). After P55, treatment was terminated, *ad libitum* food access was reinstated, and no environmental enrichment was provided in the home cage. The 3-day acclimation period is standard protocol in animal studies assessing cocaine self-administration (Roberts *et al*, 2002), neurochemistry (Mateo *et al*, 2005) as well as in studies using adolescent rats (Levin *et al*, 2007). Since the objective of the current study was to identify the effects ATO treatment across the entire period of adolescence, the treatment was initiated at postnatal day 28. One way to include a longer acclimation period in the experimental design would be to wean the rat pups early prior to shipment. However, early weaning itself is stressful and is known to produce neurological modifications (Ferdman *et al*, 2007). Of particular concern is that maternal separation in SHR is known to decrease striatal DAT function (Womersley *et al*, 2011).

Surgery for Behavioral Studies: Animals were anesthetized with i.p. injections of 90 mg/kg ketamine and 10 mg/kg xylazine. Buprenex (0.05 mg/kg s.c.; Bulter Schein, Columbus, OH, USA) was given as a preemptive analgesic 5 min prior to anesthetization. Incisions were made to expose the right femoral vein and skull. A catheter constructed of silicon tubing (I.D. = 0.020 in, O.D. = 0.037 in) was inserted s.c. between these incisions. The proximal end of the catheter was inserted into the right femoral vein and anchored to tissue underlying the vein using surgical silk and 0.5 mm square Teflon mesh (C.R. Bard Inc., Charlotte, N.C.). The distal end of the catheter was attached to a 22-gauge pedestal mount (Plastics One, Roanoke, VA). The pedestal and four stainless-steel screws were attached to the skull with dental cement. The stainless steel tubing exiting the pedestal was plugged with a sealed piece of Teflon tubing and covered with a protective plastic outer screw cap. Rats were treated for 3-5 days post-surgery with i.v. Baytril (5 mg/kg; Bayer Health Supply, Kansas City, KS) to reduce risk of systemic infection, for 3 days with s.c. Meloxicam (0.3 mg/kg; Butler Schein, Columbus, OH, USA) to reduce inflammation, as well as twice daily with s.c. Buprenex (0.025 mg/kg; Bulter Schein, Columbus, OH, USA) for 48 hr following surgery, and as needed. Catheters were flushed daily Monday-Friday with 0.1 ml of 0.9% saline solution containing 0.3 IU heparin (Baxter Healthcare, Deerfield, IL, USA) and 6.7 mg of Timentin (Glaxo-SmithKline, Research Triangle Park, NC, USA). On weekends, a locking solution containing 100% glycerol and 1000 IU/ml of undiluted heparin (3:1) was infused into the catheters to fill dead space and minimize the occurrence of blockages, thus prolonging the longevity of the catheter. On Mondays, the locking solution was withdrawn and replaced with 0.1 ml of 0.9% saline and 0.3 IU heparin. Catheter patency was checked weekly both by withdrawal of blood and by monitoring for sedation following an intravenous infusion of 1.0 mg of methohexital sodium (0.1 ml, Brevital, JHP Pharmaceuticals, Rochester, MI). If a catheter was found to be non-functional, a new catheter was implanted into the left femoral vein or into the right or left jugular vein. The animals were allowed to recover before resuming cocaine self-administration

experiments. As a result, no animals were excluded from statistical analyses due to loss of patency.

Apparatus

For behavioral studies, eight experimental chambers (model ENV-008CT; Med Associates, St. Albans, VT) were equipped with two response levers, a white stimulus light above the active lever, and a house light. Chambers were outfitted with a single-channel fluid swivel (Instech Solomon, Plymouth Meeting, PA, USA) connected to a spring leash and counterbalanced arm assembly (Med Associates). Motor-driven syringe pumps located outside the chamber were used for i.v. drug delivery. Chambers were enclosed in a sound-attenuating cubicle (model ENV-018 M; Med Associates) equipped with a ventilation fan and 8-ohm speaker to provide background white noise. A personal computer programmed in Medstate Notation and connected to a Med Associates interface controlled experimental events.

Neurochemical Studies

Materials: For the dopamine uptake assay, desipramine hydrochloride, paroxetine hydrochloride, nomifensine maleate, pargyline, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), sucrose, β -mercaptoethanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-hydroxytyramine (dopamine), sodium chloride and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO). α -d-Glucose, L-ascorbic acid, and monobasic potassium phosphate were purchased from Aldrich Chemical Co. (Milwaukee, WI), AnalaR-BHD Ltd. (Poole, UK) and Mallinckrodt (St. Louis, MO), respectively. [^3H]Dopamine (dihydroxyphenylethylamine,3,4-[7- ^3H]; specific activity, 30.3 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences Inc. (Boston, MA). All other chemicals in the uptake assay buffers were purchased from Fisher Scientific Co. (Pittsburgh, PA).

For the cell surface localization assays, antibodies recognizing rat DAT (C-20; goat polyclonal antibody), demethylated protein phosphatase 2A-C (PP2A-C; 4B7; mouse

monoclonal antibody), anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP; sc2954 chicken polyclonal antibody) and anti-goat IgG-HRP (sc2020; donkey polyclonal antibody) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against alpha-1 Na⁺/K⁺ ATPase type-1 (mouse monoclonal antibody) were obtained from Abcam (Cambridge, MA). Antibodies recognizing β-actin (A 5441, mouse monoclonal antibody) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfosuccinimidobiotin (sulfo-NHS-biotin), d-biotin and immunoPure immobilized monomeric avidin gel were purchased from Pierce Chemical (Rockford, IL). Complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Indianapolis, IN). Immunobilon-P PVDF membranes were purchased from Millipore. HyGLO Quickspray Chemiluminescent HRP Antibody Detection Reagent and HyblotCL autoradiography films were purchased from Denville Scientific Inc. (Metuchen, NJ).

Dopamine Uptake Assay: DAT function was assessed using kinetic analysis of [³H]dopamine uptake into mPFC, OFC and striatal synaptosomes employing a previously published procedure (Marusich *et al*, 2011) with minor modifications. For each experiment, mPFC, OFC and striatum of one ATO-treated and one vehicle-treated rat of the same strain and age (P77-P84) were homogenized in separate glass homogenizers, each containing 20 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM sodium bicarbonate, pH 7.4) with 16-20 passes of a Teflon pestle. Synaptosomal suspensions were subjected to two centrifugation steps (2,000g, 10 min, 4 °C followed by 20,000g, 17 min, 4 °C) using an Avanti-J30I centrifuge (Beckman Coulter, Brea, CA). Resulting pellets were resuspended in 2.2 ml (mPFC and OFC synaptosomes) or 2.4 ml (striatal synaptosomes) ice-cold uptake buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO₄, 1.25 mM CaCl₂, 1.5 mM KH₂PO₄, 10 mM glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM L-ascorbic acid, saturated with 95% O₂/5% CO₂, pH 7.4). mPFC and OFC samples (100 μl aliquots of the 2.2 ml synaptosomal suspensions) and striatal samples (30 μl aliquot of the 2.4 ml synaptosomal suspension) were incubated for 5 min in a Dubnoff metabolic shaker (Precision Scientific, Winchester, VA) at 34 °C in a

saturated 95% O₂/5% CO₂ atmosphere in the absence or presence of 10 μM nomifensine, a DAT inhibitor, which was used to obtain nonspecific [³H]dopamine uptake. mPFC and OFC suspensions also contained 5 nM each of paroxetine and desipramine to prevent [³H]dopamine uptake by serotonin transporters and NETs, respectively. Subsequently, 1 of 7 final concentrations (0.01-1.0 μM) of [³H]dopamine was added to the buffer and incubation of the mPFC, OFC and striatal synaptosomal suspensions continued for 5, 5 and 10 min, respectively. [³H]Dopamine uptake was terminated by addition of 3 ml of ice-cold assay buffer, immediately followed by filtration through Whatman GF/B glass fiber filters (presoaked with 1 mM pyrocatechol for 3 hr at 4 °C) using a cell harvester (Biochemical Research and Development Laboratories, Gaithersburg, MD). Values for total and nonspecific [³H]dopamine uptake were obtained from the amount of radioactivity retained on the filters as determined by liquid scintillation spectrometry (model B1600TR; PerkinElmer Life and Analytical Sciences, Downers Grove, IL). Protein concentrations were determined with bovine serum albumin standards using the Bradford method (Bradford, 1976). Specific [³H]dopamine uptake was obtained by subtracting nonspecific uptake from total uptake, and the values were used to determine kinetic parameters (V_{max} and K_m) by employing the commercially-available GraphPad Prism 5.0 program (GraphPad Software Inc., San Diego, CA).

DAT cellular distribution assay: Synaptosomal pellets were resuspended in ice-cold sucrose solution (1.25 ml for mPFC and OFC, and 3 ml for striatum). Synaptosomal protein concentrations were determined as previously described. Biotinylation and Western blotting assay were performed using a previously published method (Somkuwar *et al*, 2013). Briefly, synaptosomal suspensions contained 1 mg protein for mPFC and OFC and 500 μg protein for striatum. Suspensions were incubated with shaking for 1 hr at 4 °C in 500 μl of 1.5 mg/ml sulfo-NHS biotin in PBS/Ca/Mg buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.4), which labels all surface proteins with biotin. Free sulfo-NHS biotin was removed

by centrifugation (8000g, 4 min, 4 °C) using a model 5417R centrifuge (Eppendorf, Hauppauge, NY) followed by washing with 1 ml ice-cold 100 mM glycine in PBS/Ca/Mg buffer, and these steps were repeated twice. Then, samples were centrifuged using the same conditions and washed with 1 ml ice-cold PBS/Ca/Mg buffer, and these steps were repeated twice. Subsequently, synaptosomes were lysed by sonication for 2-4 s followed by incubation, with continuous shaking, for 20 min at 4 °C in Triton X-100 buffer (150 µl for mPFC and OFC synaptosomes, and 300 µl for striatal synaptosomes; 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 250 µM phenylmethanesulfonyl fluoride, pH 7.4). Lysates underwent centrifugation (21,000g, 20 min, 4 °C). Supernatants constituted the total protein fraction. To obtain the cell surface and intracellular fractions, 2/3 of the supernatant was incubated with Avidin beads with shaking for 1 hr at room temperature, and samples centrifuged (17,000g, 4 min, 4 °C). Supernatants constituted the non-biotinylated fraction (intracellular fraction). Pellets contained the Avidin-conjugated biotinylated proteins (cell surface fraction). Pellets were washed three times with 1% Triton-X-100 and then incubated for 20 min at room temperature with Lamelli buffer containing 5% v/v β-mercaptoethanol to free the cell surface proteins from the Avidin-biotin complex. Total, non-biotinylated and biotinylated fractions were frozen at -20 °C until Western blot analysis.

Total, non-biotinylated and biotinylated fractions were thawed and subjected to gel electrophoresis using a gel running apparatus Western blotting using Trans-Blot® SD Semi-Dry Transfer Cells (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were incubated (overnight, 4 °C) with primary antibody for DAT, followed by secondary antibody (1 hr, room temperature). DAT protein (72 kDa) was detected using enhanced chemiluminescence and HyblotCL autoradiography film (Denville Scientific Inc.). Blots were probed simultaneously for detection of Na⁺/K⁺ ATPase (a plasma-membrane enriched protein; 100 kDa), PP2A (an intracellular protein; 34 kDa) for determining efficiency of biotinylation of surface proteins, and β-actin (a cytoskeletal protein; 42 kDa) to control for loading of proteins. Band densities, expressed as relative optical

density, were determined for DAT and β -actin in total, non-biotinylated and biotinylated fractions using ImageJ software (<http://imagej.nih.gov/ij>).

Supplementary Results

Experiment 1: Acquisition of Cocaine Self-Administration (Fixed-Ratio).

The number of cocaine infusions earned at criterion for the 0.3 mg/kg training dose of cocaine is shown in Figure S1. Strains differed significantly ($F [2, 47] = 6.2, p \leq 0.004$). The treatment factor and its interaction with strain were not significant. Post-hoc comparisons showed that, overall, SHR earned more infusions than WKY ($p \leq 0.003$), and there was a trend towards more infusions compared to WIS ($p \leq 0.09$). Active lever responses at criterion are shown in Table S1. Strains significantly differed ($F [2, 47] = 4.5, p \leq 0.02$), and there was a trend towards a strain X treatment interaction ($F [2, 47] = 2.7, p \leq 0.08$). Post-hoc comparisons showed that, overall, SHR made more active lever responses than WKY ($p \leq .02$), and there was a trend towards more active lever responses compared to WIS ($p \leq .07$). Treatment comparisons within each strain revealed that in SHR, active lever responses were greater after ATO than vehicle ($p \leq 0.006$). Strain comparisons within each treatment condition revealed that in ATO-treated rats, SHR made more active lever responses than WKY and WIS ($p \leq 0.003$ and 0.006 , respectively). There were no strain differences in active lever responses among vehicle-treated rats at criterion. With respect to inactive lever responses, there were no strain or treatment differences at criterion (Table S1). In addition, each strain discriminated the active from inactive lever by a factor of 2 or greater (9:1 for WKY, 20:1 for WIS, and 4:1 for SHR, on average).

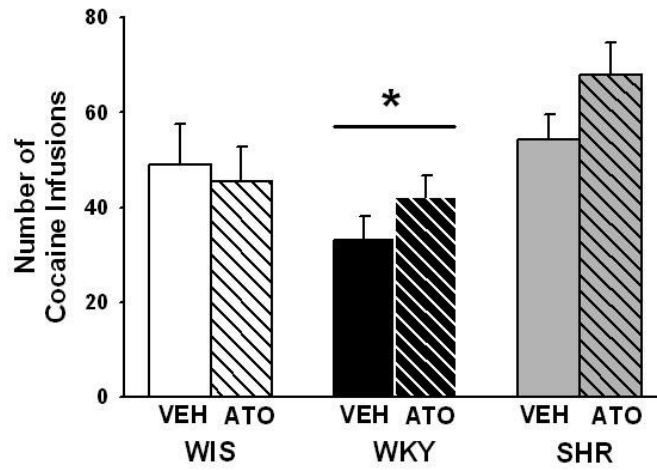


Figure S1. Infusions earned at the 0.3 mg/kg cocaine training dose after reaching the acquisition criterion. Values were obtained in adult Wistar-Kyoto (WKY; white bars), Wistar (WIS; black bars), and Spontaneously Hypertensive (SHR; grey bars) rats after discontinuation of adolescent treatment with atomoxetine (ATO; striped bars) or vehicle (VEH; solid bars). Values are mean \pm S.E.M. (n=8-11/strain and treatment). * $p \leq 0.05$ compared to SHR overall (denoted by horizontal line under the symbol).

Table S1. Active and inactive lever responses maintained by 0.3 mg/kg cocaine after reaching the acquisition criterion in adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats following discontinuation of adolescent treatment with atomoxetine or vehicle. Values are mean \pm S.E.M. (n=8-11/strain and treatment). * $p \leq 0.05$ compared to vehicle treatment in SHR.

Strain	Treatment	Lever Responses	
		Active	Inactive
WIS	Vehicle	70 \pm 17	4.0 \pm 1.3
	Atomoxetine	70 \pm 21	3.0 \pm 1.6
WKY	Vehicle	51 \pm 18	6.0 \pm 2.0
	Atomoxetine	51 \pm 6.3	5.0 \pm 1.9
SHR	Vehicle	74 \pm 7.9	37 \pm 34
	Atomoxetine	180 \pm 46 *	30 \pm 24

Experiment 2: Cocaine Dose-Response Functions (Fixed-Ratio).

The cocaine dose-response functions based on the number of active lever responses are shown in Figure S2. For the 3-way ANOVA, strain ($F [2, 43] = 4.9, p \leq 0.01$) and dose ($F [5, 215] = 36.9, p \leq 0.001$) differed significantly, and there was a dose X strain interaction ($F [10, 215] = 3.2, p \leq 0.001$). The treatment factor and its interactions with strain and/or dose were not significant. Post-hoc comparisons showed that SHR overall made more active lever responses than WKY ($p \leq 0.01$), but SHR were not different from WIS. Further testing of the strain X dose interaction revealed that SHR made more active lever responses at the 0.03 and 0.1 mg/kg doses than WKY and WIS ($p \leq 0.05$). Furthermore, WIS made more active lever responses at 0.1 mg/kg than WKY ($p \leq 0.003$). No strain differences at the other doses were found.

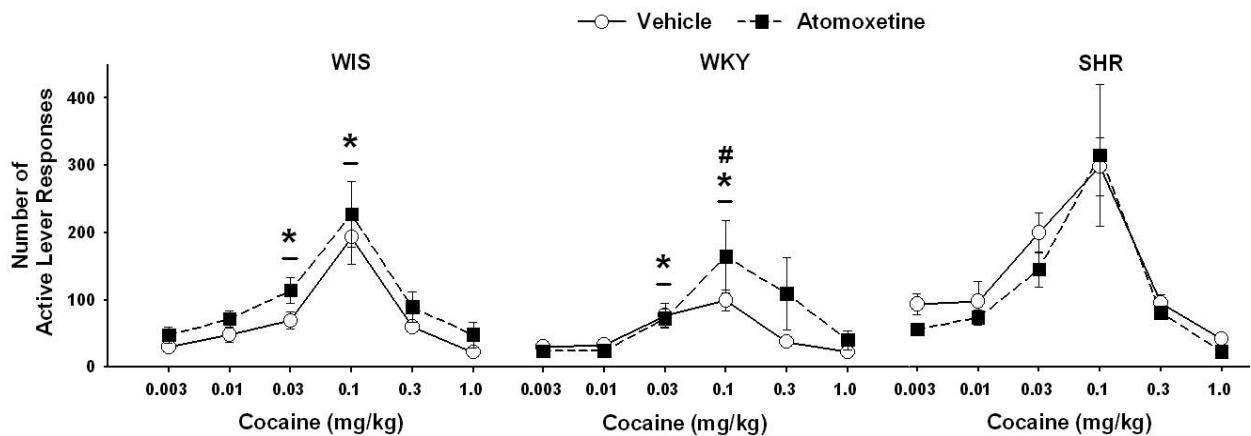


Figure S2. Cocaine dose-response functions under the FR1 schedule, based on the number of active lever responses. Values were obtained in adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats after discontinuation of adolescent treatment with atomoxetine (ATO; squares, dashed lines) or vehicle (VEH; circles, solid lines). Values are mean \pm S.E.M. ($n=8-9$ /strain and treatment). * $p \leq 0.05$ compared to SHR overall (denoted by horizontal line under the symbol). # $p \leq 0.05$ compared to the same dose in WIS.

Experiment 3: Cocaine Dose-Response Functions (Progressive Ratio).

The PR breakpoints based on the number of infusions earned are shown in Figure S3. For the 3-way ANOVA, strain ($F [2, 42] = 17.1, p \leq 0.001$) and dose differed significantly ($F [3, 126] = 158.4, p \leq 0.001$). The treatment factor and its interactions with strain and/or dose were not significant. Post-hoc tests showed that, overall, SHR had higher PR breakpoints than WKY and WIS ($p \leq 0.001$). Further analysis of the dose factor showed that animals had the highest PR breakpoint at the 1.0 mg/kg dose compared to the three other doses ($p \leq 0.001$), and the lowest PR breakpoint at the 0.01 mg/kg dose compared to the three other doses ($p \leq 0.001$). In addition, there was a higher PR breakpoint at the 0.3 mg/kg dose than at the 0.1 mg/kg dose ($p \leq 0.001$).

The PR breakpoints based on active lever responses are shown in Figure S4. For the 3-way ANOVA, strain ($F [2, 42] = 10.5, p \leq 0.001$) and dose ($F [3, 126] = 51.5, p \leq 0.001$) differed significantly. The treatment factor and its interactions with strain and/or dose were not significant. Post-hoc testing showed that, overall, SHR had higher PR breakpoints than WKY and WIS ($p \leq 0.001$ and $p \leq 0.008$, respectively). Further analysis of the dose factor revealed that animals had the highest PR breakpoint at the 1.0 mg/kg cocaine dose, compared to the three other doses ($p \leq 0.001$), and the lowest PR breakpoint at the 0.01 mg/kg cocaine dose, compared to the three other doses ($p \leq 0.001$, except at the 0.1 mg/kg dose where $p \leq 0.05$). PR breakpoints at the two intermediate cocaine doses, 0.3 mg/kg and 0.1 mg/kg, did not differ from each other.

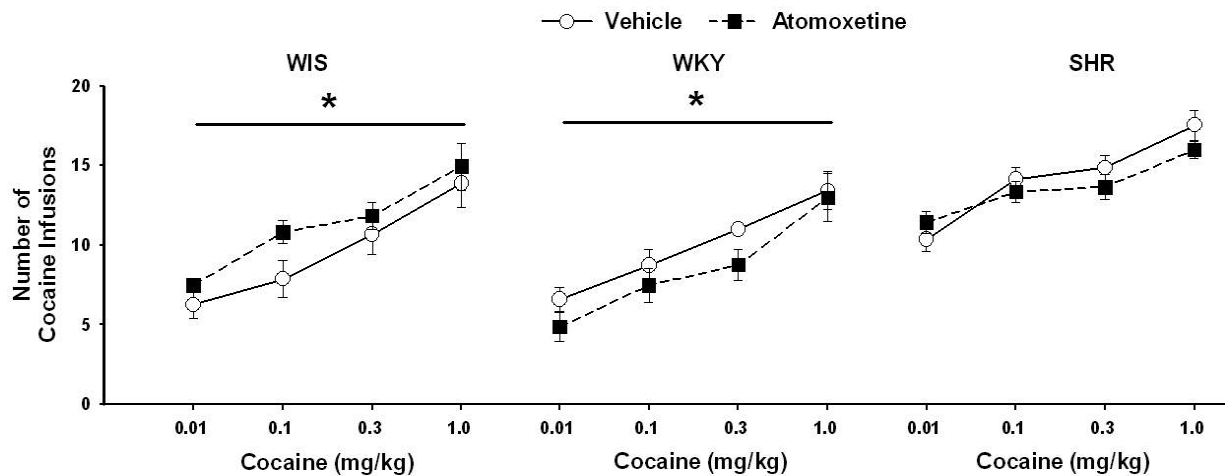


Figure S3. Progressive ratio breakpoints based on the number of cocaine infusions earned. Values were obtained in adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats after discontinuation of adolescent treatment with atomoxetine (ATO; squares, dashed lines) or vehicle (VEH; circles, solid lines). Values are mean \pm S.E.M. (n=8/strain and treatment). * $p \leq 0.05$ compared to SHR overall (denoted by horizontal line under symbol).

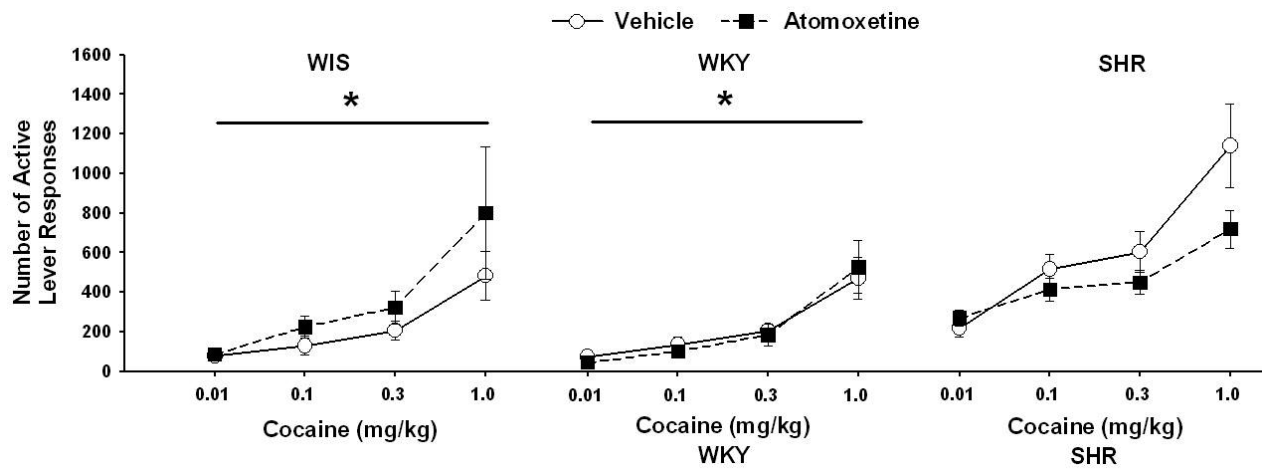


Figure S4. Progressive ratio breakpoints based on the number of active lever responses. Values were obtained in adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats after discontinuation of adolescent treatment with atomoxetine (ATO; squares, dashed lines) or vehicle (VEH; circles, solid lines). Values are mean \pm S.E.M. (n = 8/strain and treatment). * $p \leq 0.05$ compared to SHR overall (denoted by horizontal line under symbol).

Experiment 4: Dopamine Uptake.

Atomoxetine did not alter affinity of dopamine for DAT. Two-factor ANOVA comparing log transforms of Km values for dopamine did not reveal significant interactions of strain X treatment in mPFC (F [2,40] = 0.20, p > 0.05), OFC (F [2,40] = 0.002, p > 0.05) or striatum (F [2,34] = 0.49, p > 0.05).

Table S2. Km values for dopamine uptake at the dopamine transporter in the medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC) and striatum of adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHRs) following atomoxetine (ATO) or vehicle (VEH) treatment during adolescence. Values (nM) are expressed as mean ± S.E.M; n = 6-10/strain and treatment.

	WIS		WKY		SHR	
	VEH	ATO	VEH	ATO	VEH	ATO
mPFC	77 ± 8.2	110 ± 27	103 ± 18	105 ± 14	75 ± 12	86 ± 4.2
OFC	79 ± 7.5	98 ± 29	81 ± 13	90 ± 16	75 ± 10	82 ± 12
Striatum	33 ± 3.8	30 ± 0.82	36 ± 3.4	36 ± 2.9	33 ± 3.3	28 ± 2.2

Experiment 5: DAT Cellular Distribution.

DAT expression and cellular distribution did not differ between vehicle-treated SHR, WKY and WIS. One-way ANOVAs comparing DAT expression in mPFC, OFC and striatum revealed no significant effect of strain in total, non-biotinylated (intracellular) and biotinylated (surface) fractions. There was a trend towards a significant strain effect in the biotinylated (surface) fraction from striatum. DAT expression in SHR and WIS striatal surface tended to be greater than in WKY (see Table S3 for F-statistics).

Table S3: Band density for DAT in the total, non-biotinylated (intracellular) and biotinylated (surface) fractions from medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC) and striatum of adult Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHRs) that were administered saline during adolescence. Values are expressed as mean \pm S.E.M in arbitrary units; n = 6-8/strain. ^a ps > 0.05; ^b p = 0.053

		WIS	WKY	SHR	F-statistics ^a
mPFC	Total	62 \pm 9.4	45 \pm 10	85 \pm 16	F [2, 17] = 2.5
	Non-biotinylated	36 \pm 7.7	20 \pm 4.8	39 \pm 11	F [2, 17] = 1.5
	Biotinylated	25 \pm 8.4	19 \pm 4.5	27 \pm 4.8	F [2, 18] = 0.41
OFC	Total	63 \pm 14	54 \pm 11	73 \pm 12	F [2, 21] = 0.63
	Non-biotinylated	31 \pm 7.7	38 \pm 7.6	43 \pm 5.5	F [2, 21] = 0.72
	Biotinylated	23 \pm 3.1	23 \pm 4.9	33 \pm 7.1	F [2, 20] = 1.2
Striatum	Total	159 \pm 13	179 \pm 9.0	182 \pm 18	F [2, 19] = 0.85
	Non-biotinylated	96 \pm 9.8	74 \pm 12	76 \pm 15	F [2, 20] = 1.1
	Biotinylated	188 \pm 11	153 \pm 6.3	186 \pm 15	F [2, 20] = 3.4 ^b

4 CHAPTER FOUR

Norepinephrine transporter function in the prefrontal cortex following methylphenidate treatment during adolescence in a rat model of attention deficit/hyperactivity disorder

4.1 Introduction

Attention deficit/hyperactivity disorder (ADHD) is one of the most prevalent neurobehavioral disorders that affect ~12-15% children worldwide and persists into adulthood in ~4-5% individuals (Froehlich *et al*, 2007; Polanczyk *et al*, 2007). Although, the disease etiology is suspected to be complex multigenetic interaction (Faraone *et al*, 2005; Gizer *et al*, 2009; Kuntsi and Klein, 2012), several lines of evidence suggest that deficits in noradrenergic neurotransmission partially underlie ADHD etiopathology (Biederman and Spencer, 1999a; Levy, 2009). Polymorphisms of the norepinephrine transporter (NET) gene have not been unequivocally associated with the etiology of ADHD (Barr *et al*, 2002; McEvoy *et al*, 2002), see also (Kollins *et al*, 2008). However, selective inhibition of NET via ATO has been established as a therapeutically efficacious strategy for ADHD children and adolescents (Garnock-Jones *et al*, 2009; Spencer *et al*, 2001; Wilens *et al*, 2006). Moreover, the currently available FDA-approved therapeutics for ADHD increase noradrenergic neurotransmission, either indirectly via inhibition of NET (e.g., methylphenidate; MPH) or by directly activating post-synaptic α 2A adrenergic receptors (e.g., guanfacine) (Berridge *et al*, 2006; Bymaster *et al*, 2002; Fernando *et al*, 2012; Ma *et al*, 2005).

Reduced noradrenergic neurotransmission, particularly in prefrontal cortex, has been strongly associated with hallmark ADHD symptoms of high impulsivity, hyperactivity and reduced attention. Inhibition of α_2 adrenergic receptors (A, B and C receptor subtypes) with intra-cortical administration of yohimbine has been shown to produce an ADHD profile in primates (Li *et al*, 1994; Ma *et al*, 2005; Ma *et al*, 2003). Further evidence for cortical involvement is provided by studies in rats in which a low dose of MPH (2.0mg/kg, i.p.) resulted in positive blood oxygen dependent (BOLD) signals in the orbitofrontal cortex (OFC), and resulted in negative BOLD signals in the motor and somatosensory cortices (Easton *et al*, 2009). At therapeutically relevant doses, MPH preferentially increased extracellular norepinephrine (NE) in the prefrontal cortex compared to both NE in subcortical areas as well as dopamine in the prefrontal cortex (Berridge *et al*, 2006). Moderate increases in extracellular NE activates α_2A receptors, decreases intracellular second messenger cyclic adenosine monophosphate (cAMP) concentrations and thereby enhances the strength and duration of firing of cortical pyramidal neurons (Wang *et al*, 2007). Enhanced pyramidal neuronal firing is hypothesized mechanism by which ADHD medications improve attention and reduce impulsivity and working memory (Sagvolden, 2006; Wang *et al*, 2007). Indeed, MPH has been shown to increase activation of cortical α_2A adrenergic receptors in non-human primates and in rats (Arnsten, 2009; Gamo *et al*, 2010). Taken together, enhancing noradrenergic neurotransmission in the cortical areas via inhibition of NET is critical for therapeutic action of MPH. However, few studies have investigated the long-term consequences of MPH treatment during childhood and adolescence on NET function during adulthood.

The central noradrenergic system undergoes dynamic changes through childhood to adulthood. Cortical NE concentration in rhesus monkeys increases steadily from birth to adulthood, and during adulthood, cortical NE far exceeded dopamine and serotonin concentrations (Goldman-Rakic *et al*, 1982). In the rodent locus coeruleus, the nucleus of origin for NE neurons in brain, NET expression was high during the early postnatal period, and significantly decreases following adolescence into early adulthood

(Sanders *et al*, 2005; Zhu *et al*, 2005b). In contrast, NET expression in the forebrain increased from birth to post-natal day 15 (PND 15), and the expression levels were maintained through adulthood (Sanders *et al*, 2005), which was consistent with previously reported developmental changes in NET function (Coyle *et al*, 1971). α_2 adrenergic receptors followed a similar developmental trajectory in rat cortex (Happe *et al*, 2004). Taken together, these studies suggest that the cortical noradrenergic system is malleable during early childhood, but not during adolescence. Thus, the cortical noradrenergic system is expected to be resilient with respect to mild pharmacological manipulations, such as treatment with therapeutically relevant low doses of MPH.

Results from clinical neuroimaging studies suggest that cortical maturation in individuals with ADHD is developmentally delayed (Chandler *et al*, 2013b; Matecka *et al*, 1997b; Sanchez-Mora *et al*, 2013). As a consequence, the noradrenergic system in the ADHD population may be more vulnerable to long-term treatment with MPH during adolescence. Previous studies in the Spontaneously Hypertensive Rat (SHR), a well-established model of ADHD (Sagvolden, 2011; Sagvolden *et al*, 2005b), have found that MPH treatment during adolescence produced lasting changes in dopamine transporter (DAT) function in prefrontal cortex and its subregions (Harvey *et al*, 2011; Somkuwar *et al*, 2013a). Further, MPH treatment during adolescence increased impulsivity and cocaine self-administration in SHR compared to control Wistar-Kyoto and Wistar rats that did not exhibit the ADHD phenotype (Harvey *et al*, 2011); Somkuwar *et al*, 2013 submitted; Chapter 7). Both MPH and cocaine inhibit NET (Richelson *et al*, 1984). NE depletion in the medial prefrontal cortex (mPFC) inhibits the cocaine-induced increase in dopamine in nucleus accumbens and cocaine reward in mice (Ventura *et al*, 2007). Taken together, these results suggest that in SHR, chronic treatment with MPH during adolescence may produce lasting alterations in NET function, which in turn may be associated with the enhanced impulsivity and reinforcing effects of cocaine during adulthood.

Clinical verification of the role of NET in the pathophysiology of ADHD is currently not well understood (Zimmer, 2009), in part, due to the paucity of suitable radioligands for NET for use in positron emitted tomography (PET) studies (for review, Ding *et al*, 2006). Evaluation of NET function has been further stymied by the high non-specific binding of the standard NET inhibitors such as nisooxetine, desipramine and oxaprotilin. The high non-specific binding of NET ligands also has been an impediment for evaluating NET function using *in vitro* preparations such as kinetic analysis of uptake of [³H]NE into brain synaptosomes. The current study is aimed at isolating NET function using an *in vivo* approach to overcome these methodological challenges.

High speed chronoamperometry is an *in vivo* electrochemical method for detecting molecules that readily undergo oxidation and reduction when coming in contact with an electrode held at an appropriate voltage (Borland and Michael, 2007). Concentrations of such electroactive molecules are directly proportional to the current produced during their oxidation and reduction at the electrode surface. NE is one such electroactive analyte in which the phenolic ring readily oxidizes to an orthoquinone and then reduces back to the phenol ring when a 0-0.55V square-wave pulse is applied in a physiological (pH 7.4) media. Under these experimental conditions, specific NET function can be inferred from real-time clearance of exogenously applied NE, circumventing the potential confound of amount released altering clearance measurements (McCrae *et al*, 2005). The latter study evaluated DAT function by determining clearance of exogenously applied dopamine. DAT and NET share functional and structural homologies and are comparable in their substrate selectivity (Miller *et al*, 2010; Moron *et al*, 2002; Whiteside *et al*, 2005). Hence, the electrochemical procedures used in the current study relied on methods and approaches of previous studies evaluating DAT function (Liu *et al*, 2009; Ram *et al*, 2004; Zhu *et al*, 2007).

The aim of the current study was to evaluate *in vivo* NET function in subregions of prefrontal cortex, including the cingulate gyrus (CG), prelimbic cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO). The experimental protocol

was optimized and employed to identify differences between SHR and controls WKY and WIS following MPH or vehicle treatment through adolescence on NET function during adulthood.

4.2 Materials and Methods

4.2.1 Drugs and Reagents

(±)-MPH was purchased from Sigma-Aldrich (St. Louis, MO). MPH was dissolved in water to obtain concentrations of 1.5 mg/ml (prepared daily) and injected into oyster crackers (Kantak *et al*, 2008) to attain a dose of 1.5 mg/kg for oral administration. (±)-Norepinephrine (+)-bitartrate (NE), GBR12909 (1-2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride), ascorbic acid, sodium chloride and Nafion perfluorinated ion-exchange resin (5% solution) were purchased from Sigma (Milwaukee, WI). Sodium phosphate dibasic and sodium phosphate monobasic were purchased from Fischer Scientific (Fair Lawn, NJ). Urethane was purchased Sigma Life-Sciences (St. Louis, MO). Perchloric acid (70%) was purchased from Sigma-Aldrich (Milwaukee, WI). Sticky wax was purchased from FDJ/On time (Winter Park, FL).

4.2.2 Animals

For optimization of the NE clearance assay, male and female adult Sprague-Dawley rats were obtained from the Division of Laboratory Animal Resources (DLAR) at University of Kentucky. These rats had history of operant conditioning or Pavlovian conditioning including acute morphine injection during adulthood. For the comorbidity experiments, drug naive male SHR, WKY and WIS rats at post natal day (P) 25 were obtained from Charles River Laboratories (Kingston, NY or Raleigh, NC). Rats were individually housed with free access to food and water (unless specified otherwise) in a

colony room maintained on a 12-hour light:dark cycle (lights on 07:00 hour) in the DLAR (University of Kentucky, Lexington, KY). Rat handling procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and were performed in accordance with the 1996 version of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

4.2.3 Treatment

Following three days of habituation, rats were administered MPH (1.5 mg/kg, p.o.) or vehicle (VEH, water, 1 ml/kg, p.o. using one oyster cracker) from P28 to P55. P28 to P55 includes a time period from early adolescence to late adolescence (Doremus-Fitzwater *et al*, 2010; Spear, 2000a), the typical age during which MPH is administered clinically. MPH or vehicle was administered from Monday to Friday to mimic the weekend medication holiday often practiced in the treatment of children and adolescents with ADHD (American Psychiatric Association., 2013a).

The dose of MPH (1.5 mg/kg, p.o.) in outbred rats provides clinically-relevant plasma concentrations of MPH (Wargin *et al*, 1983), that increases extracellular dopamine and norepinephrine in the prefrontal cortex, but are below threshold for producing locomotor activation or increasing striatal dopamine concentration (Berridge *et al*, 2006; Kuczenski *et al*, 2002). In the SHR, MPH at 1.5 mg/kg, administered orally, through adolescence improves attention and behavioral flexibility (Harvey *et al*, 2013; Harvey *et al*, 2011; Kantak *et al*, 2008). From P56 onwards, the rats were individually housed with free access to food and water in a colony room until the electrochemical experiments were conducted. Between P77-91 rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed on a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Urethane was chosen because it did not alter DAT function *in vivo* (Weed, 2004). Body temperature was maintained at 34-35°C (the rat core temperature) (DeBow *et al*, 2003) using a heating pad coupled with a rectal thermometer (Harvard Apparatus, Holliston,

MA). A longitudinal incision on the skin over the scalp and the skin was retracted. Bleeding was stopped by local application of epinephrine (5 μ g/ml, in saline) and a styptic-powder with benzocaine (Kwikstop, ARC Laboratories, Atlanta, GA). The excess epinephrine was washed away and the exposed skull was air-dried. A small hole was drilled in the skull over the posterior cortex for placement of an Ag/AgCl silver reference electrode. A larger hole was drilled in the skull overlying the mPFC and OFC and the dura removed to expose the cortex. The exposed brain was cleaned of the blood using sterile saline. The electrode micropipette assembly was lowered into the CG (+ 2.9mm AP, \pm 0.8 mm ML, -1.6 mm DV), PrL (dorsal +2.9 mm AP, \pm 0.8 mm ML, -2.5 mm DV and ventral +2.9 mm AP, \pm 0.8 mm ML, -3.2 mm DV), IL (+2.9 mm AP, \pm 0.8 mm ML, -4.2 mm DV) and LO (+3.2 mm AP, \pm 2.6 mm ML, -4.2 mm DV). We chose to target the CG, PrL and the IL cortex subregions of mPFC separately because these regions have distinct intracortical and subcortical connections (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007; Vertes, 2006). Specifically, the CG and the dorsal coordinate of PrL are thought to project to the motor and sensory cortices, while the ventral PrL and IL project to the limbic and associative areas.

4.2.4 *In vivo* electrochemical measurements

To assess NET function, NE clearance from the extracellular space was determined following local ejection of NE into the mPFC or the OFC in urethane anesthetized rats using previously published method for evaluation of dopamine clearance (Ram *et al*, 2004; Zhu *et al*, 2007) with a few modifications. The electrochemical recording electrodes (Center for Microelectrode Technology, Lexington, KY) consisted of a single carbon fiber (30 μ m diameter) sealed in a glass capillary with 150- to 200- μ m length exposed. To enhance the selectivity of the electrode for the positively-charged catecholamines over the negatively-charged endogenous moieties (e.g., ascorbic acid), the exposed region of the carbon-fiber electrode was coated with

Nafion (10 swirls) and cured by heating at 200 °C for 5 min (Gerhardt *et al*, 1984). Electrodes were calibrated in vitro in 0.1 M phosphate buffer saline (pH 7.4) at room temperature and showed a linear current by concentration response for NE (2-8 μ M) with correlation coefficients ranging from 0.98 to 1.00. A Fast16 mkIII system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, KY) was used for electrode calibration. The range of selectivity for NE over ascorbic acid was 30 - 6800 to 1 (n=9/group). The Nafion coating process described above was repeated until the selectivity for NE over ascorbic acid reached >30:1. The range of the limit of detection for the electrodes used in the current study was 0.01 – 0.51 μ M (average = 0.11 μ M). Each electrode was attached to a single-barrel micropipette (internal diameter of tip, 10 – 20 μ m) with sticky-wax such that the electrode and the micropipette were 100 – 400 μ m apart.

Immediately before the lowering the recording assembly into the region of interest, the micropipette was filled with a non-saturating NE solution (100 or 200 μ M) with ascorbic acid (100 μ M) in saline (0.9% sodium chloride; Hospira Inc, IL) at pH 7.2-7.4. Although, NET expression is greater than DAT in the prefrontal cortex, NE has a high affinity for DAT, and therefore NE may be non-specifically cleared by DAT (Moron *et al*, 2002). Thus, clearance of NE by DAT was inhibited using a specific DAT inhibitor GBR12909 (Andersen, 1989) to isolate NET function. GBR12909 (50 nM) was used at a concentration ~15 times the K_i for inhibition of dopamine uptake at DAT (Matecka *et al*, 1997a). For the optimization experiments, the concentration of NE (200 μ M) selected was based on prior studies evaluating dopamine clearance in the mPFC using *in vivo* voltammetry (Zhu *et al*, 2007). For the comorbidity experiments, the concentration of NE (100 μ M) was reduced to improve the resolution of the volume of NE applied.

At 5 min intervals, NE solution was pressure-ejected (20-25 psi) from the micropipette using a Picospritzer II (General Valve Corporation, Fairfield, NJ). Volume of NE ejected (264 nl/mm) was measured using a reticule with a millimeter scale fitted in the eyepiece of a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL) that was focused on the meniscus of the NE solution in the micropipette. Oxidation potential

(+0.55 V for 100 msec) and reduction potential (0 V for 100 msec) were applied alternately to produce a square-wave potential. Electrochemical measurements of oxidation and reduction currents were made at 5 Hz and averaged to 1 Hz using Fast16 mkIII system. Stable baseline was defined as 3 consecutive signals for which the maximal amplitudes did not differ by $\pm 10\%$ following a constant ejection volume of NE solution. For each experiment 3 to 5 stable baseline signals were recorded.

The optimization experiments (n=3-4/group) evaluated the effects of DAT inhibition on NE clearance in mPFC and OFC. For these experiments, NE solution included GBR12909 (50 nM) for recording from the one hemisphere and did not include GBR12909 for recording in the opposite hemisphere. The sequence of the solution applied (with or without GBR12909) as well as brain hemisphere (left or right) was randomized between experiments.

For the comorbidity experiments (n = 6-9/group), a single-barrel micropipette containing NE solution (100 μ M) with GBR12909 (50 nM) and ascorbic acid (100 μ M) in saline (pH 7.2-7.4) was attached to the nafion-coated carbon-fiber electrode was lowered into mPFC or OFC. NE clearance was recorded from the four regions of interest using a within-subject design. The sequence for recording from mPFC and OFC was randomized between experiments. Recording from the CG, PrL and IL subregions of mPFC were conducted in that sequence by increasing the depths at the same AV-ML coordinates. Following 3 to 5 stable baseline signals from one region of interest, the electrode-pipette assembly was moved ventral to the next region of interest.

4.2.5 Verification of electrode placement

Immediately after each recording session, brains were removed and flash-frozen using 2-methylbutane, stored at -80°C for histological evaluation of microelectrode recording tracks. Brains were sectioned along the coronal plane (20 μ m sections) using a

Leica 1850 M cryostat (Nussloch, Germany) and sections were thaw mounted onto Fisher SuperFrost Plus® slides. The slides were stored overnight at room temperature under desiccation and stained with cresyl violet for determination of electrode placement. Probe placements were evaluated under a light microscope (1 X magnification) by observing the stained sections for tissue displacement (Fig 4.1). Only data from histologically confirmed microelectrode placements (Paxinos, 1991) were included in the subsequent analyses of results.

4.2.6 Electrochemical data acquisition and statistical analysis

Data were processed using a customized Matlab®-based analysis package, FastAnalysis Version 5.0 (Jason Burmeister Consulting, LLC). The primary parameters for evaluating NE clearance obtained from the data were peak amplitude (A_{\max} , in μM), the first-order rate constant of the signal (k_{-1} , in sec^{-1} , Liu *et al*, 2009) and the area under curve for the NE signal (AUC). NE signals were matched for amplitude (ranging from 0.6 to 1.5 μM). Range of A_{\max} was selected a priori to ensure that the Michaelis-Menten kinetics properties for NET were not altered by excessive concentration of extracellular NE. The rationale for the range of the NE A_{\max} selected were made based on prior studies evaluating DA clearance by DAT in striatum (Hebert *et al*, 1999a). Data from each brain region for each rat was averaged to one value. First order uptake rate was calculated as the product of peak amplitude and first order rate constant ($A_{\max} \times k_{-1}$, in nM/sec) (Ram *et al*, 2004). Clearance was calculated by dividing the amount of NE applied by the area under curve ($\text{NE}_{\text{amt}}/\text{AUC}$, in L/sec; Zhu *et al*, 2007). Space between the electrode and the micropipette was inferred from the ratio of the amount of NE applied through the micropipette and the peak amplitude of NE recorded at the electrode ($\text{NE}_{\text{amt}}/A_{\max}$, in L). The latter parameter is proposed in the current work based on the assumption that in a given brain region, $\text{NE}_{\text{amt}}/A_{\max}$ will increase proportionately with the electrode-micropipette distance.

Statistical analyses were conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). Data are reported as mean \pm S.E.M. and n represents the number of rats per group. For the optimization experiments, the dependent variables were compared using paired t-tests. For the comorbidity experiments, all the dependent variables, except for clearance, were compared using two-factor ANOVA (strain X treatment), and followed by Bonferroni's post-hoc comparisons. Outliers were omitted using the Grubbs test (GraphPad; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>). For comparing clearance in the comorbidity experiments two-factor ANCOVA were used with strain and treatment as between-subject factors and space between electrode and micropipette as the covariate. Significance was set at $p < 0.05$. Significant interactions were probed further using one-factor ANCOVAs to evaluate the effects of MPH treatment for individual strain.

4.3 Results

4.3.1 Histological assessment of electrode placements in mPFC and OFC

Examples of electrode placement based on tissue-displacement in mPFC and OFC are presented in Fig 4.1. Approximate location of the electrode tips in the LO and IL are presented in Fig 4.1 middle and bottom panels, respectively. For LO, localization of electrodes was between +3.2 to + 4.0 mm anterior to bregma and between 2.4 to 2.7 mm lateral to bregma. For mPFC, electrode localization was between +2.7 to +3.2 mm anterior to bregma and between 0.5 and 1.0 mm lateral to bregma. One rat was excluded from the mPFC analyses due to microelectrode placement error, because the track marks were outside the range for ML co-ordinates (at + 1.3 mm ML, and not within ± 1.0 mm ML).

4.3.2 Optimization experiments

No differences in amount of NE applied and A_{\max} were found between the hemispheres in which NE was applied alone and in which NE was applied with GBR12909 (50 nM) (Table 1). Average volume of solution that was pressure ejected into the brain was 28.6 ± 2.14 nL. In CG, dorsal PrL, ventral PrL, IL and LO subregions, no differences were found between the hemispheres in which NE was applied alone and in which NE was applied with GBR12909 for either uptake rate or clearance of NE (Fig 4.2, top panel and bottom panel, respectively).

4.3.3 Comorbidity experiments

No differences were found between MPH and VEH administered SHR, WKY and WIS in the amount of NE applied and in the A_{\max} (Table 3) for CG, dorsal PrL, ventral PrL, IL and LO subregions. The average volume of solution that was pressure ejected into the brain regions for all the experiments conducted was 47.8 ± 4.61 nL.

No differences were found between MPH and VEH administered SHR, WKY and WIS in the first order uptake rate of NE in CG, dorsal PrL, ventral PrL and IL (Fig 4.3). In contrast, a strain x treatment interaction was obtained for NE clearance in the IL after accounting for the between-experiment variation in electrode-micropipette space ($F_{\text{interaction}}[2,40] = 5.10$, $p < 0.05$; $F_{\text{covariate}} [1,40] = 144$, $p < 0.0001$; Fig 4.4). Post-hoc comparison revealed that MPH treatment during adolescence decreased NE clearance in the IL cortex of WIS. For the other subregions of mPFC, no effect of MPH treatment, strain and interaction were obtained for NE clearance, but a significant effect of experimental variation in the electrode-micropipette distance was obtained in CG (see Table 4 for statistics).

For uptake rate in the LO, a significant strain X treatment interaction was obtained ($F_{\text{interaction}}[2,45] = 4.38$, $p < 0.05$; Fig 4.5). Post-hoc comparisons revealed

that the uptake rate for vehicle administered SHR was greater than vehicle administered WKY and WIS, and MPH treatment during adolescence reduced the uptake rate in SHR. Further, the uptake rate of MPH-treated SHR was not different from MPH-treated WKY and WIS. In contrast, no difference was obtained for NE clearance in LO after accounting for the between-experiment variation in the electrode-micropipette space ($F_{interaction}[2,44] = 1.33, p > 0.05$; $F_{covariate} [1,44] = 113, p < 0.0001$). However, slopes for NE clearance in the LO against volume of brain between electrode-micropipette assembly indicated a trend in the same direction as for uptake rate (Fig 4.6).

4.4 Discussion

The optimization experiments revealed that inhibition of DAT by GBR12909 in mPFC and OFC subregions did not alter first order uptake rate of NE or NE clearance. These results are in agreement with previous findings that GBR12909 did not inhibit dopamine uptake into frontal cortical synaptosomes of mice (Moron *et al*, 2002). Compared to WKY and WIS, the uptake rate of NE in the LO subregion of OFC was greater in SHR, suggesting that NET function was elevated in SHR. Further, MPH treatment during adolescence followed by cessation of treatment during adulthood decreased the uptake rate of NE in LO of SHR, such that uptake rate in MPH-treated SHR was not different from MPH-treated WKY and WIS rats. Also, MPH treatment during adolescence decreased NE clearance in the IL-PFC of outbred WIS rats. No other effects of strain or treatment were obtained in either NE uptake rate or in NE clearance. These results suggest that increased NET function in the LO may underlie the behavioral deficits in SHR and that MPH treatment normalized LO NET function and that this normalization persisted long after discontinuation of MPH treatment.

Although, MPH treatment in adolescent SHR decreased the slope of the linear regression for NE clearance from the LO from 0.05 to 0.01 (Fig 4.6) suggesting a

decrease in NET function, statistical significance was not reached. In contrast, results for the IL-PFC reveal that NE clearance, but not uptake rate of NE in outbred WIS rats was significantly decreased by MPH treatment during adolescence. Taken together, these results suggest that although uptake rate and clearance of NE both indicate NET function, the current study found the two measures to be differentially sensitive to the effects of MPH between LO and IL-PFC. These differences may be explained partially by methodological consideration. Uptake rate is limited because it does not account for the amount of NE applied via the micropipette; amount of NE applied alters A_{\max} which is used to calculate uptake rate (Zhu *et al*, 2007). In contrast, clearance is a classical pharmacokinetic parameter providing the efficiency of NE removal by NET; theoretically, clearance is expected to a constant value independent of the amount of NE applied (Zhu *et al*, 2007). However, in the current study, the between-experiment variation in the volume (or space) between the microelectrode and the micropipette altered the observed clearance of NE (evidenced by significant effect of covariate). Therefore, clearance was compared between treatment and strains with the electrode-pipette volume as a covariate. The lack of statistical significance of strain and treatment interaction for clearance of NE in the LO may be explained by the relatively poor fit of the regression to the data (R^2 ; Fig 4.6). Thus, the interpretation of the results in this brain region is limited by the extent to which NE uptake rate represents NET function.

Based on uptake rate of NE, SHR have a hypofunctional noradrenergic system in the OFC (specifically, the LO), which may underlie some of the behavioral deficits in SHR such as impaired reversal learning, strategy shifting, working memory and attention (Harvey *et al*, 2013; Kantak *et al*, 2008; Sagvolden, 2006). These behavioral deficits may be mediated, in part, by weakened OFC projections to the locus coeruleus and ventral tegmental area which modulate neuronal firing of noradrenergic and dopaminergic neurons, respectively (Aston-Jones *et al*, 2005; Chandler *et al*, 2013a). MPH exerts its therapeutic effects by increasing extracellular NE in the prefrontal cortex and thereby, increasing $\alpha 2A$ receptor activation (Berridge *et al*, 2006; Wang *et al*, 2007). The current results indicate that chronic MPH treatment during adolescence normalizes the elevated

NET function in the SHR that persists long after treatment discontinuation. Clinically, MPH treatment is often discontinued because several ADHD symptoms diminish with age (Faraone *et al*, 2006; McCarthy *et al*, 2009). The current results suggest that MPH treatment during adolescence may contribute to the observed age-related reduction in ADHD symptoms.

Therapeutically relevant MPH treatment in adolescent SHR followed by treatment discontinuation increased cocaine self-administration during adulthood (Harvey *et al*, 2011). In contrast, MPH treatment during adolescence reduced the rewarding effects of cocaine in adult SHR as evidenced by rightward shifts in the dose-response curves for cocaine-induced conditioned place preference (Augustyniak *et al*, 2006). The latter study did not find a change in cocaine-evoked dopamine release in nucleus accumbens, which is known to be associated with the reinforcing effects of cocaine (Dewit *et al*, 1977; Ritz *et al*, 1987). These apparent disparities between the rewarding and the reinforcing effects of cocaine can be explained by the dichotomy of the role of NE neurotransmission between these behaviors. Specifically, NE neurotransmission is thought to be engaged by conditioned appetitive (as well as aversive) stimuli, but is not the primary mediator of the reinforcing effects of drugs of abuse (Feenstra *et al*, 1999; Mingote *et al*, 2004; Ritz *et al*, 1987). Previous studies suggest that in the mPFC, NE depletion inhibits cocaine conditioned place preference, but inhibition of $\alpha 1$ receptors does not alter cocaine self-administration (Ecke *et al*, 2012; Ventura *et al*, 2007). However, the implications of hypofunctional NE in the OFC, and normalization of NE uptake by MPH treatment on cocaine self-administration behavior in SHR are not well understood.

In the prefrontal cortex, NET function contributes to dopamine clearance (Moron *et al*, 2002). Thus, elevated NET function in the SHR may contribute to hypo-dopaminergic state in the LO. Mechanistically, impulsivity is associated with reduced dopamine transmission in the OFC (Winstanley *et al*, 2010b; Zeeb *et al*, 2010). Increased impulsivity, in turn, is associated with increased cocaine self-administration (Dalley *et al*,

2007; Dalley *et al*, 2008). Taken together, the elevated NET function in the LO observed in the current study may contribute to the increased impulsivity (Chapter 7) and increased cocaine self-administration (Harvey *et al*, 2011; Somkuwar *et al*, 2013b); see Chapter 1) in adult SHR compared to both WKY and WIS.

Impulsivity is a broad psychological construct that includes impulsive decision making, defined by increased choice of a smaller immediate reward over a larger delayed reward, as well as response inhibition deficit, defined by a reduced capacity to inhibit a prepotent response (Broos *et al*, 2012; Evenden, 1999b). Chronic NET inhibition during adolescence as well as increased dopamine receptor activation in the OFC decreased impulsive decision making (Mobini *et al*, 2002; Pardey *et al*, 2013; Sun *et al*, 2012). However, neither chronic NET inhibition during adolescence nor activation of dopaminergic receptors in the OFC altered response inhibition capacity in rats (Sun *et al*, 2012; Winstanley *et al*, 2010b). Alternate neurochemical mechanisms like increased DAT function in the mPFC and decreased mPFC dopamine (Sokolowski and Salamone, 1994; Somkuwar *et al*, 2013a) may underlie the increased response inhibition deficit in MPH-treated SHR (Chapter 7). Initiation of drug taking behavior (rate of acquisition of self-administration) is associated with increased response inhibition deficit (Diergaarde *et al*, 2008). However, maintenance of drug-taking behavior is associated with both impulsive decision making and response inhibition deficits. Although normalization of NET function in the LO of MPH-treated SHR may be partially protective against impulsive decision making, MPH treatment is not protective against cocaine abuse liability because of the increased in response inhibition deficit (Chapter 7).

In contrast the SHR, MPH treatment in adolescent WIS decreased acquisition of cocaine self-administration (Harvey *et al*, 2011). In terms of neurochemistry, MPH treatment during adolescence increased DAT function in mPFC of SHR, but not WIS (Somkuwar *et al*, 2013a). In contrast, only in WIS, NET function was decreased in the IL sub-region of mPFC by MPH treatment during adolescence, suggesting a localized increase in dopaminergic signaling in WIS IL-PFC (Moron *et al*, 2002). Currently, no

empirical evidence is available to support the involvement of IL-PFC in acquisition of cocaine self-administration. However, IL-PFC sends projections to the nucleus accumbens shell and shares reciprocal innervations with the amygdala (McDonald, 1996). This particular circuitry has been implicated in extinction learning in both fear conditioning as well as cocaine-seeking (Peters *et al*, 2008; Quirk and Mueller, 2008). With respect to cocaine-seeking, reversible inactivation of IL-PFC induced reinstatement of cocaine-seeking, while activation of IL-PFC (via AMPA infusion) attenuated cocaine-induced reinstatement (Peters *et al*, 2008). Further, disconnection between IL-PFC and nucleus accumbens shell induced reinstatement of cocaine-seeking (Peters *et al*, 2008). Therefore, IL-PFC is essential for expression of extinction learning as well as consolidation of extinction of cocaine-seeking (LaLumiere *et al*, 2010). These may be indirect mechanisms that contribute to the protective effects of MPH treatment in subjects without an ADHD phenotype. Additionally, the PL-PFC is involved in reinstatement of responding for cues associated with cocaine (Ball and Slane, 2012; McLaughlin and See, 2003). NET function was not altered in the PL-PFC or in the IL-PFC of MPH-treated SHR, which support for the observation that MPH treatment during adolescence does not alter extinction of cocaine self-administration or cocaine cue-reactivity in SHR (Fig 8.5; Jordan et al. submitted). Taken together, NET function in the mPFC subregions are not associated with the previously observed increased cocaine self-administration in SHR (Harvey *et al*, 2011).

In conclusion, the current study suggests that increased NET function in the LO underlies behavioral deficits in SHR, and chronic MPH treatment during adolescence normalized NET function that persisted into adulthood. Although, MPH-treatment mediated decreased NET function may be beneficial for reducing some behavioral deficits in SHR, MPH-treatment did not confer protection against increased cocaine self-administration. Thus, alternate therapeutic strategies need to be explored for treating adolescents with ADHD in whom increased cocaine abuse liability is a concern.

4.5 Tables

Table 4.1 Maximum amplitude of NE (A_{max}) and amount of NE applied for the optimization experiment

Maximum amplitude of NE (A_{max}) and amount of NE applied to cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO) for the optimization experiments. Norepinephrine (NE, 200 μ M) was applied alone (NE alone) or with GBR 12909 (50 nM; NE + GBR) in these brain regions on opposite hemispheres of the brain of individual rats. n = 3-4/group.

	Amax (μM)		Amount of NE applied (pmol)	
	NE alone	NE + GBR	NE alone	NE + GBR
CG	0.67 \pm 0.09 ^a	0.79 \pm 0.17	7.2 \pm 2.6	8.1 \pm 2.9
Dorsal PrL	1.1 \pm 0.04	0.80 \pm 0.04	8.3 \pm 1.6	4.5 \pm 1.1
Ventral PrL	1.0 \pm 0.25	0.83 \pm 0.11	4.0 \pm 1.4	3.4 \pm 1.1
IL	0.80 \pm 0.21	0.99 \pm 0.10	2.2 \pm 0.86	2.3 \pm 0.61
LO	1.3 \pm 0.28	1.7 \pm 0.30	6.1 \pm 1.8	11 \pm 2.2

^a Values are mean \pm S.E.M.

Table 4.2 Amount of NE applied and maximum amplitude of NE (A_{max}) for the comorbidity experiment

Amount of NE applied and maximum amplitude of NE (A_{max}) for cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO) of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH; 1.5 mg/kg, p.o.) or vehicle (VEH) during adolescence. Norepinephrine (NE, 100 μ M) was applied with GBR 12909 (50 nM) in these brain regions for individual rats using a within-subject design. n = 7–9/group for CG, dorsal and ventral PrL and LO; n = 6–8/group for IL.

		CG	Dorsal PrL	Ventral PrL	IL	LO
NE applied (in pmol)	SHR-VEH	7.2 \pm 2.6 ^a	3.7 \pm 0.90	3.2 \pm 1.2	2.3 \pm 0.61	7.4 \pm 5.0
	SHR-MPH	2.8 \pm 1.3	7.6 \pm 5.7	2.2 \pm 0.86	1.8 \pm 0.81	4.9 \pm 2.6
	WKY-VEH	4.4 \pm 1.3	4.4 \pm 0.84	7.3 \pm 3.0	4.1 \pm 1.4	5.6 \pm 2.0
	WKY-MPH	8.1 \pm 2.9	3.4 \pm 1.1	4.5 \pm 1.6	3.2 \pm 1.1	4.0 \pm 1.8
	WIS-VEH	10 \pm 7.0	4.0 \pm 1.4	8.3 \pm 4.5	5.0 \pm 3.1	4.8 \pm 1.0
	WIS-MPH	4.1 \pm 2.9	2.3 \pm 0.82	4.1 \pm 1.2	6.1 \pm 2.2	4.3 \pm 1.9
Amax (in μM)	SHR-VEH	0.57 \pm 0.11	0.68 \pm 0.13	0.69 \pm 0.13	0.67 \pm 0.09	0.89 \pm 0.15
	SHR-MPH	0.74 \pm 0.13	0.57 \pm 0.07	0.68 \pm 0.10	0.83 \pm 0.17	0.87 \pm 0.16
	WKY-VEH	1.2 \pm 0.33	0.81 \pm 0.23	0.97 \pm 0.37	0.65 \pm 0.12	0.69 \pm 0.15
	WKY-MPH	0.67 \pm 0.12	0.81 \pm 1.4	0.86 \pm 0.13	0.72 \pm 0.11	0.62 \pm 0.11
	WIS-VEH	1.2 \pm 0.28	1.2 \pm 0.23	1.3 \pm 0.25	1.2 \pm 0.38	0.61 \pm 0.09
	WIS-MPH	1.5 \pm 0.52	0.96 \pm 0.25	0.73 \pm 0.12	0.71 \pm 0.07	0.95 \pm 0.24

^a Values are mean \pm S.E.M.

Table 4.3 Clearance of NE in cingulate gyrus (CG), and dorsal and ventral prelimbic cortex (PrL)

Linear regression of clearance for NE (y) by Volume (or space between micropipette and electrode; x) and goodness-of-fit (R^2) for cingulate gyrus (CG), and dorsal and ventral prelimbic cortex (PrL) of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH; 1.5 mg/kg, p.o.) or vehicle (VEH) during adolescence. Norepinephrine (NE, 100 μ M) was applied with GBR 12909 (50 nM) in these brain regions for individual rats using a within-subject design. Clearance was calculated as amount of NE applied through the micropipette divided by the area-under-curve for the NE signal at the carbon-fiber electrode. Volume was calculated as amount of NE applied through the micropipette divided by the maximum amplitude of NE detected at the carbon-fiber electrode. n = 7-9/group for CG, dorsal and ventral PrL.

		SHR		WKY		WIS		F-statistics
		Regression	R^2	Regression	R^2	Regression	R^2	
CG	VEH	$y = 0.02x - 0.08$	0.86	$y = 0.02x - 0.01$	0.99	$y = 0.02x + 0.02$	0.98	<i>Finteraction</i> [2,45]=0.74
	MPH	$y = 0.01x + 0.01$	0.98	$y = 0.02x - 0.04$	0.98	$y = 0.01x + 0.00$	1.0	<i>Fcovariate</i> [1,45]=400*
Dorsal PrL	VEH	$y = 0.01x + 0.01$	0.94	$y = 0.01x - 0.00$	0.86	$y = 0.01x + 0.02$	0.70	<i>Finteraction</i> [2,42]=0.39
	MPH	$y = 0.02x - 0.01$	0.70	$y = 0.01x + 0.01$	0.93	$y = 0.02x - 0.02$	0.95	<i>Fcovariate</i> [1,42]=200*
Ventral PrL	VEH	$y = 0.01x + 0.01$	0.97	$y = 0.01x + 0.00$	0.99	$y = 0.01x + 0.01$	0.95	<i>Finteraction</i> [2,46]=3.1
	MPH	$y = 0.01x + 0.00$	0.93	$y = 0.01x + 0.00$	0.92	$y = 0.01x - 0.00$	0.93	<i>Fcovariate</i> [1,46]=650*

* $p < 0.0001$

4.6 Figures

Figure 4.1 Electrode placement

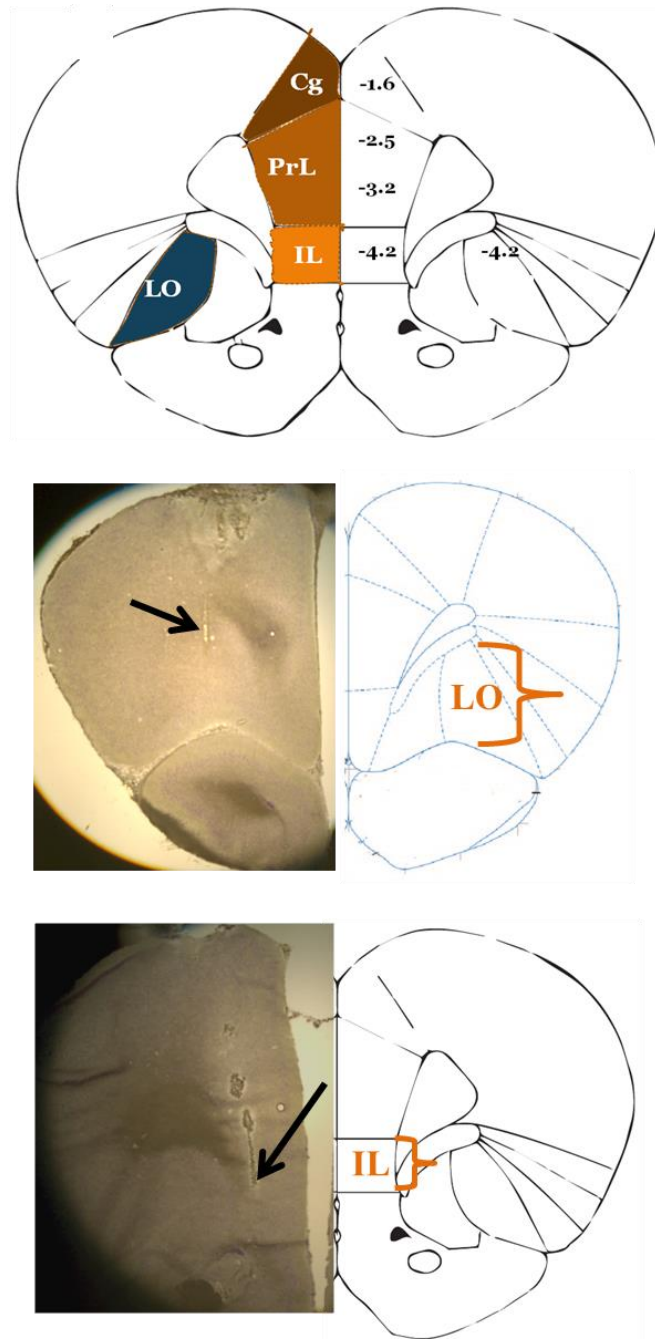


Fig 4.1 Neuroanatomical localization of electrodes. The regions of interest for evaluating NET function were cingulate gyrus (CG), dorsal and ventral prelimbic cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO; Top panel). Dorsoventral coordinates are presented (in mm) from the surface of the rat brain (Paxinos, 1991). The placement of the tip of carbon-fiber electrodes within the rodent LO (middle panel) and IL (bottom panel). The figure on the left side is a representative of the actual track-marks from a cresyl violet stained 20 μm slice for LO and mPFC (middle and bottom panels, respectively). The diagram on the right shows the approximate location of the tip of the carbon-fiber microelectrodes in the LO and the IL PFC (middle and bottom panels, respectively).

Figure 4.2 Uptake rate and clearance of NE for the optimization experiments

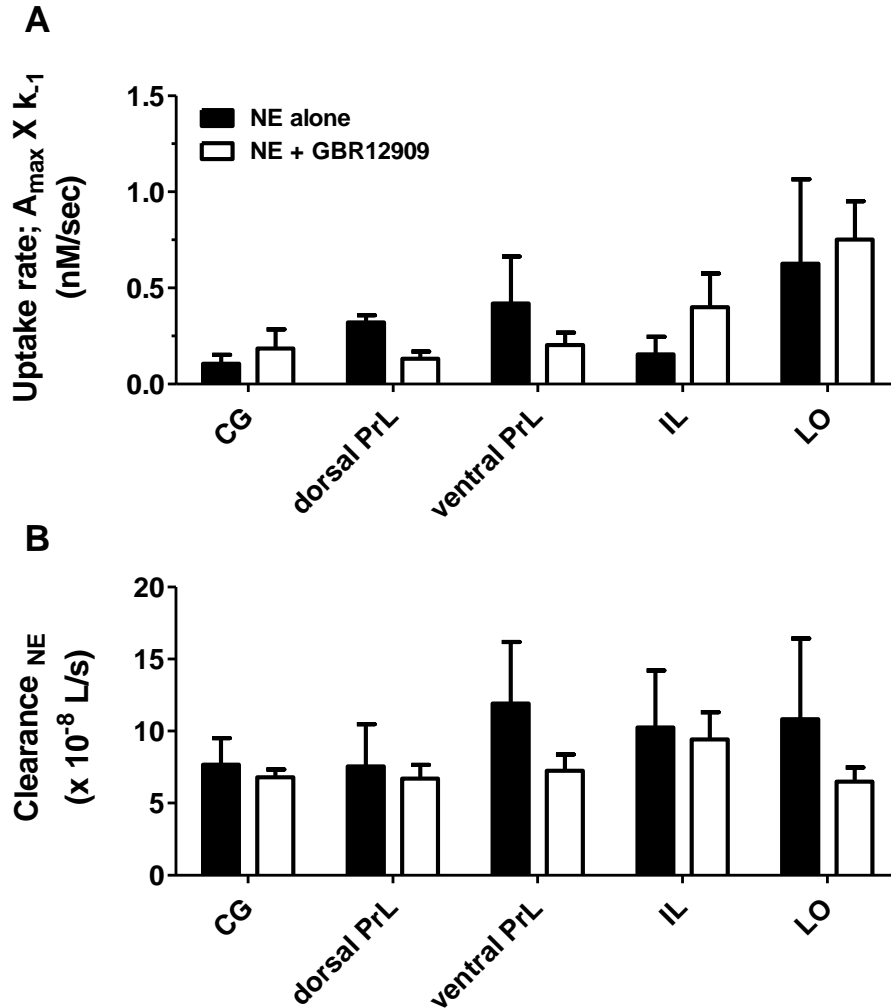


Fig 4.2 Optimization of norepinephrine transporter (NET) function. NET function was evaluated using uptake rate (A) and clearance (B) of norepinephrine (NE; 200 μ M) applied locally to cingulate gyrus (CG), dorsal and ventral prefrontal cortex (PrL), infralimbic cortex (IL) and lateral orbitofrontal cortex (LO). For all the brain regions evaluated, NE was applied alone (NE alone) or with GBR 12909 (50 nM; NE + GBR) on opposite hemispheres of the brain of individual rats. Uptake rate (μ M/sec; mean \pm S.E.M.) as well as clearance (L/sec; mean \pm S.E.M.) did not differ between hemispheres. n = 3-4/group.

Figure 4.3 Uptake rate of NE in the subregions of mPFC for the comorbidity experiments

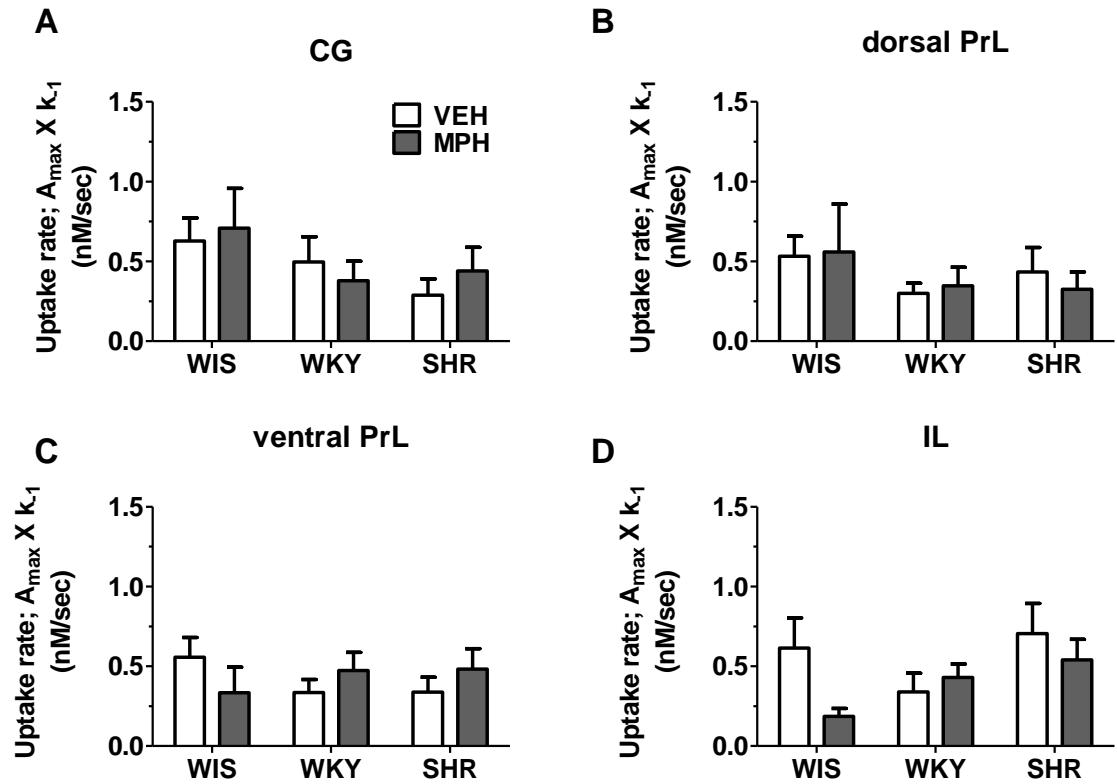


Fig 4.3 Uptake rate of norepinephrine (NE) in the mPFC subregions of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH) or vehicle (VEH) during adolescence. NE (100 μ M; GBR 12909 50 nM) was applied locally to cingulate gyrus (CG; A), dorsal and ventral prelimbic cortex (PrL; B and C, respectively) and infralimbic cortex (IL; D) of individual rats using a within-subject design. Uptake rate was mean \pm S.E.M. of the product of the maximum amplitude of the NE signal at the electrode (A_{max}) and the first order fitting of the signal decay (k_{-1}) for group. n = 7-9/group for CG, dorsal and ventral PrL; n = 6 – 8/group for IL.

Figure 4.4 Clearance of NE in the IL-PFC for the comorbidity experiments

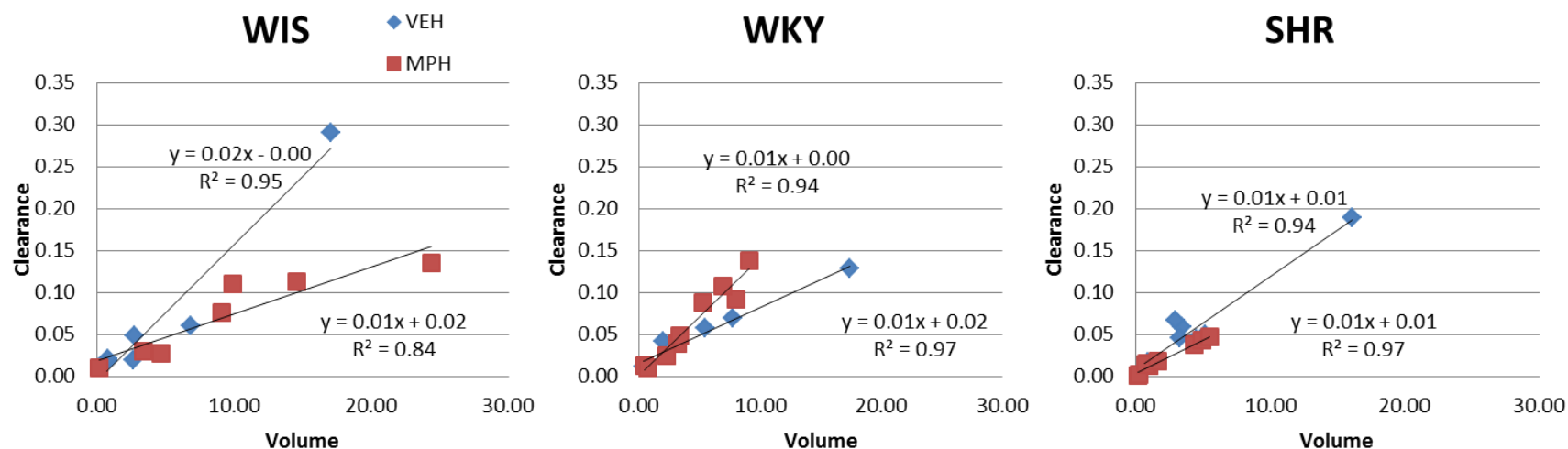


Fig 4.4 Clearance of NE in the infralimbic cortex (IL) of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH) or vehicle (VEH) during adolescence. Clearance was calculated as amount of NE (100 μ M, with GBR 12909, 50 nM) applied through the micropipette divided by the area-under-curve for the NE signal at the carbon-fiber electrode. Volume (or space between electrode and micropipette) calculated as amount of NE applied through the micropipette divided by the maximum amplitude of NE detected at the carbon-fiber electrode. $n = 6 - 8$ /group; linear regression of clearance (y) as a function of volume (x) and the goodness-of-fit (R^2) of the regressions are presented. Clearance of NE from the IL of MPH-treated WIS was significantly different from the VEH control.

Figure 4.5 Uptake rate of NE in the lateral OFC for the comorbidity experiments

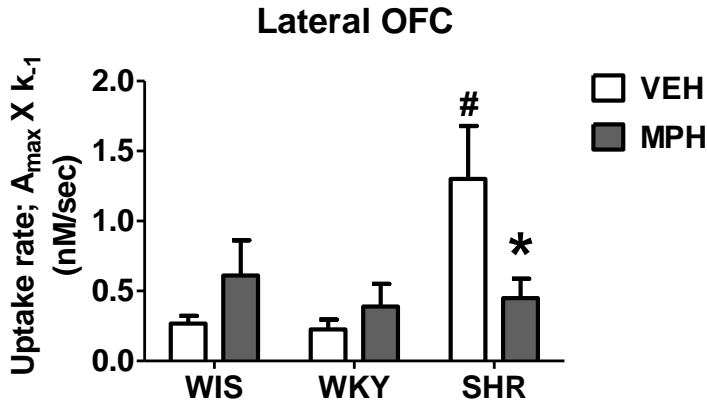


Fig 4.5 Uptake rate of norepinephrine (NE) in the lateral orbitofrontal cortex (LO) of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH) or vehicle (VEH) during adolescence. NE (100 μ M; GBR 12909 50 nM) was applied locally to LO of individual. Uptake rate was mean \pm S.E.M. of the product of the maximum amplitude of the NE signal at the electrode (A_{max}) and the first order fitting of the signal decay (k_{-1}) for group. $n = 8-9$ /group; * $p < 0.05$ different from the respective vehicle control; # $p < 0.05$ different from the VEH administered WKY and WIS.

Figure 4.6 Clearance of NE in the lateral OFC for the comorbidity experiments

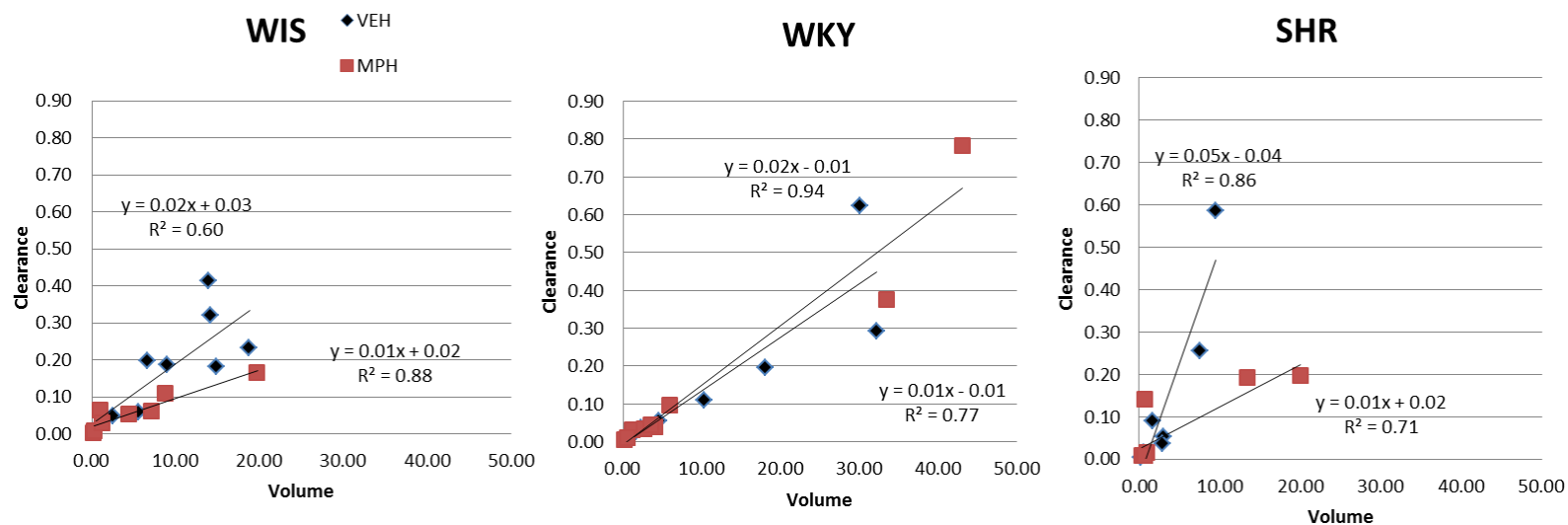


Fig 4.6 Clearance of NE in the lateral orbitofrontal cortex (LO) of the adult Spontaneously Hypertensive Rat (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered methylphenidate (MPH) or vehicle (VEH) during adolescence. Clearance was calculated as amount of NE (100 μ M, with GBR 12909, 50 nM) applied through the micropipette divided by the area-under-curve for the NE signal at the carbon-fiber electrode. Volume was calculated as amount of NE applied through the micropipette divided by the maximum amplitude of NE detected at the carbon-fiber electrode. n = 8-9/group; linear regression of clearance (y) as a function of volume (x) and the goodness-of-fit (R^2) of the regressions are presented.

5 CHAPTER FIVE

Rat strain differences, but not prior methylphenidate treatment, influences sensitization to cocaine during adulthood.

5.1 Introduction

Individuals with attention deficit/hyperactivity disorder (ADHD) are more likely to develop cocaine addiction as adults compared to demographically matched controls (Levin *et al*, 1999; Wilens *et al*, 1998b). Clinical studies suggest that the age of initiation of methylphenidate (MPH) treatment in ADHD may influence the outcome of later cocaine abuse, such that treatment initiated in childhood may be protective against future cocaine addiction (Fischer *et al*, 2003; Wilens *et al*, 2003) or not alter cocaine abuse liability (Molina *et al*, 2013), while treatment initiated in adolescence may increase cocaine abuse liability (Lambert *et al*, 1998; Mannuzza *et al*, 2008).

Preclinical studies using outbred rats indicate that MPH treatment in juvenile (postnatal day 20-35; P20-35) rats decreases conditioned place preference to cocaine during adulthood (Andersen *et al*, 2002). In contrast, MPH administered to adolescent rats increased acquisition rate of cocaine self-administration (Brandon *et al*, 2001) and progressive ratio (PR) breakpoints for cocaine self-administration (Crawford *et al*, 2011) assessed during adulthood.

Studies employing a widely accepted animal model of ADHD, the spontaneously hypertensive rats (Russell *et al*, 2005), indicate that cocaine self-administration is greater in SHR than controls Wistar-Kyoto (WKY; inbred) and Wistar (WIS; outbred) rats (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). Further, MPH treatment during

adolescence improved learning in a visual discrimination task in SHR; however, discontinuation of MPH treatment in adulthood enhanced cocaine self-administration in SHR compared to vehicle-administered SHR and MPH-treated WKY and WIS (Harvey *et al*, 2011). In the latter study, during adulthood, MPH-treated SHRs acquired cocaine self-administration behavior more rapidly, maintained greater responding and intake across a range of cocaine doses, and exhibited greater PR breakpoints (motivation) for cocaine compared to the control groups. Using a comparable MPH treatment regimen, dopamine transporter (DAT) function was increased in the medial prefrontal cortex (mPFC) of adult SHR compared to vehicle control and MPH-treated WKY and WIS (Somkuwar *et al*, 2013a). Taken together, cortical dopamine regulation may contribute to the enhanced cocaine abuse vulnerability following MPH treatment.

Dopamine in the mPFC is considered to exert inhibitory control over dopamine release in the nucleus accumbens (Beyer and Steketee, 1999), thereby, modulating reward and reinforcement of natural reinforcers and drugs of abuse (Tzschentke, 2000; Tzschentke and Schmidt, 2000). Dopamine depletion, via 6-hydroxydopamine lesions in mPFC increases cocaine self-administration under fixed-ratio schedules (Schenk *et al*, 1991). Another behavioral effect of cocaine mediated by accumbal dopaminergic function is hyperactivity, or increase in locomotor activity following cocaine injection (Cailhol and Mormede, 1999; Frankowska *et al*, 2009; Pulvirenti *et al*, 1989). Dopamine depletion in the mPFC enhances cocaine-induced hyperactivity in the open-field (Beyer *et al*, 1999). Further, repeated cocaine exposure progressively enhances cocaine-induced hyperactivity, this is termed behavioral sensitization (Dumars *et al*, 1988). Behavioral sensitization is associated with a decrease in prefrontal cortical dopaminergic function (Liu *et al*, 2011). Another feature of sensitization is that the context in which cocaine is repeatedly administered gets integrated into the sensitization process (Fraioli *et al*, 1999; Robinson *et al*, 1998). The context acquires incentive salience and eventually, can elicit a conditioned response (Robinson *et al*, 2000, 2001). This process is cocaine conditioning, and accumbal dopamine, in part, modulates this effect (Berridge and Robinson, 1998). Therefore, increased mPFC DAT function observed in adult SHR

following MPH treatment during adolescence may increase sensitivity of SHR to cocaine, which in turn, may underlie the previously observed enhanced cocaine self-administration in SHR (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). Further, the enhancement in cocaine-induced hyperactivity, cocaine sensitization and conditioned response to the cocaine-paired context may serve as surrogates for identifying enhanced sensitivity to cocaine in MPH-treated SHRs.

The current study aims to determine whether MPH treatment during adolescence alters sensitivity to cocaine in adult SHR, WKY and WIS. The hypothesis tested herein is that MPH treatment during adolescence leads to enhanced cocaine-induced hyperactivity in adult SHR and augments sensitization to repeated cocaine injections in SHR compared to vehicle-administered SHR and MPH-treated WKY and WIS.

5.2 Methods

5.2.1 Drugs

(±)-Methylphenidate was purchased from Sigma-Aldrich (St. Louis, MO). MPH was dissolved in water to obtain concentrations of 1.5 mg/ml (prepared daily) and injected into oyster crackers (Kantak *et al*, 2008) to attain a dose of 1.5 mg/kg for oral administration. Cocaine HCl was a gift from the National Institute on Drug Abuse (Bethesda, MD, USA) and was dissolved in sterile saline (NaCl, 0.9% w/v).

5.2.2 Animals and Treatments

Male SHR and WKY at P25 were obtained from Charles River Laboratories (Kingston, NY) and male WIS rats at P25 were obtained from Charles River Laboratories (Raleigh, NC). The locations listed above are where Charles River Laboratories maintain

the breeding colony for the specific strains employed in this study. Rats were individually housed with free access to food and water in a colony room maintained on a 12-hour light:dark cycle (lights on 07:00 hour) in the Division of Laboratory Animal Resources (University of Kentucky, Lexington, KY). Following three days of habituation, these rats were orally administered MPH (1.5 mg/kg, p.o.) or vehicle (VEH, water, 1 ml/kg, p.o. using one oyster cracker) from P28 to P55. This dose of MPH (1.5 mg/kg, p.o.) produces clinically-relevant plasma concentrations of MPH (Wargin *et al*, 1983), that increase extracellular dopamine and norepinephrine in prefrontal cortex, but are below threshold for producing locomotor activation or increasing striatal dopamine concentration (Berridge *et al*, 2006; Kuczenski *et al*, 2002). This MPH dose has been shown to improve attention and behavioral flexibility in SHR (Harvey *et al*, 2013; Harvey *et al*, 2011). P28 to P55 includes a time period from early adolescence to late adolescence (Doremus-Fitzwater *et al*, 2010; Spear, 2000a), the typical age during which MPH is administered clinically. Rat handling procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and were performed in accordance with the 1996 version of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

5.2.3 Acute cocaine-induced hyperactivity

Typically, when exposed to a novel environment, rats express hyperactivity (Ho *et al*, 2000; Laviola and Adriani, 1998). The novelty-induced hyperactivity decreases following repeated exposure to the same environment, which is termed habituation (Green *et al*, 2003a). During adulthood (P134), SHR, WKY and WIS rats were habituated for 3 days in the acrylic open-field locomotor chambers (42 × 42 × 30 cm), each with a 16 × 16 grid of photobeam sensors. Locomotor activity was recorded with a monitoring system (AccuScan Instruments Inc., Columbus, OH) and was expressed as total number of horizontal beam breaks recorded during each 1-hour session. To determine cocaine-induced hyperactivity, rats were administered a single injection cocaine (10 mg/kg, i.p.)

or saline (1ml/kg, i.p.) in a randomized manner and locomotor activity was recorded for an hour. The dose of cocaine chosen has been shown to induce locomotor sensitization in adult SHR, WKY and WIS (Cailhol *et al*, 1999; Frankowska *et al*, 2009; Frantz *et al*, 2007).

5.2.4 Sensitization to repeated cocaine administration

To evaluate induction of sensitization to repeated cocaine, rats were administered cocaine (10 mg/kg, i.p.) and allowed to explore the locomotor chambers for 1-hour daily for 10 consecutive days. Upon completion of the 10-day repeated treatment phase, rats remained undisturbed in their home cages for a 14-day cocaine-free period. During this period, rats were not given access to the locomotor chambers.

On the 15th day, expression of sensitization to cocaine was tested with cocaine, 0, 5, 10 and 20 mg/kg, i.p. in an ascending dose order within a single session. The half-life of cocaine in rats is 30 minutes (Pan and Hedaya, 1999). Thus, to obtain the desired plasma levels of cocaine at each dose, the following cumulative dosing regimen was employed: saline, cocaine 5 mg/kg, 7.5 mg/kg, and 15 mg/kg, i.p. injected at 30-min intervals. Following each injection, locomotor activity was monitored for 30 min.

5.2.5 Statistical Analysis

Outcomes were analyzed using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). Data are reported as mean \pm S.E.M, number of horizontal beam breaks during 1 hour for cocaine-induced hyperactivity and for the induction of sensitization to cocaine. For the expression of sensitization assay, mean \pm S.E.M. of the number of horizontal beam breaks during the 30-min period after each dose of cocaine are reported; n represents the number of rats per group. Mauchly's tests of Sphericity

were employed to validate repeated-measures ANOVA. When the sphericity assumption was violated, Greenhouse-Geisser correction was used to determine within-subject effects. Data were analyzed using 3-factor ANOVAs with adolescent treatment (2 levels) and strain (3 levels) as between-subject factors. Cocaine dose served as within-subject factor for hyperactivity (2 levels) and expression of sensitization (4 levels) assays, and session number was a within-subject factor in the induction of sensitization assay (10 levels). Significant main effects and interactions were evaluated using appropriate *post-hoc* analyses.

Linear mixed model analyses were conducted (Verbeke *et al*, 2000) as a complementary approach for evaluating induction of sensitization using session as a continuous variable, and not as a discrete variable as in the 3-factor ANOVA. Significant session X strain and session x treatment interactions were further probed using linear regression analyses, whereby deviation from zero served as an estimate for the induction of sensitization. A significant strain x treatment interaction in the absence of significant session x strain x treatment interaction indicate a difference in the y-intercept of the linear regressions between strain and treatment groups (Verbeke and Molenberghs, 2000), suggesting that the response to the first cocaine injection was different between strain and treatment. Therefore, a significant strain x treatment interaction in the linear mixed model analysis was further investigated using a separate 2-factor ANOVA, with appropriate *post-hoc* tests, of the horizontal beam-breaks during the first session of induction of sensitization assay.

To evaluate conditioning to cocaine-paired context, total number of horizontal beam-breaks following saline injection on the expression of sensitization was compared with beam-breaks during the first 30-min of access to the open-field chambers for the first day of habituation, following saline injection prior to induction of sensitization and that following cocaine injection on the 1st session of induction of sensitization using a separate 2-factor ANOVA with strain (3 levels) as a between-subject factor and session-

type (4 levels) as within-subject factor. Significant interactions and main effects were evaluated further using Bonferroni's *post-hoc* analyses.

5.3 Results

5.3.1 Acute cocaine-induced hyperactivity

In the acute cocaine-induced hyperactivity assay, the interactions of treatment X strain X dose ($F[2,29] = 0.259, p > 0.05$; Fig 5.1) and treatment X dose ($F[1,29] = 0.003, p > 0.05$) and strain X dose ($F[2,29] = 0.515, p > 0.05$) were not significant. However, a main effect of cocaine dose was found ($F[1,29] = 19.0, p < 0.001$). Horizontal locomotor activity following a single cocaine injection was greater than that following saline injection for all three strains ($ps < 0.05$). The increase in locomotor activity following acute cocaine injection in WIS, WKY and SHR was 56%, 83% and 33%, respectively, of that after saline injection within-subject.

5.3.2 Induction of locomotor sensitization to cocaine

During the induction of sensitization, treatment X strain X session ($F[18,261] = 0.919, p > 0.05$; Fig 5.2A) and the treatment X session ($F[9,261] = 1.83, p > 0.05$) interactions were not significant. However, the strain X session interaction ($F[18,261] = 3.21, p < 0.001$) and the main effect of session ($F[9,261] = 14.4, p < 0.001$) were significant. MPH treatment did not alter the induction of sensitization to repeated cocaine. Therefore, the sensitization data were collapsed between adolescent VEH and MPH groups. Induction of sensitization was evaluated separately for individual strains using repeated-measures one-way ANOVAs with Dunnett's *post-hoc* analysis compared to the first session of cocaine administration. For SHR, locomotor activity was elevated ($F[9,99] = 15.2, p < 0.0001$ Fig 5.2A, right panel) from the 4th to the 10th day compared to

the 1st day of repeated cocaine injection ($p < 0.05$). For WKY, locomotor activity differed by session ($F[9,90] = 3.64, p < 0.001$; Fig 5.2A, middle panel), but Dunnett's *post-hoc* analysis did not reveal significant pairwise differences compared to the 1st day of repeated cocaine injection. For WIS, locomotor activity was not elevated by repeated cocaine injection ($F[9,99] = 1.92, p > 0.05$ Fig 5.2A, left panel).

Linear mixed model analysis revealed a significant strain X session interaction ($F[2,39.1] = 6.21; p < 0.01$; Fig 5.2B), but no significant interactions of treatment X session ($F[1,39.1] = 1.80; p > 0.05$) or strain X treatment X session ($F[2,39.1] = 0.886; p > 0.05$), indicating that the slope for cocaine sensitization differs between strains, but not between treatment groups. Linear regression analyses of the significant strain X session interaction revealed that the slopes for induction of sensitization to cocaine were significantly different from zero in SHR ($1390 \pm 185; p < 0.0001$; Fig 5.2B), WKY ($337 \pm 132, p < 0.05$) and WIS ($445 \pm 193, p < 0.05$). Further, the slope was greater for SHR compared to both WKY and WIS ($F[2,344] = 11.9, p < 0.0001$).

The linear mixed model analysis also revealed a significant strain X treatment interaction ($F[2,124.7] = 3.38; p < 0.05$), indicating differences in locomotor activity on session #1 of repeated cocaine administration. Two-way ANOVA evaluating locomotor activity on session #1 of the induction of sensitization revealed a significant main effect of strain ($F[2,29] = 4.87, p < 0.05$; Fig 5.2C), but not of treatment ($F[1,29] = 0.020, p > 0.05$) and not for the strain x treatment interaction ($F[2,29] = 0.773, p > 0.05$). SHRs exhibited greater locomotor activity on session #1 compared to WKY ($p < 0.05$, Fig 5.2C) but not WIS.

5.3.3 Expression of locomotor sensitization to cocaine

On the test-day for expression of cocaine sensitization, the treatment X strain X dose ($F[6,87] = 0.374, p > 0.05$; Fig 5.3) and the treatment X dose ($F[3,87] = 0.862, p >$

0.05) interactions were not significant. However, a significant strain X dose interaction ($F[6,87] = 7.12, p < 0.05$) was found. Further, a main effect of cocaine dose was found ($F[3,87] = 28.4, p < 0.001$). *Post-hoc* analyses revealed that in WIS, cocaine 20 mg/kg produced greater locomotor activity than 5 mg/kg, but not saline ($p < 0.05$). In WKY, repeated cocaine resulted in a dose-dependent increase in locomotor activity, such that saline < cocaine 10 and 20 mg/kg, and cocaine 5 mg/kg < cocaine 20 mg/kg ($ps < 0.05$). In SHR, administration of cocaine resulted in a dose-dependent increase in locomotor activity, such that activity after cocaine 5 mg/kg < 10mg/kg < 20 mg/kg; however, only the 20 mg/kg dose of cocaine was different from saline ($ps < 0.05$).

5.3.4 Conditioning to context-paired with repeated cocaine administration

For conditioning to cocaine-paired context, significant strain X test-day interaction ($F[6,96] = 6.51, p < 0.0001$; Fig 5.4), and significant main effects of strain ($F[2,96] = 45.3, p < 0.0001$) and test-day ($F[3,96] = 29.1, p < 0.0001$) were obtained. Bonferroni's *post hoc* analyses for WKY revealed no significant pairwise differences between test-days. For WIS, locomotor activity following conditioning to cocaine-paired context was greater than locomotor activity after 3 days of habituation ($p < 0.05$); no other significant differences were found. For SHR, locomotor activity following cocaine-conditioning was greater than locomotor activity with saline injection after habituation but prior to induction of sensitization ($p < 0.05$; Fig 5.4). Also, the conditioned response was greater than cocaine-induced hyperactivity (session#1 of induction of sensitization; $p < 0.05$). Further, locomotor activity following conditioned response was greater than locomotor activity on the first day of habituation ($p < 0.05$; Fig 5.4).

5.4 Discussion

The current study tested the hypothesis that chronic MPH treatment in adolescent SHR, followed by treatment discontinuation in early adulthood increases the sensitivity of adult SHR to the behavioral effects of acute and repeated cocaine administration. Contrary to the hypothesis, acute cocaine-induced hyperactivity, and induction and expression of sensitization as well as the conditioned response to cocaine were not altered in adult SHR following chronic MPH treatment during adolescence. The current study only identified strain differences in sensitivity to cocaine. Specifically, acute cocaine administration induced hyperactivity in all three rat strains; surprisingly, the cocaine induced hyperactivity was the greatest increase in the genetic control, WKY, and the smallest increase in SHR. In contrast, repeated administration of a moderate dose of cocaine (10 mg/kg, i.p.) produced robust sensitization in SHR, but not in either of the control strains. Furthermore, SHR also exhibited an increased conditioned response to the cocaine-paired context compared to WKY and WIS. Thus, SHR sensitize to cocaine and cocaine-paired context more readily compared to control. Taken together, the development of psychomotor sensitization to cocaine is influenced by strain/genetic differences between subjects, but MPH treatment in during adolescence does not alter the sensitivity to the psychomimetic effects of cocaine.

Cocaine administered intraperitoneally at the 10 mg/kg dose was reported to induce hyperactivity following a single administration and induce behavioral sensitization following repeated administration in SHR, WKY and WIS (Cailhol *et al*, 1999; Frankowska *et al*, 2009). In the current study, this dose of cocaine induced hyperactivity in all three strains (Fig 5.1), however, sensitization was modest in WKY and WIS (Fig 5.2). Specifically, linear regression for the induction of sensitization was significantly different from zero, but cocaine induced hyperactivity was not significantly increased from session #1 of repeated cocaine administration. One explanation for modest effect of cocaine in WIS may be the variability in the induction of sensitization to repeated cocaine in outbred rats. Support for this explanation is provided by reports of

individual differences in induction of sensitization with low doses of cocaine (Perez *et al*, 2010; Pierce *et al*, 1996a). The latter studies suggest that the expression of sensitization depends on increased accumbal glutamatergic transmission. The accumbens of SHR is less sensitive to glutamate stimulated dopamine and acetylcholine release compared to WKY (Russell, 2003; Tsuda *et al*, 1996). Taken together, these results suggest that the development of sensitization to repeated administration of a low dose of cocaine depends on deficient accumbal glutamatergic function.

Differences in acute cocaine-induced hyperactivity has been employed to segregate outbred rats into low-cocaine responders (LCR) and high cocaine responders (HCR; Gulley *et al*, 2003; Mandt *et al*, 2008). Compared to HCR, LCR develop robust behavioral sensitization to repeated cocaine administration, and exhibit greater motivation for cocaine self-administration (Mandt *et al*, 2008). LCRs also express greater striatal DAT and reduced D2 function (Mandt *et al*, 2010; Merritt *et al*, 2013). SHR have several similarities with the LCR; compared to WKY and outbred control. SHR exhibit greater striatal DAT function and reduced D2 function compared to WKY and outbred rats (Fujita *et al*, 2003; Miller *et al*, 2012). Also, SHRs had a higher rate of acquisition of cocaine self-administration with higher PR breakpoints compared to both WKY and WIS (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). In the current study, SHR exhibited lower cocaine-induced hyperactivity and greater induction of sensitization following repeated cocaine administration compared to WKY and WIS. Taken together, these reports suggest that striatal dopaminergic function contributes to the behavioral profile predicting enhanced vulnerability to cocaine.

Chronic MPH treatment in adolescent SHR, followed by treatment discontinuation in early adulthood, resulted in increased cocaine self-administration and increased motivation for cocaine, compared to vehicle control and MPH-treated WKY and WIS (Harvey *et al*, 2011). MPH treatment in adolescent SHR increased DAT function in mPFC, but did not alter DAT function in striatum (Somkuwar *et al*, 2013a). As discussed above, striatal dopaminergic function has been associated with cocaine

sensitization and conditioning to cocaine paired context and cues (Berridge *et al*, 1998; Mandt *et al*, 2010; Merritt *et al*, 2013). Cocaine cue-reactivity, evaluated as reinstatement of second order responding for cocaine-cues, in adult SHR was not increased following MPH treatment during adolescence (Jordan *et al.*, submitted). Taken together, these results suggest that chronic MPH treatment during adolescence may not increase the sensitivity of SHR to the psychomotor effects of cocaine. The current results are in agreement with this hypothesis. In contrast to MPH, chronic ATO treatment during adolescence was reported to decrease striatal DAT function as well as cocaine cue-reactivity in adult SHR (Somkuwar *et al*, 2013b; Jordan *et al.*, submitted). Taken together, these results further suggest that striatal DAT function is critically involved in the conditioned response to cocaine paired context and cues.

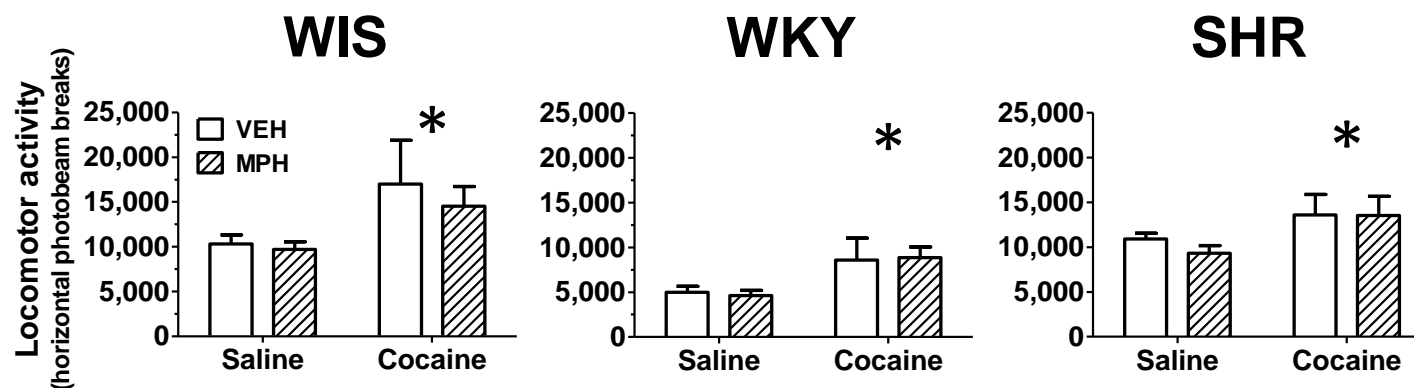
Self-administration and sensitization models different aspects of cocaine abuse. The former models stimulus-response learning in cocaine addiction and evaluates the reinforcing properties of a drug under a non-dependent state (Collins *et al*, 1984; O'Connor *et al*, 2011). The latter models the development of sensitization to the incentive motivational effects for cocaine and cocaine-associated cues (Robinson *et al*, 1993; Robinson and Berridge, 2008b). Sensitization has been suggested to mediate transition from recreational drug use to addiction (Ferrario *et al*, 2005; Robinson *et al*, 2001) but see (Ahmed and Cador, 2006). Thus, neurochemical changes produced by prior MPH treatment may enhance future cocaine abuse liability, but may not be directly responsible for the development of addiction. The core tenet of the incentive sensitization theory is that in susceptible individuals, repeated administration of an addictive substance, such as cocaine, leads to attribution of incentive salience to stimuli (Brown *et al*, 2011; Tindell *et al*, 2005). These changes may then render the subjects hypersensitive to cocaine and cocaine-associated cues (Di Ciano, 2008), and the enhanced sensitivity persists long after the discontinuation of repeated drug treatment (Antelman, 1988; Di Ciano, 2008). SHR show greater motivation for cocaine self-administration compared to WKY and WIS (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). SHR were reported to exhibit greater cocaine cue-reactivity than WKY and WIS (Jordan

et al., submitted). The importance of context in sensitization is well established, particularly with psychostimulants (Fraioli *et al*, 1999; Robinson *et al*, 1998). In the current study, SHR exhibited greater conditioning to the cocaine-paired context compared to WKY and WIS. This increased responding in the cocaine-paired context was greater than that elicited by saline injection, suggesting that the enhanced locomotor activity was not due to stress associated with injection. Furthermore, only in the SHRs, hyperactivity in the open-field chamber following repeated cocaine administration was greater than both novelty-induced hyperactivity as well as acute cocaine-induced hyperactivity, suggesting a robust effect of cocaine conditioning. Taken together with the current results, genetic differences confer sensitivity to the behavioral effects of cocaine, however prior MPH treatment does not alter the incentive motivational effects of cocaine sensitization.

In conclusion, the SHR strain is vulnerable to development of psychomotor sensitization to cocaine, and prior MPH treatment during adolescence does not alter the sensitivity to the psychomotor effects of cocaine in any of the strains. Locomotor sensitization to cocaine in SHR may be mediated by increased sensitivity to cocaine and by increased sensitivity to the associated context. The increased cocaine sensitivity in SHR may be associated with the previously reported higher cocaine self-administration and cocaine cue-reactivity in SHR and by extrapolation, may mediate, partially, the high cocaine abuse liability in ADHD.

5.5 Figures

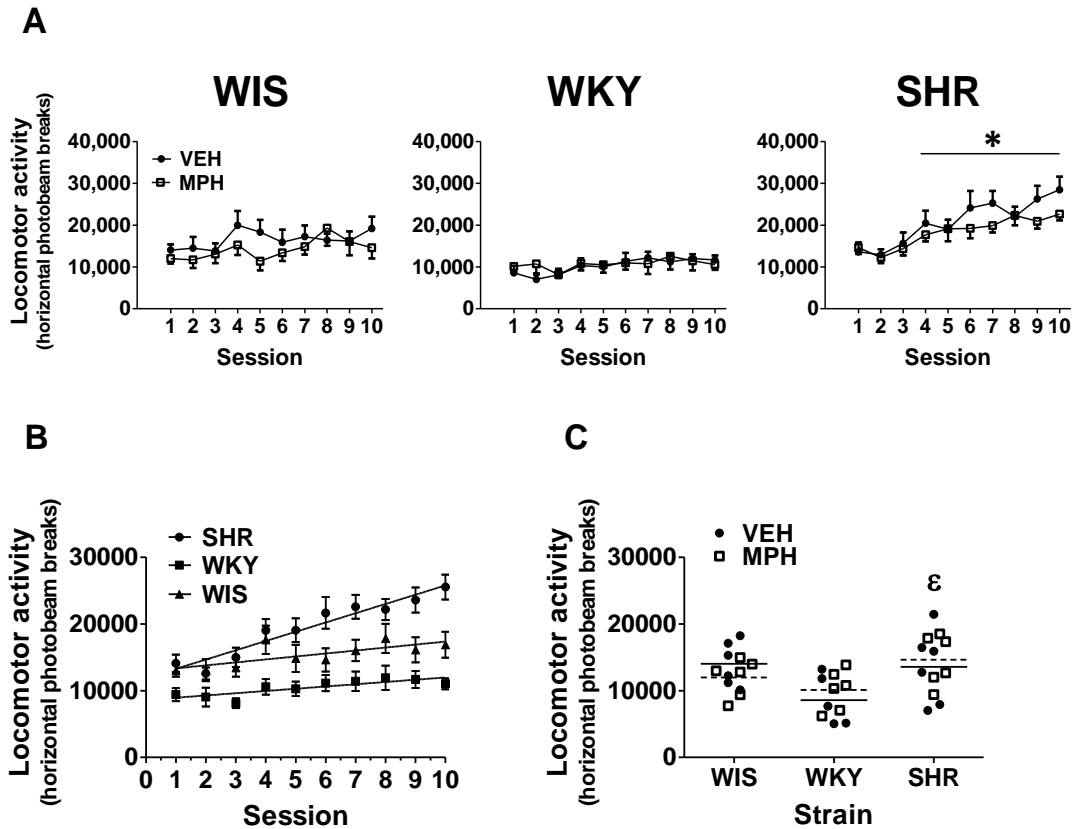
Figure 5.1. Cocaine-induced hyperactivity



Locomotor activity in open-field chambers following cocaine or saline injections in methylphenidate-treated (MPH; striped) and vehicle-treated (VEH; plain) WIS, WKY and SHR. Values are mean \pm S.E.M. for number of horizontal photobeam breaks.

* $p < 0.05$ compared to respective saline control; $n = 6$ /group for all treatment and strain groups, except vehicle treated WKY, where $n=5$.

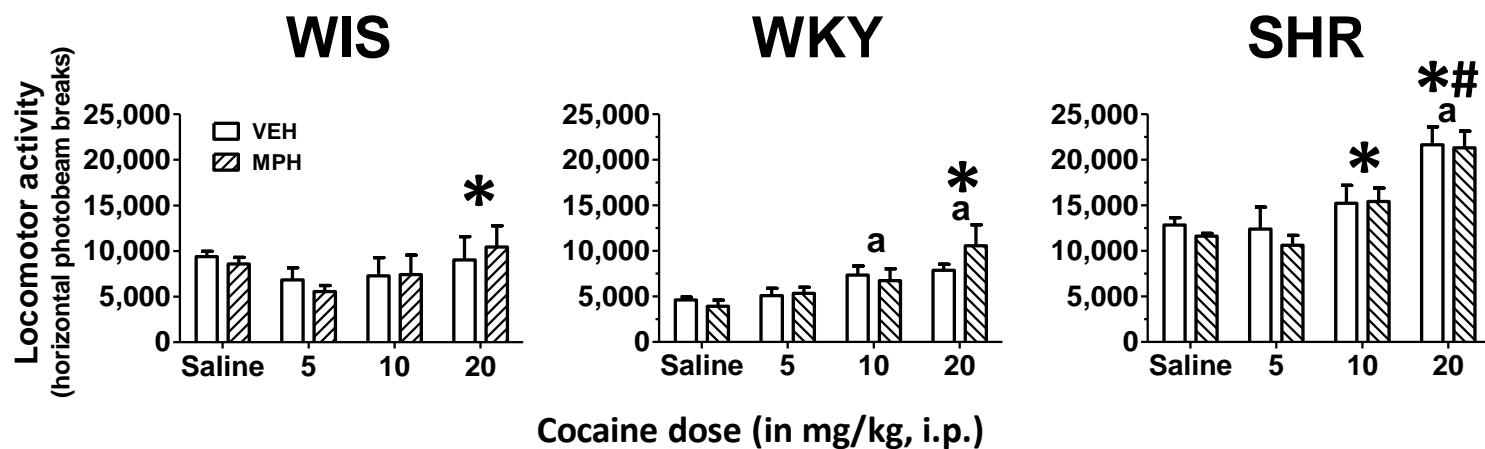
Figure 5.2 Induction of sensitization to repeated cocaine



Locomotor activity in open-field chambers following cocaine injections in methylphenidate-treated (MPH; open squares) and vehicle-treated (VEH; closed circles) WIS, WKY and SHR for sessions 1-10 (panel A). Values are mean \pm S.E.M. for number of horizontal photobeam breaks. Linear regression for locomotor activity across 10 sessions of repeated cocaine administration for SHR, WKY and WIS (B); values are collapsed across VEH and MPH groups. Locomotor activity for individual rats following cocaine on session#1 of induction of sensitization (C).

* $p < 0.05$ compared to session#1; ^ε $p < 0.05$ compared to WKY; n = 6/group for all treatment and strain groups, except vehicle treated WKY, where n = 5.

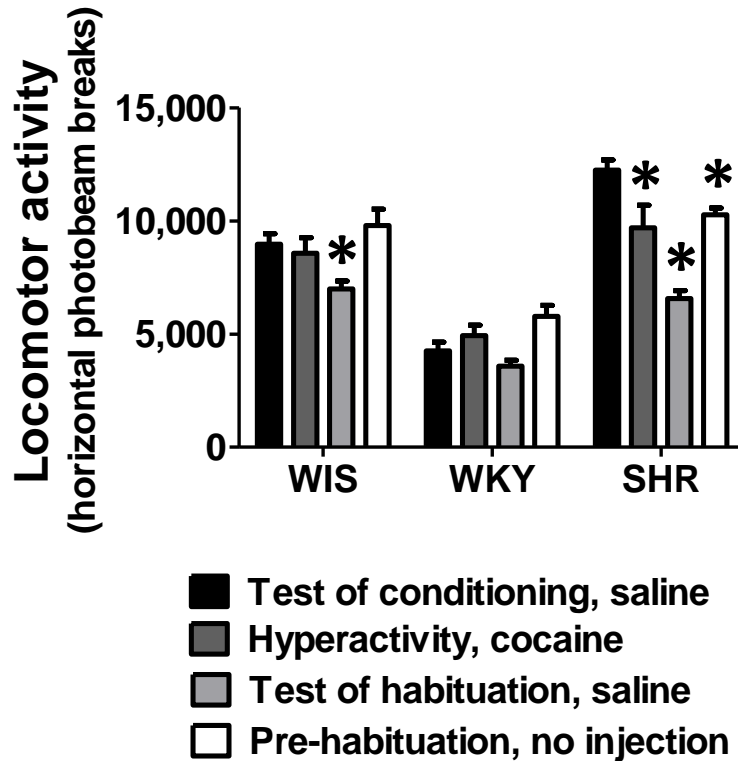
Figure 5.3. Expression of sensitization to cocaine



Locomotor activity in open-field chambers following cocaine (5 – 20 mg/kg, i.p.) or saline injections in methylphenidate-treated (MPH; striped) and vehicle-treated (VEH; plain) WIS, WKY and SHR. Values are mean \pm S.E.M. for number of horizontal photobeam breaks

^a $p < 0.05$ compared to saline; * $p < 0.05$ compared to 5 mg/kg cocaine; # $p < 0.05$ compared to 10 mg/kg cocaine; n = 6/group for all treatment and strain groups, except vehicle treated WKY, where n=5.

Figure 5.4. Conditioning to the context paired with cocaine



Locomotor activity in open-field chambers following saline injections 14 days after induction of sensitization (test of conditioning; black bars), following cocaine injection during session#1 of induction of sensitization (hyperactivity; dark grey bars), following saline injection after 3 days of habituation to the open-field chambers (test of habituation; light grey bars) and on the first day of habituation to the locomotor chambers (pre-habituatation; white bars) in WIS, WKY and SHR. Values are mean \pm S.E.M. for number of horizontal photobeam breaks, and are collapsed between methylphenidate and vehicle groups.

* $p < 0.05$ compared to test of conditioning; $n = 12$ /group for all treatment and strain groups, except vehicle treated WKY, where $n=11$.

Impulsivity endophenotypes identified using differential reinforcement of low rate schedules in a rodent model of attention deficit hyperactivity disorder

6.1 Introduction

Impulsivity is a complex multidimensional phenotype encompassing a wide variety of maladaptive behaviors including, but not limited to, actions that are poorly conceived, prematurely executed, unnecessarily risky, inappropriate to the situation, and often with undesirable outcomes (Evenden, 1999b). Impulsivity is a hallmark of attention deficit/hyperactivity disorder (ADHD). Aspects of impulsivity, including having “difficulty waiting his/her turn”, “impatience”, and feeling “uncomfortable doing things slowly and systematically” serve as diagnostic criteria for ADHD (American Psychiatric Association., 2000).

Impulsivity has been ascribed to impaired response inhibition (Barkley, 1997; Dalley *et al*, 2008). Response inhibition is an executive control mechanism that prevents the execution of an undesirable action (Aron, 2007). Also, impulsivity in ADHD has been suggested to be an outcome of a deficiency in timing (Rubia *et al*, 2009a), in which deficits in either motor timing or time perception are expressed as premature responding. Certain features of impulsivity are accounted for by either poor inhibitory control or poor timing. For instance, “impatience” is an outward manifestation of either reluctance to wait (poor response inhibition) or a deficient timing perception (durations appear longer than they are in actuality). ADHD patients display deficits in both response inhibition and temporal processing (Sonuga-Barke *et al*, 2010), indicating that deficits in response inhibition and timing may be two endophenotypes underlying

impulsivity in ADHD. Furthermore, impulsivity associated with ADHD is ameliorated by methylphenidate (MPH), a stimulant ADHD medication, which enhances response inhibition and reduces neuronal dysfunction associated with disturbed temporal processing (Broyd *et al*, 2005).

The spontaneously hypertensive rat (SHR) is the most widely used model of ADHD (Sagvolden *et al*, 2005b). However, controversy exists as to whether the SHR appropriately models impulsivity associated with ADHD (Wickens *et al*, 2011). Generally, studies using response-withholding preparations indicate that SHR have reduced response inhibition, and thus, exhibit greater impulsivity compared to their inbred progenitor strain Wistar-Kyoto (WKY), as well as compared to outbred strains (Evenden and Meyerson, 1999; Sanabria *et al*, 2008). However, impulsivity in SHR measured by reduced reinforcement in response-withholding preparations such as the differential reinforcement of low rate (DRL) schedule and the 5-choice serial reaction time tasks, was not decreased as expected by MPH (Ferguson *et al*, 2007; van den Bergh *et al*, 2006), suggesting that the SHR model of ADHD has limitations or that the assessment of impulsivity in the latter studies was not adequate (Hill *et al*, 2012a).

Clinical studies report an improvement in response inhibition tasks following acute MPH administration only in patients who had been prescribed MPH previously (Broyd *et al*, 2005; DeVito *et al*, 2009), but not in drug naïve ADHD subjects (Rhodes *et al*, 2006; Rubia *et al*, 2009a). In drug naïve ADHD subjects, chronic MPH treatment produced observable improvements in response inhibition and other executive functions (Coghill *et al*, 2007). Further, MPH is most often administered to children and adolescents diagnosed with ADHD (Robison *et al*, 1999), MPH effects may be more prominent in younger subjects. MPH reduced impulsivity in adolescent, but not in adult WIS rats in a delay-discounting task, where impulsivity is measured as increased choice of a smaller immediate reward compared to a larger delayed reward (Bizot *et al*, 2011). In adolescent SHR, acute MPH administration has been reported to reduce the ADHD-

like phenotype, including impulsivity using a delay-discounting schedule (Adriani *et al*, 2003; Umehara *et al*, 2013b). Chronic MPH administration has been shown to reduce working memory deficits and inattention in adolescent SHR (Harvey *et al*, 2013; Kantak *et al*, 2008). The current research evaluated the effects of chronic MPH treatment on impulsivity in adolescent SHR.

The current research employed DRL schedules, an operant conditioning procedure that reinforces only responses (e.g., lever presses) that are separated by an experimenter-defined minimum time since the previous response. In this paradigm, impulsivity is measured as a reduced efficiency of reinforcement as a consequence of early inappropriate responding (Stein and Landis, 1975). Reduced efficiency on a DRL schedule may result from reduced response inhibition and/or timing disturbances (Doughty and Richards, 2002a; Wiley *et al*, 2000). SHR show reduced efficiency compared to WKY and outbred rats (Bull *et al*, 2000; Sanabria *et al*, 2008); extensive training on the schedule reduces this difference (Orduna *et al*, 2009). However, information regarding the behavioral response output is lost when only efficiency or molar patterns of behavior, defined using arbitrary inter-response time (IRT) cut-offs, are considered (Richards *et al*, 1993). Furthermore, efficiency does not differentiate between errors due to response inhibition and timing deficiency.

IRT distributions obtained under DRL schedules provide valuable information for the characterization of the temporal occurrence of behavior (Williams, 1968). Burst responding is characterized by short IRTs (generally <2 sec) and indicate a reduced capacity to withhold responding. Burst responding may be a mechanism contributing to reduced efficiency (Sagvolden *et al*, 2005a). SHR exhibit higher burst responding compared to control (Boix *et al*, 1998; Sagvolden *et al*, 2005a; van den Bergh *et al*, 2006); however, other studies report contrasting results (Bull *et al*, 2000; Ferguson *et al*, 2007; Orduna *et al*, 2009), which may be due to differences in experimental procedures and in the molar definition of 'bursts'. Mathematical modeling approaches offer a

quantitative analysis of IRT distributions by taking into consideration the overall distribution of IRTs, and not simply arbitrary summary statistics. As such, response inhibition and timing deficiencies in DRL behavior in SHR have been parsed out using a modeling approach (Orduna *et al*, 2009; Sanabria *et al*, 2008).

The current study aims to optimize the quantification of IRT distributions to parse out individual processes underlying SHR behavior under two DRL schedules, a short DRL 5 second with limited hold (DRL5LH) and a long DRL 30 second (DRL30) schedule. Optimization also included the comparison of molar patterns (alterations in reinforcement efficacy) with mathematical modeling parameters. Thus, the current research determined the contribution of DRL interval on impulsivity endophenotypes expressed by SHR by examining global performance metrics and IRT distribution parameters.

6.2 Material and methods

6.2.1 Subjects

Male SHR and WKY rats (Charles River Laboratories, Kingston, NY) and male Wistar rats (WIS; Raleigh, NC) arrived on postnatal day 70 (P70) or P25. WKY and WIS were used as inbred and outbred comparator strains, respectively. Twelve adult rats from each strain were used to identify strain differences in endophenotypes of impulsivity, except where noted. Twenty four adolescent rats from each strain were used to identify effects of MPH on endophenotypes of impulsivity. Rats were maintained on a 12-h light:dark cycle with lights on 07:00 h, individually housed with free access to food and water in a colony room (Division of Laboratory Animal Resources, University of Kentucky, Lexington, KY, USA). The experiments were

conducted during the light-cycle of the rats. After 3 days of habituation, rats were food restricted to 90-95% of their expected free-feeding body weight. All experimental protocols were conducted according to the 1996 NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

6.2.2 Apparatus

Experiments were conducted in operant conditioning chambers (ENV-001; MED Associates, St. Albans, VT, USA) housed in sound attenuating compartments (ENV-018 M, MED Associates). Operant chambers were connected to a PC interface (SG-6080D, MED Associates) and operated using MED-PC™ software. Chambers were equipped with a 5 cm × 4.2 cm recessed food receptacle, two retractable metal levers located on either side of the food tray and 7.3 cm above a metal grid floor, and a house light mounted on the wall opposite the food receptacle. Food pellet reinforcers (45-mg Noyes Precision Pellets; Research Diets, Inc., New Brunswick, NJ, USA) were delivered via a dispenser (ENV-203, MED Associates) mounted outside of the operant chamber.

6.2.3 Drugs

(±)-Methylphenidate hydrochloride (Sigma-Aldrich, St Louis, MO) was dissolved in water at concentration of 1.5 mg/ml and injected into oyster crackers (Kantak *et al*, 2008) providing oral doses of 1.5 mg/kg. Oyster crackers injected with water (1 ml/kg) were used as the vehicle control. Solutions were prepared fresh on each experimental day and the cracker was provided in the homepage 30 min prior to the operant session.

The MPH dose employed results in clinically relevant plasma concentrations (Wargin *et al*, 1983), and was below the threshold for producing locomotor activation (Kuczenski *et al*, 2002). Rats consumed the oyster cracker within 5 min.

6.2.4 Differential reinforcement of low rate schedules

For evaluating strain differences adult SHR, WKY and WIS were trained on daily 55-min sessions of DRL5LH, followed by DRL30 schedule, using a previously reported training schedule (Sanabria *et al*, 2008). For both DRL5LH and DRL30 schedules, consecutive responses on the active lever greater than 5 or 30 sec apart, respectively, were reinforced by delivery of a food pellet reinforcer. Responses on the inactive lever were recorded, but had no programmed consequence. An adjusting limited hold (LH) condition was included in the DRL5LH schedule to reduce the probability of reinforcing nonscheduled-directed alternate behaviors, such as grooming, which result in long time lapses between consecutive responses (McClure and McMillan, 1997). LH was initially set at 10 sec. Responding within the LH resulted in a decrease in LH duration by 0.01 sec. Lack of responding within the LH resulted in an increase in LH duration by 0.03 sec. Following stable performance on DRL5LH, which required 12-16 sessions, the dose-response for MPH treatment was evaluated. Subsequently, the DRL interval was increased gradually by 0.75% following each reinforced response within-session. The adjusted DRL interval was carried forward from the previous session until DRL30 was reached. In DRL30, a LH was not used because the probability of reinforcement of a time-insensitive IRT is lower with this schedule. Rats were maintained on DRL30 until stable responding was reached, requiring 12-22 sessions.

Stability under both DRL5LH and DRL30 schedules was defined as less than a 20% change in IRT at 5 and 30 sec, respectively, across 5 consecutive sessions. Number of

active and inactive lever responses, number of reinforcers earned and IRTs were recorded over 5 consecutive sessions of stable behavior. Efficiency was defined as pellets earned, calculated as a percent of the total number of responses on the active lever. Cut-offs of IRT durations for burst responding for DRL5LH and DRL30 were set at 1.4 and 2.0 sec, respectively, to segregate the waiting distribution based on procedures described in previous reports (Richards *et al*, 1993). Task delinquent behavior was defined for DRL5LH as IRTs longer than 100 sec, which were not reinforced. For DRL30, delinquent behavior, defined as IRTs longer than 120 sec, were reinforced.

For evaluating the effects of MPH on impulsivity, adolescent SHR, WKY and WIS were administered MPH (1.5 mg/kg p.o.) or VEH (water, 1 ml/kg, p.o.) daily from P28-55 and trained on either DRL5LH or DRL30 (n=6/group/experiment). Training protocols used were the same as that described above, with minor modifications. One cohort of adolescent rats (n=6/group) was trained to stability on DRL5LH schedule, which required 12-18 sessions. A separate cohort of adolescent rats (n=6/group) was trained from DRL3 to DRL30, and then maintained on DRL30 until stable responding was reached, requiring 12-25 sessions.

6.2.5 Modified temporal regulation (TR) model

Two non-linear mathematical models were utilized to identify endophenotypes of impulsivity in SHR (Hill *et al*, 2012a; Mika *et al*, 2012; Sanabria *et al*, 2008). Both models were fit to the IRT distribution across 5 sessions of stable responding. The first model posits that the distribution of IRTs is a mixture of two independent probability distributions (see equation 1). One distribution is sensitive to the DRL contingency (*waiting-related*) and predicted by a gamma function theoretically centered near the DRL target time (Supplemental Fig. 1). The other distribution is not sensitive to the DRL

contingency (*non-waiting related*), and is predicted by an exponential function. The second model (see equation 2) posits that the distribution of non-timed IRTs are a composite of two processes. One process results in burst responses characterized by an exponential function with a high rate-of-decay (Supplemental Fig. 1). The second process results in task delinquent behavior characterized by an exponential function with a low rate-of-decay (Supplemental Fig. 1).

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + (1 - p)\lambda e^{-\lambda(t-\delta)}; \quad 0 < \delta < t. \quad (1)$$

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + q(1 - p)L e^{-L(t-\delta)} + (1 - q)(1 - p)L' e^{-L'(t-\delta)}; \quad 0 < \delta < t. \quad (2)$$

Equation 1 indicates that the probability of IRT duration t sec is a function of five parameters: p , N , c , λ and δ ; where p is the proportion of timed IRTs and $(1-p)$ is the proportion of non-timed IRTs. The shape and scale parameters of the gamma distribution Γ are N and c , respectively. λ is the rate of decay of the non-timed IRT or the inverse of the mean non-timed IRT. δ is the shortest interval possible and was estimated as the shortest IRT emitted (Brackney *et al*, 2011); it was not a free parameter. Equation 2 is a modified version of equation 1, where λ has been replaced by three other parameters. Here the proportion and rate-of-decay of short non-timed IRTs are q and L , respectively, $(1-q)$ and L' are the proportion and rate-of-decay of the long non-timed IRTs. TR parameters were estimated using the method of maximum likelihood (Myung, 2003). Models were compared for predicting behavior under the two DRL schedules for each rat using Akaike's Information Criterion (AIC; Burnham, 2002); equation 3 was used to compare the two AIC values.

$$\Delta AIC = 2 * (\text{number of parameters}_{\text{equation 2}} - \text{number of parameters}_{\text{equation 1}}) - 2 * (\text{loglikelihood}_{\text{equation 2}} - \text{loglikelihood}_{\text{equation 1}})$$

(3)

Free parameters were estimated for each rat x schedule combination according to the model selected by ΔAIC . Equation 1 was selected if the ΔAIC for an individual was greater than 10. Equation 2 was used if ΔAIC for an individual was less than 10. The composite ΔAIC for each strain x schedule combination was reported. Response threshold θ was calculated as the mean timed IRT divided by the target time, $\left[\frac{N \times c}{5}\right]$ and $\left[\frac{N \times c}{30}\right]$ for DRL5LH and DRL30, respectively, with c estimated in sec. The Weber fraction or coefficient of variation of timed IRTs, ω , was computed as the standard deviation divided by the estimated mean, which reduces to $\left[\sqrt{\frac{1}{N}}\right]$. Further, p, q, N, c, λ, L and L' were considered primary TR parameters and θ and ω were considered secondary or derived TR parameters.

6.2.6 Open-field locomotor activity

Increases in burst responding under the DRL schedules following MPH administration may be due to MPH-induced hyperactivity and not due to the effects of MPH on response inhibition. To evaluate this possibility, adolescent SHR, WKY and WIS were administered MPH or VEH and 30 min later tested for activity in open-field chambers. Of note, the rats used in this assay were trained previously on DRL5LH. Three days after the completion of DRL studies, locomotor activity was evaluated. Rats were habituated for 3 days, for 1-hr each day, in one of 12 open-field chambers (42 × 42 × 30 cm), each with a 16 × 16 grid of photobeam sensors with monitoring

system (AccuScan Instruments Inc., Columbus, OH). Total number of horizontal beam breaks during the third day were recorded and compared between groups.

6.2.7 Data analysis

Analyses were conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). For evaluating strain differences, dependent variables were as follows: 1) active lever responses; 2) reinforcers earned; 3) sessions to train from DRL5LH to DRL30; 4) efficiency (% of responses reinforced); 5) limited hold (in sec); 6) rate of reinforcement (pellets per min); 7) proportion of burst responses (IRT<1.4 sec for DRL5LH and IRT<2.0 sec for DRL30); 8) task delinquency (IRT>100 sec for DRL5LH and IRT>120 sec for DRL30); 9) location of the timed peak (x-coordinate of the maxima of waiting peak obtained by plotting 1-second and 4-second moving averages for DRL5LH and DRL30, respectively); 10) peak spread (width of the waiting-related peak at half-maximal peak height); and 11) primary and derived parameters from modified TR models. For evaluating effects of MPH, dependent variables were as follows: 1) efficiency (% of responses reinforced), 2) latency to respond following the delivery of reinforcer under DRL5LH, 3) sessions to train from DRL3 to DRL30; 4) primary and derived parameters from modified TR models and 5) horizontal activity in open-field chambers.

All data are reported as the mean \pm S.E.M. and n represents the number of rats per group. Estimates of L , L' , and λ were log transformed to maintain homogeneity of variance. Strain differences were determined using either one-factor ANOVA with Tukey's post-hoc test or unpaired t-test, as appropriate. Further, θ estimates were analyzed using Student's one-sample t-test for matched subjects for comparison with the hypothetical value of 1 (Vadum *et al*, 1998). Reduced accuracy for a strain was

indicated by θ less than 1. Strain difference in task delinquency under the DRL30 schedule was evaluated using a Kruskal-Wallis test with Dunn's Multiple Comparisons post-hoc test. Acute MPH treatment effects for each rat strain were determined using repeated-measure one-way ANOVA and Dunnett's post hoc analysis. Test of sphericity was conducted to validate the repeated measures ANOVA (Mauchly, 1940). When the assumption of sphericity was violated, Huynh-Feldt correction was applied to adjust the F-statistic (Huynh and Feldt, 1980). Effects of MPH treatment were determined using two-factor ANOVA (strain X treatment), followed by Bonferroni's post-hoc comparisons. Main effects of strain or treatment were evaluated using Tukey's post-hoc comparison. Outliers were omitted using the Grubbs test (GraphPad; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>).

6.3 Results

6.3.1 Between-strain differences in behavior in adult rats under DRL

No strain differences were found in the number of sessions required to train rats from the DRL5LH to the DRL30 schedule (Supplementary Fig. 2). Number of active lever responses and reinforcers earned by SHR under the DRL5LH schedule were greater than for WKY, but fewer than WIS (for active lever responses, $F_s[2,33]=71.6$, $p<0.0001$; for reinforcers earned $F_s[2,33]=124.2$, $p<0.0001$; Table 6.1). In contrast, under DRL30, SHR emitted a greater number of active lever responses ($F_s[2,33]=40.9$, $p<0.0001$), but earned fewer reinforcers compared to both WKY and WIS ($F_s[2,33]=43.5$, $p<0.0001$). Visual inspection of the mean IRT frequency distributions for SHR, WKY and WIS under DRL5LH and DRL30 reveals several strain differences (Fig 6.1A-B). Premature responses (left of the dotted line) resulted in loss of reinforcement. Responses (right of the dotted line) were reinforced under DRL30; however, only responses within the limited hold

were reinforced under DRL5LH. Under DRL5LH, SHR emitted a lower frequency of responding at the peak IRT location compared to WIS, and SHR emitted a greater frequency of burst responding (IRTs<1.4 sec) compared to WKY (Fig 6.1A, left). Quantification of the reinforced responses (efficiency) for DRL5LH revealed no strain differences ($F_s[2,33]=0.642$, $p>0.05$; Fig 6.1A, right). The rank order for adjusted LH and rate of reinforcement under DRL5LH schedule was WIS<SHR<WKY ($F_s[2,33]=37.4$, $p<0.0001$) and WKY<SHR<WIS ($F_s[2,33]=124$, $p<0.0001$), respectively (Fig 6.2). Compared to both WKY and WIS, SHR under DRL30 had a lower frequency of reinforcement (right of the dotted line), a skewed leftward-shifted waiting distribution, and a greater frequency of burst responding (IRTs<2 sec; Fig 6.1B, left). Under DRL30, SHR exhibited a lower reinforcement efficiency compared to WKY and WIS ($F_s[2,33]=56.0$, $p<0.001$); further, WKY had a higher efficiency than WIS (Fig 6.1B, right).

6.3.2 Strain differences in TR parameters following mathematical modeling of IRT distributions

Because the ΔAIC values for DRL5LH for SHR, WKY and WIS were less than 10 (ΔAIC values = -1974, -549.7 and -3947, respectively), behavior was predicted by equation (2). As expected, both timed responding (i.e., sensitive to the DRL contingency) and non-timed responding (exponential functions) were observed under DRL5LH. As illustrated by data obtained from representative rats (Supplementary Fig. 3), four IRT distribution patterns were observed under DRL5LH. The waiting pattern was a uni-modal distribution with responding centered near the 5-second waiting-time that achieved an asymptote by 7.5 sec (Supplementary Fig. 3A). The burst pattern was a bi-modal distribution with the rise to an early plateau delineating burst-related responding, followed by waiting (Supplementary Fig. 3B). The task-delinquency pattern was a uni-modal distribution in which the waiting IRTs required >15 sec to reach

asymptote (Supplementary Fig. 3C). The combination pattern was a composite of a bi-modal IRT distribution (burst pattern) that required >15 sec to reach asymptote (task-delinquency pattern; Supplementary Fig. 3D).

TR parameters for the IRT distributions from SHR, WKY and WIS under the DRL5LH schedule illustrated in Fig 6.1A are provided in Table 6.2. With respect to the waiting-related distribution, SHR had a higher response threshold (θ ; $F_s[2,33]=6.33$, $p<0.005$) compared to WKY. Also, θ was lower than the hypothetical value of 1 for WKY and WIS ($t[11]=4.05$ and $t[11]=3.46$, respectively, $ps<0.01$), demonstrating reduced accuracy of estimating the 5-sec waiting time. The coefficient of variation for waiting IRT (ω) for SHR was not different from that for the control strains, demonstrating no differences in precision; however, ω was greater for WKY compared to WIS ($F_s[2,33]=3.50$, $p<0.05$). SHR had a lower proportion of waiting-related responding (p) compared to WIS, but not compared to WKY ($F_s[2,33]=3.36$, $p<0.05$).

For non-waiting-related short-IRTs emitted under the DRL5LH schedule, the rate of decay (L) was faster for SHR compared to WKY ($F_s[2,33]=4.02$, $p<0.05$). SHR emitted shorter bursts (mean length of bursts; $1/L=3.85$ sec) than WKY (7.69 sec), but not significantly different from WIS (5.26 sec). However, the proportion of short-IRTs was not different between strains ($q^*(1-p)$; $F_s[2,33]=0.149$, $p>0.05$). The between-strain differences in the non-waiting-related IRT parameters (L and $q^*(1-p)$) are presented as log-transformed exponential functions illustrating that the SHR emitted shorter burst responses compared to control (Fig 6.3A).

For non-waiting-related long-IRTs emitted under the DRL5LH schedule, the rate of decay (L') was slower for SHR (mean length, $1/L'=66.7$ sec) and WKY (100 sec) compared to WIS (31.3 sec; $F_s[2,32]=9.53$, $p<0.001$). Also, the proportion of long-IRTs ($(1-q)^*(1-p)$) was greater in SHR and WKY compared to WIS ($F_s[2,32]=6.48$, $p<0.005$). The between-strain differences in the non-waiting-related IRT parameters (L' and $(1-q)^*(1-p)$)

p) are presented as log-transformed exponential functions illustrating that the SHR and WKY emitted longer and more frequent task-delinquent responses than WIS (Fig 6.3B).

Under DRL30, the ΔAIC for SHR, WKY and WIS were -31.6, -120, and +48.0, respectively. For SHR and WKY ($\Delta AICs < 10$), behavior was predicted by equation (2), whereas equation (1) predicted behavior emitted by WIS ($\Delta AIC > 10$). Further, the ΔAIC for one SHR and two WKY rats were > 10 ; as a consequence, θ , ω , λ and p were determined from equation (1). Thus, for WIS rats and for the two WKY and one SHR, the non-timed IRTs were not a composite of burst (function of q and L) and task-delinquency (function of $1-q$ and L') processes. Rather, a single burst-related exponential function (λ) sufficiently described the non-timed responding. For statistical analyses, $L = \lambda$, and $q=1$ for these three rats and for WIS. TR parameters for IRT distributions from SHR, WKY and WIS under the DRL30 schedule are provided in Table 6.2. With respect to waiting-related distributions, WKY had a greater θ than SHR and WIS ($F_s[2,33]=44.7$, $p < 0.0001$). Further, θ deviated from 1 for SHR and WIS ($t[11]=16.8$ and $t[11]=7.26$, respectively, $ps < 0.0001$), demonstrating reduced accuracy of estimating the 30-sec waiting time. No strain differences were found in the coefficient of variation of waiting IRTs (ω ; $F_s[2,33]=0.085$, $p > 0.05$), revealing no strain differences in precision. For SHR and WKY, the proportion of waiting-related responding (p) was lower than those obtained for WIS ($F_s[2,33]=7.80$, $p < 0.01$).

For non-waiting-related short-IRTs emitted under the DRL30 schedule, the rate of decay was faster for SHR ($1/L=0.59$ sec) than for WKY (1.27 sec), and slower for WKY compared to WIS (0.45 sec; $F_s[2,31]=8.13$, $p < 0.0015$). Further, the proportion of short IRTs was greater for SHR compared to WIS, but not different from those for WKY ($F_s[2,32]=7.74$, $p < 0.005$). Between-strain differences in the non-waiting-related IRT parameters (L , λ and $q^*(1-p)$, $(1-p)$) are presented as log-transformed exponential functions illustrating that SHR emitted shorter and more frequent bursts compared to controls (Fig 6.3C).

For non-waiting-related long-IRTs emitted under the DRL30 schedule, the rate of decay was faster for SHR ($1/L'=0.91$ sec) than for WKY (3.75 sec; $t[18]=2.10$ $p<0.05$), but the proportion of these IRTs was not different between strains ($t[21]=1.49$, $p>0.05$). The non-waiting-related IRT parameters (L' , and $(1-q)*(1-p)$) are presented as log-transformed exponential functions (Fig 6.3D), illustrating that the distribution for “long-IRTs” resembled burst-like IRTs in SHR.

6.3.3 Molar IRT distribution parameters

To determine strain differences for waiting-related responding under DRL5LH and DRL30 schedules, the IRT frequency distributions illustrated in Fig 6.1A-B were fractionated using visually-defined “molar” behavioral patterns (peak location and peak width; Fig 6.4A-D). For waiting-related responding under the DRL5LH schedule, the peak location was not different between SHR and WIS; however, the peak location for SHR and WIS was longer than that for WKY ($F_s[2,33]=7.63$, $p<0.0005$; Fig 6.4A). Further, the peak width was greater for SHR compared to WIS ($F_s[2,33]=9.67$, $p<0.0005$; Fig 6.4B). For waiting-related responding under the DRL30 schedule, the peak location for SHR was shorter than that for WKY, but not compared to that for WIS ($F_s[2,33]=11.2$, $p<0.0005$, Fig 6.4C). Further, the peak location for WKY was greater than that for WIS. The peak width under DRL30 was shorter in SHR compared to WKY, but not compared to WIS, and peak width for WKY was wider than that for WIS ($F_s[2,33]=7.08$, $p<0.005$, Fig 6.4D).

Strain differences in non-waiting-related responding under DRL5LH and DRL30 schedules were characterized using percentage of short-IRTs and long-IRTs (Fig 6.4E-H). SHR emitted a greater percentage of short-IRTs (bursts) under DRL5LH (IRT<1.4 sec; $F_s[2,31]=7.99$, $p<0.005$; Fig 6.4E) and DRL30 (IRT<2.0 sec, $F_s[2,32]=17.0$, $p<0.0001$, Fig

6.4G) schedules compared to WKY and WIS. Under DRL5LH, both SHR and WKY emitted a greater percentage of long-IRTs (task-delinquent responding) compared to WIS (IRT>100 sec, $F_s[2,33]=10.8$, $p<0.0001$, Fig 6.4F). Under DRL30, long-IRTs were emitted by only one SHR, one WIS and nine WKY rats. Between-strain comparisons of the percentage of long-IRTs revealed that WKY emitted a greater proportion of long-IRTs compared to WIS and SHR (IRT>120 sec; Kruskal-Wallis test, $\chi^2(3)=18$, $p<0.0001$; Fig 6.4H).

6.3.4 Effects of chronic MPH in adolescent rats under DRL5LH

Chronic MPH treatment decreased latency to respond following reinforcer delivery in the three strains of adolescent rats ($F_t[1,29] = 7.86$, $p<0.01$; $F_i[2, 29] = 0.842$, $p>0.05$; Fig 6.5A), and adolescent SHRs had a lower response latency compared to WKY and WIS ($F_s[2, 29] = 7.14$, $p<0.01$). Furthermore, the MPH-induced decrease in response latency was more pronounced in adolescent SHR (83% of vehicle control; $t[10] = 2.64$, $p<0.05$) than WKY and WIS (91% and 95%, respectively). Efficiency under DRL5LH was not altered by chronic MPH treatment (Table 6.3) in any strain of rat; SHR had reduced efficiency compared to WKY and WIS. However, a visual inspection of the mean IRT distributions revealed that frequency of waiting-related responding was decreased with chronic MPH treatment in all three strains (Fig 6.6A). With respect to modeling parameters, chronic MPH treatment decreased the proportion of waiting IRTs ($F_t[1, 30] = 7.42$, $p<0.05$; Fig 6.6B) and increased burst responses ($F_t[1, 30] = 5.54$, $p<0.05$; Fig 6.6C) in all three strains. No interaction or main effect of strain was revealed for proportion of waiting and burst IRTs ($F_i[2,30] = 0.792$, $F_i[2,30] = 0.0774$, for waiting and burst IRTs, respectively, $ps>0.05$). Further, no interactions and main effects were revealed for mean and proportion of task-delinquent IRTs (Table 6.4), and for precision of timed IRTs (Table 6.5).

6.3.5 Effects of chronic MPH in adolescent rats under DRL30

Adolescent SHR required more sessions to train from DRL3 to DRL30 compared to adolescent WKY and WIS ($F_i[2,30] = 1.25, p > 0.05$; $F_s[2, 30] = 14.1, p < 0.0001$; Fig 6.5B). Chronic MPH did not alter the number of training sessions in any of the strains ($F_t[1,30] = 1.41, p > 0.05$). Efficiency under DRL30 schedule was not altered by chronic MPH treatment (Table 6.3) in any of the rat strains; SHR had reduced efficiency compared to WKY and WIS, and WKY exhibited greater efficiency compared to WIS (Table 6.3). Visual inspection of the mean IRT distributions revealed that frequency of burst responding was increased and the peak of timed IRTs was shifted leftwards in adolescent SHR treated with MPH; no other differences were observed in the IRT distribution patterns between MPH and VEH groups (Fig 6.7A). The parameters derived from fitting the mathematical model to the IRT distribution are reported below.

With respect to the modeling parameters, adolescent SHR showed a reduced accuracy of timed IRTs (i.e. mean timed IRTs) compared to WKY and WIS (θ ; $F_s[2, 30] = 50.0, p < 0.001$; Fig 6.7B). Accuracy was further decreased by chronic MPH treatment ($F_t[1, 30] = 4.71, p < 0.05$), and this decrease was more pronounced for SHR (78% of vehicle control; $t[10] = 3.07, p < 0.05$) than for WKY and WIS (98% and 94%, respectively). The mean burst IRTs was increased in MPH-treated SHR compared to vehicle control as well as MPH-treated WKY and WIS ($1/L$; $F_i[1, 30] = 3.85, p < 0.05$; Fig 6.7C). Compared to adolescent WKY and WIS, adolescent SHR emitted a greater proportion of burst responses, which was further increased by MPH treatment ($1-p$; $F_i[2, 28] = 3.58, p < 0.05$; Fig 6.7D). Further, no interactions nor main effects were revealed for precision of timed IRTs for adolescent SHR (Table 6.5)

6.3.6 Effects of chronic MPH on locomotor activity in adolescent rats

In the open-field chambers, the adolescent SHR exhibited greater locomotor activity compared to WKY and WIS ($F_s[2, 30] = 21.5, p < 0.0001$; Fig 6.7E). Further, locomotor activity for the adolescent WKY was reduced compared to the adolescent WIS. However, no interaction or main effect of treatment ($F_i[2, 30] = 3.27, F_t[1, 30] = 3.22$, respectively, $p > 0.05$) was obtained in open field locomotor activity in adolescent rats.

6.4 Discussion

In the current study, impulsivity endophenotypes expressed by SHR included increased burst responding in both DRL5LH and DRL30 schedules, and reduced wait-time accuracy in the DRL30 schedule. These endophenotypes contribute to reduced reinforcement efficiency, a conventional measure of impulsivity using the DRL task. Reinforcement efficiency values for adult SHR were consistent with previous studies using DRL30 and DRL5LH (Ferguson *et al*, 2007; Sanabria *et al*, 2008; van den Bergh *et al*, 2006), but not consistent with DRL5 without a LH condition (Orduna *et al*, 2009; Sanabria *et al*, 2008). In the current study, SHR attained higher reinforcement rates than WKY under the DRL5LH schedule, which was an expected consequence of the observed hyperactivity on the active lever in SHRs, and in agreement with previously reported hyperactivity in this strain (Hill *et al*, 2012b). Under the DRL30 schedule, hyperactivity in SHR led to a reduced efficiency compared to WKY and WIS. Under the current DRL30 schedule, WKY were hypoactive compared to WIS, in agreement with previous reports (van den Bergh *et al*, 2006). Also, as a result of fewer active lever responses, WKY had the highest reinforcement efficiency under the DRL30 schedule. As a consequence, outbred WIS rats proved to be an important control for evaluating impulsivity in SHR,

particularly when measures (e.g., efficiency) are normalized by response rates (Alsop, 2007b). Thus, the current study describes optimized procedures for delineating different components of impulsivity, a complex and multifactorial construct.

6.4.1 Optimization using mathematical modeling

Endophenotypes of impulsivity that contribute to reduced reinforcement efficiency in SHR were evaluated by quantification of IRT distribution patterns. Typically, IRT patterns have been quantified using molar parameters such as burst ratios and peak location, which fragment the IRT distribution (Richards *et al*, 1993). Although the latter molar approach has been informative, current state-of-the-art methods for analyzing large data sets often include mathematical modeling, which accounts for the complete IRT distribution pattern, rather than simply a fragment of the pattern. Mathematical modeling allows informative comparisons of results between different laboratories by eliminating the non-standardized cut-offs for IRTs to define the impulsivity endophenotypes. In the current study, the results from the mathematical modeling approach provided a more informative description than did the molar approach and identified the endophenotype of impulsivity expressed as reduced response inhibition in the SHR under both schedules.

Mathematical modeling has been used previously to describe SHR behavior under DRL schedules. In the current study, SHR had higher response threshold (θ) estimates than WKY under DRL5LH, contrary to previous reports (Orduna *et al*, 2009; Sanabria *et al*, 2008). This discrepancy may arise from the type of distribution the model used to describe the waiting, i.e., normal distribution in the earlier studies versus gamma distribution used herein. A recent report suggests that gamma distributions, modified from the Erlang distribution for waiting-time, more accurately model waiting

behavior under DRL schedules compared with normal distributions (Hill *et al*, 2012a). In the current study, comparison of AIC values from the modified TR equation using the normal function (Orduna *et al*, 2009; Sanabria *et al*, 2008) with AIC values from Equation 1 using a gamma function revealed that the latter provided a better fit the waiting distribution (Supplementary Table 1). Another possible reason for the inconsistency in response threshold may be that Equation (2) includes a second exponential distribution of longer non-waiting IRTs, which may have been included with the waiting IRTs in the previous studies. Thus, when the long task-delinquency related IRTs were excluded from the waiting IRTs, as in the current model (Equation 2), SHR did not differ from WIS in the accuracy of estimating the 5 sec waiting time under DRL5LH, emphasizing the importance of selecting appropriate modeling tools to draw inferences about impulsivity.

6.4.2 Impulsivity endophenotypes

For waiting-related responses under DRL5LH, mathematical modeling showed that SHR had the highest accuracy of timing (θ), which was different from WKY, but not different from WIS. The current models also showed that SHR were less likely to produce waiting responses (p). Thus, SHR exhibited high accuracy, but a reduced tendency to wait. For non-timed responses, the molar parameter (i.e., percentage short-IRTs) indicates that the SHR produced a greater frequency of burst responses. However, the mathematical model which accounts for the entire responding pattern reveals that SHR did not emit a greater frequency of bursts (i.e., $q*(1-p)$), but rather, emitted shorter bursts (i.e., $1/L$), contributing to a reduced reinforcement efficiency. In contrast, in the evaluation of task-delinquency, both molar parameters (i.e., percentage long-IRTs) and modeling parameters (i.e., $(1-p)*(1-q)$) were in agreement and indicated that SHR and WKY emitted a greater proportion of delinquent responses compared to WIS. Thus, results from the model show that SHR obtain fewer reinforcers under a DRL5LH schedule due to a reduced tendency to wait, shorter burst responding, and

more frequent and longer task-delinquency behavior. Reduced tendency to wait and shorter bursts indicate that SHR express a specific impulsivity endophenotype.

Under the DRL30 schedule, SHR exhibited reduced response thresholds, indicating either reduced ability to estimate wait-time or impaired time perception. Previous research using peak-interval procedures indicate that SHR, WKY and WIS do not differ in ability to time 30 sec (Fox *et al*, 2009; Orduna *et al*, 2008). Thus, the low response threshold (θ) in SHR and WIS may be an outcome of reduced ability to wait, indicative of aversion to wait. Moreover, SHR produced a lower proportion (p) of waiting-related responses compared with WIS, indicating that reduced response inhibition contributed to the low reinforcement efficiency in SHR. Another contributor to loss in efficiency was the more frequent burst responding (i.e., $q^*(1-p)$) emitted by SHR. Thus, under the DRL30 schedule, SHR obtained fewer reinforcers due to a reduced wait-time accuracy (θ) and reduced response inhibition (fewer waiting-related responses and more frequent bursts), all of which contribute to impulsivity in SHR. Thus, increasing the DRL interval further reveals behavioral deficits associated with impulsivity. The current results are consistent with previous findings that low rates of reinforcement with variable interval schedules reveal behavioral deficits in SHR (Hill *et al*, 2012a; Williams *et al*, 2009a). The current results further validate the SHR model of ADHD since behavioral deficits in ADHD patients are manifest only under schedules providing infrequent reinforcement (Aase and Sagvolden, 2006).

A limitation of exceedingly-long DRL intervals is the risk of reducing the reinforcement frequency to the point where responding is no longer maintained. This limitation has been overcome by extensive training (Orduna *et al*, 2009). Further, not all behavioral changes associated with reduced reinforcement frequency are specific to the ADHD model. Under low rates of reinforcement, outbred rats are unable to inhibit rapid-responding on the active lever (Stein *et al*, 1975; Williams *et al*, 2009a; Wultz and

Sagvolden, 1992). In agreement, increasing wait-time from 5 to 30 sec in the DRL schedule resulted in shortening of the burst IRTs in all three rat strains.

6.4.3 Methylphenidate and DRL behavior

For animal models of ADHD, the face-validity criterion posits that low doses of MPH will reduce impulsivity (Willner, 1986). In adolescent outbred rats, MPH administered repeatedly decreased home-cage activity (Kuczenski *et al*, 2002), but not locomotor activity in an open field (Yang *et al*, 2003; Yang *et al*, 2011). In adolescent SHR, therapeutically relevant low doses of MPH decreased hyperactivity in open-field chambers when administered acutely (Umehara *et al*, 2013a; Umehara *et al*, 2013b), but this effect did not persist following chronic treatment (Yang *et al*, 2011; Yetnikoff *et al*, 2013). Consistent with these reports, the current study found that chronic MPH treatment did not alter locomotor activity in adolescent rats. MPH has been shown to improve several prefrontal cortical functions such as behavioral flexibility and motivation in outbred rats (Floresco *et al*, 2009; Yamagata *et al*, 2012). In adolescent SHRs, chronic MPH administration improved learning, attention and non-spatial working memory in visual discrimination tasks (Harvey *et al*, 2011; Kantak *et al*, 2008), as well as behavioral flexibility in strategy set-shifting tasks (Harvey *et al*, 2013). The current study extends the previous findings by showing that chronic MPH treatment in adolescent SHR increased motivation to respond (reduced response latency, Fig 6.5A) under DRL5LH. Taken together, these results support the adolescent SHR as a model of ADHD.

In contrast to the effects of MPH on response latency, chronic MPH increased impulsivity in adolescent rats. Specifically, MPH increased burst responding in adolescent SHR, WKY and WIS under DRL5LH, and also increased burst responding and decreased accuracy of estimating the 30-sec wait-time under DRL30 only in SHR. These results were contradictory to clinical observation that response inhibition is decreased by repeated MPH treatment in children and adolescents with ADHD (Broyd *et al*, 2005;

Lee *et al*, 2010), and thus may suggest a limitation of the SHR model of ADHD or of DRL schedules as a measure of impulsivity.

The 5-choice serial reaction time task (5CSRT) is a response inhibition task that measures impulsivity as increased premature responding (Puumala *et al*, 1996; Robbins, 2002). In adult as well as adolescent outbred rats, premature responding under 5CSRT schedules has been reported to be increased or not altered by acute MPH at therapeutically relevant doses (Economidou *et al*, 2012; Navarra *et al*, 2008). In contrast to MPH, the non-stimulant ATO decreased premature responding in the 5CSRT (Economidou *et al*, 2012; Navarra *et al*, 2008). In adult SHR, MPH was found to not alter premature responding (van den Bergh *et al*, 2006); however, effects of MPH or ATO in adolescent SHR under 5CSRT tasks have not been reported. Taken together with the current results, response inhibition tasks, such as 5CSRT and the DRL schedules appear to be more sensitive to the stimulant properties of MPH, even with doses that were below the threshold for increasing locomotor activity (Berridge *et al*, 2006; Kuczenski *et al*, 2002).

In conclusion, the current study supports the use of adult and adolescent SHR as a model of ADHD. However, the DRL schedules were limited in their ability to model the effects MPH on impulsivity. This study extends the literature by demonstrating that reduced accuracy of estimating waiting-time and reduced response inhibition (composite of reduced proportion of waiting responses and increased burst responses) contribute to impulsivity in SHR. These individual processes are differentially sensitive to strain, MPH and schedule of reinforcement. Thus, mathematical modeling approach may prove to be a valuable tool and may have substantial translational value to extend our understanding of DRL behavior in the clinical samples.

Table 6.1 Responses on the active and the number of pellets earned by WIS, WKY and SHR under the DRL5LH and DRL30 schedules of responding.

		Active lever response	Reinforcers earned
DRL5LH	SHR	274 ± 10.4 * ^{#a}	67.7 ± 2.17 * [#]
	WKY	121 ± 11.4 *	30.4 ± 2.54 *
	WIS	475 ± 32.9	109 ± 5.07
DRL30	SHR	258 ± 13.6 * [#]	13.8 ± 2.25 * [#]
	WKY	107 ± 8.19 *	45.5 ± 1.52 *
	WIS	184 ± 13.1	23.5 ± 3.28

^a All values are mean ± S.E.M; * p < 0.05, different from WIS; [#] p < 0.05 different from WKY; n=12/strain.

Table 6.2 Estimates of TR parameters obtained from IRT data from SHR, WKY and WIS on DRL5LH and DRL30 schedules.

Distribution	Waiting-related or timed IRT			Burst		Task-delinquency		
	Accuracy (seconds)	θ ω (seconds)	Precision Proportion p	Rate of decay L (second ⁻¹)	Proportion $q * (1-p)$	Rate of decay L' (minute ⁻¹)	Proportion $(1-q)*(1-p) \times 10^{-1}$	
DRL5LH	SHR ^b	0.99 ± 0.031 ^{#a}	0.37 ± 0.053	0.50 ± 0.053 [*]	0.26 ± 0.091 [#]	0.44 ± 0.058	0.93 ± 0.12 [*]	0.60 ± 0.13 ^{**}
	WKY ^b	0.85 ± 0.038 ^{* †}	0.37 ± 0.027 [*]	0.57 ± 0.042	0.13 ± 0.017	0.41 ± 0.055	0.63 ± 0.20 ^{***}	0.49 ± 0.10 [*]
	WIS ^b	0.95 ± 0.015 [†]	0.27 ± 0.015	0.67 ± 0.036	0.19 ± 0.016	0.30 ± 0.034	1.9 ± 0.42	0.24 ± 0.042
DRL30	SHR ^b	0.55 ± 0.027 ^{* #### †}	0.51 ± 0.018	0.72 ± 0.044 ^{**}	1.7 ± 0.20 ^{##}	0.22 ± 0.018 ^{**}	66 ± 19 [#]	0.42 ± 0.22
	WKY ^b	1.1 ± 0.059 ^{***}	0.51 ± 0.030	0.75 ± 0.037 [*]	0.73 ± 0.22 ^{**}	0.15 ± 0.025	16 ± 11	0.98 ± 0.29
	WIS ^c	0.68 ± 0.044 [†]	0.50 ± 0.021	0.91 ± 0.024	2.2 ± 0.40 ^c	0.091 ± 0.024	N.A.	N.A.

^a Values are mean ± S.E.M. All comparisons are within the same schedule. ^b Values for the strain for each parameter were obtained from equation (2). ^c Values for the strain for each parameter were obtained from equation (1). * p < 0.05, ** p < 0.01 and *** p < 0.0001, different from WIS; # p < 0.05, ## p < 0.01 and #### p < 0.0001, different from WKY; † p < 0.05, different from unity. N.A. Not applicable, since equation (1) does not include L' and q .

Table 6.3 Adolescent SHR exhibited reduced efficiency under DRL5LH and DRL30 schedules.

Adolescent SHR exhibited reduced efficiency of earning reinforcers compared to WKY and WIS under DRL5LH and DRL30 schedules. Under DRL30, adolescent WKY exhibited the greater efficiency compared to WIS. Chronic MPH treatment did not alter efficiency of earning reinforcers under either DRL5LH or DRL30 schedules

			WIS	WKY	SHR	F _{interaction}	F _{strain}
Efficiency	DRL5LH	VEH	27.4 ± 2.08 ^a	27.8 ± 1.15	24.4 ± 1.50	F[2,30] = 2.32	F[2,30] = 6.03 ^b
		MPH	23.6 ± 1.15	31.6 ± 1.35	22.4 ± 2.89		SHR < WIS = WKY
	DRL30	VEH	13.1 ± 3.11	16.7 ± 3.14	3.11 ± 0.55	F[2,30] = 0.038	F[2,30] = 23.1 ^b
		MPH	11.0 ± 1.79	15.7 ± 1.84	1.40 ± 0.32		SHR < WIS < WKY

^a Values are mean ± S.E.M.

Table 6.4 For task-delinquent responding, adolescent SHR exhibited shorter mean delinquent IRTs compared to WKY and WIS under DRL5LH

Adolescent WKY exhibited the longest mean task-delinquent IRTs. Chronic MPH treatment did not alter mean task-delinquent IRTs. Further, no effects of strain or treatment were obtained for proportion of task-delinquent IRTs under DRL5LH.

			WIS	WKY	SHR	F _{interaction}	F _{strain}	
Task-delinquent responding	DRL5LH	Proportion	VEH	0.095 ± 0.036	0.12 ± 0.052	0.13 ± 0.048	F[2,30] = 0.832	F[2,30] = 0.149
		(1-p)*(1-q)	MPH	0.15 ± 0.052	0.078 ± 0.032	0.079 ± 0.040		
		Mean IRT	VEH	22.3 ± 8.1	42.6 ± 10.9	6.5 ± 1.8	F[2,26] = 1.39	F[2,26] = 12.1 ^b
		1/L' (in sec)	MPH	20.7 ± 5.8	49.0 ± 21.0	13.1 ± 4.7		SHR < WIS < WKY

^a Values are mean ± S.E. M.

^b $p < 0.05$

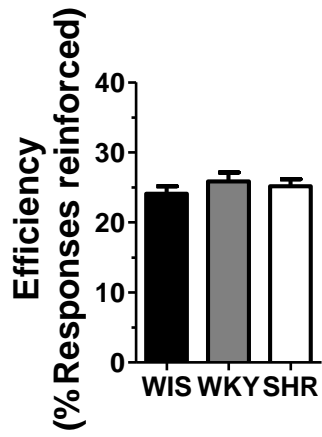
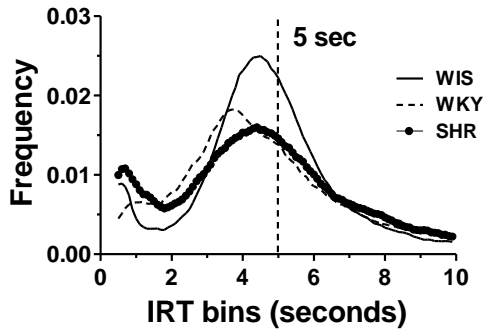
Table 6.5 For precision of waiting-related responding, no differences were found between MPH and VEH treated groups of adolescent SHR, WKY and WIS rats under DRL5LH and DRL30 schedules

			WIS	WKY	SHR	F_{interaction}	F_{strain}
Precision	DRL5LH	VEH	0.41 ± 0.068 ^a	0.45 ± 0.074	0.37 ± 0.056	F[2,30] = 0.371	F[2,30] = 0.300
		MPH	0.33 ± 0.027	0.39 ± 0.074	0.39 ± 0.082		
(ω)	DRL30	VEH	0.45 ± 0.032	0.50 ± 0.051	0.54 ± 0.036	F[2,30] = 0.271	F[2,30] = 0.966
		MPH	0.49 ± 0.025	0.50 ± 0.034	0.52 ± 0.079		

^a Values are mean ± S. E.M.

Figure 6.1

A DRL5LH



B DRL30

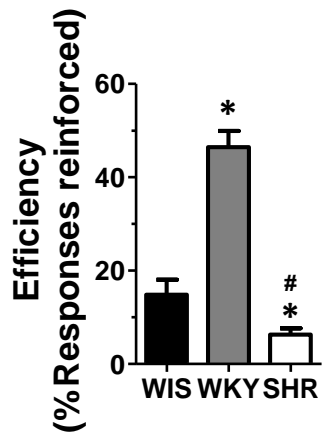
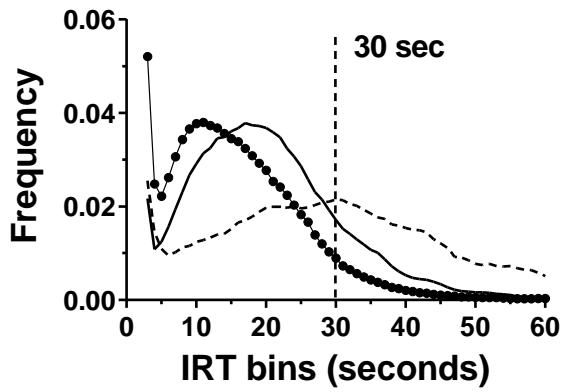


Fig. 6.1: Average frequency distribution of the inter-response times (IRTs) and percentage of responses reinforced during responding under DRL5LH (panel A) and DRL30 (panel B) schedules for adult Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto rats (WKY) and Wistar rats (WIS). The frequency of IRTs are presented as the mean number of responses in each IRT bin divided by the total number of IRTs emitted for WIS (solid curve), WKY (dashed curve) and SHR (dotted curve). The IRT curves are presented as 1-second and 5-second moving averages for DRL5LH and DRL30, respectively. The vertical dotted lines indicate the minimum waiting time for earning reinforcers for each schedule. Premature responses left of the vertical dotted line resulted in loss of reinforcement. Responses to the right of the dotted line were reinforced under DRL30; however, only responses within the limited hold were reinforced under DRL5LH. Efficiency is presented as the total number of pellets earned (mean \pm S.E.M) as percentage of the total number of responses on the active lever for WIS (black bars), WKY (grey bars) and SHR (white bars).

* $p < 0.05$, different from WIS;

$p < 0.0001$, different from WKY;

n = 12/strain.

Figure 6.2

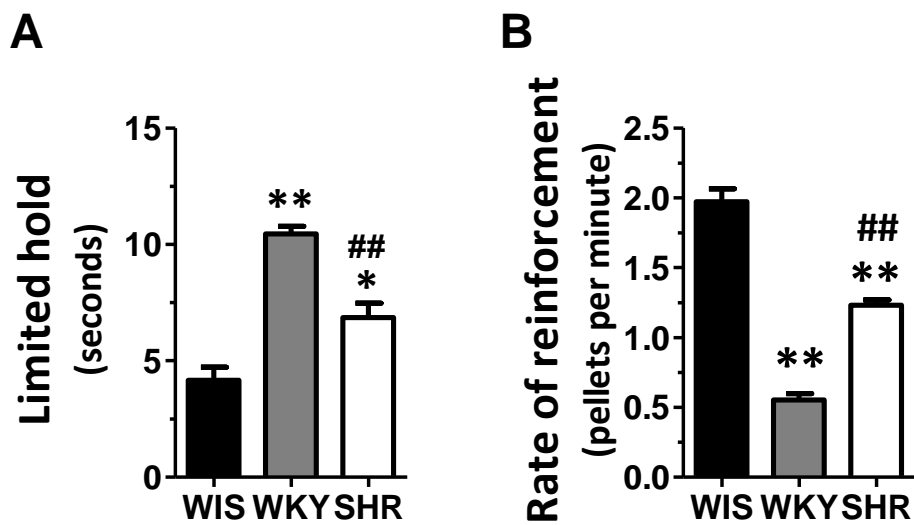


Fig 6.2 Stable adjusted limited hold (A) and rate of reinforcement (B) for adult Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats under DRL5LH schedule. Stable adjusted limited hold values are mean \pm S.E.M. in seconds for WIS (black bars), WKY (grey bars) and SHR (white bars). Rate of reinforcement for WIS, WKY and SHR are number of pellets earned (mean \pm S.E.M.) normalized per minute of each 55-minute session (right);

* $p < 0.01$, ** $p < 0.001$, different from WIS;

$p < 0.001$, different from WKY;

n = 12/strain.

Figure 6.3

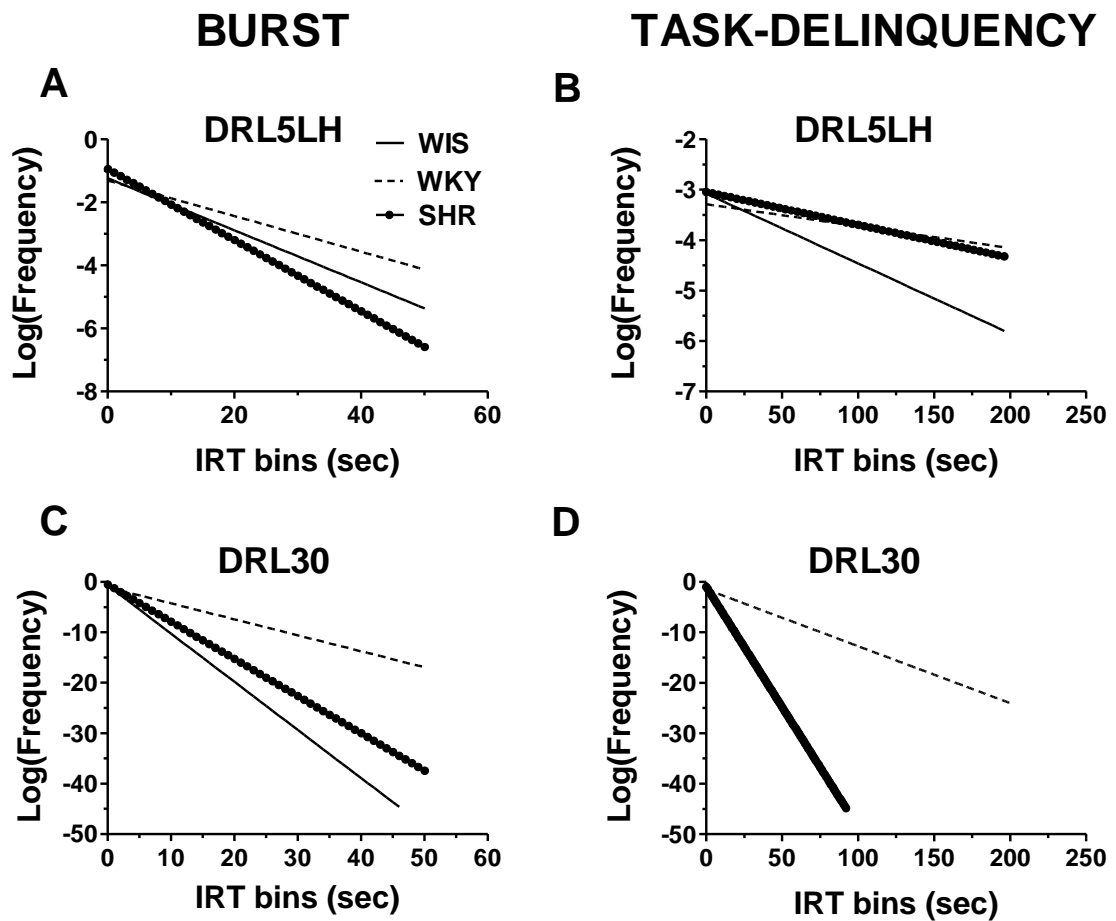
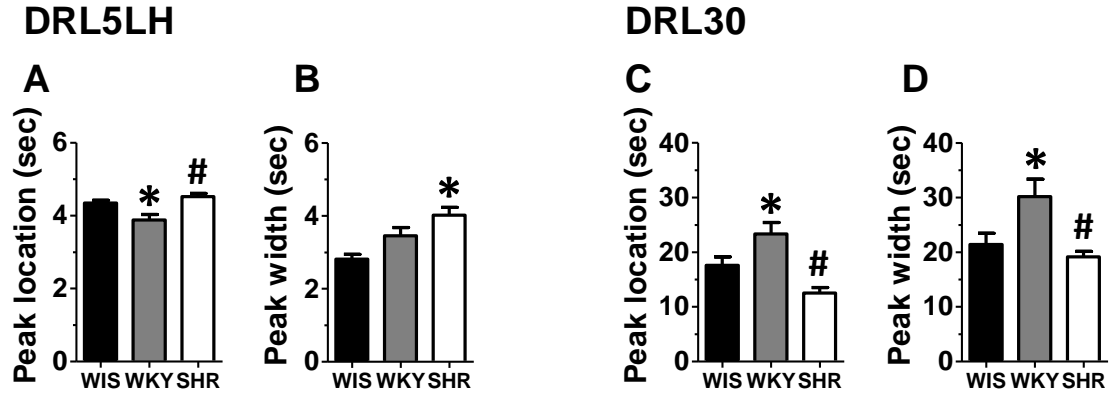


Fig 6.3 The non-waiting-related IRT parameters depicting bursts (left) and task-delinquent responding (right) emitted by adult Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto rats (WKY) and Wistar rats (WIS) under DRL5LH (A and B) and DRL30 (C and D) schedules are presented as hypothetical exponential frequency-distribution semi-log plots. The curves were generated from the mean parameter estimates for bursts (L and $q \cdot (1-p)$) and for task delinquency (L' and $(1-q) \cdot (1-p)$) under both schedules for SHR (dotted curve), WKY (dashed curve) and WIS (solid curve); $n = 12/\text{strain}$.

Figure 6.4

Waiting-related responding



Non-waiting-related responding

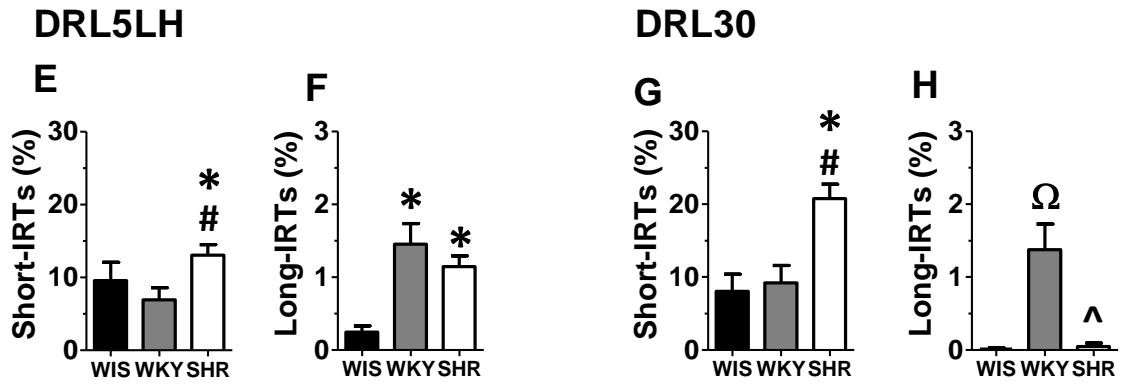


Fig 6.4 Molar descriptors for the waiting-related (top) and non-waiting-related (bottom) responding emitted by adult Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats under DRL5LH and DRL30 schedules. For waiting-related responding under DRL5LH (A, B) and DRL30 schedules (C, D), the peak location (A, C) and peak width (B, D) are mean \pm S.E.M. sec for WIS (black bars), WKY (grey bars) and SHR (white bars). For non-waiting-related responding, short- and long-IRTs under DRL5LH (E, F) and short-IRTs under DRL30 (G) are mean \pm S.E.M. expressed as a percentage of the total number of IRTs for WIS (black bars), WKY (grey bars) and SHR (white bars). Under DRL30 (H), between-strain differences in long-IRTs (>120 sec) were found.

* $p < 0.05$, different from WIS;

$p < 0.05$, different from WKY.

n = 12/strain (A-G).

Ω $p < 0.0001$, different from WIS;

\wedge $p < 0.0001$, different from WKY.

n = 1 SHR, 1 WIS, 9 WKY (H).

Figure 6.5

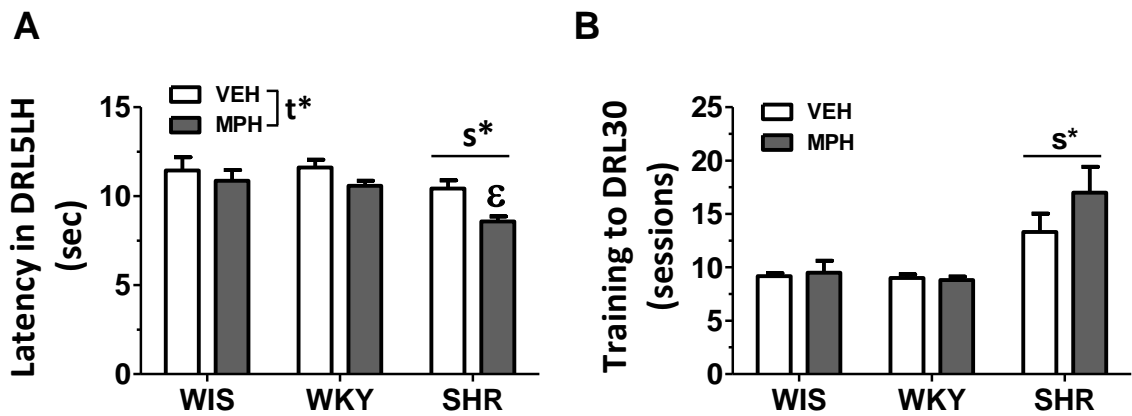


Fig 6.5 Latency for responding under DRL5LH (A) and number of sessions to train from DRL3 to DRL30 (B) for adolescent Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto (WKY) and Wistar (WIS) rats that were administered chronic methylphenidate (MPH; grey bars) or vehicle (VEH; white bars) 30 min prior to the behavioral session. Latency for responding (in sec) and number of training sessions are mean \pm S.E.M. in seconds for WIS, WKY and SHR.

s* $p < 0.05$, main effect of strain, different from WKY and WIS;

t* $p < 0.05$, main effect of treatment;

^ε $p < 0.05$ compared to the respective vehicle control;

n = 6/group.

Figure 6.6

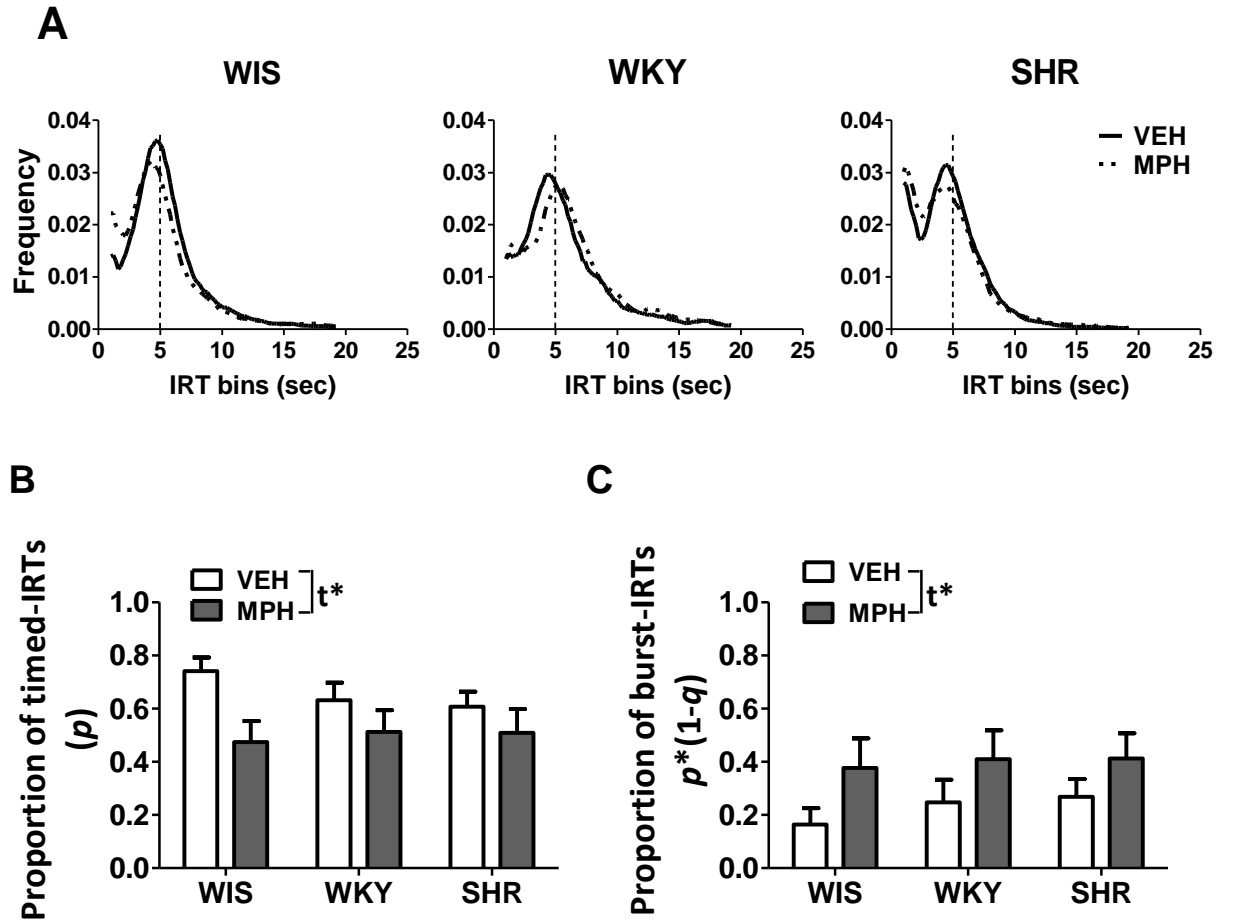


Fig 6.6 Average frequency distribution of the inter-response times (IRTs, A) and proportion of timed-IRTs (B) and burst-IRTs (C) for adolescent Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto rats (WKY) and Wistar rats (WIS) under the DRL5LH schedule that were administered chronic methylphenidate (MPH; grey bars) or vehicle (VEH; white bars). The frequency of IRTs are presented as the as 1-sec moving averages of mean number of responses in each IRT bin divided by the total number of IRTs emitted following chronic MPH (dotted curve) or VEH (solid curve) treatments for adolescent WIS, WKY and SHR under DRL5LH. The vertical dotted lines indicate the minimum waiting time for earning reinforcers for each schedule. Premature responses left of the vertical dotted line resulted in loss of reinforcement. Values are mean \pm S.E.M. for proportion of timed-IRTs and proportion of burst-IRTs.

t* $p < 0.05$, main effect of treatment;

n = 6/group.

Figure 6.7

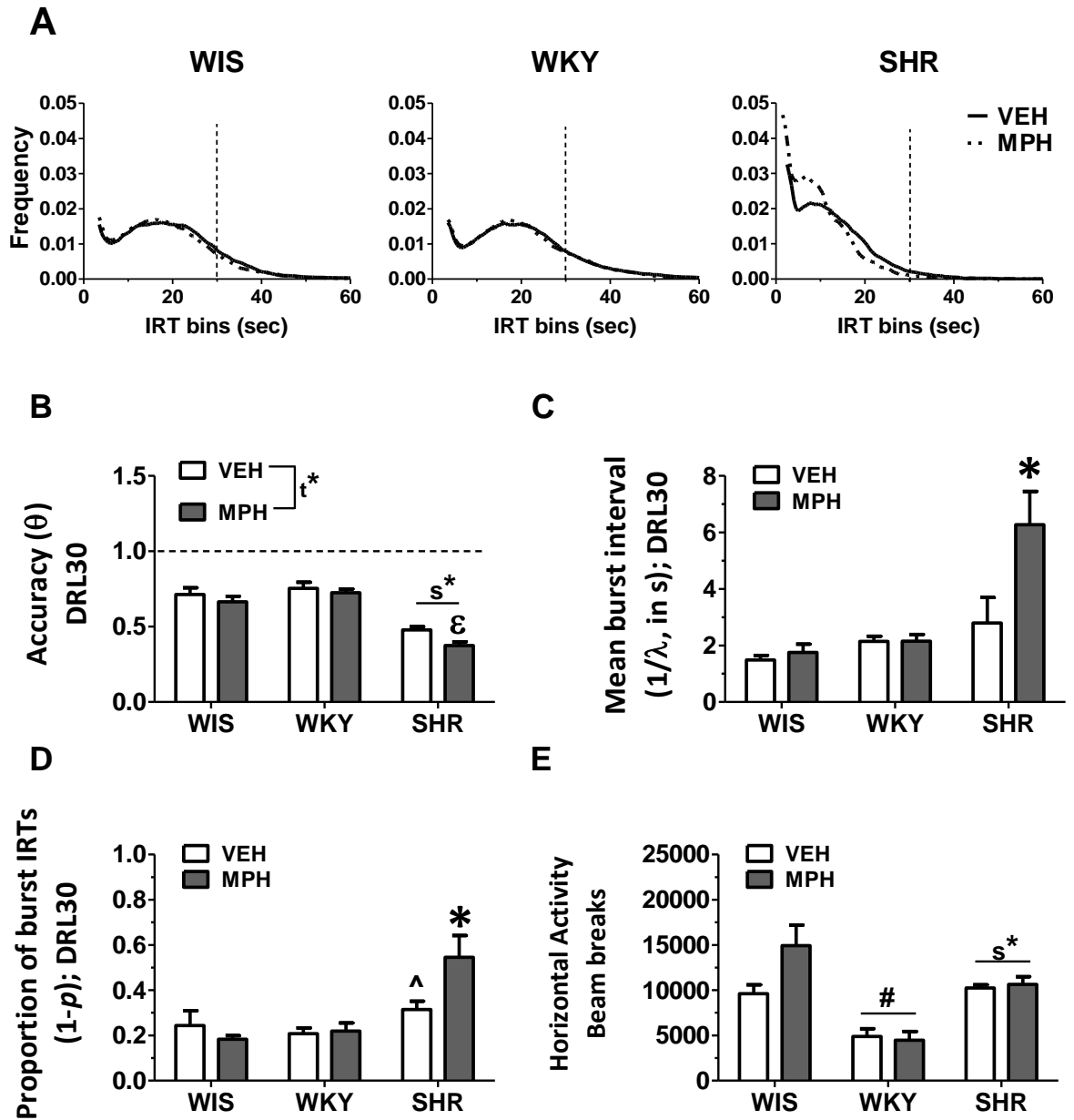


Fig 6.7 Average frequency distribution of the inter-response times (IRTs, A) and mathematically modeled impulsivity parameters (B-D), and hyperactivity (E) for adolescent Spontaneously Hypertensive Rats (SHR), Wistar-Kyoto rats (WKY) and Wistar rats (WIS) under the DRL30 schedule that were administered chronic methylphenidate (MPH; grey bars) or vehicle (VEH; white bars). The frequency of IRTs are presented as the as 5-sec moving averages of mean number of responses in each IRT bin divided by the total number of IRTs emitted following chronic MPH (dotted curve) or VEH (solid curve) treatments for adolescent WIS, WKY and SHR under DRL30. The vertical dotted lines indicate the minimum waiting time for earning reinforcers for each schedule. Premature responses left of the vertical dotted line resulted in loss of reinforcement. Values are mean \pm S.E.M. for accuracy of mean timed-IRTs (B), mean burst IRTs (C; in sec) and proportion of burst-IRTs (D) under DRL30 schedule and number of horizontal beam-breaks (E) in open-field chambers.

* $p \leq 0.05$ compared to the respective VEH control as well as MPH-treated WKY and WIS;

^ $p \leq 0.05$ compared to VEH-treated WKY and WIS;

ϵ $p \leq 0.05$ compared to the respective VEH

s* $p \leq 0.05$ main effect of strain, different from WKY and WIS;

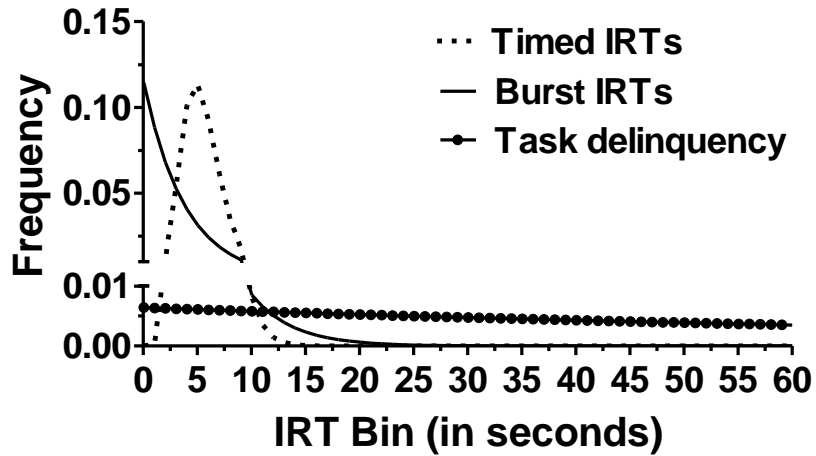
$p \leq 0.05$ main effect of strain, different from WIS;

t* $p < 0.05$, main effect of treatment;

n = 6/group.

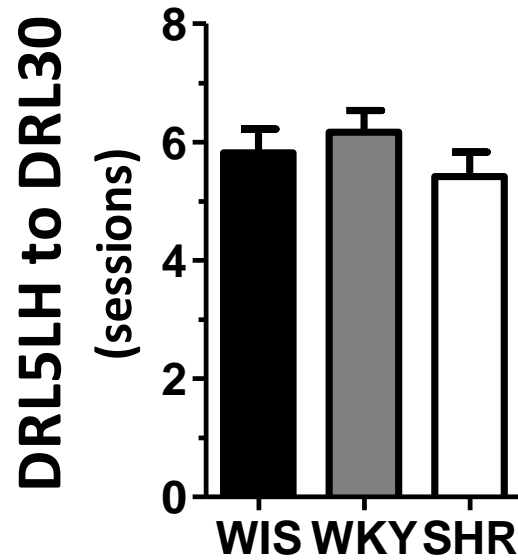
6.5 Supplementary Materials

Supplementary Fig. 1



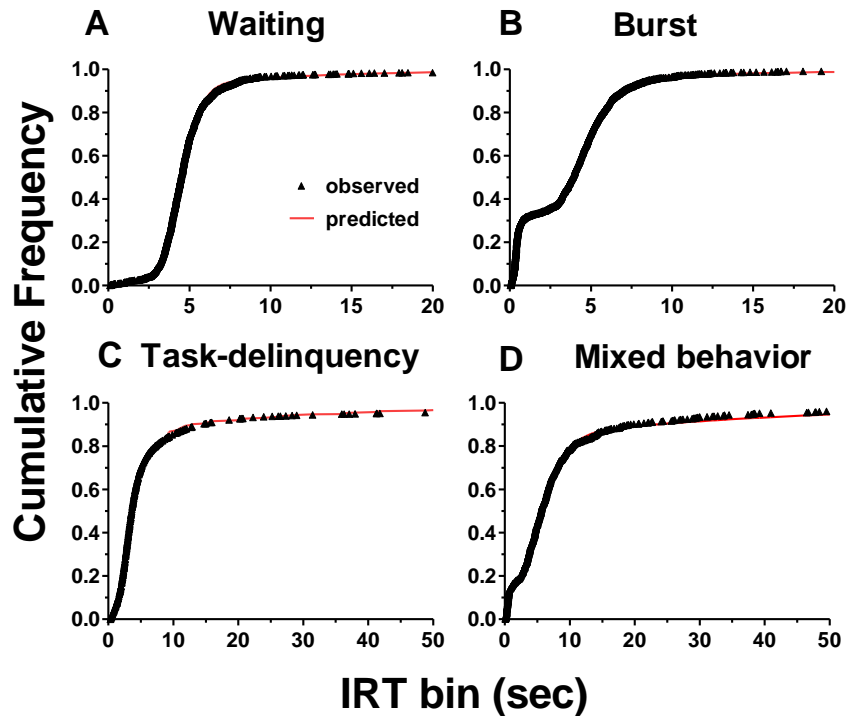
Supplementary Fig. 1 Theoretical data representing the individual components of **equation 2**; a timing-related gamma-function (dashed line) that is typically centered on the DRL wait time, a fast-decaying exponential function (solid line) describing burst of iterative responding and a slow-decaying exponential function (beaded line) describing iterative behavior with long pauses between subsequent lever responses. For equation 1, a single exponential function describes both burst and task-delinquent responding (representation not shown).

Supplementary Fig. 2



Supplementary Fig 2: The number of sessions to train adult rats from DRL5LH to DRL30 did not differ between Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR). Training required by WIS (black bars), WKY (grey bars) and SHR (white bars) are mean \pm S.E.M. in sessions; n=12/strain.

Supplementary Fig. 3



Supplementary Fig. 3 Representative IRT data from individual rats under DRL5LH schedule plotted as cumulative frequency distributions (A-D) illustrating that the observed values (closed triangles) are accurately predicted by the mathematical model (equation 2; solid red curve). Observed and predicted lines overlap such that they cannot be distinguished individually. As illustrated, four IRT distribution patterns were observed under DRL5LH. (A) The waiting pattern was a uni-modal distribution with responding centered at the 5-second waiting-time that achieved an asymptote by 7.5 seconds. (B) The burst pattern was a bi-modal distribution with the rise to the early plateau delineating burst-related responding, followed by waiting. (C) The task-delinquency pattern was a uni-modal distribution in which the waiting IRTs required >15 seconds to reach asymptote. (D) The combination pattern was a composite of a bi-modal IRT distribution (burst pattern) and required >15 seconds to reach asymptote (task-delinquency pattern).

Supplementary Table 1: Comparison of gamma- and normal-distribution for predicting behavior for Wistar (WIS), Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR) under DRL5LH schedule of responding. The IRT data for WIS, WKY and SHR under DRL5LH schedule were used for mathematical modeling using two equations. In equation 1), waiting-related responding was predicted using a normal distribution (Orduna *et al*, 2009; Sanabria *et al*, 2008). In equation 2), waiting-related responding was predicted using a gamma distribution (Hill *et al*, 2012a). Subsequently, the data-fit for equations 1) and 2) were compared using the Akieke Information Criterion (AIC), given in equation 3).

$$P(IRT = t) = p\phi(t - \delta; \mu, \sigma^2) + (1 - p)\lambda e^{-\lambda(t-\delta)}; \quad 0 < \delta < t.$$

1)

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + (1 - p)\lambda e^{-\lambda(t-\delta)}; \quad 0 < \delta < t.$$

2)

$$\Delta AIC = 2 * (\text{number of parameters}_{\text{equation 2}} - \text{number of parameters}_{\text{equation 1}}) - 2 * (\text{loglikelihood}_{\text{equation 2}} - \text{loglikelihood}_{\text{equation 1}})$$

3)

	WIS	WKY	SHR
DRL5LH	-437 ^a	-872	-788

^a Values are ΔAIC for each strain, obtained from equation 3

Since the ΔAIC values for DRL5LH for SHR, WKY and WIS were less than 10, behavior was predicted by equation 2). These results are consistent with the suggestions from a recent report (Hill *et al*, 2012), gamma distributions, modified from

the Erlang distribution for waiting-time, more accurately modeled waiting behavior under DRL5LH compared with normal distributions.

7 CHAPTER SEVEN

Chronic methylphenidate treatment during adolescence increases impulsivity that persists into adulthood in the Spontaneously Hypertensive Rat model of ADHD

7.1 Introduction

Attention deficit/hyperactivity disorder (ADHD) is a neurobehavioral disorders affecting 8-12% of children and about 5% of adults worldwide (Biederman *et al*, 2010a). The diagnosis of ADHD is based on observable clinical descriptors (hyperactivity, impulsivity and inattention) rather than founded on genetic or pathophysiological markers (Bearden *et al*, 2004; Castellanos and Tannock, 2002). Adults with ADHD have a higher risk of developing substance use disorders compared to individuals without ADHD (Biederman *et al*, 2010b; Lee *et al*, 2011; Wilens *et al*, 1998a). In particular, individuals with ADHD have a 35% higher incidence of cocaine abuse compared to the general population (Carroll *et al*, 1993; Levin *et al*, 1999). Mechanisms underlying the comorbidity of ADHD and cocaine abuse have not been elucidated. Methylphenidate (MPH) is the most widely prescribed pharmacotherapy for children and adolescents with ADHD (Chai *et al*, 2012; Goldman *et al*, 1998; Robison *et al*, 1999). Controversy exists regarding the effects of MPH treatment during childhood and adolescence on subsequent cocaine abuse liability (Kollins, 2008b), such that increases, decreases as well as no effect have been reported (Barkley *et al*, 2003; Biederman *et al*, 1999b; Fischer *et al*, 2003; Lambert *et al*, 1998; Molina *et al*, 2013; Wilens *et al*, 2003). These discrepancies may be due to inherent heterogeneity in the ADHD population, variations in age and duration of MPH treatment, and complexities associated with comorbid conditions such as conduct disorder (Barkley *et al*, 2003; Molina *et al*, 2013). A prospective longitudinal study in children with ADHD, but without conduct disorder,

reported that initiation of MPH treatment in early adolescence was associated with higher lifetime rates of cocaine abuse compared to initiation during childhood (Mannuzza *et al*, 2008). Thus, the age of initiation of MPH treatment may be a critical factor modulating cocaine abuse liability in individuals with ADHD.

Impulsivity, a hallmark of ADHD (American Psychiatric Association., 2000), is an established predictor for drug abuse liability, including for cocaine (Belin *et al*, 2008b; Everitt *et al*, 2008; Winstanley *et al*, 2010a). Impulsivity is a broad psychological construct that encompasses a variety of maladaptive behaviors including, but not limited to, actions that are poorly conceived, unnecessarily risky and prematurely executed without sufficient forethought (Evenden, 1999b). Experimental measures evaluating these facets of impulsivity do not correlate with each other, indicating that the construct of impulsivity consists of orthogonal factors (Block, 1995; Broos *et al*; Whiteside *et al*, 2001; Winstanley *et al*, 2006a).

One approach to simplify the construct of impulsivity in ADHD is via the identification of endophenotypes, which by definition are proximal markers of gene action (Almasy *et al*, 2001; Gottesman *et al*, 2003), such as a deficit in response inhibition capacity (Crosbie *et al*, 2008; Slaats-Willemse *et al*, 2003; Willner, 1986). Response inhibition capacity is an executive control mechanism to prevent premature execution of an intermittently reinforced action (Aron, 2007; Barkley, 1997). Response inhibition capacity was increased in children and adolescents with ADHD during chronic MPH treatment (Coghill *et al*, 2007; Rhodes *et al*, 2006). However, the long-term effects of discontinuation of MPH treatment on response inhibition capacity have not been reported (Chen *et al*, 2011; McCarthy *et al*, 2009). Cocaine abuse is associated with response inhibition deficits (Fillmore and Rush, 2002; Li *et al*, 2006). Individuals with comorbid ADHD and cocaine dependence exhibit greater deficits compared to those with ADHD but not cocaine dependence, and compared to demographically-matched healthy controls (Crunelle *et al*, 2013). Thus, response inhibition deficits may be the

facet of impulsivity that underlies the greater cocaine abuse liability in individuals with ADHD.

Preclinical studies support that reduced response inhibition is associated with increased drug-taking and drug-seeking behavior (Dalley *et al*, 2011; Diergaarde *et al*, 2008; Groman *et al*, 2009). In outbred animals, low therapeutic doses of MPH decreased impulsivity ((Eagle *et al*, 2007; Hill *et al*, 2012a; Kuczenski *et al*, 2002; van den Bergh *et al*, 2006), also see (Fernando *et al*, 2012; Navarra *et al*, 2008)). MPH treatment in adolescent outbred animals increased sensitivity to aversive effects of cocaine and decreased cocaine self-administration during adulthood (Andersen *et al*, 2002; Carlezon *et al*, 2003; Thanos *et al*, 2007). Latter results suggest that MPH treatment may be protective against cocaine abuse liability; however, outbred animals do not adequately represent the deficits associated with ADHD. Subjects with the ADHD-like phenotype are needed to evaluate the complex interaction between chronic MPH treatment and response inhibition deficits on cocaine abuse liability.

The Spontaneously Hypertensive Rat (SHR), the most widely accepted inbred rat model of ADHD, displays many of the phenotypic characteristics of ADHD, including impulsivity (Adriani *et al*, 2003; Kantak *et al*, 2008; Mill *et al*, 2005; Sagvolden *et al*, 2005b; Sanabria *et al*, 2008). The inbred progenitor rat strain, Wistar-Kyoto (WKY), also exhibits behavioral deficits such as hypoactivity, anxiety and depression (De La Garza *et al*, 2004; Langen and Dost, 2011; van den Bergh *et al*, 2006). Thus, outbred rat strains, such as Wistar (WIS) rats, are included to disambiguate differences between SHR and WKY (Alsop, 2007b; Langen *et al*, 2011; Somkuwar *et al*, 2013a). Chronic MPH treatment in adolescent SHRs improved behavioral deficits including working memory and behavioral flexibility relative to vehicle control as well as WKY and WIS (Harvey *et al*, 2013; Kantak *et al*, 2008).

Compared to WKY and WIS, adult SHR acquire cocaine self-administration faster and exhibit upward-shifted dose-response functions for cocaine (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). Chronic MPH treatment through adolescence followed by

treatment discontinuation in early adulthood resulted in a further increase in cocaine self-administration in adult SHR compared to SHR administered vehicle and compared to the control strains administered MPH (Harvey *et al*, 2011). Consistent with previous studies in outbred animals (Carlezon *et al*, 2003; Thanos *et al*, 2007), chronic MPH treatment in the WIS rats reduced acquisition of cocaine self-administration compared to its vehicle control. Chronic MPH treatment during adolescence in rats with an ADHD phenotype (SHR) also increased anxiety, decreased the rewarding effect of MPH, increased the cross-sensitization to cocaine, and increased dopamine transporter function in the medial prefrontal cortex (dela Pena *et al*, 2012a; Somkuwar *et al*, 2013a; Vendruscolo *et al*, 2008; Yetnikoff *et al*, 2013). The prefrontal cortex undergoes rapid development during adolescence, and thus, adolescents differ from adults in their response to rewarding as well as aversive stimuli (Casey *et al*, 2010; Somerville *et al*, 2010b). Also, adolescent rats are typically more impulsive compared to adult rats (Adriani *et al*, 2004; Burton and Fletcher, 2012; Proal *et al*, 2011). Chronic MPH treatment in adolescent SHR altered neuronal excitability in adolescent and adult prefrontal cortical neurons as well as an increased in cocaine self-administration during adulthood (Harvey *et al*, 2011; Somkuwar *et al*, 2013a; Urban *et al*, 2013; Urban *et al*, 2012). Thus, chronic MPH treatment during adolescence may have altered the developmental trajectory in the prefrontal cortex, ultimately resulting in increased cocaine abuse.

The current study used differential reinforcement of low rate (DRL) schedules to determine effects of treatment with a therapeutically relevant dose of MPH during adolescence on response inhibition capacity in adult SHR. MPH-induced changes in response inhibition capacity may mediate the increase in cocaine self-administration observed previously by altering prefrontal cortical function during development (Harvey *et al*, 2011; Somkuwar *et al*, 2013a; Urban *et al*, 2012). Under a DRL schedule, the subject must wait a defined time interval between consecutive responses to earn reinforcers, i.e., inter-response times (IRT) greater than an experimenter-defined minimum time are reinforced (Monterosso and Ainslie, 1999). Under DRL schedules,

response inhibition deficit (or premature responding) is inferred from reduced efficiency of earning reinforcers (Stein *et al*, 1975). Compared to WKY and several outbred strains, adult SHR showed reduced efficiency under several DRL schedules (Bull *et al*, 2000; Ferguson *et al*, 2007; Orduna *et al*, 2009; Sanabria *et al*, 2008; van den Bergh *et al*, 2006). Surprisingly, therapeutically relevant MPH doses administered acutely did not increase efficiency in adult SHR (Ferguson *et al*, 2007; Orduna *et al*, 2009; van den Bergh *et al*, 2006), which may be attributed to acute, rather than chronic administration. Effects of chronic MPH in either adolescent or adult rats responding on DRL schedules have not been evaluated. Additionally, efficiency may not be sensitive to the effects of MPH on deficits in response inhibition capacity. Quantitative modeling of IRT-distribution patterns under DRL schedules provide theoretically based measures of response inhibition capacity and may be more sensitive than efficiency to the effects of MPH (Hill *et al*, 2012a; Orduna *et al*, 2009; Sanabria *et al*, 2008).

7.2 Methods

7.2.1 Subjects/Animals

Three separate cohorts of male SHR and WKY rats (Charles River Laboratories, Kingston, NY) and male Wistar rats (WIS; Raleigh, NC) were employed. WKY and WIS were used as inbred and outbred comparator strains, respectively. Rats were maintained on a 12-h light:dark cycle with lights on 07:00 h, individually housed with free access to food and water in a colony room (Division of Laboratory Animal Resources, University of Kentucky, Lexington, KY, USA). The experiments were conducted during the light-cycle of the rats. Adult rats obtained at postnatal day 70 (P70), were employed for the pilot experiments (n=12/strain) and for experiment II (n=6/group). For experiment I, rats arrived at P25 (n=6/group). After 3 days of habituation, rats were food restricted to 90-95% of their expected free-feeding body weight. All experimental protocols were conducted according to the 1996 *National*

Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

7.2.2 Drugs and treatments

(±)-Methylphenidate hydrochloride (Sigma-Aldrich, St Louis, MO) was dissolved in water at concentrations of 0.3, 0.75, 1.5, 3.0 and 5.0 mg/ml and injected into oyster crackers (Kantak *et al*, 2008) providing oral doses of 0.3 - 5.0 mg/kg. Oyster crackers injected with water (1 ml/kg) were used as the vehicle control. Solutions were prepared fresh on each experimental day. The MPH dose range results in clinically relevant plasma concentrations (Kuczenski *et al*, 2002; Wargin *et al*, 1983). With the exception of the highest MPH dose, doses administered were below the threshold for producing locomotor activation (Kuczenski *et al*, 2002). For the pilot experiments, the cracker was provided in the home-cage 45 min prior to the operant session to attain peak plasma levels of MPH immediately before behavioral sessions (Kuczenski *et al*, 2002). Rats consumed the oyster cracker within 5 min of its presentation.

Previous studies have reported that MPH at 1.5 mg/kg, p.o. reduce working memory and learning deficits in the SHR, but not in WKY (Harvey *et al*, 2011; Kantak *et al*, 2008). Based on the latter reports as well as results from the pilot study (detailed in the results section), the 1.5 mg/kg, p.o., dose of MPH was used for experiments I and II. For experiment I, rats were treated with MPH or vehicle between P28 to P55, which includes early to late adolescence (Doremus-Fitzwater *et al*, 2010; Spear, 2000a). Rats were dosed Monday to Friday to model the weekend “medication holiday” often recommended for individuals with ADHD (1996), and evaluated on DRL schedules from P77. In experiment II, starting from P77, daily dosing and behavior testing regimen was employed to obtain stable operant behavior. Based on preliminary results, MPH was administered 30 min before the session.

7.2.3 Differential reinforcement of low rate schedules

Using a previously reported procedure, adult SHR, WKY and WIS were trained on daily 55-min sessions of a differential reinforcement of low-rate 5 sec with limited hold (DRL5LH) schedule (Sanabria *et al*, 2008) starting at P77. Experiments were conducted in operant conditioning chambers (detailed in Supplementary Materials). For DRL5LH schedule, consecutive responses on the active lever greater than 5 sec apart were reinforced by delivery of a food pellet. Responses on the inactive lever were recorded, but had no programmed consequence. An adjusting limited hold (LH) condition was included in the DRL5LH schedule to reduce the probability of reinforcing nonscheduled-directed alternate behaviors, such as grooming, which result in long time lapses between consecutive responses (McClure *et al*, 1997). LH was initially set at 10 sec. Responding within the LH resulted in a decrease in LH duration by 0.01 sec. Lack of responding within the LH resulted in an increase in LH duration by 0.03 sec. Stability under the DRL5LH schedule was defined as less than a 10% change in IRT at 5 sec across 5 consecutive sessions, and typically required 12-16 sessions.

For the rats in the pilot experiments, after reaching stability, MPH (0.3-5.0 mg/kg, p.o.) or vehicle (1 ml/kg water in oyster crackers, p.o.) was administered 45 min before the session using a randomized Latin-square design with three drug-free sessions between each MPH test session. Dose-response function for MPH was not evaluated in adolescent SHR because the short four-week window for adolescence (Doremus-Fitzwater *et al*, 2012; Spear, 2000b) is inadequate for training and testing rats on the DRL schedule using the above experimental design. During these three days of 'wash-out' period between each MPH dose, rats were tested on the DRL5LH schedule without any drug treatment to reestablish baseline behavior. Number of active and inactive lever responses, number of reinforcers earned and IRTs were recorded. Efficiency was defined as pellets earned, calculated as a percentage of the total number of responses on the active lever. Following oral delivery, MPH achieves peak plasma concentration

within 35-45 min (Gerasimov *et al*, 2000). MPH is metabolized rapidly in rats, having a reported brain and serum half-life of 20 and 50 min, respectively (Patrick *et al*, 1984). Since the purpose of the pilot experiment was to identify an efficacious dose of MPH, efficiency during the first 15 min of the DRL5LH session (i.e., during peak plasma levels of MPH) was compared between doses for SHR.

Previous studies suggest that, similar to ADHD individuals, deficits in SHR are normalized by higher rates of reinforcement (Aase *et al*, 2006; Wultz *et al*, 1992). Thus, for experiments I and II, two DRL schedules were used for evaluating response inhibition capacity. In the DRL5LH schedule, IRTs > 5 sec were reinforced by a food pellet, resulting in relatively high reinforcement rates (Sanabria *et al*, 2008). The training protocol was that described above. The timer recording IRT reset to zero following the delivery of a pellet. The time between reinforcer delivery and the subsequent lever-response was defined as 'response latency', which is an indicator of motivation for obtaining the reinforcer (Mattila *et al*, 2011; Meyer *et al*, 2012). The number of active and inactive lever responses, number of pellets earned, response latencies and IRTs were recorded over 5 sessions of stable performance.

For the second schedule, the DRL interval was increased to 30 sec (DRL30), to make "waiting" more challenging. A within-session incremental training protocol was used in which rats progressed to the 30 sec waiting time at their own pace (Sanabria *et al*, 2008). The timer recording IRT restarted following each response on the active lever, and response latency measure and limited hold were not incorporated. The number of active and inactive lever responses, number of pellets earned and IRTs were recorded over 5 sessions of stable performance. Efficiency was calculated as the number of pellets earned expressed as a percent of active lever responses.

7.2.4 Open-field locomotor activity

The purpose of this experiment was to evaluate the effects of chronic MPH during adolescence or adulthood on activity in adult rats. Three-five days after the completion of DRL studies, locomotor activity was evaluated. Rats were habituated for 3 days, for 1-hr each day, in one of 12 open-field chambers (detailed in Supplementary Materials). Total number of horizontal beam breaks during the third day was recorded and compared between groups.

7.2.5 Data analysis

For pilot experiments

Analyses were conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). Data are reported as the mean \pm S.E.M. and n represents the number of rats per group. A repeated-measure two-way ANOVA was conducted to evaluate the interaction of rat strain and MPH dose on percentage of responses reinforced (efficiency). Test of sphericity was conducted to validate the repeated measures ANOVA (Mauchly, 1940). When the assumption of sphericity was violated, Huyhn-Feldt correction was applied to adjust the F-statistic (Huynh *et al*, 1980). Since the objective of the pilot study was to identify a dose of MPH for the subsequent impulsivity studies, effects of acute MPH treatment were determined using repeated-measure one-way ANOVAs for each rat strain and Dunnett's post hoc analysis. Furthermore, the initial 15 min of the behavioral session was evaluated for efficiency (i.e., 45 min after administration), when MPH was at peak plasma levels.

For experiments I and II:

Modified temporal regulation (TR) model: Non-linear mathematical models in Equation 1 and 2 (detailed in Supplementary Materials) were fit to the cumulative-frequency distribution of IRTs for each rat under DRL5LH and DRL30, respectively (Hill *et al*, 2012a; Mika *et al*, 2012; Sanabria *et al*, 2008). Both of the models segregate the IRT distribution between timed-IRTs and non-timed IRTs. The timed-IRTs congregate near the target time (for example, at 5 sec for DRL5LH) and the non-timed IRTs are exponentially distributed.

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + q(1 - p)Le^{-L(t-\delta)} + (1 - q)(1 - p)L'e^{-L'(t-\delta)}$$

(EQ 1; DRL5LH)

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + (1 - p)\lambda e^{-\lambda(t-\delta)} \quad 0 < \delta < t. \quad (\text{EQ 2;}$$

DRL30)

The free parameters obtained from the mathematical models served as dependent variables, and are described below:

1. Response threshold θ , $\theta = (Nxc)/5$ sec and $(Nxc)/30$ sec for DRL5LH and DRL30, respectively, where (Nxc) is the mean of timed-IRTs. Smaller value of θ compared to 1, indicates reduced accuracy of timed-IRTs.
2. Proportion of timed-IRTs (p), expressed as a fraction of all IRTs emitted by individual rats. Greater value of p indicates greater likelihood of producing timed-responses.
3. Proportion of burst-IRTs ($q^*(1-p)$) and $(1-p)$ for DRL5LH and DRL30, respectively, expressed as a fraction of all IRTs emitted by individual rats. Burst IRTs are a component of the non-timed IRTs with a relatively short average duration and occur under both the DRL schedules. A greater proportion of burst IRTs indicates reduced response inhibition capacity. For DRL30, burst responses are complementary to timed IRTs; thus, only bursts IRTs have been reported to avoid redundancy.

4. In DRL5LH and DRL30, the rate-of-decay of burst-IRTs (L and λ , respectively) is expressed as sec^{-1} ; the inverse of the rate-of-decay ($1/L$ and $1/\lambda$) is the mean burst IRT in sec.
5. The model also provides the Weber-fraction (ω) which is an index of the precision of timing. Other parameters include the fraction $((1-p)*(1-q))$ and rate-of-decay (L') of the long-IRTs under DRL5LH that characterize task-delinquent responses (McClure *et al*, 1997). Since, task-delinquent responses are not as prevalent under the DRL30 schedule, the simpler previously published mathematical model was used for quantifying IRT distribution for this schedule (Hill *et al*, 2012a).

Data are reported as the mean \pm S.E.M. and n represents the number of rats per group. The data were log-transformed to maintain homogeneity of variance when necessary. For evaluating effects of MPH treatment, dependent variables were compared using two-factor ANOVA (strain X treatment), followed by Bonferroni's post-hoc comparisons. Main effects of strain or treatment were evaluated using Tukey's post-hoc comparison. One WKY in experiment I died due to factors not related to treatment prior to obtaining any data. In experiment I, the number of burst IRTs under the DRL30 schedule emitted by one WKY administered vehicle was identified as an outlier using the Grubbs test (GraphPad; <http://www.graphpad.com/quickcalcs/Grubbs1.cfm>).

7.3 Results

7.3.1 Pilot experiments

To determine the appropriate dose and time of administration of MPH, percent of responses reinforced (or efficiency) was evaluated over the 55-min DRL5LH session. No strain X dose interaction was obtained ($F[10,165]=1.65$, $p>0.05$); however, the main effect of MPH tended to be significant ($F[5,165]=2.24$, $p=0.053$). One-way ANOVAs revealed that MPH modestly increased the efficiency for SHR and WKY, but not for WIS (Table 1). Analysis of the data during the first 15-min of the DRL5LH session revealed that MPH (1.5 mg/kg, p.o.) increased efficiency in SHR compared to vehicle ($F[5,55]=2.70$, $p<0.05$; Fig 1). Therefore, the dose MPH chosen was 1.5 mg/kg for Experiments I and II, and the pretreatment time chosen was 30 min for Experiment II.

7.3.2 Methylphenidate treatment during adolescence on behavior during adulthood

The mean IRT distribution patterns for adult rats under DRL5LH were not different between groups administered MPH or vehicle during adolescence (Fig 2A). Efficiency was lower in SHR compared to WIS and WKY; however MPH treatment did not alter efficiency (Table 3). The proportion of timed-IRTs was increased by MPH treatment during adolescence only in WKY (p ; $F_{\text{interaction}[2, 29]}=5.19$, $p<0.05$; Fig 2B). Furthermore, WKY emitted a lesser proportion of timed-IRTs compared to WIS and SHR ($F_{\text{strain}[2, 29]}=5.11$, $p<0.05$). No strain X treatment interactions or main effects of treatment were obtained for the proportion of burst IRTs ($q^*(1-p)$; Fig 2C), as well as for mean timed-IRTs and mean burst-IRTs (Table 4). Also, parameters from the DRL5LH experiment that were not related to impulsivity showed strain differences, but not treatment differences (Supplementary Table T1). Specifically, response latency, proportion and mean of task delinquent IRTs, precision of timed-IRTs, and number of sessions to train from DRL5LH to DRL30 were not different between treatment groups. However, strain differences were found for proportion and mean task delinquent IRTs, which were greater for SHR and WKY compared to WIS.

Under DRL30, differences in the mean IRT distribution pattern were found only in adult SHR between groups administered MPH and vehicle during adolescence. Visual inspection of the IRT distribution patterns (Fig 3A) reveals that MPH-treated SHR emitted a greater proportion of burst responses and fewer timed-IRTs compared to vehicle control. Only a main effect of strain was found for efficiency, with lower efficiency for SHR than for WKY and WIS (Table 3). With respect to modeling parameters, no interactions or main effects were obtained for mean burst-IRTs (Table 4) or any other dependent variable under DRL30 (Supplementary Table T1). However, main effects of strain were found, such that accuracy of timed IRTs for SHR was lower than for WKY and WIS, and accuracy for WKY was greater than for WIS (θ ; $F_{\text{strain}[2, 29]}=32.9$, $p<0.0001$; Fig 3B). However, there was no strain X treatment interaction or

main effect of treatment. In contrast, for proportion of burst IRTs, a strain X treatment interaction was found ($(1-p)$; $F_{\text{interaction}}[2, 28]=3.58, p<0.05$). Importantly, the vehicle-administered SHR showed burst IRTs greater than vehicle administered WKY and WIS (Fig 3C). Moreover, MPH during adolescence increased burst IRTs only in adult SHR significantly above that for the vehicle-administered SHR group (Fig 3C).

For locomotor activity, main effects of treatment and strain were obtained but no interaction was found ($F_{\text{treatment}}[1, 29]=12.3, p<0.01$; $F_{\text{strain}}[2, 29]=40.4, p<0.0001$). Locomotor activity was decreased by MPH treatment during adolescence in all three strains (Fig 4), but the decrease was greater in MPH-treated SHR (75% of vehicle control; $t_{\text{SHR}}[10] = 3.00, p<0.05$) than WKY and WIS (82% and 84%, respectively). Also, SHR was hyperactive compared to both WKY and WIS, and WKY were hypoactive compared to WIS.

7.3.3 Methylphenidate treatment in adult rats on behavior during adulthood

For experiment II, mean IRT distribution patterns for adult SHR, WKY and WIS under DRL5LH and DRL30 schedules were not different between MPH and vehicle administered groups (Figures S2). For DRL5LH, evaluation of efficiency (Table 3), proportion of timed-IRTs (p ; Fig 5A), proportion of burst-IRTs ($q^*(1-p)$; Fig 5B), mean timed-IRTs and mean burst IRTs (Table 4) revealed no interactions or main effects. Similarly, no effects were found for other modeling parameters and for the number of sessions to train from DRL5LH to DRL30 (Supplementary Table T2). Under DRL30 schedule, efficiency was lower in SHR compared to WKY and WIS, and greater in WKY compared to WIS; however, no interaction or main effect of treatment was revealed (Table 3). With respect to modeling parameters, a main effect of strain was revealed for accuracy of timed-IRTs, such that SHR exhibited reduced accuracy compared to WKY and WIS, and WKY exhibited greater accuracy compared to WIS ($F_{\text{strain}}[2, 30] = 80.3, p<0.0001$; Fig 5C). For proportion of burst-IRTs ($1-p$; Fig 5D) and mean burst-IRTs (Table

3) as well as for other dependent variable unrelated to impulsivity, no interactions or main effects were found (Supplementary Table T2).

Locomotor activity was greater in SHR compared to WKY and WIS ($F_{\text{strain}[2, 30]} = 49.7, p < 0.0001$; Fig 6); also activity in WKY was lower than WIS. However, no strain x treatment interaction or main effect of treatment was found.

7.4 Discussion

The current results reveal that in SHR, chronic MPH treatment during adolescence followed by discontinuation in early adulthood increased response inhibition deficits in a DRL schedule, modeling a facet of impulsivity. Although, these effects of MPH were not evident as changes in efficiency, mathematical modeling of the entire responding pattern under DRL revealed an increase in proportion of burst IRTs and a corresponding decrease in proportion of timed IRTs, and thus, these parameters were more sensitive to the effects of prior MPH treatment. In contrast, chronic MPH during adulthood did not alter these parameters of impulsivity in SHR suggesting an age-dependent effect of MPH. Furthermore, the increased burst responding in MPH-treated SHR was not due to a generalized increase in activity. Thus, evidence is provided that chronic MPH treatment during adolescence increases impulsivity that persists into adulthood. Furthermore, ongoing chronic MPH treatment during adulthood did not alter impulsivity in adult rats.

Clinical evidence indicates that with increasing age and MPH treatment during childhood and adolescence, ADHD symptoms including hyperactivity and impulsivity decrease in severity leading to discontinuation of treatment (Faraone *et al*, 2006; Mannuzza *et al*, 1998; McCarthy *et al*, 2009). In animal models, treatment with acute or several injections of a therapeutically-relevant dose of MPH during adolescence reduced hyperactivity in both adolescent outbred Sprague-Dawley and SHR (Kuczenski *et al*,

2002; Umehara *et al*, 2013a; Umehara *et al*, 2013b). Consistent with clinical observations, the current study shows that chronic oral MPH treatment during adolescence (P28-55) resulted in a decreased locomotor activity in adult SHR, providing further support for the SHR model of ADHD.

MPH is an efficacious therapeutic for reducing both hyperactivity and impulsivity exhibited in adults with ADHD (Boonstra *et al*, 2005; Castells *et al*, 2011). In SHR treated during adulthood, neither acute nor chronic MPH reduced hyperactivity in an open field (van den Bergh *et al*, 2006; Yang *et al*, 2011), suggesting that the adult SHR may not adequately model this symptom of ADHD. Consistent with these previous findings, the current results show no change in hyperactivity in adult SHR administered chronic oral MPH. With respect to impulsivity, both acute and chronic MPH administration to adult SHR have been reported to increase choice for a larger delayed reinforcer, and reduce choice for a smaller immediate reinforcer in a delay-discounting task, suggesting that MPH reduced impulsive choice in adult SHR (Slezak and Anderson, 2011). In the current study, acute but not chronic MPH produced a modest, but significant, increase in efficiency in the DRL5LH schedule in the adult SHR. However, the modest effect of MPH on efficiency, taken together with the absence of effect following chronic MPH, is interpreted as insufficient evidence to suggest a reliable reduction in impulsivity using the DRL5LH schedule. Moreover, acute and chronic MPH treatment in adult SHR did not reduce response inhibition deficits, indicating a lack of effect in this model of impulsivity (Ferguson *et al*, 2007; Orduna *et al*, 2009; van den Bergh *et al*, 2006; Yang *et al*, 2011). In the current study, chronic MPH treatment in adult SHR similarly did not alter either efficiency or the mathematically-modeled parameters of impulsivity using the DRL5LH and DRL30 schedules. Thus, adult SHR appear to have limited utility for translational studies evaluating effects of ADHD pharmacotherapeutics for behavior such as hyperactivity and response inhibition deficits.

Impulsivity, including that measured as response inhibition deficits under DRL schedules, has been associated with decreased dopaminergic function in prefrontal

cortex of adult outbred rats (Antonelli *et al*, 2013; Pardey *et al*, 2013; Simon *et al*, 2013; Sokolowski *et al*, 1994). Age-dependent decreases in impulsivity have been associated with developmental maturation of prefrontal cortex, particularly with respect to the dopaminergic system (Brenhouse *et al*, 2008; Burton *et al*, 2012; Doremus-Fitzwater *et al*, 2012; Rothmond *et al*, 2012; Somerville *et al*, 2011). Repeated peripheral administration of low doses of MPH from the juvenile period through adolescence resulted in long-lasting changes in firing patterns of prefrontal cortical pyramidal cells that persisted into adulthood in outbreds (Fumagalli *et al*, 2010; Urban *et al*, 2012). The neurochemical mechanism by which chronic MPH treatment during adolescence alters the developmental trajectory of the prefrontal cortex in SHR has not been elucidated in detail. However, some evidence suggests that chronic MPH treatment during adolescence (P28-55) increased dopamine transporter function in medial prefrontal cortex (mPFC) of adult SHR relative to vehicle control (Somkuwar *et al*, 2013a). Importantly, chronic MPH did not alter transporter function in mPFC of WKY and WIS controls (Somkuwar *et al*, 2013a). Taken together with the current findings, the mPFC dopaminergic system likely contributes to the underlying mechanism responsible for the increased response inhibition deficits observed herein in the MPH-treated SHR. Furthermore, the increased response inhibition deficit may be the behavioral mechanism underlying the enhanced cocaine self-administration following chronic MPH treatment during adolescence (Harvey *et al*, 2011). Thus, the current study advances the understanding of the impact of MPH treatment during adolescence on outcomes during adulthood in ADHD.

7.5 Tables

Table 7.1 Experimental design and methods summary

Two separate cohorts (n=6/group/experiment) of male Spontaneously Hypertensive Rats, Wistar-Kyoto and Wistar rats were used for the current study. For experiment I, rats were administered methylphenidate (1.5mg/kg/day, p.o.) or vehicle (water, 1 ml/kg/day, p.o. in oyster-crackers) Monday to Friday between P28 to P55. After P55, methylphenidate treatment ended. From P77, the rats were evaluated on the behavioral assays. Rats were trained to stability on differential reinforcement of low-rates (DRL) of responding with 5-sec with limited-hold (DRL5LH), followed by DRL with 30-sec (DRL30) and then evaluated on open-field locomotor activity for 3 days. In experiment II, rats were administered methylphenidate or vehicle from P77 onwards and thirty minutes later evaluated in the behavioral assays, i.e. DRL5LH, DRL30 and locomotor activity, in that order.

	P28	P55	P77	P130
	Adolescence			Adulthood
Exp I	Methylphenidate or vehicle			DRL5LH → DRL30 → Locomotor activity
Exp II				Methylphenidate or vehicle DRL5LH → DRL30 → Locomotor activity

Table 7.2 Therapeutically relevant low doses of MPH (0.3 - 5.0 mg/kg, p.o.) increased efficiency (% responses reinforced) in SHR and WKY, but not WIS under DRL5LH schedule of reinforcements.

	Vehicle	0.3	0.75	1.5	3.0	5.0	F-statistics ^b
SHR	22.5 ± 1.48 ^a	25.1 ± 1.28	25.1 ± 1.06	24.8 ± 1.21	22.4 ± 1.54	23.6 ± 1.38	2.50 *
WKY	25.6 ± 1.72	27.4 ± 1.40	28.9 ± 1.10	27.7 ± 1.17	28.1 ± 1.19	24.1 ± 1.25	2.40 *
WIS	25.5 ± 1.34	24.5 ± 1.71	25.5 ± 1.88	25.6 ± 1.99	23.9 ± 1.28	25.8 ± 0.97	0.704

^a All values are mean ± S.E.M.;

^b For all the statistics, the within-subject degrees of freedom = 5, and residual degrees of freedom = 55.

*Significant main effect of MPH, $p < 0.05$

Table 7.3 SHR exhibited reduced efficiency (% responses reinforced) compared to controls, but chronic methylphenidate (MPH) treatment during adolescence or adulthood did not alter efficiency in adult SHR, WKY and WIS rats under DRL5LH and DRL30 schedules.

		Efficiency under DRL5LH		Efficiency under DRL30	
		VEH	MPH	VEH	MPH
Experiment I	SHR	23.2 ± 1.00 ^a	22.6 ± 0.92	2.52 ± 0.83	2.81 ± 0.92
	WKY	25.7 ± 1.56	29.7 ± 1.59	33.8 ± 6.86	41.6 ± 7.60
	WIS	27.1 ± 1.06	28.0 ± 0.96	10.8 ± 2.86	12.4 ± 2.43
	F_{strain}	F[2,29] = 10.5 s*; SHR < WIS = WKY		F[2,29] = 32.9 s*; SHR < WIS < WKY	
Experiment II	SHR	27.1 ± 2.18	24.7 ± 0.37	5.35 ± 0.41	4.65 ± 0.84
	WKY	26.8 ± 1.76	26.2 ± 1.35	45.9 ± 2.47	50.3 ± 3.29
	WIS	25.3 ± 0.55	26.0 ± 1.09	17.9 ± 5.73	15.9 ± 4.84
	F_{strain}	F[2,30] = 0.208		F[2,30] = 80.3 s*; SHR < WIS < WKY	

^a All values are mean ± S.E.M.;

s* Significant main effect of strain, p < 0.05

Table 7.4 Chronic MPH treatment during adolescence or adulthood did not alter accuracy of timed-IRTs and mean burst IRT under DRL5LH schedule for adult SHR, WKY and WIS rats.

Under DRL5LH, methylphenidate (MPH) treatment and rat strain did not alter accuracy of timed-IRTs as well as mean duration of burst IRTs in adult SHR, WKY and WIS. Chronic MPH treatment during adolescence or adulthood did not alter precision of timed-IRTs as well as mean duration and proportion of task-delinquent responses under DRL5LH in adult SHR, WKY and WIS; strain differences have been tabulated (Supplementary Table S1 and S2).

		Accuracy of timed-IRTs under DRL5LH		Mean burst IRTs under DRL5LH (sec)	
		VEH	MPH	VEH	MPH
Experiment I	SHR	0.94 ± 0.04 ^a	0.97 ± 0.03	2.3 ± 1.1	2.1 ± 0.8
	WKY	0.97 ± 0.09	1.05 ± 0.07	5.8 ± 1.1	8.7 ± 2.9
	WIS	1.03 ± 0.05	1.01 ± 0.02	3.1 ± 1.2	4.8 ± 1.4
	F_{strain}	F[2,29] = 0.866		F[2,29] = 0.879	
Experiment II	SHR	1.01 ± 0.06	0.96 ± 0.03	2.5 ± 1.2	3.2 ± 1.3
	WKY	0.96 ± 0.06	0.91 ± 0.05	3.7 ± 2.1	3.4 ± 1.7
	WIS	0.97 ± 0.01	0.97 ± 0.01	1.8 ± 0.8	1.7 ± 1.0
	F_{strain}	F[2,30] = 0.805		F[2,30] = 0.825	

^a All values are mean ± S.E.M.

Table 7.5 Chronic MPH treatment during adolescence or adulthood did not alter accuracy of timed-IRTs or mean burst IRT under DRL30 schedule for adult SHR, WKY and WIS rats.

Under DRL30, methylphenidate (MPH) treatment did not alter accuracy of timed-IRTs as well as mean duration of burst IRTs in adult SHR, WKY and WIS; main effects of strain are tabulated below. Chronic methylphenidate treatment did not alter precision of timed-IRTs under DRL30; strain differences have been tabulated (Supplementary Table S1 and S2).

		Mean burst IRTs under DRL30 (sec)	
		VEH	MPH
Experiment I	SHR	2.4 ± 1.7 ^a	1.9 ± 1.0
	WKY	1.7 ± 0.34	1.6 ± 0.50
	WIS	0.81 ± 0.30	1.5 ± 0.64
	F_{strain}	F[2,29] = 0.662	
Experiment II	SHR	4.7 ± 1.9	2.7 ± 1.5
	WKY	11.3 ± 6.1	2.2 ± 1.8
	WIS	15.4 ± 7.0	10.1 ± 8.2
	F_{strain}	F[2,30] = 0.288	

^a All values are mean ± S.E.M.

7.6 Figures

Figure 7.1

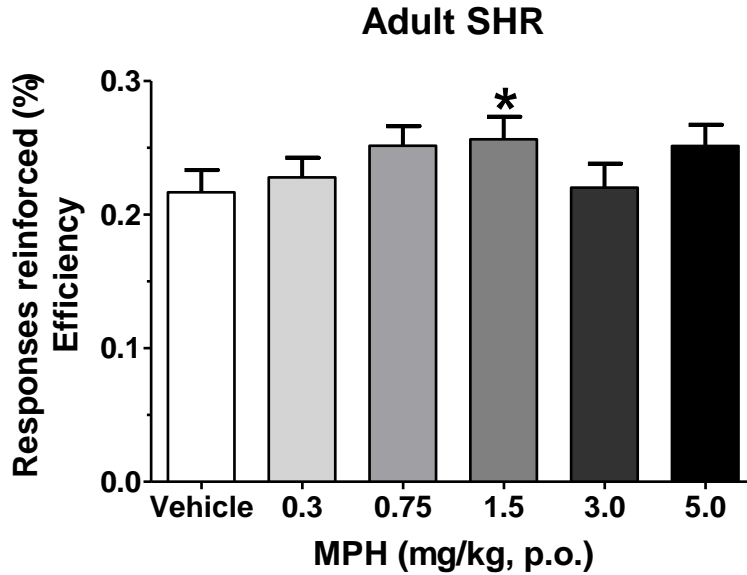


Fig 7.1: At a therapeutically relevant dose, methylphenidate (MPH) increased efficiency (% responses reinforced) in adult Spontaneously Hypertensive Rat (SHR) under a DRL5LH schedule. Values are number of pellets earned (mean \pm S.E.M.), expressed as a % of responses on the active lever by adult SHR under DRL5LH schedule, evaluated between 45-60 min after oral administration of one of the six doses of MPH (0-5.0 mg/kg, p.o.) using a randomized Latin-square design. n= 12; *p < 0.05 compared to vehicle (0 mg/kg).

Figure 7.2

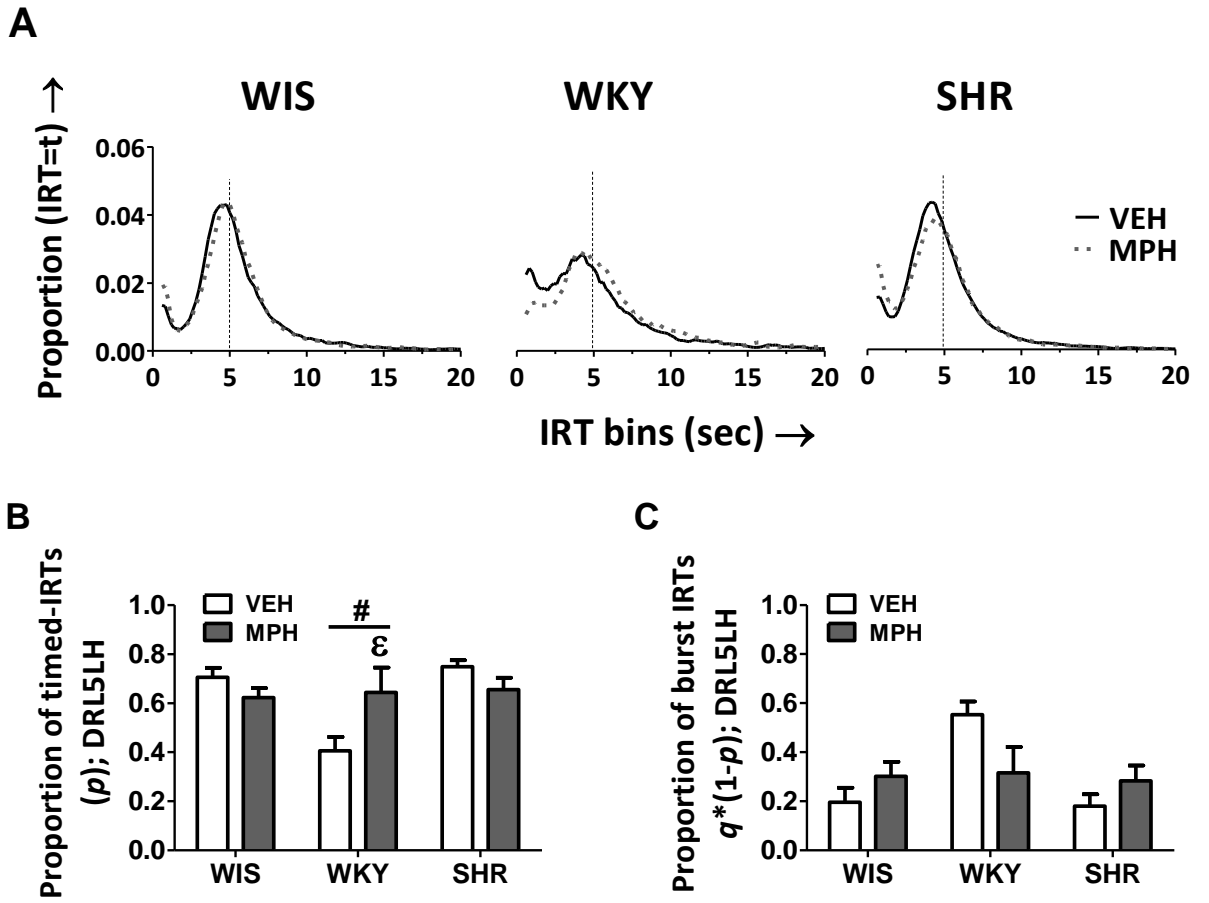


Fig 7.2: Under DRL5LH, discontinuation of chronic methylphenidate (MPH) treatment during adolescence did not alter response inhibition capacity in adult Spontaneously Hypertensive Rat (SHR). (A) Mean inter-response time (IRT) distributions were not different between MPH (dotted curves) and vehicle (VEH, solid curves) groups, except for Wistar-Kyoto (WKY; middle panel). (B) Proportion of timed IRTs (mean \pm S.E.M.) was increased in adult WKY following discontinuation of adolescent treatment with MPH (grey bars) compared to its VEH control (open bars). (C) Proportion of burst IRTs (mean \pm S.E.M.) was not different between adult SHR, WKY and Wistar (WIS) under DRL5LH schedule following discontinuation of adolescent treatment with MPH and VEH. Mean IRT distributions are presented as 1-sec moving averages of IRTs for SHR, WKY and WIS under DRL5LH. n = 5-6/ group; ^ε p \leq 0.05 compared to the respective VEH control. [#] p \leq 0.05 main effect of strain, different from WIS.

Figure 7.3

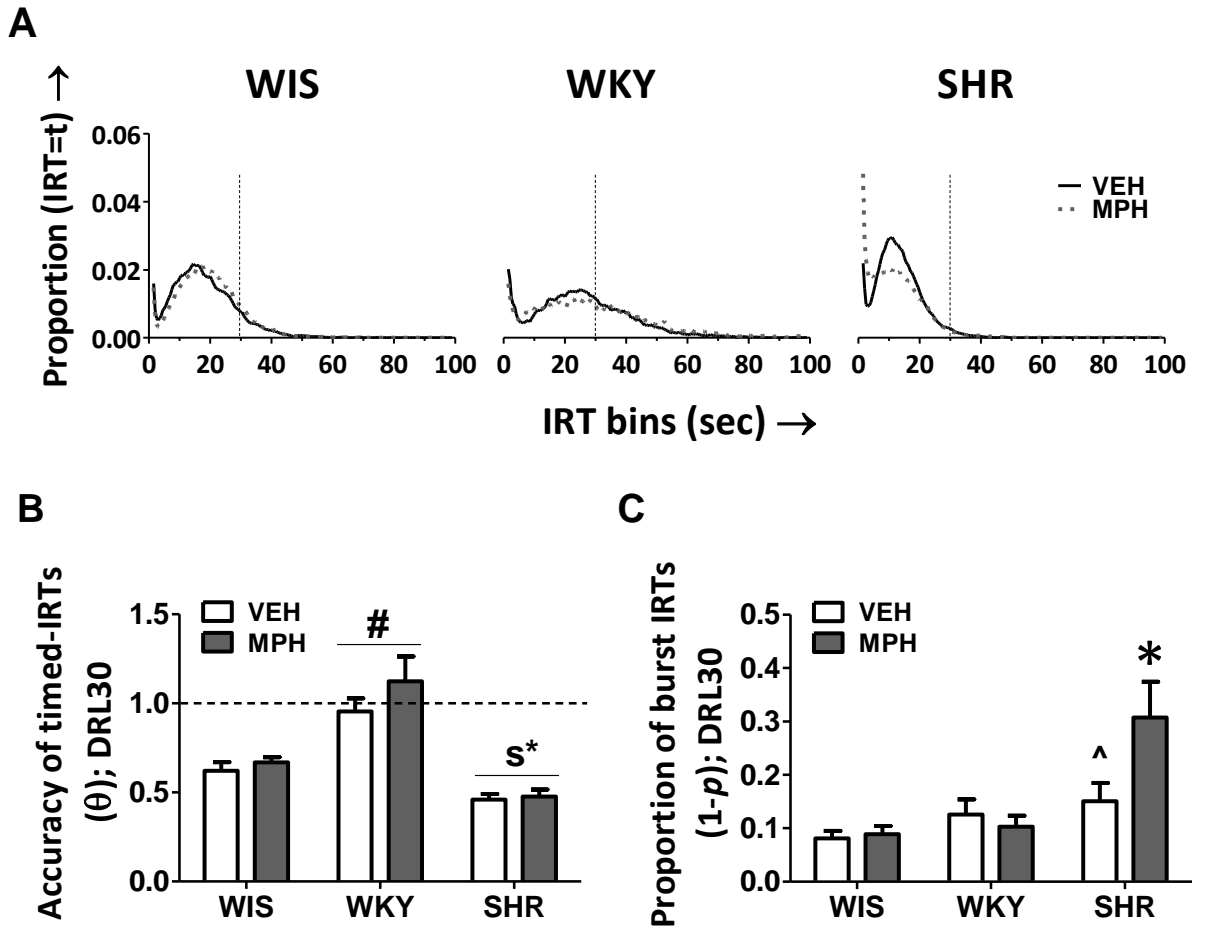


Fig 7.3: Under DRL30, discontinuation of chronic methylphenidate (MPH) treatment during adolescence increased response inhibition deficit in adult Spontaneously Hypertensive Rat (SHR). (A) In adult SHR (right panel), but not in control strains, discontinuation of adolescent treatment with MPH (dotted curves) altered inter-response time (IRT) distribution by increasing burst IRTs and proportionately decreasing timed IRTs. (B) Compared to Wistar-Kyoto (WKY) and Wistar (WIS) rats, adult SHR under the DRL30 schedule exhibited reduced accuracy of timed IRTs (mean \pm S.E.M.) irrespective of adolescent treatment with MPH (grey bars) or vehicle (VEH, open bars). Also, compared to WIS, accuracy of timed IRTs was greater for WKY. (C) Compared to WKY and WIS, adult SHR administered VEH during adolescence emitted greater proportion of burst IRTs (mean \pm S.E.M.), and MPH treatment during adolescence further increased burst IRTs during adulthood. Mean IRT distributions are presented as 5-sec moving averages of IRTs for SHR, WKY and WIS under DRL30. n = 5-6/ group; * p \leq 0.05 compared to the respective VEH control as well as MPH-treated WKY and WIS; ^ p \leq 0.05 compared to VEH-treated WKY and WIS; ^{s*} p \leq 0.05 main effect of strain, different from WKY and WIS; # p \leq 0.05 main effect of strain, different from WIS.

Figure 7.4

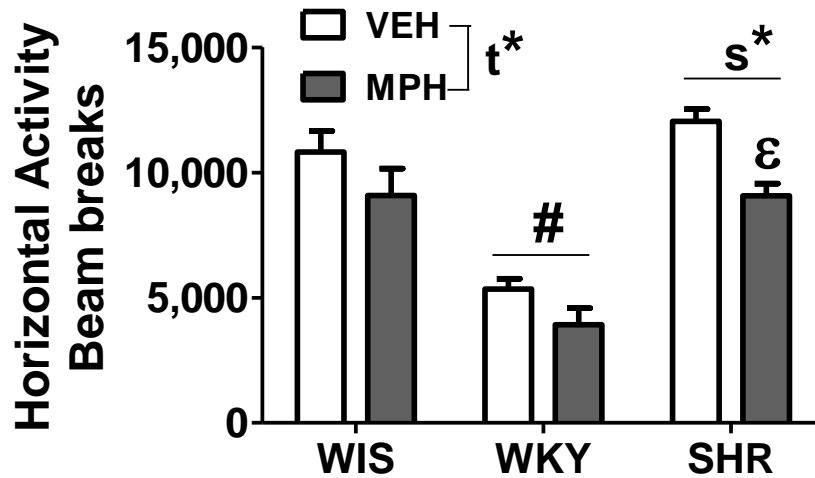


Fig 7.4: Discontinuation of chronic methylphenidate (MPH) treatment during adolescence decreased locomotor activity in adult Spontaneously Hypertensive Rat (SHR). SHR exhibited greater horizontal activity (mean \pm S.E.M.) in open-field chambers compared to Wistar-Kyoto (WKY) and Wistar (WIS) rats. Further, horizontal activity in adult WKY was reduced compared to WIS. Discontinuation of adolescent treatment with MPH (grey bars) decreased horizontal activity in adult rats compared to vehicle control (VEH, open bars). $n = 5-6/$ group; $^{\epsilon} p \leq 0.05$ compared to the respective VEH control; $^{t*} p \leq 0.05$ main effect of treatment; $^{s*} p \leq 0.05$ main effect of strain, different from WKY and WIS; $^{\#} p \leq 0.05$ main effect of strain, different from WIS.

Figure 7.5

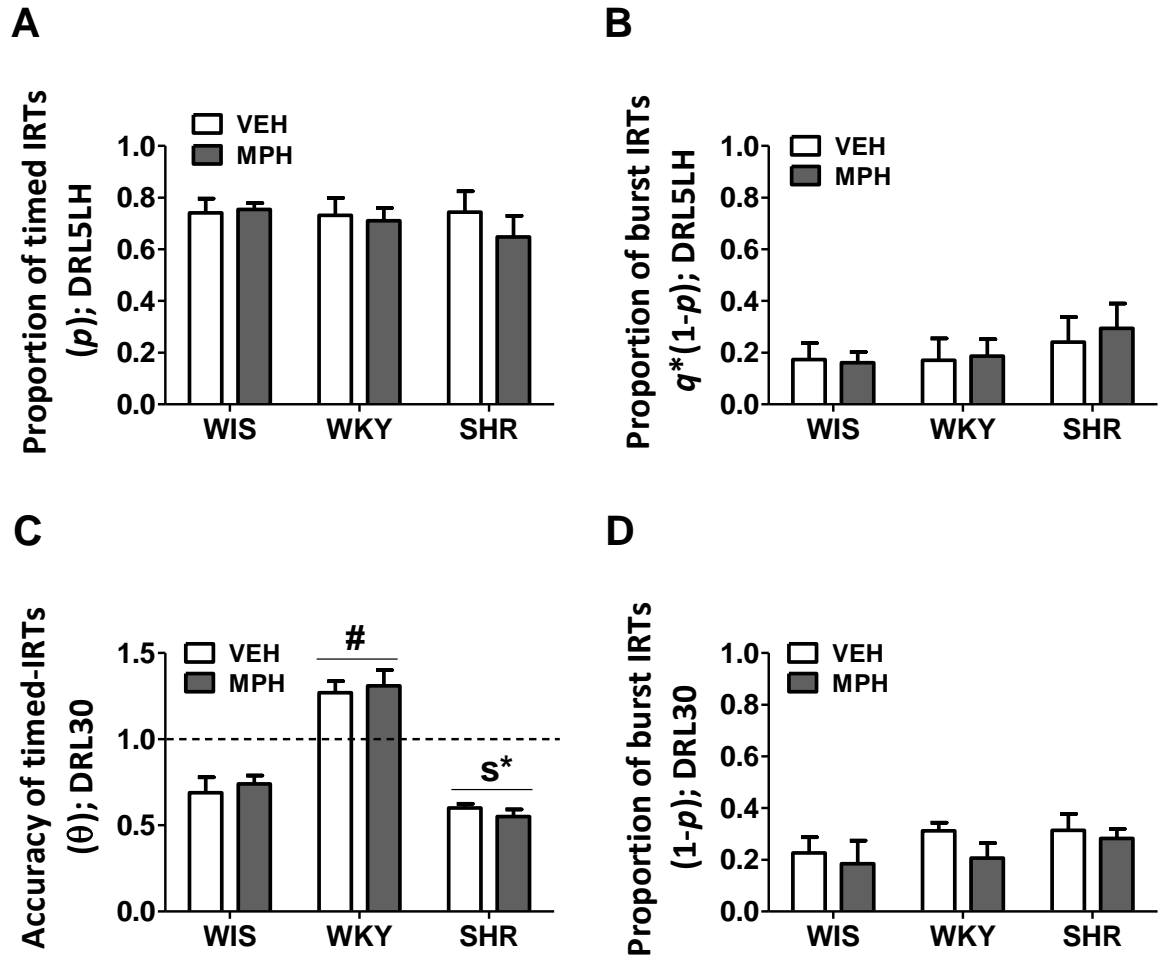


Fig 7.5. Chronic methylphenidate (MPH) treatment during adulthood did not alter response inhibition capacity in adult Wistar (WIS), Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs). Mean inter-response time (IRT) distributions were not different between MPH and vehicle (VEH) groups (Supplementary Materials Fig 2). For SHR, WKY and WIS rats under the DRL5LH schedule, (A) proportion of timed IRTs (mean \pm S.E.M.) and (B) proportion of burst IRTs did not differ between MPH- and VEH-treated groups. (C) Under the DRL30 schedule, adult SHR exhibited reduced accuracy of timed IRTs (mean \pm S.E.M.) compared to WKY and WIS, irrespective of adolescent treatment with MPH (grey bars) or vehicle (VEH, open bars). Also, compared to WIS, accuracy of timed IRTs was greater for WKY. (D) For SHR, WKY and WIS rats under the DRL30 schedule, proportion of burst IRTs (mean \pm S.E.M.) did not differ between MPH- and VEH-treated groups. $n = 6/$ group; ^{s*} $p \leq 0.05$ main effect of strain, different from WKY and WIS; # $p \leq 0.05$ main effect of strain, different from WIS.

Figure 7.6

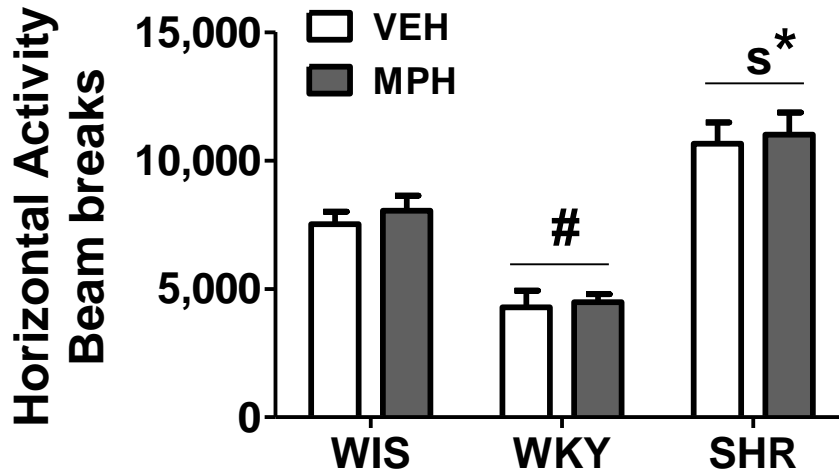


Fig 7.6: Chronic methylphenidate (MPH) treatment during adulthood did not alter locomotor activity in adult Wistar (WIS), Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHRs). SHR exhibited greater horizontal activity (mean \pm S.E.M.) in open-field chambers compared to WKY and WIS rats. Further, horizontal activity in adult WKY was reduced compared to WIS. $n = 6/\text{group}$; $^{\#} p \leq 0.05$ main effect of strain, different from WKY and WIS; $^{\#} p \leq 0.05$ main effect of strain, different from WIS.

7.7 Supplementary Materials

Preliminary studies for optimizing the dose of methylphenidate (MPH)

For animal models of ADHD, a face-validity criterion posits that low doses of MPH should reduce impulsivity (Willner, 1986). In previous work, therapeutically relevant doses of MPH (0.5-2 mg/kg, i.p.) increased reinforcers/hr in WIS rats in a response-withholding task, employing a fixed minimum interval schedule (Hill *et al*, 2012a). In another response withholding task, the 5-choice serial reaction time task, MPH (0.1 mg/kg, i.p.) reduced premature responses in WIS. In contrast, higher doses of MPH in WIS reduced efficiency, peak time and response threshold (θ) (Emmett-Oglesby *et al*, 1980; Orduna *et al*, 2009; van den Bergh *et al*, 2006). Thus, MPH appears to produce a U-shaped dose-response curve for reducing impulsivity in outbred rats. The current pilot study was aimed at identifying the dose of MPH that reduced impulsivity in the SHR rat model of ADHD.

Supplementary Methods

Apparatus

Differential reinforcement of low rate (DRL) experiments were conducted in operant conditioning chambers (ENV-001; MED Associates, St. Albans, VT, USA) housed in sound attenuating compartments (ENV-018 M, MED Associates). Operant chambers were connected to a PC interface (SG-6080D, MED Associates) and operated using MED-PC™ software. Chambers were equipped with a 5 cm × 4.2 cm recessed food receptacle, two retractable metal levers located on either side of the food tray and 7.3 cm above a metal grid floor, and a house light mounted on the wall opposite the food receptacle. Food pellet reinforcers (45-mg Noyes Precision Pellets; Research Diets, Inc., New Brunswick, NJ, USA) were delivered via a dispenser (ENV-203, MED Associates) mounted outside of the operant chamber.

Locomotor activity was assessed in acrylic open-field locomotor chambers (42 × 42 × 30 cm), each with a 16 × 16 grid of photobeam sensors with monitoring system (AccuScan Instruments Inc., Columbus, OH). Locomotor activity was recorded and was expressed as total number of horizontal beam breaks recorded over each 1-hour session.

Differential reinforcement of low rate schedules details

Starting from P77, rats from experiment I and experiment II were trained on daily 55-min sessions of DRL5LH, followed by DRL30 schedule as published previously (Sanabria *et al*, 2008).

Protocol for training from DRL5LH to DRL30: Following stable performance on DRL5LH, the rats from experiment I and II were trained to DRL30 by gradually increasing the schedule requirement by 0.75% within-session following each reinforced response. The adjusted DRL interval was carried over from session to session until DRL30 was reached.

Stability criteria the DRL schedules: Stability under both DRL5LH and DRL30 schedules was defined as less than a 20% change in IRT at 5 and 30 sec, respectively, across 5 consecutive sessions.

Modified temporal regulation (TR) model

Two non-linear mathematical models were utilized to evaluate impulsivity in SHR (Hill *et al*, 2012a; Mika *et al*, 2012; Sanabria *et al*, 2008). Both models were fit to the IRT distribution data across 5 sessions of stable responding. The first model posits that the distribution of IRTs is a mixture of two independent probability distributions (see equation 1). One distribution is sensitive to the DRL contingency (*timed-IRTs*) and predicted by a gamma function theoretically centered near the DRL target time (Supplemental Fig. S1). The other distribution is not sensitive to the DRL contingency (*non-timed-IRTs*), and is predicted by an exponential function. The second model (see

equation 2) posits that the distribution of non-timed IRTs is a composite of two processes. One process results in burst responses characterized by an exponential function with a high rate-of-decay (Supplemental Fig. S1). The second process results in task delinquent behavior characterized by an exponential function with a low rate-of-decay (Supplemental Fig. S2).

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + (1 - p)\lambda e^{-\lambda(t-\delta)}; \quad 0 < \delta < t.$$

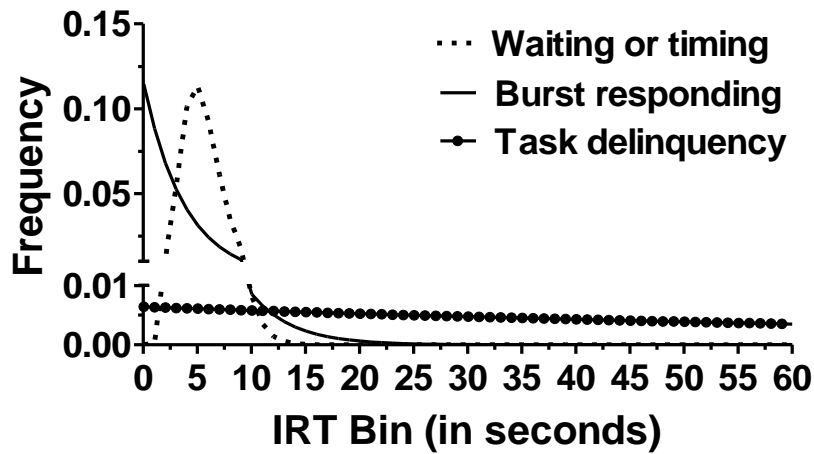
(1)

$$P(IRT = t) = p\Gamma(t - \delta; N, c) + q(1 - p)L e^{-L(t-\delta)} + (1 - q)(1 - p)L' e^{-L'(t-\delta)};$$

$0 < \delta <$

$t.$

(2)



Supplementary Fig. S1 Theoretical data representing the individual components of equation 2; a timing-IRT gamma-function (dashed line) that is typically centered on the DRL wait time, a fast-decaying exponential function (solid line) describing burst responding and a slow-decaying exponential function (beaded line) describing iterative

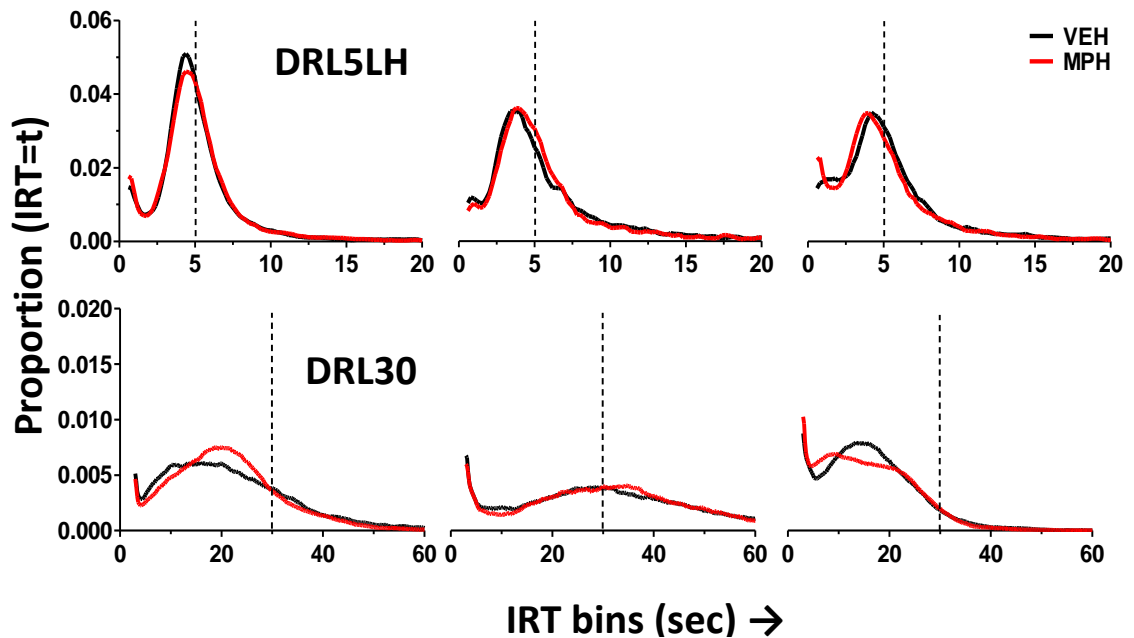
behavior with long pauses between subsequent lever responses. For equation 1, a single exponential function describes both burst and task-delinquent responding (representation not shown).

Statistical analysis: Analysis was conducted using SPSS Statistics Version 19 (SPSS Inc., IBM Company, Armonk, NY). The IRT frequency distribution for each session was determined as a fraction of total number of active lever responses using the equation below.

$$\text{Frequency of IRTs} = \frac{\text{IRTs of a given interval}}{\text{Total number of IRTs}}$$

The frequency distribution was then smoothed out (reduce noise due to the session-to-session variation) using moving averages for 0.5 sec intervals and then averaged over 5 sessions of stable performance for each individual rat. The IRT frequency distributions are reported as mean of the moving averages of IRT distributions response for rats in each group; n represents the number of rats per group.

Supplementary Results



Supplementary Figure S2: IRT distributions for adult WIS, WKY and SHR treated with chronic methylphenidate (MPH, 1.5 mg/kg/day, p.o.) or vehicle (VEH, water 1 ml/kg/day in oyster crackers, p.o.) administered 30 minutes before testing under DRL5LH (top) and DRL30 (bottom) schedules. The values are mean moving averages for each group, $n=6$ /group. The dotted line represents the DRL interval; all responses to the left of the line lead to loss of reinforcement due to some form of impulsivity. Under DRL30, area under the curve for responses right of the dotted line represent “efficiency” of earning reinforcers.

Table 1: Chronic MPH treatment during adolescence or adulthood did not alter latency to respond under DRL5LH and number of sessions to train to DRL30 for adult SHR, WKY and WIS rats. MPH treatment did not alter latency of responding following reinforcer delivery as well as number of sessions to train from DRL5LH to DRL30 in either of the two cohorts of rats.

		Latency to respond in DRL5LH (sec)		Session to train to DRL30	
		VEH	MPH	VEH	MPH
Experiment I	SHR	16 ± 1.1 ^a	14 ± 1.8	12 ± 1.8	8.7 ± 1.1
	WKY	13 ± 1.3	12 ± 0.69	6.0 ± 0.3	6.2 ± 0.3
	WIS	10 ± 0.65	9.7 ± 0.70	6.0 ± 0.9	5.3 ± 0.4
	F_{strain}	F[2,29] = 11.1 s*; SHR > WIS		F[2,29] = 12.3 s*; SHR > WIS = WKY	
Experiment II	SHR	14 ± 1.0	14 ± 0.32	6.2 ± 0.3	5.8 ± 0.4
	WKY	9.8 ± 1.3	12 ± 0.58	6.5 ± 0.5	6.3 ± 0.6
	WIS	10 ± 0.24	10 ± 0.52	5.2 ± 0.3	5.3 ± 0.3
	F_{strain}	F[2,30] = 15.8 s*; SHR > WIS = WKY		F[2,30] = 3.85 s*; WKY > WIS	

^a All values are mean ± S.E.M.;

s* Significant main effect of strain, p < 0.05

Supplementary Table 2: Other dependent variables for adult SHR, WKY and WIS rats under DRL5LH and DRL30 schedules in experiment I. Chronic MPH treatment did not alter efficiency of earning reinforcers, accuracy and precision of timing and mean duration of burst responses under either DRL5LH or DRL30. MPH treatment during adolescence did not alter latency of responding following reinforcer delivery as well as mean duration and proportion of task-delinquent responses under DRL5LH in adult SHR, WKY and WIS. Strain differences in latency of responding, precision of timing and mean duration of delinquency under DRL5LH schedule as well as in sessions to reach DRL30, efficiency of earning reinforcers and accuracy of estimating 30 sec under DRL30 schedule have been described.

			WIS	WKY	SHR	F _{interaction}	F _{strain}
DRL5LH	Latency (in s)	VEH	10 ± 0.24 ^a	9.8 ± 1.3	14 ± 1.0	F[2,30] = 0.907	F[2,30] = 15.8 *
		MPH	10 ± 0.52	12 ± 0.58	14 ± 0.32		SHR >WIS, WKY
	Precision (ω) (in sec)	VEH	0.30 0.022	0.52 0.035	0.47 ± 0.077	F[2,30] = 0.270	F[2,30] = 9.32*
		MPH	0.31 0.014	0.47 0.031	0.44 ± 0.065		SHR, WKY>WIS
	Delinquency (1-p)*(1-q)	VEH	0.084 ± 0.012	0.098 ± 0.032	0.055 ± 0.021	F[2,30] = 0.005	F[2,30] = 1.79
		MPH	0.085 ± 0.019	0.103 ± 0.030	0.059 ± 0.020		
1/L' (in sec)	VEH	20.8 ± 1.4	268 ± 125	85.5 ± 18.5	F[2,26] = 1.32	F[2,26] = 9.86*	
	MPH	30.1 ± 8.1	87.2 ± 37.8	64.4 ± 22.8		SHR, WKY>WIS	
DRL30	Session to reach DRL30	VEH	5.2 ± 0.3	6.5 ± 0.5	6.2 ± 0.3	F[2,30] = 0.179	F[2,30] = 3.85*
		MPH	5.3 ± 0.3	6.3 ± 0.6	5.8 ± 0.4		WKY > WIS
	Precision (ω) (in sec)	VEH	0.50 ± 0.029	0.46 ± 0.048	0.44 ± 0.018	F[2,30] = 1.81	F[2,30] = 0.146
		MPH	0.44 ± 0.038	0.53 ± 0.055	0.51 ± 0.051		

^a Values are mean ± S.E.M.

* $p < 0.05$

Supplementary Table 3: Other dependent variables for adult WIS, WKY and SHR rats in experiment II. Chronic MPH treatment did not alter efficiency of earning reinforcers, accuracy and precision of timing and mean duration of burst responses under either DRL5LH or DRL30 in WIS, WKY or SHR. Methylphenidate treatment during adulthood did not alter latency of responding following reinforcer delivery as well as mean duration and proportion of task-delinquent responses under DRL5LH in adult, WIS, WKY or SHR. Strain differences in latency of responding, efficiency of earning reinforcers and mean duration of task-delinquency under DRL5LH schedule as well as in sessions to reach DRL30, efficiency of earning reinforcers under DRL30 schedule and accuracy of estimating 30 sec have been described.

			WIS	WKY	SHR	F _{interaction}	F _{strain}
DRL5LH	Latency (in s)	VEH	10 ± 0.65 ^a	13 ± 1.3	16 ± 1.1	F[2,29] = 0.471	F[2,29] = 11.1 *
		MPH	9.7 ± 0.70	12 ± 0.69	14 ± 1.8		
	Precision (ω) (in s)	VEH	0.32 ± 0.04	0.47 ± 0.12	0.37 ± 0.02	F[2,29] = 0.843	F[2,29] = 6.89*
		MPH	0.27 ± 0.01	0.48 ± 0.08	0.34 ± 0.01		
	Delinquency (1-p)*(1-q)	VEH	0.098 ± 0.033	0.041 ± 0.007	0.071 ± 0.024	F[2,29] = 0.081	F[2,29] = 2.24
		MPH	0.075 ± 0.022	0.034 ± 0.006	0.062 ± 0.023		
	1/L' (in s)	VEH	36.4 ± 7.2	200 ± 27.9	30.6 ± 5.9	F[2,29] = 0.159	F[2,29] = 8.15 *
		MPH	43.5 ± 15.4	222 ± 39.9	27.9 ± 6.2		
DRL30	Session to reach DRL30	VEH	6.0 ± 0.9	6.0 ± 0.3	12 ± 1.8	F[2,29] = 1.31	F[2,29] = 12.3*
		MPH	5.3 ± 0.4	6.2 ± 0.3	8.7 ± 1.1		
	Precision (ω) (in s)	VEH	0.51 ± 0.03	0.47 ± 0.06	0.47 ± 0.02	F[2,29] = 2.60	F[2,29] = 0.615
		MPH	0.46 ± 0.01	0.59 ± 0.03	0.55 ± 0.07		

^a Values are mean ± S.E.M.

* $p < 0.05$

8 CHAPTER EIGHT: OVERALL DISCUSSION

8.1 Review

Although several advances have been made in ADHD research, the etiology underlying the disorder is not clearly understood (Tripp *et al*, 2009). The most widely accepted theory suggests that ADHD is a consequence of catecholaminergic dysregulation particularly in the prefrontal cortex (PFC; Arnsten, 2009). ADHD is typically treated with stimulant medications such as methylphenidate (MPH), or with non-stimulant medications like ATO. These drugs inhibit dopamine transporters (DAT) and/or norepinephrine transporter (NET), and thus enhance dopaminergic and noradrenergic neurotransmission in the PFC (Arnsten, 2009; Levy, 2008). However, controversy exists as to whether chronic treatments with modulators of catecholamine neurotransmitter systems are completely safe, especially in children and adolescents (Kollins, 2008b).

A major concern in evaluating therapeutic strategies for ADHD is the high substance use liability in ADHD. ADHD children are twice as likely as their non-ADHD counterparts to develop substance use disorders, including cocaine abuse, later in life (Lee *et al*, 2011). The prevalence of cocaine abuse is 35% greater in ADHD individuals compared to non-ADHD individuals (Carroll *et al*, 1993). However, the factors mediating the high comorbidity of cocaine abuse and ADHD are unclear. Concerns have been raised regarding MPH because the pharmacological profile and mechanism of action of the therapeutic is comparable to cocaine (Kollins *et al*, 2001; Volkow *et al*, 1995). Further, MPH treatment may potentiate susceptibility for cocaine abuse, because repeated administration of MPH produces the similar neuroadaptations as does cocaine (Brandon and Steiner, 2003; Zahniser *et al*, 2009). For example, cocaine and MPH, both increase neuronal activation in the dorsal striatum (Brandon *et al*, 2003), a component of the frontostriatal attention circuitry affected in ADHD as well as in drug abuse (Robbins *et al*, 1998). MPH is efficacious by inhibiting DAT and NET function (Berridge *et*

al, 2006; Oades *et al*, 2005). Cocaine produces its addictive properties by enhancing dopaminergic neurotransmission also via inhibition of DAT. Further, cocaine enhances noradrenergic neurotransmission via inhibition of NET function, which is thought to play a modulatory role in addiction (Koob *et al*, 2010; Steiner and Van Waes, 2013; Wee *et al*, 2008). However, clinical studies evaluating the effects of MPH treatment on future cocaine abuse outcomes have been inconclusive and controversial. While some studies suggest that MPH treatment is protective against future cocaine abuse liability (Wilens *et al*, 2003), others indicate that the treatment does not exacerbate the comorbidity of ADHD and cocaine abuse (Molina *et al*, 2013). Another study reported a positive correlation between age of initiation of MPH treatment and future cocaine abuse liability (Mannuzza *et al*, 2008). Specifically, when treatment was initiated late, i.e. during early adolescence instead of childhood, cocaine abuse liability was elevated in adulthood.

Adolescence appears to be a developmentally critical period during, which MPH treatment produces lasting alterations that increase the reinforcing properties of cocaine in adulthood in Sprague-Dawley rats (Brandon *et al*, 2001). In contrast, MPH treatment during peri-adolescence reduces the sensitivity to cocaine as well as natural rewards (Andersen *et al*, 2002; Bolanos *et al*, 2003; Carlezon *et al*, 2003). However, these studies are limited since the experimental models employed do not account for the preexisting deficits associated with ADHD.

Neurobehavioral deficits in ADHD further enhance the susceptibility of ADHD individuals to drug abuse. Altered processing of rewards and mechanisms of reinforcement in ADHD have been suggested to confer the drug abuse vulnerability in ADHD individuals (Sagvolden *et al*, 1998a). Enhanced impulsivity, a hallmark of ADHD, is also a well-established predictor of cocaine abuse liability. Individuals with comorbid ADHD and cocaine dependence are more impulsive than ADHD individuals without this comorbidity (Crunelle *et al*, 2013; Groman *et al*, 2009). Further, ADHD etiology and

cocaine abuse impinge on overlapping neurological substrates. PFC subregions including medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) regulate cognitive and executive function and working memory, which when impaired, increase cocaine seeking and cocaine taking behavior (Di Pietro *et al*, 2004; Di Pietro *et al*, 2006; Grakalic *et al*, 2010). Taken together, these results emphasize the importance of the use of an animal model of ADHD to evaluate the impact of ADHD therapeutics on future cocaine abuse liability.

Our previous studies using the Spontaneously Hypertensive Rats (SHR) model of ADHD demonstrate greater cocaine self-administration in SHR compared to control strains Wistar-Kyoto and Wistar (Harvey *et al*, 2011). Treatment during adolescence (postnatal day 28-55) with a therapeutically relevant dose of MPH (1.5 mg/kg/day, p.o.; Gerasimov *et al*, 2000; Kuczenski *et al*, 2002), followed by treatment discontinuation in early adulthood led to an enhanced cocaine self-administration during adulthood (>77 days old) in the MPH-treated SHR compared to control (Harvey *et al*, 2011). Importantly, cocaine self-administration was not altered in MPH-treated Wistar-Kyoto (WKY; inbred control) and Wistar (WIS; outbred control) rats compared to the respective vehicle controls (Harvey *et al*, 2011). Taken together, these results established SHRs as a model for the clinically observed comorbidity of ADHD and cocaine abuse and for the enhanced cocaine abuse liability observed in ADHD individuals who initiated MPH treatment during adolescence (Klein *et al*, 2012; Mannuzza *et al*, 2008). The purpose of the current study was to delineate the neurochemical and behavioral mechanisms potentially underlying with the increased cocaine self-administration in MPH-treated SHR.

The first and second aims of this dissertation (Chapters 2 and 3, respectively) determined the effects of treatment during adolescence with MPH and ATO on DAT function and cellular distribution in mPFC, OFC and striatum during adulthood. The maximal velocity of [³H]dopamine uptake (V_{max}) and cellular distribution of DAT did not differ between vehicle administered SHR, WKY and WIS (Chapters 2 and 3). Importantly,

V_{max} was increased in MPH-treated SHR compared to vehicle control and MPH-treated Wistar-Kyoto and Wistar rats (Chapter 2). Further the increased DAT function was not associated with a corresponding increase in surface DAT, suggesting that the effects of MPH were trafficking independent (Fig 8.1). On the other hand, treatment with the non-stimulant ATO did not increase either cocaine-self administration in SHR (Somkuwar *et al*, 2013b) or DAT function in mPFC, OFC and striatum (Chapter 3). In contrast, DAT function in the OFC and striatum obtained from ATO-treated SHRs was decreased compared to controls. Furthermore, the decreased DAT function in OFC was associated with a corresponding decrease in cell surface expression of DAT (Fig 8.2), indicating that the effects of ATO were trafficking independent in the OFC. Also, the decreased DAT function in the striatum was associated with no difference in cell surface expression DAT; however total DAT expression in the striatum was increased (Fig 8.3) which is also suggestive of trafficking mediated mechanisms. Taken together, MPH treatment during adolescence increased DAT function in mPFC which may contribute to the enhanced cocaine self-administration in SHR. Also, ATO's protection from enhanced cocaine self-administration (compared to MPH) may be due to the reduced DAT function in the OFC and striatum.

The third aim determined the effects of MPH during adolescence on NET function in mPFC and OFC during adulthood (Chapter 4). In the PFC, NET is involved in clearance of both norepinephrine and dopamine (Moron *et al*, 2002). Thus, changes in NET function will have implications on extracellular norepinephrine as well as dopamine in PFC. NET function was assessed using *in vivo* voltammetry in anesthetized rats to quantify the clearance kinetics of exogenously applied norepinephrine. NET function was measured using two parameters: 1) Uptake rate, defined as the peak amplitude of norepinephrine (or A_{max}) X first-order removal kinetics of norepinephrine (k_{-1}), and 2) Clearance, defined as the amount of norepinephrine applied/area under curve for the norepinephrine signal. In SHR, NET function in the mPFC in adults was not different from WKY and WIS and was not altered by MPH treatment during adolescence. These results

suggest that the increased DAT function in mPFC of MPH-treated SHR (Chapter 2) was neither accentuated nor compensated by a change in NET function. In contrast to mPFC, in the OFC, specifically the lateral OFC (LO), NET function was greater in SHR compared to WKY and WIS. The elevated NET function in lateral OFC may contribute to the behavioral deficits in SHR such as impulsivity, inattention, impaired behavioral flexibility and working memory (Harvey *et al*, 2013; Kantak *et al*, 2008; Sagvolden, 2006; Winstanley *et al*, 2006b; Zeeb *et al*, 2010). MPH treatment during adolescence followed by treatment discontinuation normalized NET function in SHR lateral OFC (Chapter 4; Fig 8.4), potentially normalizing the disrupted noradrenergic as well dopaminergic function in lateral OFC. Thus, some of the beneficial effects of MPH treatment during adolescence persist well into adulthood, even after treatment is discontinued. One example of a lasting beneficial behavioral effect of MPH treatment during adolescence identified in the current study was a reduction of the hyperactivity in SHR (Chapter 7). However, empirical evidence is not available currently to ascribe this decreased hyperactivity with the decreased NET function in the lateral OFC of the MPH-treated SHR.

The fourth aim was to determine the effects of MPH treatment during adolescence on sensitivity to cocaine-induced psychomotor response. The results indicate that while the sensitivity to acute cocaine-induced locomotor activation was least in SHR, the magnitude of locomotor sensitization to repeated cocaine administration was greatest in SHR (Chapter 5). The association between strain-dependent differences in cocaine self-administration and psychomotor sensitization to cocaine suggests that subjects that readily develop sensitization to cocaine are more likely to demonstrate greater motivation for cocaine (Mandt *et al*, 2008). However, contrary to the hypothesis, MPH treatment during adolescence, followed by treatment discontinuation, increased motivation for cocaine (Harvey *et al*, 2011), but did not increase sensitivity to the psychomotor effects of cocaine in adult SHR.

The fifth aim was to determine the effects of MPH during adolescence on impulsivity during adulthood evaluated using differential reinforcement of low-rate (DRL) schedules. The DRL schedule investigates the response inhibition deficit, a facet of impulsivity that is thought to mediate the comorbidity of substance abuse and ADHD (Groman *et al*, 2009; Orduna *et al*, 2009). The fifth specific aim was executed through two experiments; the first experiment was aimed at optimizing a behavioral preparation and analytical technique to identify effects of strain as well as MPH treatment on response inhibition capacity (Chapter 6). The second aim applied the optimized procedure to determine whether MPH treatment during adolescence produced persistent alterations in response inhibition capacity in SHR (Chapter 7).

The first set of experiments revealed strain differences between adult SHR, Wistar-Kyoto and Wistar under DRL 5 sec with limited hold (DRL5LH) and DRL 30 sec (DRL30) schedules (Chapter 6). Compared to Wistar-Kyoto and Wistar rats, adult SHR exhibited a greater frequency of burst responding and fewer timed responses under both schedules, indicating that SHRs have reduced response inhibition capacity compared to controls. Further, adult SHRs exhibited reduced accuracy of timing (reduced response threshold) under the DRL30 schedule, suggesting that response inhibition deficits in SHR become more evident under schedules where waiting is more challenging.

Adolescent rats are known to be more impulsive than adult rats (Burton *et al*, 2012; Sagvolden, 2011). Previous studies revealed that chronic MPH treatment in adolescent SHR improved behavioral flexibility and working memory compared to control (Harvey *et al*, 2013; Kantak *et al*, 2008). In the current study, the effects of chronic MPH treatment on responding under DRL schedules were evaluated in a separate cohort of adolescent rats (Chapter 6). Under the DRL5LH schedule, chronic MPH treatment increased burst responding and decreased proportion of timed responding in all three strains of adolescent rats. Under the DRL30 schedule, burst

responding was increased and accuracy of timing was decreased in all three strains, but the effects were most pronounced in the SHR. In contrast, response latency was decreased in MPH treated SHR, suggesting an increased motivation for earning the reinforcer (Mattila *et al*, 2011; Meyer *et al*, 2012). These results suggest that MPH produces mixed effects on SHR behavior. That is, MPH treatment decreased response inhibition capacity while simultaneously improving motivation to work for reinforcement (Chapter 6).

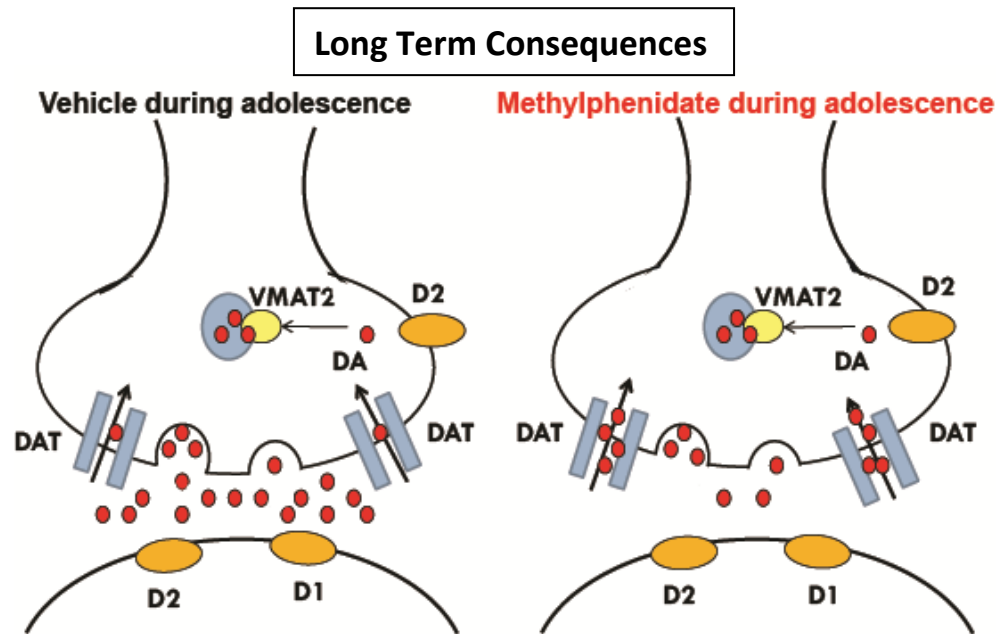
The second set of experiments evaluated whether MPH treatment during adolescence altered response inhibition capacity in adult rats. The results revealed that only in SHR, MPH treatment during adolescence, followed by treatment discontinuation, further increase in burst responding and decrease timed responding under the DRL30 schedule (Chapter 7). These results suggest that, in accordance with the hypothesis of the current experiment, chronic MPH treatment during adolescence increases response inhibition deficits in adult SHR. Furthermore, chronic MPH treatment during adulthood did not alter response inhibition capacity in adult SHRs. Taken together, MPH when administered during the developmentally vulnerable period (adolescence) is associated with this deleterious sequel. Finally, the enhanced response inhibition deficit may underlie the previously observed enhanced cocaine self-administration in MPH-treated SHR.

Table 8.1 Summary of results of methylphenidate (MPH) or atomoxetine (ATO) treatment during adolescence, followed by treatment discontinuation, on neurobehavioral outcomes during adulthood

		Cocaine's effects				Impulsivity		Hyperactivity	Neurotransmitter transporter function					
		Self-administration	Locomotor activation	Sensitization		Response inhibition deficit	Deficit response threshold		DAT			NET		
				To drug	To context				Str	OFC	mPFC	OFC	mPFC	
WIS	VEH													
	MPH	↓↓ ^A	↔	↔	↔	↔	↔	↔	↔	↔*	↔	↔	↔	↔
WKY	VEH													
	MPH	↔ ^{↑↑}	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
SHR	VEH	↑	↓	↑	↑	↑	↑	↑	↔	↔	↔	↑	↔	
	MPH	↑↑ ^A				↑↑		↓↓	*	*	↑↑ ^A	↓↓		

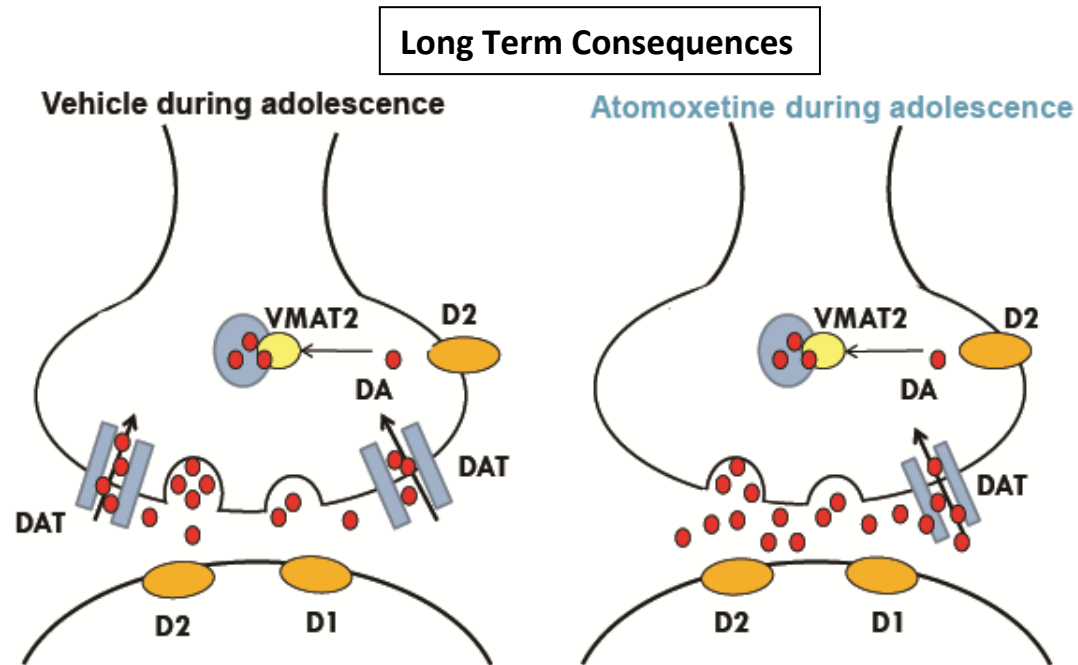
↔ not different between strains or treatment groups; ↑ higher than control WKY and WIS rats; ↑↑ increased with MPH treatment; ↓↓ decreased with MPH treatment; ^A not changed with non-stimulant ATO treatment; * decreased with ATO treatment, ↑↑ increased with ATO treatment

Figure 8.1 Dopaminergic terminals in the medial prefrontal cortex of adult SHR



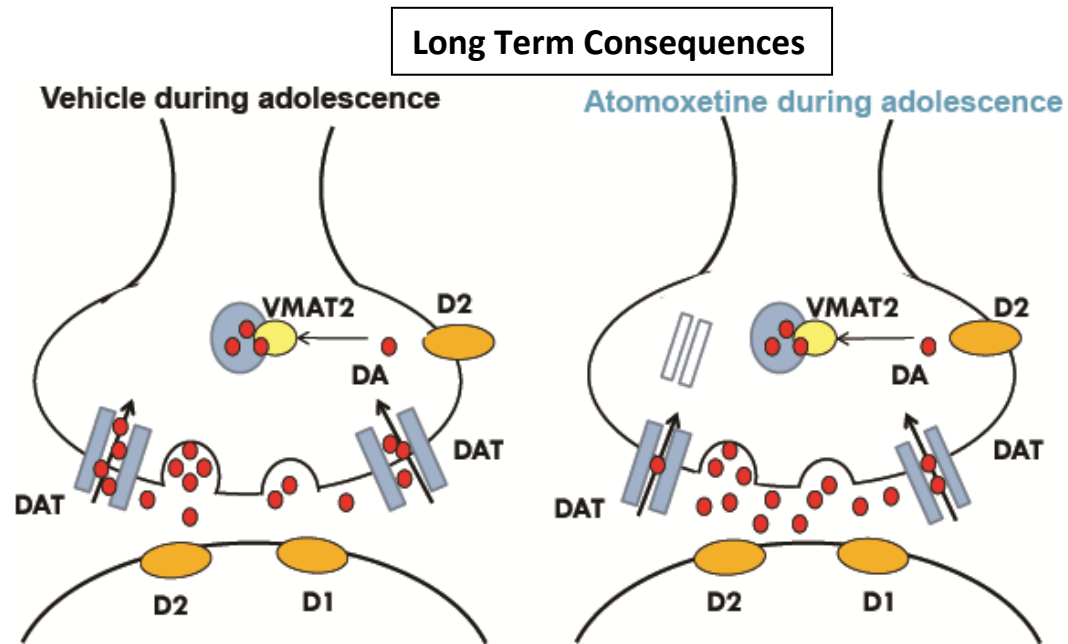
Graphical illustration: MPH treatment during adolescence increases dopamine transporter function in the medial prefrontal cortex of adult SHR without increasing the cell surface expression of dopamine transporters, indicating a trafficking-independent mechanism of regulation by MPH. MPH treatment did not alter dopamine transporter function in the orbitofrontal cortex or striatum of adult SHR. As such, the increase in dopamine transporter function in the medial prefrontal cortex may underlie the enhanced cocaine self-administration in MPH-treated SHRs.

Figure 8.2 Dopaminergic terminals in the orbitofrontal cortex of adult SHR



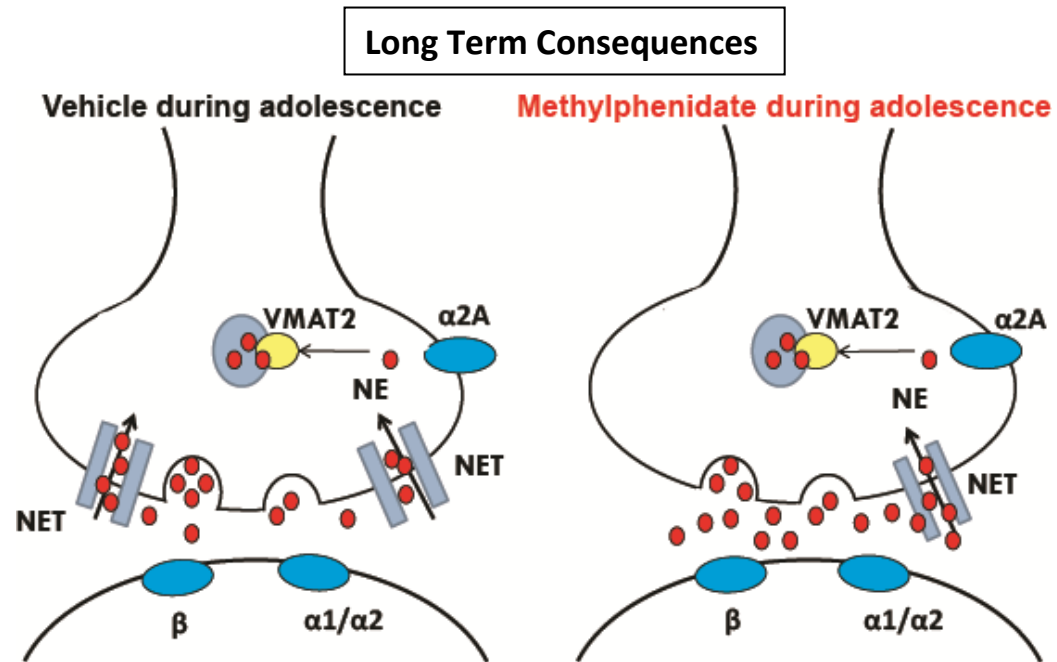
Graphical illustration: Treatment with atomoxetine (ATO) during adolescence decreases dopamine transporter function and cell surface expression in the orbitofrontal cortex of adult SHR, indicating a trafficking-dependent mechanism of regulation by ATO. ATO treatment did not alter dopamine transporter function in the medial prefrontal cortex of adult SHR, and did not increase cocaine self-administration in adult SHR.

Figure 8.3 Dopaminergic terminals in the striatum of adult SHR



Graphical illustration: Atomoxetine (ATO) treatment during adolescence decreases dopamine transporter function in the striatum of adult SHR. The decreased transporter function is associated with an increase in total expression of dopamine transporters, indicating a compensatory mechanism.

Figure 8.4 Noradrenergic terminals in the lateral orbitofrontal cortex of adult SHR



Graphical illustration: Treatment with MPH during adolescence decreases norepinephrine transporter function in the lateral orbitofrontal cortex of adult SHR. In the vehicle administered-SHR, norepinephrine transporter function in the lateral orbitofrontal cortex was greater than that in WKY and WIS controls. Also, MPH treatment did not alter norepinephrine transporter function in the medial prefrontal cortex of adult SHR.

8.2 Integration of results

8.2.1 DAT function in mPFC, OFC and striatum and behavior

Evidence that mPFC, OFC and striatum are critically involved in cocaine self-administration comes from studies in which local inactivation or lesions decreased cocaine-seeking and taking behavior (Di Ciano *et al*, 2008; Di Pietro *et al*, 2006; Grakalic *et al*, 2010). Following MPH treatment during adolescence, the observed increase in mPFC DAT function (Chapter 2) would be expected to decrease extracellular dopamine concentration in mPFC. Previous studies show that mPFC DA depletion results in an increased motivation for and increased sensitivity to cocaine (McGregor *et al*, 1996; Schenk *et al*, 1991). Thus, increased DAT function in mPFC may underlie the increased cocaine self-administration observed following adolescent MPH treatment (Harvey *et al*, 2011). Furthermore, reduced dopamine in the mPFC was associated with increased burst responding on a DRL30 schedule (Sokolowski *et al*, 1994). Taken together, these results suggest that increased DAT function in the mPFC leads to increased response inhibition deficits in MPH-treated SHR (Chapter 7), and the increased response inhibition deficits may underlie the enhanced cocaine self-administration, as evidenced by upward shifts in FR and PR responding (Harvey *et al*, 2011) as well as greater cocaine intake during adulthood (Jordan *et al*, submitted).

The relative protection rendered by ATO may be mediated partially by the absence of effects on DAT function in mPFC (Chapter 3). The decrease in DAT function in the OFC and striatum was not associated with alterations in rate of acquisition of cocaine self-administration or dose response functions for cocaine self-administration under FR and PR schedules (Somkuwar *et al*, 2013b). The OFC is known to process information regarding the reinforcing value of cocaine (Kantak *et al*, 2009). Specifically, OFC inactivation results in a right-shift in the cocaine dose-response under FR schedules. Therefore, the lack of effect of ATO treatment on cocaine self-administration

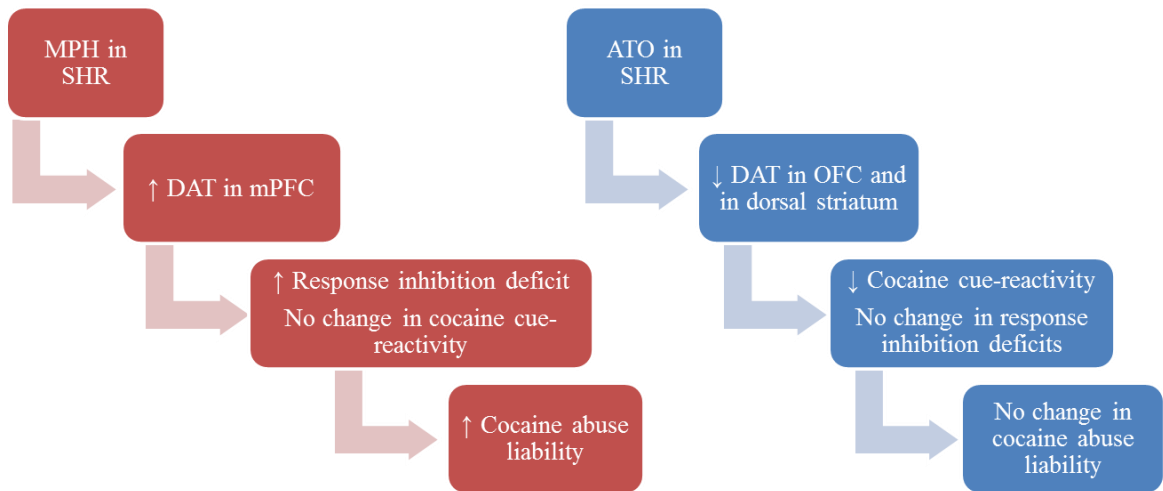
(Somkuwar *et al*, 2013b) despite the decreased DAT function in the OFC of ATO-treated SHR (Chapter 3) was unexpected. One explanation for the apparent disconnect between cocaine self-administration and DAT function in the OFC may be that OFC codes information regarding reward value by modulating specific post-synaptic dopaminergic receptor and not via modulation of DAT function per se (Zeeb *et al*, 2010). Further support for this idea comes from the DRL experiments (Chapter 6). The rank order for the decrease in accuracy of estimating waiting time from DRL5 to DRL30 was SHR > WIS > WKY, suggesting that SHR are most sensitive to the delay of reinforcement, a function mediated by OFC (Dalley *et al*, 2004). However, no strain differences were observed with respect to OFC DAT function (Chapter 2 and 3). Furthermore, decreased OFC dopamine receptor expression and/or activation increases impulsive choice in delay discounting schedules, but have limited effects on response inhibition deficits as measured using the 5-choice serial reaction time (5-CSRT) task (Winstanley *et al*, 2010b; Zeeb *et al*, 2010). Therefore, OFC may mediate impulsivity observed in the current study (Chapter 6) in conjunction with other neurotransmitter systems such as norepinephrine and serotonin (Bari *et al*, 2011; Jupp *et al*, 2013a; Wischhof *et al*, 2011).

The dorsal striatum is involved in habit learning as well as habitual drug seeking as evaluated using instrumental learning paradigms (Everitt and Robbins, 2005; Yin *et al*, 2004). Decreasing dopamine signal in the dorsal striatum alters acquisition of second-order responding, but decreases responding once the behavior is established (Taylor and Robbins, 1986; Vanderschuren *et al*, 2005). Furthermore, responding for a conditioned stimulus is concurrent with increases in extracellular dopamine in the dorsal striatum (Ito *et al*, 2002). In the current study, ATO treatment during adolescence followed by treatment discontinuation in early adulthood decreased DAT function in the striatum of adult SHR (Chapter 3). The decreased striatal DAT function in the ATO-treated may result in elevated extracellular dopamine during adulthood which may therefore, blunt the dopaminergic signal while responding for cocaine cues in adult SHRs. Taken together, the attenuated signal to noise ratio in the striatum of adult SHR

may underlie the observed decrease in cocaine cue-reactivity under second-order schedule in ATO-treated SHR (Jordan *et al*, submitted).

In conclusion, the increased DAT function in the MPH-treated SHR may underlie the increased response inhibition deficits as well as cocaine self-administration during adulthood (Fig 8.5). In contrast, the decreased DAT function in the OFC and dorsal striatum of ATO-treated SHR may underlie the decreased cocaine cue-reactivity as well as the protection from further increase in cocaine abuse risk.

Figure 8.5 Potential mechanism integrating changes in DAT function with behavioral outcomes in adult SHR



Schematic representation of potential dopaminergic mechanism contributing to the opposite effects of methylphenidate (MPH) and atomoxetine (ATO) treatments during adolescence on cocaine abuse during adulthood evaluated using a heuristically useful model of ADHD (SHR).

8.2.2 NET function in mPFC and OFC and behavior

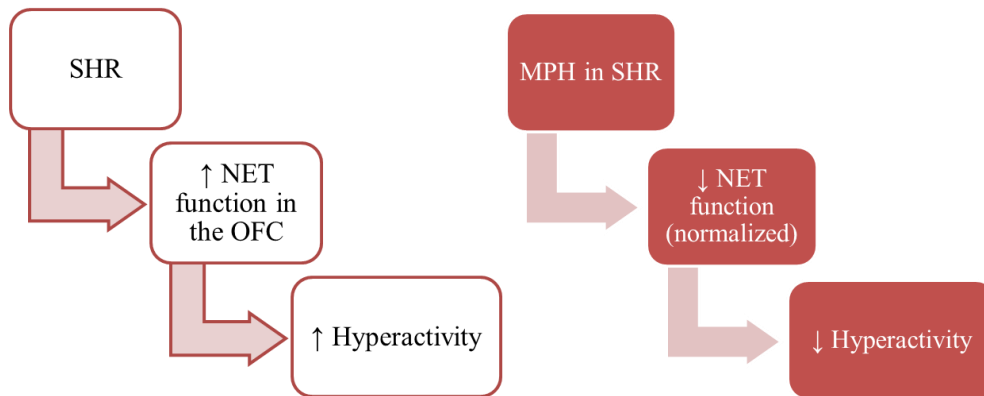
Hyperactivity is a hallmark of ADHD (American Psychiatric Association., 2013a). Hyperactivity as well as other behavioral deficits in ADHD decrease in severity with increasing age (Faraone *et al*, 2006; Mannuzza *et al*, 1998; Mannuzza *et al*, 2002). Also,

individuals with ADHD show delayed cortical maturation that is associated with impaired control of inappropriate behavior (Eshel *et al*, 2007; McLaughlin *et al*, 2010). In the current study, adolescent and adult SHR showed greater locomotor activity in open-field chambers compared to control Wistar-Kyoto and Wistar rats (Chapter 6 and 7). These results were consistent with previous reports (van den Bergh *et al*, 2006; Yang *et al*, 2011). Previous studies suggest that in SHR, open-field activity did not decrease till 6 months after birth, while in Wistar-Kyoto, locomotor activity decreased from 1 to 3 months (Hsieh and Yang, 2008). In contrast, low doses of MPH (0.3, and 1 mg/kg, i.p.) administered acutely, reduced open-field activity in adolescent SHRs, but not WKY and WIS (Umehara *et al*, 2013a; Umehara *et al*, 2013b). In the current study, locomotor activity was decreased by the age of ~3.5 months in adult SHRs that were administered MPH during adolescence (Chapter 7). These current results complement the clinical findings that MPH treatment during development, in part, may be responsible for the age-dependent decrease in ADHD symptoms.

Mechanistically, the MPH-induced decrease in hyperactivity in adolescent SHRs was blocked by idazoxan, an α 2A receptor antagonist, at doses that were sufficient to block MPH-induced cognitive enhancement (Gamo *et al*, 2010; Umehara *et al*, 2013b). Further guanfacine, α 2A agonist, reduced open-field activity while A-68930, a selective D1 agonist increased activity in SHR (Langen *et al*, 2011). Taken together, these current results and those from the literature suggest that attenuation of hyperactivity by MPH is mediated by activation α 2A noradrenergic receptors. In the current study, NET function in the lateral orbitofrontal cortex of SHR was elevated compared to Wistar-Kyoto and Wistar. Chronic MPH treatment during adolescence normalized NET function in the lateral orbitofrontal cortex of SHRs (Chapter 4; Fig 8.4). Normalization of NET function in the lateral orbitofrontal cortex may increase extracellular norepinephrine concentrations and thereby enhance post-synaptic α 2A receptor signaling, which may have contributed to the reduced hyperactivity in adult SHR following discontinuation of adolescent treatment with MPH.

In conclusion, NET function is elevated in the lateral orbitofrontal cortex of SHR compared to Wistar-Kyoto and Wistar. MPH treatment during adolescence followed by treatment discontinuation in early adulthood normalized NET function in lateral orbitofrontal cortex of SHR, which may contribute to the reduced locomotor activity in adult SHR compared to vehicle control (Fig 8.6).

Figure 8.6 Potential mechanism integrating changes in NET function with hyperactivity in adult SHR following methylphenidate treatment



Schematic representation of potential noradrenergic mechanism contributing to the decreased hyperactivity in adult SHR that were administered methylphenidate (MPH) during adolescence.

8.2.3 Differences in neuropsychopharmacology of methylphenidate and atomoxetine

The current in vitro neurochemistry results show that MPH and ATO have distinct profiles for altering DAT function and expression in mPFC, OFC and striatum. MPH treatment during adolescence resulted in an increase in DAT function in the mPFC

of adult SHR (Chapter 2). Since clearance of extracellular dopamine is regulated by DAT localized at the cell surface of dopaminergic neurons (Zahniser and Sorkin, 2004), the cell surface expression of DAT was expected to be altered in the direction corresponding with the change in DAT function in the MPH and ATO treated-SHR. However, in the current study, DAT cell surface expression was not altered by MPH during adolescence (Chapter 2). Previous studies reported that chronic exposure to DAT inhibitors results in post-translational modifications or protein-protein associations that alter transporter function without changing cell-surface expression of DAT (Foster *et al*, 2008; Ramamoorthy *et al*, 2010). Taken together, MPH treatment during adolescence increased DAT function in adult SHR in a trafficking-independent manner.

In contrast to effects of MPH, ATO decreased DAT function in OFC and striatum of SHR in a trafficking-dependent manner (Chapter 3). The effect of ATO, a selective NET inhibitor, on DAT in OFC was not surprising since the dose of ATO used in the current studies increases extracellular dopamine in the prefrontal cortex by inhibiting dopamine clearance via NET (Bymaster *et al*, 2002; Moron *et al*, 2002). As expected with the current treatment regimen, chronic ATO has been shown to persistently increase extracellular dopamine in the PFC (Koda *et al*, 2010). Further, repeated exposure to DAT substrates, including dopamine results in persistent trafficking-dependent downregulation of the transporter (Zahniser *et al*, 2009). Taken together, chronic ATO treatment during adolescence inhibits dopamine clearance by NET, which persistently increased extracellular dopamine and consequently, resulted in reduced DAT function and surface expression in the OFC (Chapter 3). In contrast, the effect of ATO on DAT in the striatum was not expected. However, frontostriatal cortical networks in OFC send extensive projections to the ventromedial caudate nucleus of the dorsal striatum (Alvarez and Emory, 2006) and are responsible for decision making and regulation of impulsivity (Feil *et al*, 2010). Thus, in the current study, the decreased DAT function in the OFC following chronic ATO treatment during adolescence may alter the connectivity between OFC and dorsal striatum and thereby, contribute to the decreased DAT function in the dorsal striatum.

Cocaine self-administration results also showed distinct profiles following discontinuation of MPH and ATO treatments in SHR. MPH treatment adolescence results in increased cocaine self-administration in adult SHR, while ATO treatment during adolescence was protective against the further increase in cocaine self-administration during adulthood (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). These results suggest that MPH, but not ATO, treatment during adolescence may increase cocaine abuse liability in adults with ADHD. One of the hallmark features of addiction is that drug-paired cues and context induces persistent drug-seeking behavior (Robinson *et al*, 1993). Conditioned stimuli (or cues) paired with drugs of abuse induce craving and contribute to relapse (Ehrman *et al*, 1992; Kosten *et al*, 2006). Another set of experiments conducted in collaboration with the current studies compared the effects of MPH and atomoxetine treatments during adolescence on cues paired with cocaine self-administration using a second-order schedule of responding during adulthood (Jordan *et al*, submitted). Second-order schedules of reinforcement were used to evaluate differential reactivity for cues that predict availability of a drug between strain and treatment groups (Di Ciano *et al*, 2005; Schindler *et al*, 2002). Under the second order schedules, animals learn a complex sequence of responding; each step in the sequence is reinforced by a conditioned reinforcer (e.g., cue-light) and completion of the sequence results in delivery of the primary reinforcer (Cain *et al*, 2004). As such, the conditioned stimulus reinforces the operant responding and more importantly, signal that completion of the successive steps will result in the delivery of the drug of abuse. The current study used a fixed interval 5 min – fixed ratio 5 (FI5 min [FR5:S]) schedule; every 5th response on the lever (i.e., fixed ratio) resulted in the presentation of a cue-light (stimulus; S). This continued for 5 min (i.e. fixed interval), after which the 5th response on the lever resulted in the presentation of the cue-light and the delivery of the primary reinforcer (i.e., cocaine; Kantak *et al*, 2001). During extinction of second-order responding, neither cocaine nor cue-light were presented during operant responding. During cue-reinstatement, every 5th response resulted in the presentation of cue-light; however, cocaine was not available to the rats (Kantak *et al*, 2002). Results

revealed that SHR earned more cocaine infusions during the maintenance of cocaine responding under the FI5min[FR5:S] schedule, required greater number of sessions to reach extinction criterion, and reinstated cue-induced cocaine seeking to a greater extent than control WKY and WIS (Jordan *et al*, submitted). Further, MPH treatment during adolescence increased the number of cocaine infusions earned during the maintenance, but did not alter extinction of responding or cue-reinstatement of cocaine seeking. In contrast, ATO did not alter responding during maintenance or extinction, but attenuated cue-induced reinstatement of cocaine seeking. These results are in agreement with the current study which shows that SHR sensitize to cocaine-induced locomotor activation more readily than WKY and WIS, but MPH treatment does not alter cocaine sensitization (Chapter 5). The effect of chronic ATO treatment on cocaine sensitization has not been evaluated, and would be interesting to pursue in future studies. Previous studies suggest a relation between sensitization and relapse, such that greater sensitization is associated with robust reinstatement of responding for context and cues associated with the drug of abuse through a process termed as incentive sensitization (Robinson *et al*, 2001). Although data supporting this hypothesis are debatable at present (Robinson *et al*, 2008b), the current study found that compared to control Wistar-Kyoto and Wistar rats, SHR sensitize more readily to repeated cocaine administration and exhibit increased conditioned response in the cocaine-paired context (Chapter 5). MPH treatment during adolescence did not alter cocaine sensitization or conditioning the adult SHR (Chapter 5), nor did it alter cocaine cue-reactivity under the second-order schedule (Jordan *et al*, submitted). Taken together, these results lend support to the incentive sensitization theory by showing that strain differences in sensitization is associated with robust reinstatement of responding for cocaine cues and context.

In conclusion, SHR exhibit enhanced sensitivity for cocaine and cocaine-cues compared to Wistar-Kyoto and Wistar rats. MPH treatment during adolescence did not alter sensitization to cocaine or cocaine cue-reactivity in adult SHR, but increased DAT function in the mPFC in a trafficking independent manner. In contrast, ATO treatment

during adolescence decreased cocaine cue-reactivity and decreased DAT function in the OFC and dorsal striatum of adult SHR.

8.3 Implications

8.3.1 How can the results with SHR be used to improve treatment outcomes in ADHD?

ADHD individuals with comorbid cocaine dependence were found to be more impulsive than non-cocaine dependent ADHD individuals and demographically-matched healthy controls (Crunelle *et al*, 2013). However, prospective experimental design is needed to evaluate whether preexisting differences in impulsivity led to cocaine dependence in individuals with ADHD or whether cocaine abuse contributed to the increased impulsivity. Using an animal model of ADHD, the current study reported that MPH treatment during adolescence increased burst responses in adult SHR under DRL30 schedule (Chapter 7), suggesting that MPH treatment increased response inhibition deficit in SHR. By extrapolation, increased response inhibition deficit following MPH treatment during adolescence may underlie increased cocaine abuse (Lambert *et al*, 1998; Mannuzza *et al*, 2008). Furthermore, the increased response inhibition deficit as evaluated by increased burst responding may be a behavioral marker for increased cocaine abuse liability in individuals with ADHD who were treated with MPH during adolescence. However, human subjects learn the DRL schedule of reinforcement to 100% efficiency in a matter of hours (Stewart *et al*, 2006). Thus, in clinical laboratory studies, individuals exhibit suboptimal performance on DRL schedules for a very limited time during which the differences between individuals with comorbid ADHD and cocaine dependence and those without the comorbid cocaine dependence may be apparent. The mathematical modeling approach optimized herein (Chapter 6) may be able to identify individual differences in responding under the DRL schedule within a limited time-window, and thus facilitate clinical research with DRL schedules. Further,

DRL schedules coupled with mathematical modeling approach may be developed as a behavioral assay or marker to identify individuals with ADHD in whom MPH treatment during adolescence increased cocaine abuse liability.

ADHD-like symptoms are often presented by individuals with neurogenetic disorders such as Tuberous Sclerosis Complex, Neurofibromatosis I, Williams Syndrome, and Fragile X Syndrome. These clinical comorbidities clearly indicate that the downstream effects of these varied genetic abnormalities converge upon common biological pathways or neural circuits (Curatolo *et al*, 2010; Lo-Castro *et al*, 2011), thus giving rise to the concept of endophenotypes. Endophenotypes are proximal markers of gene action, and comorbid conditions may help identify such genetically homogeneous subgroups (Bearden *et al*, 2004; Gottesman *et al*, 2003). Furthermore, pharmacogenomics research in ADHD for predicting response to treatment and side effects of treatment has been lagging (Kieling *et al*, 2010). Improved understanding of the effects of ADHD therapeutics on endophenotypes will advance the field of ADHD pharmacogenomics. Impulsivity under DRL schedules may isolate potential endophenotypes associated with the increased cocaine abuse liability in individuals with ADHD (Almasy *et al*, 2001; Bearden *et al*, 2004; Crosbie *et al*, 2008; Winstanley *et al*, 2010a). With the two DRL schedules used herein, response inhibition deficits (burst responding) and sensitivity to delayed reinforcement (accuracy of estimating wait-time) were identified as two facets of impulsivity that may underlie the comorbidity between ADHD and cocaine abuse. A key finding of the current study was that MPH treatment during adolescence resulted in decreased response inhibition (increased burst responding, Chapter 7) in adulthood. Furthermore, converging evidence from the DAT function assays presented herein and studies from the literature reporting effects of 6-hydroxydopamine lesion suggest that reduced dopaminergic signaling in the mPFC may be the neurological basis for reduced response inhibition. Taken together, increased burst responding under DRL30 and the associated decreased dopaminergic signaling in the mPFC may be the neurobehavioral marker for the increased cocaine abuse liability.

The current results have several implications for clinical practice of therapeutic strategies for ADHD. Firstly, given the causal relation between reduced response inhibition and increased cocaine abuse liability (Fillmore *et al*, 2002; Li *et al*, 2006), our studies suggest that the increased cocaine abuse liability in adulthood following MPH treatment during adolescence (Mannuzza *et al*, 2008) may be mediated by decreased response inhibition in the ADHD individuals. Thus, therapeutic approaches that reduce response inhibition deficits will be protective against cocaine abuse liability in MPH-treated ADHD individuals.

Secondly, an improved understanding of the underlying neurochemistry associated with discontinuation of MPH treatment may enable identification of appropriate therapeutic strategies for ADHD individuals with comorbid cocaine abuse. In the current study, SHR displayed elevated NET function in the lateral orbitofrontal cortex (Chapter 4), which is expected to lead to decreased norepinephrine transmission as well as decreased dopamine transmission by inhibiting dopamine clearance via NET (Moron *et al*, 2002). MPH treatment during adolescence normalized NET function in SHR (Chapter 4), suggesting that both norepinephrine and dopamine transmission in the OFC are normalized. Therefore, OFC mediated function such as attention, working memory and impulsive decision making are expected to be normalized following MPH treatment during adolescence (Harvey *et al*, 2013; Kantak *et al*, 2008; Sun *et al*, 2012; Zeeb *et al*, 2010). Prophylactic strategies that capitalize on these behavioral facets may provide better outcomes in terms of reducing cocaine abuse risk in individuals with ADHD who had been treated with MPH during adolescence.

Dopaminergic function in the OFC is implicated in ascribing the relative 'value' of a larger delayed reward compared to a smaller immediate reward (Kheramin *et al*, 2004; Kheramin *et al*, 2002). In delay discounting studies, inhibition of D1 and/or D2 receptors in the OFC increases the choice for small immediate reward over a delayed but larger reward, and increasing D1/D2 receptor activation produced the opposite effect (Pardey *et al*, 2013; Wade *et al*, 2000). In the current study, ATO treatment during adolescence

did not increase DAT function in the mPFC, but decreased DAT function in the OFC of SHR during adulthood (Chapter 3). Thus, an ATO-induced decrease in DAT function in the OFC of SHR would be expected to decrease impulsive choice. A previous study reported that chronic ATO administration to adolescent outbred rats decreased impulsivity on a delay discounting assay, that was associated with changes in neuronal plasticity markers in the OFC (Sun *et al*, 2012). Thus, the protective effect of ATO against further increasing cocaine self-administration (Somkuwar *et al*, 2013b) may be mediated partially by decreased impulsivity. However, whether ATO treatment during adolescence is associated with decreased impulsive choice during adulthood needs to be empirically evaluated in future work. Importantly, ATO decreased cue-induced reinstatement of second order responding (Jordan *et al*, submitted), which was associated with a decreased striatal DAT function (Chapter 3). Taken together, ATO may be a safer treatment alternative for ADHD adolescents in whom cocaine abuse liability is a concern.

8.3.2 Predictions for future studies

8.3.2.1 Other facets of impulsivity

SHR show greater sensitivity to delayed reinforcement (reduced response threshold under DRL30; Chapter 7) compared to WKY and WIS, which is consistent with strain differences in delay discounting studies (Wooters *et al*, 2011). MPH treatment during adolescence does not alter sensitivity to delayed reinforcement in adult SHR under DRL schedules (Chapter 7). However, the effects of MPH treatment on SHR behavior under delay discounting schedules, including a 'choice' to obtain a smaller reinforcement as opposed to a delayed larger reward has not been evaluated.

The neurochemical results in the current study reveal that MPH treatment increased DAT function in mPFC and normalized the previously elevated NET function in the OFC in SHR (Chapters 2 and 4, respectively). The role of mPFC dopaminergic signaling on impulsive choice is complex. Previous studies report that D1 antagonists as well as high doses D1 agonists applied into the mPFC increase impulsive choice, i.e. choice of a the smaller immediate reinforcement (Loos *et al*, 2010; Pardey *et al*, 2013). Thus, tonic D1 activation promotes choice of delayed larger reward; decreased D1 receptor activation increases impulsive choice due to ineffective suppression of cortical inputs (i.e. noise; Arnsten *et al*, 2005). In the current study, increased DAT function in the mPFC of MPH-treated SHR may lead to impulsive choice in this ADHD model.

On the other hand, inhibition of D1 and/or D2 receptors in OFC increase the choice of the small immediate reward over delayed but larger reward, and increasing D1/D2 receptor signaling by increasing extracellular dopamine has the opposite effect on impulsive choice (Pardey *et al*, 2013; Wade *et al*, 2000). In the current study, NET function was greater in the OFC of SHR compared to Wistar-Kyoto and Wistar rats (Chapter 4). Since NET in cortical areas is responsible for regulating extracellular dopamine (Moron *et al*, 2002), the increased NET function in the OFC of SHR reported herein may be the neurochemical basis for the previously reported increased impulsive choice in SHR (Wooters *et al*, 2011). Furthermore, MPH treatment during adolescence normalized NET function in the OFC of adult SHR (Chapter 4). Therefore, MPH-treated SHR are expected to exhibit reduced impulsivity on a delay discounting paradigm during adulthood.

8.3.2.2 Other neurochemical mechanisms

Compared to adult WKY and WIS, SHR display several behavioral deficits that may underlie increased cocaine self-administration in SHR (Harvey *et al*, 2011; Somkuwar *et al*, 2013b). SHR display reduced response inhibition during the DRL30

schedule (increased bursts, Chapters 6 and 7), as well as an elevated sensitivity to delayed reinforcement under DRL schedules (reduced response threshold under DRL30, Chapter 6 and 7) and delayed reward choice paradigms (Slezak *et al*, 2011; Wooters *et al*, 2011), all of which confer the vulnerability for acquiring drug-taking behavior in SHR. Further studies are required to obtain a more comprehensive understanding of the neurochemical basis for the strain differences between SHR and control in sensitivity to delayed reinforcement (accuracy of estimating wait-time), cocaine self-administration as well as cocaine sensitization. Catecholaminergic receptors, other neurotransmitter systems (e.g. serotonin) as well as other brain regions (e.g., nucleus accumbens; NAc) have been implicated in these behaviors (Pattij and Vanderschuren, 2008).

Increased sensitization to the psychomotor effects of cocaine has been suggested to mediate the transition from recreational drug use to addiction (Ferrario *et al*, 2005; Robinson *et al*, 2001; but see Ahmed *et al*, 2006). Compared to Wistar-Kyoto and Wistar, SHR were less sensitive to the locomotor-activating effect of cocaine, but readily sensitized to repeated cocaine administration and reinstated responding for cocaine cues (Chapter 5 and Jordan *et al*, submitted). Dopamine in the NAc is implicated in cocaine sensitization and cue-induced reinstatement of cocaine seeking behavior (Di Ciano *et al*, 2008; Pierce *et al*, 1996a; Pierce *et al*, 1996b; Robinson *et al*, 1993). Compared to WKY controls, SHR show attenuated dopamine release in the NAc that was reversed by D2 receptor antagonists to a greater extent in SHR than in WKY (Russell *et al*, 1995, 1998). Dopamine in the mPFC modulates dopamine release in the NAc (Beyer *et al*, 1999). Following MPH treatment during adolescence, DAT function in the mPFC and cocaine self-administration were both increased (Chapter 2; Harvey *et al*, 2011; Somkuwar *et al*, 2013a). Surprisingly, locomotor sensitization to cocaine and second order reinstatement for cocaine cues were not altered by MPH treatment (Chapter 5 and Jordan *et al*, submitted). Self-administration may be modulated by phasic dopamine release in the mPFC, while sensitization and cue-reactivity may be modulated by tonic dopamine through D2 receptor-mediated mechanisms. Support for this hypothesis is that decreased D2 function in the mPFC facilitated development of sensitization by

increasing glutamatergic signaling in the mPFC and NAc (Liu *et al*, 2011). D2 antagonists did not alter cocaine-induced reinstatement but attenuated cue-induced reinstatement (Capriles *et al*, 2003; Cervo *et al*, 2003). Taken together, SHR may have lower D2 function in mPFC and greater D2 function in the NAc compared to control. Also, the lack of effect of MPH on sensitization and cue-reactivity suggests that MPH treatment does not alter D2 function in either areas of the brain.

The dorsolateral subregion of the striatum is implicated in cue-reactivity under second-order schedules (Vanderschuren *et al*, 2005), and dopaminergic cross-talk between the dorsolateral striatum and NAc modulates cocaine cue-reactivity (Belin and Everitt, 2008a). ATO treatment during adolescence decreased striatal DAT function in SHR (Chapter 3), which may underlie the attenuated cocaine cue-reactivity during adulthood. In the current study, strain differences in striatal DAT function were not obtained (Chapter 2 and 3), but SHR exhibited greater cocaine cue-reactivity compared to Wistar-Kyoto and Wistar rats (Jordan *et al*, submitted). In contrast to the results for striatal DAT function reported herein, a recent study using *in vivo* voltammetry suggested that compared to Wistar-Kyoto and outbred rats, SHR exhibit differences in DAT function within the subregions of the dorsal striatum (Miller *et al*, 2012). The latter study evaluated uptake of exogenously applied dopamine for three depths of the dorsal striatum and for NAc. SHR exhibited faster dopamine uptake in the NAc and in the ventral portions of the dorsal striatum, while DAT function did not differ in the dorsal coordinates of dorsal striatum. Taken together, the increased DAT function in the ventral portions of the striatum and in the NAc may underlie the increased cocaine cue-reactivity in SHR compared to controls. Extending these results to the increased cocaine abuse liability in individuals with ADHD suggests that hypodopaminergic striatum and accumbens may contribute to increased sensitivity for cocaine cues.

Serotonergic tone and receptor function also have been implicated in impulsivity (Dalley *et al*, 2002; Perry *et al*, 2011; Winstanley *et al*, 2006b). A dysfunctional serotonergic system is not thought to underlie ADHD, since serotonergic modulators do

not ameliorate ADHD symptoms (Adriani *et al*, 2004; Winstanley *et al*, 2006a). As a consequence, serotonergic involvement ADHD etiopathology has not been investigated extensively. However, a recent genetic association study suggests that serotonergic receptor systems may be involved in ADHD and substance use disorder comorbidity (Sanchez-Mora *et al*, 2013). Although, serotonin transporters have not been associated with response inhibition deficits (Jupp *et al*, 2013b), serotonin transporter polymorphisms have been associated with greater impulsive choice in delay discounting paradigms in individuals with ADHD (Sonuga-Barke *et al*, 2011). Furthermore, individuals with comorbid ADHD and cocaine dependence are a more impulsive subpopulation of adults with ADHD (Crunelle *et al*, 2013). Taken together, these results suggest that serotonergic mechanisms need to be evaluated further with respect to control of impulsivity in ADHD, which also may be a valuable therapeutic strategy for managing comorbid ADHD and cocaine abuse.

8.3.3 Implications of the 5th edition of Diagnostic and Statistical Manual of Mental Disorders (DSM V, American Psychiatric Association., 2013b)

In the new edition of the DSM-V, the age of expression of ADHD symptoms was increased from 7 years to 12 years. The increase in age of diagnosis is of particular concern taking into account the results of the current study that shows that MPH treatment initiation during adolescence is associated with several deleterious consequences with respect to future cocaine abuse liability. However, the current study does not eliminate the possibility that the behavioral and neurochemical sequel of MPH treatment during adolescence was a consequence of 'discontinuation' of the treatment. Clinical report suggests that continued stimulant treatment may be protective against development of future cocaine abuse liability (Biederman *et al*, 1999b).

A recent study reported that in 15 to 21 year old individuals with ADHD, the prescription rate of MPH and ATO declines more rapidly than the rate of decline of

ADHD symptoms (McCarthy *et al*, 2009). These results suggest the possibility that treatment is prematurely discontinued in some young adults. One reason for these clinical practices may be the reluctance of clinicians to diagnose ADHD in older adolescents due to the unclear definitions of remission in DSM IV (American Psychiatric Association., 2000). In DSM IV, partial remission status was applied for individuals who exhibited symptoms that “no longer meet full criteria” (American Psychiatric Association., 2000). In the DSM V, the definition and diagnostic criteria for ADHD have been updated to facilitate characterization of ADHD in older adolescents (age >17 years) and adults (American Psychiatric Association., 2013b). Specifically, partial remission was applied when individuals who met the full ADHD criteria in the past (i.e., during childhood or adolescence) exhibited fewer symptoms than the full criteria for the past 6 months, and the symptoms still contribute to impaired function in social, academic and occupational settings. Furthermore, provisions have been included to specify the severity of the impairments under the new DSM V. Again, studies using the SHR model may reveal whether continued MPH treatment leads to different outcomes in terms of abuse liability evaluated using cocaine self-administration, response inhibition deficit and DAT function in the mPFC.

8.4 Limitations

8.4.1 General limitations of the overall experimental design

One limitation of the overall experimental design was that single doses of MPH and ATO were evaluated in the current study. MPH dose was chosen based on previous studies that demonstrated efficacy as well as therapeutically relevant plasma levels of MPH in rats (Berridge *et al*, 2006; Harvey *et al*, 2011; Kantak *et al*, 2008; Kuczenski *et al*, 2002; Umehara *et al*, 2013b). Unlike MPH, the ATO dose employed may not be referred to as being ‘therapeutically relevant’ because the plasma concentration of ATO in rats has not been empirically compared with the therapeutically efficacious plasma levels

observed in the clinical studies. However, the dose used herein was established as pharmacologically efficacious and mechanistically relevant as an ADHD therapeutic (Bymaster *et al*, 2002; Levy, 2008; Robinson *et al*, 2008a; Swanson *et al*, 2006). Specifically, ATO at 0.3 mg/kg, i.p. increased extracellular dopamine and norepinephrine in the PFC, decreased hyperactivity and impulsivity and increased attention in rats (Bymaster *et al*, 2002; Gamo *et al*, 2010; Robinson *et al*, 2008a; Umehara *et al*, 2013a).

The oral route of administration is clinically relevant for both MPH and ATO. Therefore, the oral dosing regimen of MPH used in the current study closely resembles the clinical situation (Gerasimov *et al*, 2000). However, ATO was administered via intraperitoneal injection in the current study. Oral bioavailability of ATO in rats is 4% of intravenous administration (Mattiuz *et al*, 2003), and hence the intraperitoneal route of administration was used in our studies to better control dosing. Taken together, although there are always limitations, the dose and route of administration of MPH and ATO were optimal for addressing the objectives of the current study.

8.4.2 Limitations in translational value of the impulsivity study

The current study revealed that in adolescent SHR, chronic treatment with a therapeutically-relevant dose of MPH did not decrease response inhibition deficit (Chapter 6 and 7). Compared to vehicle control, burst responding was increased and response threshold was decrease with chronic MPH in adolescent SHRs (Chapter 6). These results were surprising since MPH reduces impulsivity in individuals with ADHD. One explanation may be limitations of the SHR model of ADHD (van den Bergh *et al*, 2006). SHR, though inbred and genetically homogeneous, have been suggested to have impulsive and non-impulsive subpopulations that differ in their response to MPH under delay discounting schedules (Adriani *et al*, 2003). In contrast to effects on response inhibition capacity, comparable dose of MPH improved several ADHD-related behavioral deficits in SHRs, including working memory, behavioral flexibility, hyperactivity as well

as impulsive decision making (Adriani *et al*, 2003; Harvey *et al*, 2013; Harvey *et al*, 2011; Kantak *et al*, 2008; Umehara *et al*, 2013b). Furthermore, in agreement with previous reports, the current study found reduced latency for responding and no change in locomotor activity with chronic MPH treatment (Harvey *et al*, 2013; Yang *et al*, 2011). Taken together, these results suggest SHR may capture limited aspects of ADHD and other models also should be explored.

Another explanation for the unexpected effects of MPH on response inhibition capacity may be that the experimental preparations for rats do not model the clinical measures of response inhibition capacity. Another schedule evaluating response inhibition capacity is the 5-choice serial reaction time task. In this preparation, MPH and d-amphetamine administered at therapeutically relevant low doses reproducibly increased premature responses (Economidou *et al*, 2012; Navarra *et al*, 2008; Paterson *et al*, 2011). These results are in agreement with the results from the current study that proportion of burst responding was increased and the proportion of timed responding was decreased in MPH-treated adolescent rats (Chapter 6). In contrast to stimulants, the NET inhibitor ATO was reported to reduce premature responding (Navarra *et al*, 2008; Paterson *et al*, 2011). Taken together, response inhibition tasks in rodents may be particularly sensitive to the 'stimulant' properties of MPH, and thus, these tasks have limited translational utility.

8.4.3 Limitations of an animal model approach

The high comorbidity of cocaine abuse in ADHD individuals is well documented in the clinical literature (see section 1.4; Carroll *et al*, 1993; Levin *et al*, 1999; Szobot *et al*, 2007). While the current study empirically evaluated mechanisms that contribute to this comorbidity, they have inherent limitations. These reductionist approach using animal models cannot capture the inherent complexity of psychiatric comorbidities being modeled. Factors such as prenatal drug exposure, environmental factors, and complex

social interactions need to be individually modeled in comparable studies to increase our understanding of the comorbidity of ADHD and cocaine abuse liability.

One of the most widely debated and highly controversial topics in the comorbidity of ADHD and cocaine abuse has been the effects of MPH treatment on cocaine abuse liability in individuals with ADHD (Kollins, 2008b). One of the early studies reporting that MPH treatment during adolescence increased the risk substance abuse and dependence (Lambert *et al*, 1998) was heavily criticized (Mick *et al*, 2000). A particular concern was that the authors did not control for comorbid conduct disorder in their subjects, which is an independent predictor for problem drug use (Burke *et al*, 2001), also see section 1.4.1 A later study found that MPH treatment during childhood was associated with a greater likelihood of ever using cocaine by adulthood only in the presence of comorbid conduct disorder (Barkley *et al*, 2003). In contrast, a study that excluded children with conduct disorder found a positive correlation between age of initiation of MPH treatment and cocaine abuse during adulthood (Mannuzza *et al*, 2008), such that lifetime rates of cocaine abuse were higher when treatment was initiated in early adolescence. Taken together, the effect of MPH treatment on the comorbidity of ADHD and cocaine abuse, appears to depend on a complex relation between age of treatment initiation and presence of comorbid conditions and the current model does not capture these complexities in its entirety.

8.5 Future directions

To complete the profile of ATO as an alternative therapeutic strategy for ADHD adolescents at a higher risk for cocaine abuse, effects of discontinuation of ATO treatments on cocaine sensitization, response inhibition capacity as well as NET function in the mPFC and OFC needs to be evaluated. Due to the non-stimulant profile, ATO may be particularly useful for individuals with ADHD and comorbid substance use disorders (Garnock-Jones *et al*, 2010). However, ATO is not efficacious in about 25% ADHD

children and 40% ADHD adults (Spencer *et al*, 2001; Surman *et al*, 2010); therefore other therapeutic options also need to be explored.

The treatment discontinuation model is clinically relevant because delayed initiation of MPH treatment has been associated with increased discontinuation of treatment (Chen *et al*, 2011). Furthermore, a recent review documented high rates of non-adherence to ADHD medications and noted peak rates of discontinuation of MPH after 5 years of treatment initiation in children and adolescents (Adler *et al*, 2010). Therefore, whether the elevated risk for cocaine abuse liability was an outcome of MPH treatment itself or of treatment discontinuation needs to be addressed in future studies. The impact of discontinuation of MPH treatment on drug abuse liability needs to be evaluated to inform new treatment strategies for ADHD individuals at an elevated risk for cocaine abuse.

Strain differences between adult SHR, WKY and WIS revealed several behavioral mechanisms that underlie the elevated cocaine self-administration in SHRs such as greater impulsivity, sensitivity to cocaine cues and context, and greater reinforcing efficacy of cocaine. Dopaminergic function in the NAc and serotonergic function in the mPFC and OFC maybe mechanisms that also contribute to the increased cocaine abuse vulnerability in ADHD individuals (Koob *et al*, 2010; Pattij *et al*, 2008) and should be evaluated in the future.

The current model of MPH treatment and discontinuation in SHR was established as a valuable model for delineating the neurobehavioral mechanisms associated the comorbidity of ADHD and cocaine abuse as well as the MPH-treatment mediated increase in future cocaine abuse liability. Based on the results of the current study, this model is recommended for future studies to identify additional neurochemical and behavioral mechanisms to assist in developing therapeutic strategies to protect individuals with ADHD from developing cocaine abuse as well as to identify measures to attenuate the risk of escalating cocaine abuse in individuals with ADHD who have been treated with methylpheniate during adolescence.

8.6 Final comments

The current study delineates the neurochemical and behavioral mechanisms associated with the increased cocaine self-administration in MPH-treated SHR. The results demonstrate that SHR were more impulsive and more sensitive to repeated cocaine administration compared to control, thus making SHR more vulnerable to cocaine self-administration and more reactive to cocaine cues, respectively. Also, SHR exhibit increased NET function in the OFC which may partially underlie the increased impulsivity and cocaine self-administration in SHR. MPH treatment during adolescence increased impulsivity and mPFC DAT function during adulthood, providing neurobehavioral mechanisms for the increased cocaine self-administration in SHR. In contrast, ATO treatment during adolescence decreased DAT function in the OFC and striatum thus providing neurochemical mechanism for reduced cocaine cue-reactivity and suggesting a decrease in impulsivity in SHRs. Also, MPH normalized NET function in the OFC and decreased locomotor activity in adult SHR, suggesting that MPH treatment during adolescence may produce some persistent neurobehavioral improvements in ADHD. Pharmacological and behavioral interventions counteracting the increased mPFC DAT function and impulsivity in MPH-treated ADHD individuals may provide protection against cocaine abuse liability. Furthermore, interventions that reduce DAT function in the PFC may provide additional protection against cocaine abuse liability in ADHD individuals.

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10 CURRICULUM VITAE

SUCHARITA SEN SOMKUWAR, B.PHARM.

Graduate Student

Department of Pharmaceutical Sciences
University of Kentucky
789 South Limestone
498 Biological Pharmaceutical Complex
Lexington, KY 40536-0596

A. Education

2008-Present	Graduate Education GPA: 4.0	University of Kentucky, Lexington, KY Advisor: Dr. Linda P. Dwoskin PhD Candidacy: December 17, 2010
2003-2007	Bachelor of Pharmaceutical Sciences GPA: 8.71/10.0	Institute of Technology, Banaras Hindu University, Varanasi, India

B. Relevant Experience

2009-Present	Graduate Student	Department of Pharmaceutical Sciences University of Kentucky, Lexington, KY Advisor: Dr. Linda P. Dwoskin
2011	Guest Instructor	Reinforcement and Addiction Advanced Behavioral Neuroscience (PSY 456), Department of Psychology University of Kentucky, Lexington, KY 30 students, 2 lectures Primary Instructor: Dr. J.S. Beckmann

2008-2009	Teaching Assistant	Department of Pharmaceutical Sciences University of Kentucky, Lexington, KY Advisor: Dr. J.Pauly, Dr. K.Kirsh
08/07 – 05/08	Research Assistant	National Center for Biological Sciences Bangalore, India Advisor: Dr. S. Chatterji
05/07 – 07/07	Summer Intern	National Center for Biological Sciences Bangalore, India Advisor: Dr. M.M. Panicker
02/07 – 04/07	B. Pharm project <i>Required for completion of the undergraduate degree</i>	Department of Pharmaceutical Sciences, Institute of Technology, Varanasi, India Advisor: Dr. Sairam Krishnamurthy
05/06 – 07/06	Summer Internship	National Center for Biological Sciences Bangalore, India Advisor: Dr. M.M. Panicker
05/05 – 07/05	Summer Internship <i>Summer Scholarship Program Indian Association for Sciences</i>	Center for DNA Fingerprinting and Diagnostics, Hyderabad, India Advisor: Dr. J. Nagaraju

C. Publications

Harvey R.C., **Sen S.**, Deaciuc A., Dwoskin L.P., Kantak K.M. Adolescent methylphenidate treatment in rats with an attention deficit/hyperactivity disorder phenotype: Cocaine addiction vulnerability and dopamine transporter function. *Neuropsychopharmacology*, 2011 March; 36(4):837-47.

Somkuwar S.S., Kantak K.M., Dwoskin L.P. Adolescence methylphenidate treatment in a rodent model of attention deficit/hyperactivity disorder: Dopamine transporter function and cellular distribution in adulthood. *Biochemical Pharmacology*, 2013 Jul; 86(2):309-16.

Somkuwar S.S., Jordan C.J., Katak K.M., Dwoskin L.P. Adolescent atomoxetine treatment in a rodent model of ADHD: Effects on cocaine self-administration and dopamine transporters in frontostriatal regions. *Neuropsychopharmacology* 2013, in press. doi: 10.1038/npp.2013.163.

Narayanaswami V., **Somkuwar S.S.,** Horton D., Cassis L.A. and Dwoskin L.P. Angiotensin AT1 and AT2 receptor antagonists modulate nicotine-evoked [3H]dopamine and [3H]norepinephrine release. *Biochemical Pharmacology* 2013 Sep; 86(5):656-65.

Manuscripts in preparation

Somkuwar S.S., Sanabria F., Beckmann J.S., Bardo M.T., Katak K.M., Dwoskin L.P. Impulsivity endophenotypes identified using differential reinforcement of low rate schedules in a rodent model of attention deficit hyperactivity disorder. To be submitted to *Neuropsychopharmacology*, 2014.

Somkuwar S.S., Katak K.M., Bardo M.T., Dwoskin L.P. Chronic methylphenidate treatment in an ADHD model during adolescence increases impulsivity that persists into adulthood. To be submitted to *Biological Psychiatry*, 2014.

Somkuwar S.S., Bardo M.T., Dwoskin L.P. Challenges in modeling the impulsivity aspect of ADHD in rats. To be submitted to *Behavioral and Brain Functions*, 2014.

D. Research Skills

Neurochemistry *in vitro*: Neurotransmitter release assays using rat striatal and hypothalamic slices; dopamine neurotransmitter uptake kinetic assays using rat medial prefrontal cortex, orbitofrontal cortex and striatal synaptosomes; competitive inhibition of dopamine neurotransmitter uptake assays using rat striatal synaptosomes.

Neurochemistry *in vivo*: Clearance of neurotransmitters - determination of transporter function from medial prefrontal cortex, orbitofrontal cortex and dorsal striatum using *in vivo* voltammetry in anesthetized rats; stereotaxic surgery.

Behavioral Psychology: Characterization of rat strains using DRL task, programming and testing operant behavioral tasks and Macros using MED-PC interface,

mathematical modeling for characterization of timing and non-timing behavior under DRL task, locomotor activity assays, psychomotor sensitization assays.

Statistical analysis: Parametric comparison using one-way and two-way analysis of variance, repeated measures analysis of variance and linear mixed model analysis; model comparison using Akieke Information Criterion.

Molecular biology and cell culture: Cell culture of embryonic stem cells; immunoprecipitation; biotinylation; gel electrophoresis; western blotting; transformation, transfection, DNA cloning and PCR.

Instrumentation: HPLC; FPLC; fluorescence and UV/Visual spectrophotometry; mass spectrometry

Others: Determination of minimum inhibitory concentration of antibiotics, bactericidal agents and disinfectants; bacterial staining techniques; biochemical characterization of bacterial species; screening of cardiac stimulants and cardiac depressants using frog heart perfusion; bioassay of acetylcholine and histamine; biochemical assays for identification and quantification of proteins, carbohydrates, amino acids, fats and nucleic acids.

E. Invited symposium

Somkuwar S.S. Age-specific effects of chronic methylphenidate treatment on impulsivity in Attention Deficit/Hyperactivity Disorder. 45th Annual Pharmaceutics Graduate Student Research Meeting, Iowa City, IA 2013

Somkuwar S.S. Comorbidity of Attention Deficit/Hyperactivity Disorder and Substance Use Disorder. Invited speaker at the Department of Pharmaceutical Sciences, Indian Institute of Technology, Banaras Hindu University, Varanasi, India 2012

Nanosymposium Session 122. ADHD, SLI, Dyslexia and Other Specific Disorders of Neurobehavior. Symposium Organizer: **Somkuwar S.S.**

Somkuwar S.S., Sanabria F., Beckmann J.S., Bardo M.T., Kantak K.M., Dwoskin L.P. Impulsivity and timing disturbances assessed by DRL schedules in a rat model of ADHD. Nanosymposium Session 122, Neuroscience 2012, Society for Neuroscience, New Orleans, LA 2012.

Somkuwar S.S. Impulsivity in an animal model of ADHD. Behavioral Neuropharmacology Seminar Series, Department of Psychology, University of Kentucky, Lexington, KY 2012.

Somkuwar S.S. Modeling impulsivity in ADHD. Center for Drug Abuse Research and Translation Trainee Research Seminar, Center for Drug Abuse Research and Translation. University of Kentucky, Lexington, KY 2012

F. Posters and Abstracts

Somkuwar S.S., Katak K.M., Bardo M.T., Dwoskin L.P. Chronic methylphenidate treatment during adolescence increases impulsivity that persists into adulthood. Neuroscience 2013, Society for Neuroscience, San Diego, CA, 2013

Somkuwar S.S., Sanabria F., Beckmann J.S., Bardo M.T., Katak K.M., Dwoskin L.P. Impulsivity in a rodent model of attention deficit/hyperactivity disorder: Impairments in response inhibition and disturbances in timing. Early Career Investigators Poster Session, NIDA Miniconvention, New Orleans, LA, 2012

Somkuwar S.S., Deaciuc A., Katak K.M., Dwoskin L.P. Adolescent methylphenidate vs. atomoxetine treatments on dopamine transporter function and expression during adulthood in an ADHD model. 74th Annual Meeting - College on Problems of Drug Dependence, Palm Springs, CA, 2012

Somkuwar S.S., Deaciuc A., Katak K.M., Dwoskin L.P. Adolescent methylphenidate vs. atomoxetine treatments on dopamine transporter function and expression during adulthood in an ADHD model. Spring Neuroscience Day, Blue Grass Society for Neuroscience, Graduate student poster session, Children at Risk Conference, Rho Chi Research Day, Lexington, KY, 2012

Sen S., Deaciuc A., Katak K.M. and Dwoskin L.P. Dopamine transporter function during adulthood in an ADHD model: adolescent methylphenidate vs. atomoxetine treatments. Neuroscience 2011, Society for Neuroscience, Washington, DC, 2011

Sen S., Deaciuc A., Katak K.M. and Dwoskin L.P. Chronic methylphenidate and atomoxetine during adolescence on dopamine transporter function in a rodent model of ADHD: Consequences of long-term treatment. Spring Neuroscience Day, Blue Grass Society for Neuroscience, Graduate student poster session, Children at

Risk Conference, Center for Clinical and Translational Sciences annual meeting, Lexington, KY, 2011

Sen S., Narayanaswami V., Horton D.B., Cassis L.A. and Dwoskin L.P. Losartan, an angiotensin II-1 receptor antagonist, inhibits nicotine-evoked [³H]dopamine and [³H]norepinephrine release from rat striatum and hypothalamus, respectively. Spring Neuroscience Day, Blue Grass Society for Neuroscience, Lexington, KY, 2010.

Sen S., Deaciuc A., Katak K.M. and Dwoskin L.P. Different long-term effect of adolescent methylphenidate and atomoxetine treatment on dopamine transporter function in rodent model of ADHD. Annual Symposium on Drug Discovery and Development, University of Kentucky, Lexington, KY, 2010.

Sen S., Narayanaswami V., Horton D.B., Cassis L.A. and Dwoskin L.P. Angiotensin AT1 and AT2 receptors modulate the effect of nicotine to evoke neurotransmitter release. Neuroscience 2010, Society for Neuroscience, San Diego, CA, 2010.

G. Awards

2013	First place	First Annual Elevator Speech Competition Department of Pharmaceutical Sciences University of Kentucky
2013	Travel Award	Bluegrass Chapter of Society for Neuroscience
2013, 2011 and 2010	Poster Award Graduate student category	Spring Neuroscience Day Bluegrass Society for Neuroscience University of Kentucky
2012	Travel Award Early Career Investigators poster session	“Frontiers in Addiction Research” NIDA Miniconvention
2012	First Place Poster Award	Graduate Student Poster Session Rho Chi Research Day
2011-12	Kentucky Opportunity Fellowship	DGS- nominated graduate student fellowship

2012, 2011 and 2010	Travel Award	Student Support, Graduate School, University of Kentucky
2011	First Place Poster Award	Graduate Student Poster Session Children at Risk Conference, 2011 University of Kentucky
2007	Salutatorian Graduating class of 2007	Department of Pharmaceutical Sciences, Institute of Technology, Banaras Hindu University
2006	Institute Color Institute of Technology Banaras Hindu University	For achievements and contributions to sports activities while representing IT- BHU in the academic year 2005-06
2006	Merit Scholarship	For outstanding academic performance during the academic year 2005-06
2005	Summer Research Scholarship	Indian Academy of Sciences
2002	Best All-Round Student Certificate of Honor	Shaw Education Foundation Award, School Awards for Excellence, The Telegraph, Kolkata, India

H. Services

2013 2012	Graduate Student Representative	Drug Discovery and Development Symposium committee
2012	AV team staff	74 th Annual Meeting - College on Problems of Drug Dependence, Palm Springs, CA 2012
2010-11 2009-10 2008-09	Event Coordinator Treasurer Treasurer	American Association of Pharmaceutical Scientists, University of Kentucky Student Chapter

I. Professional Affiliations

2008 – Present	American Association of Pharmaceutical Scientists (AAPS)
2008 – Present	AAPS Student Chapter, University of Kentucky
2009 – Present	American Association for the Advancement of Sciences (AAAS)
2010 – Present	Bluegrass Society for Neuroscience
2010 – Present	Society for Neuroscience (SFN)
2010 – Present	The Honor Society of Phi Kappa Phi

K. Research Presentations

2013	Chronic methylphenidate treatment during adolescence increases impulsivity that persists into adulthood. Graduate Student and Post-doctoral Fellow Presentations, Annual Symposium on Drug Discovery and Development, University of Kentucky, Lexington, KY (October)
2013	Effects of methylphenidate treatment on impulsivity and sensitization to cocaine in an animal model of ADHD. Departmental Seminar, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky (January)
2012	Modeling impulsivity aspect of ADHD in rodents. Departmental Seminar, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky (March)

- 2011 Effect of chronic methylphenidate on dopamine transporter function and expression in a rat model of ADHD. Graduate Student Presentations, Annual Symposium on Drug Discovery and Development, University of Kentucky, Lexington, KY (October)
- 2011 Methylphenidate treatment in adolescence and effects on impulsivity Departmental Seminar, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky (March)
- 2010 Effect of a stimulant ADHD medication on dopamine transporter (DAT) function in an animal model of ADHD Departmental Seminar, Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky (April)
- 2007 Study of anxiolytic and anti-ulcer activity of risperidone Departmental Seminar, Department of Pharmaceutical Sciences Institute of Technology, Banaras Hindu University, Varanasi, India, (May)
- 2006 Quantification of serotonin in embryonic stem cells using HPLC-FD National Centre for Biological Studies, Tata Institute of Fundamental Research Bangalore, India, (July)
- 2005 Standardization of expression of silkworm lysozyme in E. coli Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India (July)

L. References

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