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PRECLINICAL EVALUATION OF LOBELINE FOR THE TREATMENT OF ADHD: COMPARISON WITH PSYCHOSTIMULANT THERAPIES

Yolanda D. Williams
University of Kentucky, ydw9696@gmail.com

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Yolanda D. Williams, Student

Dr. Linda P. Dwoskin, Major Professor

Jim Pauly, Ph.D., Director of Graduate Studies

PRECLINICAL EVALUATION OF LOBELINE FOR THE TREATMENT OF ADHD: COMPARISON WITH PSYCHOSTIMULANT THERAPIES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
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By
Yolanda D. Williams

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Director: Dr. Linda P. Dwoskin, Professor of Pharmaceutical Sciences

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ABSTRACT OF DISSERTATION

PRECLINICAL EVALUATION OF LOBELINE FOR THE TREATMENT OF ADHD: COMPARISON WITH PSYCHOSTIMULANT THERAPIES

This dissertation work investigated the effect of acute and repeated in vivo administration of lobeline on dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2) function. The effects of lobeline were then compared to the effects of acute and repeated in vivo administration of methylphenidate and amphetamine to determine if lobeline produced similar effects compared to these Attention Deficit Hyperactivity Disorder (ADHD) medications. These medications are considered the first line of pharmacotherapy for ADHD, although there is a growing concern associated with their potential for abuse and other side effects. This merits the need for novel ADHD treatments that have a safer side effect profile. If lobeline alters DAT and VMAT2 function in the same way as methylphenidate or amphetamine, further investigation may be necessary to evaluate lobeline as a potential treatment for ADHD. Kinetic analysis of [^{3}H]dopamine (DA) was utilized to determine the effect on DAT and VMAT2 function in rat striatum. Results from the DAT experiments, revealed that lobeline as well as amphetamine had no effect on DAT function. However, methylphenidate increased DAT function after acute and 7-day treatment. None of the drug treatment regimens altered K_m. To determine if the methylphenidate-induced increase in DAT function was due to DAT trafficking, biotinylation and Western blot analyses were performed. Acute administration of methylphenidate did not alter surface DAT, however repeated administration of methylphenidate for 7 days decreased intracellular DAT, suggesting that methylphenidate redistributes DAT in a time-dependent manner. Similar results were found in the VMAT2 experiments. Lobeline and amphetamine had no effect on VMAT2 function after acute or repeated administration. Amphetamine decreased the K_m after repeated administration for 7 days. Methylphenidate increased VMAT2 function after acute and repeated administration for 7 days. The overall results of these experiments suggest that methylphenidate interacts with DAT and VMAT2 in a different manner than amphetamine and lobeline. In addition, since lobeline and amphetamine had no effect on DAT and VMAT2 function, further investigation is warranted to elucidate the underlying mechanisms of the therapeutic actions of these agents. This additional information will aid in the development of novel treatments for ADHD.

Keywords: lobeline, methylphenidate, amphetamine, striatum, ADHD, dopamine transporter, vesicular monoamine transporter

Yolanda D. Williams
Student's Signature

October 25, 2011__
Date

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By

Yolanda D. Williams

Jim Pauly, Ph.D._____
Director of Graduate Studies

Linda P. Dwoskin, Ph.D.____
Director of Dissertation

October 25, 2011_____

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Hebrews 12:1b-3
“And let us run with perseverance the race marked out for us, fixing our eyes on Jesus, the pioneer and perfecter of faith. For the joy set before Him He endured the cross, scorning its shame, and sat down at the right hand of God. Consider Him who endured such opposition from sinners, so that you will not grow weary and lose heart.”

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Chapter One

Introduction and Background

A. Attention-Deficit Hyperactivity Disorder

Attention-Deficit Hyperactivity Disorder (ADHD) is defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) as primarily a childhood psychiatric disorder; however, 30-60% of childhood cases continue into adulthood (American Psychiatric Association, 2000; Himelstein & Halperin; 2000). Historically, this disease was labeled a “mental restlessness or a disease of attention”, specifically in 1798 by Sir Alexander Crichton, a Scottish author and physician (Palmer & Finger, 2001). However, Sir George Fredrick Still, a British pediatrician, is most well-known for describing in 1902 the clinical manifestations of ADHD, based on his observations of over 40 children with serious problems of sustained attention and self-control. Sir Still described the behavior of these children as a “quite abnormal incapacity for sustained attention” (Still, 1902). Over the years, the classification of this condition has evolved from hyperkinetic reaction of childhood disorder to attention deficit disorder in 1980 to ADHD, which includes the hyperactivity component of the behavior (American Psychiatric Association, 1987).

1. Prevalence

ADHD is a major clinical and public health problem due to its disruptive nature in school-age children, and rising prevalence and impact on the economy (Meijer et al., 2009). Research has shown that boys are 3 times more likely to have ADHD compared to girls (Barkley et al., 1990; Faraone et al., 2003). The world-wide prevalence of ADHD is 5-12% and 3-5% in children and adults, respectively, in the United States, depending on the diagnostic criteria utilized (Brown et al., 2001b; Faraone et al., 2003; Kessler et al., 2005; Kessler et al., 2006; Polanczyk et al., 2007). The estimated mean annual cost for a child/adolescent with ADHD in the United States is $14,576 which includes health and mental healthcare costs ($2,636), education ($4,900), and crime and

delinquency ($7,040) with a range of $12,005 to $17,458 (Pelham et al., 2007). The overall cost of illness based on the 2000 census of 60 million school-aged children was estimated to be $42.5 billion annually, of which $7.9 billion is health and mental healthcare costs, $13.6 billion is educational cost, and $21.1 billion is crime and delinquency costs (Pelham et al., 2007). Based on the prevalence in adults, the estimated cost of ADHD is between $36-77 billion, which is close to the costs associated with alcohol abuse ($85 billion); greater than the costs associated with drugs of abuse such as cocaine and marijuana ($58 billion);and greater than costs associated with depression ($43 billion) (Pelham et al., 2007; Reinberg, 2004). Furthermore, the National Health Statistics Report estimated that there were as many as 7 million ambulatory care visits for ADHD in 2006 (Schappert, 2008). In addition, a study that included 10 countries concluded that adult ADHD was associated with 143.8 million lost days of productivity each year (de Graaf et al., 2008) and another study reported a work loss cost of adults with ADHD that is approximately $3.7 billion in the US (Birnbaum et al., 2005). Taken together, it is clear that ADHD has a huge economic impact on the health and economy for the US, as well as the world.

2. Etiology

The etiology of ADHD is unknown; however, there has been an enormous amount of research conducted on this topic. Generally, the overall conclusion is that the etiology of ADHD is complex and involves a combination of factors. These factors include genetics, prenatal exposure to tobacco smoking, or alcohol, or to both tobacco smoking and alcohol simultaneously (Betel, 1995; Dawson, 2000; Mayfield, 2008).

As stated above, an important factor is genetic predisposition. Twin and adoption studies show that genes play a vital role in the familial transmission of ADHD (Biederman & Faraone, 2005). For example, adoptive relatives are less likely than biological relatives to have the disorder or associated symptoms (Sprich et al., 2000). Numerous family studies have shown that there is a genetic link to ADHD, such that 25-33% of parents of ADHD children have ADHD themselves (Cormier, 2008). Specifically, variants of genes associated with

dopamine (DA) neurotransmission have been investigated most commonly with respect to the etiology of ADHD (Faraone & Khan, 2006). These polymorphic genes include the DA D4 receptor gene (DRD4) (Benjamin et al., 1996; Gabriela et al., 2009; Rubinstein et al., 1997; Tahir et al., 2000), the DA D_5 receptor gene (DRD5) (Hawi et al., 2005; Manor et al., 2004; Mill et al., 2004; Tahir et al., 2000), and the DA transporter gene (Barr et al., 2001; Cook et al., 1995; Giros et al., 1996; Hawi et al., 2009; Henriquez et al., 2008; Hiongwa et al., 2004).

Research has been conducted on ADHD using D4 knock-out (D4KO) mice. D4KO mice are genetically altered mice that have had the D4 receptor gene deleted. D4KO mice were found to have a 32% decreased basal locomotor activity compared to wild-type (Rubinstein et al., 1997). In addition, this study revealed that the D4KO mice were more sensitive to cocaine (15 and 30 mg/kg) and methamphetamine (1 and 2 mg/kg) administration, as demonstrated by a two-fold increase in stimulant response in D4KO mice compared to WT. These results suggest that the DRD4 gene is involved in hyperactivity, an ADHD hallmark symptom, as well as response to psychostimulants that are used as treatments for ADHD.

To follow up on the results of this preclinical study employing D4KO mice, relationships between the DRD4 exon III sequence variants and personality test scores were evaluated in 315 mostly male siblings and other family members of individuals with ADHD (Benjamin et al., 1996). An association was found between the DRD4 variants and the novelty seeking personality trait in relatives of the ADHD individuals. Another study examined the association of the DRD4-7 repeat (DRD4-7r) allele with novelty seeking in a sample of 303 15-year old adolescents (144 males and 159 females) using data from a high-risk community sample (Becker et al., 2005). Males with the DRD4-7r allele polymorphism scored significantly higher with respect to the novelty seeking personality trait ($p=0.002$) of the Junior Temperament and Character Inventory and no association was found in females. However, a meta-analysis of 20 studies conducted by Kluger et. al., 2002, found no association between the DRD4 polymorphism and novelty seeking. A meta-analysis combines the results of

several studies that address a set of related research hypotheses. In its simplest form, this is normally by identification of a common measure of effect size, for which a weighted average might be the output of a meta-analyses. Here the weighting might be related to sample sizes within the individual studies. More generally there are other differences between the studies that need to be allowed for, but the general aim of a meta-analysis is to more powerfully estimate the true "effect size" as opposed to a smaller "effect size" derived in a single study under a given single set of assumptions and conditions. It is only a statistical examination of scientific studies, not an actual scientific study, itself.

Nevertheless, the presence of high variability of the association between DRD4 polymorphism and novelty seeking suggests that there are unknown causes for observing weak to moderate positive effects in some studies. This issue could be addressed more fully by utilizing more advanced statistical techniques, high-through output genotyping and large numbers of polymorphoric markers such as single nucleotide polymorphisms.

With respect to the DRD5 gene polymorphism, a transmission disequilibrium test (TDT) was used to evaluate the linkage of the DRD5 gene variant and ADHD (Hawi et al., 2005). ADHD was associated with over transmission of the variant paternal alleles of both DRD5 and DRD4 genes. Another study using TDT found only a trend for an association with the DRD5 gene, but a significant increase in transmission of the DRD4-7r allele in ADHD individuals (Tahir et al., 2000). Also, the role of DRD5 gene in ADHD individuals was studied by genotyping ADHD families and searching for the 148 base pair allele of DRD5 (Manor et al., 2004). A preferential transmission of this variant DRD5 gene was confirmed using TDT with the Family Based Association Test, suggesting that the DRD5 repeat polymorphism confers a small, but significant ($p=0.037$) risk for ADHD (Manor et al., 2004). Taken together, the results of these studies suggest that the DRD5 gene also plays a role as a genetic factor contributing to ADHD, which needs to be further evaluated.

The DAT1 gene is another gene that has been found to be associated with ADHD. The association between the presence of ADHD and the 480-bp DAT1

allele was determined in 49 subjects with ADHD based on DSM-IV criteria using the haplotype-based haplotype relative risk (HHRR) method to avoid effects associated with the small sample of subjects with regards to population stratification (Cook et al., 1995). This study found a significant association between the 480-bp DAT1 allele and ADHD. As a preclinical correlate, a disruption by homologous recombination of the mouse DAT gene knock-out (DAT-KO) resulted in 3-fold greater spontaneous hyperactivity, demonstrated as immediate hyperactivity when placed in a chamber with no treatment given, compared to heterozygotes and WT mice. Since hyperactivity is a hallmark symptom of ADHD, the DAT gene was suggested to be involved in the etiology of ADHD (Giros et al., 1996). Another study investigated the variable number tandem repeats (VNTR) polymorphism of DAT1, which is a location on the genome where a short nucleotide sequence is organized as a tandem repeat, where a pattern of 2 or more nucleotides is repeated and the repetitions are directly adjacent to one another (Barr et al., 2001). To determine if the VNTR was associated with ADHD; 333 subjects from 102 nuclear families were genotyped. A linkage of the 480-bp allele of the DAT1 gene with ADHD was found, suggesting that molecular analysis of the DAT gene may identify mutations that increase the risk for ADHD.

The utilization of pharmacogenomics, which is a branch of pharmacology involving the influence of genetic variation on drug response by relating gene expression or polymorphisms with a drug's efficacy or toxicity, may be a potential method in developing a gene therapy for ADHD. For example, pharmacogenomics is used in cancer research to determine which patient will have the best response to a certain treatment. Hopefully, in the same way biochemical analysis of these mutations in specific patients could lead to the development of more efficacious therapeutic treatment options for individuals with ADHD.

In addition to DAT, the norepinephrine transporter (NET) gene is of interest due to the efficacy of atomoxetine, a selective norepinephrine uptake inhibitor, as a treatment for ADHD. In addition, NET is primarily responsible for

the reuptake of DA in the frontal cortex due to the low density of DAT in this brain region. In one study, 184 unrelated males (mean age 34.1 yrs) with adult ADHD were assessed according to the International Classification of Diseases (ICD-10), 10^{th} revision: Clinical Modifications, 6^{th} (Greydanus et al., 2007), criteria for ADHD (Retz et al., 2008). The results of this study found negative evidence of an association between the NET gene and ADHD. However, this result could be due to the fact that this was a study of adults in Germany with ADHD who may have less severe ADHD symptoms compared to adolescents; such that the age of the subjects and location of the study could play a role in the results. In contrast, Kim et al., (2006, 2008) observed in two separate studies a significant association between the polymorphism of the NET gene and ADHD, suggesting that anomalous transcription factor-based repression of NET may increase risk for the development of ADHD (Kim et al., 2006; Kim et al., 2008). Given the controversial results found in the investigation of the NET gene in regards to the etiology of ADHD, it is difficult to draw any conclusions as to its association with ADHD.

Based on the evidence provided, genetics may play a vital role in the etiology of ADHD. There is still much to be determined in regards to which specific polymorphisms are more responsible for the development of ADHD, however, future advances that will be made in this area will help gain a better understanding of the role that genetics play in the etiology of ADHD.

Other studies have shown that environmental conditions such as prenatal exposure to alcohol and tobacco smoking may be associated with the development of ADHD in childhood (Linnet et al., 2003; Mick et al., 2002; Milberger et al., 1996; Milberger et al., 1998). One study assessed 1,452 twin pairs and found that maternal smoking shows an association with ADHD symptoms in the offspring (Thapar et al., 2003). A meta-analysis on literature published before 2005 revealed a pooled odds ratio indicating more than a 2-fold increase in the risk for ADHD in individuals whose mothers smoked tobacco during pregnancy (Langley et al., 2005), suggesting that maternal smoking during pregnancy is a risk factor for ADHD. With respect to alcohol exposure, one

study assessed 68 children who were born into three separate groups, women who reported not drinking during pregnancy, women who reported drinking during pregnancy, and women who reported drinking an equivalent amount, but who stopped after an educational intervention during the second trimester (Brown et al., 1991). The results showed those children who were exposed to alcohol throughout pregnancy showed deficits in the ability to sustain attention. Another study analyzed a twin cohort consisting of 922 children with a history of maternal alcohol use (Knopik et al., 2006). The study used a children-of-twins design, which is a design that incorporates twins in order to explore the effects of genetics and environmental variance on a phenotype. In addition, diagnostic telephone interview data from high-risk families and control families targeted from a large Australian twin cohort were employed. The calculated odds ratio (OR=2.53) suggested that children exposed to prenatal alcohol were two-times more likely to exhibit ADHD compared to those not exposed to prenatal alcohol. The findings from the aforementioned studies imply that maternal alcohol and tobacco use may play a key role in the etiology of ADHD.

Evidence has shown that these risk factors of maternal tobacco and alcohol use may be important etiologies of ADHD. These risk factors may work in concert with genetic factors, in that the exposure to nicotine and alcohol may interact with genetic polymorphisms, making these children more at risk for ADHD. In contrast, there are children who were not exposed prenatally to nicotine or alcohol that have ADHD. With that in mind, further research is necessary to fully address the complexity of the etiology of ADHD.

3. Symptomology and Diagnosis

ADHD presents as a myriad of symptoms including the hallmarks, hyperactivity, impulsivity and inattention, with 30-60% of childhood cases extending into adulthood (American Psychiatric Association, 2000; Himelstein & Halperin, 2000). The specific symptoms listed as diagnostic criteria for ADHD in the DSM-IV-TR include:

1) Six or more of the following symptoms of inattention have persisted for at least 6 months to a degree that is maladaptive and inconsistent with the developmental level:

a) Often fails to pay close attention to details or makes careless mistakes in schoolwork, work, or other activities

b) Often has difficulty sustaining attention in tasks or play activities.

c) Often does not seem to listen when spoken to directly

d) Often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (not due oppositional behavior or failure to understand instructions)

e) Often has difficulty organizing tasks and activities

f) Often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (such as schoolwork or homework)

g) Often loses things necessary for tasks or activities (i.e., toys, school assignments, pencils, books, or tools)

h) Is often easily distracted by extraneous stimuli

i) Is often forgetful in daily activities

2) Six or more of the following symptoms of hyperactivity/impulsivity have persisted for at least 6 months to a degree that is maladaptive and inconsistent with the developmental level:

a) Often fidgets with hands or feet or squirms in seat

b) Often leaves seat in classroom or in other situations in which remaining seated is required

c) Often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings of restlessness)

d) Often has difficulty playing or engaging in leisure activities quietly
e) Is often “on the go” or often acts as if “driven by a motor”
f) Often talks excessively
g) Often blurts out answers before questions have been completed
h) Often has difficulty waiting
i) Often interrupts or intrudes on others (butts into conversations or games)

(American Psychiatric Association, 2000; Greydanus et al., 2007)

Some of the hyperactive-impulsive or inattentive symptoms that cause impairment were present before 7 years of age. In addition, some impairment from the symptoms is observed in 2 or more settings (i.e., at school, work, or at home). Due to the complex symptomology, ADHD has been divided into three subtypes: a) predominantly inattentive (known as Attention-Deficit Disorder (ADD), b) predominantly hyperactive/impulsive, and c) combined hyperactive/impulsive and inattentive subtypes, to help obtain a more accurate diagnosis and treatment of the disorder (American Psychiatric Association, 2000). The inattentive subtype is characterized by inattention, distractibility, disorganization, forgetfulness, and lethargy. The hyperactive-impulsive subtype includes symptoms of interrupting, impatience, and fidgetiness. The combined subtype is a combination of both the inattentive and hyperactive-impulsive subtypes (Tamm et al., 2001). Dividing ADHD into these subtypes is helpful with respect to treatment, because ADHD symptoms are unique to each patient depending on their specific subtype and their individual response to the medication. Russell Barkley, a renowned ADHD researcher believes that methylphenidate is more effective in treating ADHD and that amphetamine is better suited for the treatment of ADD. This is based on the mechanism of action of amphetamine causing it to have a greater effect on norepinephrine than

dopamine, therefore, having more of an effect on the inattentive symptom (Barkley, 2001).

Impulsivity and sensation seeking are personality traits that have been linked together for various reasons (Nower & Blaszczynski, 2006). Impulsivity is defined as “the failure to resist an impulse, drive, or temptation to perform an act that is harmful to the person or others” (American Psychiatric Association, 2000). Sensation seeking is defined as “the seeking of varied, novel, complex and intense sensations and experiences, and the willingness to take physical, social, legal, and financial risks for the sake of such experiences (Zuckerman, 1994). Adolescence appears to be a critical period of development for these traits. Two systems that are involved in the development of adolescent behavior are the subcortical socioemotional system and the cognitive control system (Nower & Blaszczynski, 2006). The subcortical socioemotional system includes the ventral striatum and the amygdala, which is responsible for emotion, novelty, and reward. The cognitive control system includes the prefrontal cortex, which is crucial for impulse control, emotion regulation, and decision-making. These systems mature at different time points throughout the course of development. For example, the socio-emotional system appears to become more sensitive in early adolescence, during the same time of the onset of puberty. However, the cognitive control system develops more gradually during the end of early adulthood (Casey et al., 2005). Therefore, adolescents may experience an increased responsiveness to rewards, affective cues, and novelty while still having immature capacities for impulse control and inhibition. A recent report conducted in humans found that mean levels of impulsivity were found to decline through adolescence and then plateau as youth reached their mid-20s; however, mean levels of sensation seeking were found to increase profoundly until mid-adolescence, peaking around the age of 16 and then slowly decreasing through the mid-20s. Various experimental methods have been developed to examine impulsivity and sensation seeking in an animal model; however, most of these methods have only used a single task as the predictor variable. A novel study determined if measuring multiple behavioral tasks simultaneously would be

useful for characterizing aspects of sensation seeking and impulsivity as predictor variables for amphetamine self-administration (Marusich et al., 2011a). This study found that combining these predictor variables into a multivariate approached failed to produce any significant correlations among predictor and outcome measures. The results imply that multivariate tests are possibly insensitive as reliable predictors of drug self-administration, which could represent a limitation of animal models for assessing drug abuse vulnerability, at least for this particular study.

In conclusion, impulsivity and sensation seeking are human traits that are multifaceted and require a great deal of skill to evaluate. Furthermore, assessing these traits in an animal model is even more of a challenge based on the difference in life span between and human and a rat. In addition, impulsivity and sensation seeking are human traits that may not be fully modeled in laboratory animals. Studies have shown a relationship between impulsivity and sensation seeking, however more research is warranted to determine the extent of this relationship.

Evaluation and diagnosis of ADHD are subjective because there are no objective diagnostic measurements to test for ADHD. However, there are numerous specific diagnostic criteria of ADHD available to assist clinicians in the diagnosis. The overall desired outcome of assessment criteria is to determine if a child or adult meets the qualifications for ADHD, which may rule out other conditions (Liu & Leslie, 2003). Other medical, psychiatric, or developmental disorders must be ruled out before the presence of developmentally inappropriate levels of inattention and/or hyperactivity/impulsivity can be diagnosed as ADHD (American Psychiatric Association, 2000). There are three commonly used diagnostic tools, including the DSM-IV-TR, Classification of Child an Adolescent Mental Diagnosis in Primary Care: Diagnosis and Statistical Manual for Primary Care (DSM-PC), and the ICD-10 (Greydanus et al., 2007). The American Psychiatric Association (APA) publishes the DSM-IV-TR and gives the most detailed list of symptoms by classification with diagnostic codes and criteria used to evaluate the disorder as stated previously. These methods for

diagnosis were developed based on different settings that patients are evaluated in, and generally are similar. Therefore, the most appropriate assessment method is chosen based on setting. The DSM-PC, intended for primary care environments, uses a developmental approach to diagnosis, and distinguishes between variations, problems, and disorders. The ICD-10 uses the term attention-deficit/hyperkinetic disorder, which is commonly used by insurance companies (Greydanus et al., 2007). Questionnaires and rating scales, such as the Connors ADHD Index and the DSM-IV Symptoms scales have been suggested by the American Academy of Pediatrics (Forsback et al., 2004) as guidelines to ensure accurate diagnosis (Tripp et al., 2006). The Connors ADHD Index has various different versions based on who is taking the assessment (Conners, 1999). For example, there is the Conners' Parenting Rating Scale Revised-Long Version (CPRS-R:L). This version contains 80 items with 10 different scales for the parents or caregivers to complete. The short version (CPRS-R:S) contains only 27 items and 4 scales. There is also the Conners-Well's Adolescent Self-Report Scale-Long Form (CASS:L). This self-report scale, which is ideal for adolescents between the ages of 12 and 17, contains 87 items and 8 scales. There is also an assessment for adults called the Conner's Adult ADHD Rating Scale-Self Report: Short Version (CAARS-S:S). It contains 26 items that quantitatively measure ADHD symptoms while looking at the manifestations of ADHD in adults. Subjects read descriptive statements that they may, or may not, be presently experiencing (i.e., I have trouble sitting still). Subjects rate each statement on a four-point Likert scale ranging from "Not at all" to "Very much". CAARS subscales include: A = Inattention/Memory Problems, B = Hyperactivity/Restlessness, C = Impulsivity/Emotional Reliability, D = Problems with Self-Concept and E = Total ADHD Index. In summary, there are several tools that clinicians can utilize to ensure accurate evaluation and diagnosis of ADHD.

4. Neuroanatomical and Neurochemical Basis of ADHD

a. Neuroanatomy

The neuroanatomical and neurochemical basis of ADHD is based on the general consensus that there is a dysregulation of the catecholaminergic neurotransmitter systems (Heal et al., 2008). Neuroimaging studies have suggested that brain regions, including the prefrontal cortex (PFC) and the basal ganglia (striatum, caudate nucleus and putamen) are the regions with dysregulation of neurotransmission in ADHD (Casey et al., 2007). The PFC and striatum are the most widely studied regions regarding ADHD, because these regions are involved in behavior, attention, motor control and cognition (Arnsten, 2006; Kieling et al., 2008). Specifically, motor control is modulated by the nigrostriatal DA pathway projecting from the substania nigra to the basal ganglia (Stanwood & Zigmond, 2000). The mesolimbic pathway, projecting from the ventral tegmental area (VTA) to the subcortical limbic regions (nucleus accumbens, olfactory tubercle and amygdala) is associated with motivated behavior (Ikemoto & Panksepp, 1999) and reinforcement (Schultz et al., 2000). The mesocortical DA pathway projects from the VTA to the PFC and is involved in cognitive function (Floresco & Magyar, 2006).

Noradrenergic neurons originate both in the locus coeruleus and the lateral tegmental area. Norepinephrine containing cell bodies are only found in the pons and medulla. However, the pontine norepinephrine-containing cells which terminate in the nucleus locus coeruleus, give rise to extensive projections to the hypothalamus, thalamus, limbic regions, and cortex. Medullary norepinephrine cells project to the hypothalamus, locus coeruleus, and spinal cord (Rinaman, 2011).

b. Neurochemistry

i. DA synthesis and metabolism

Research on the neurochemical basis of ADHD has focused primarily on dopaminergic neurotransmission and function (Figure 1). DA is transported by DAT and pharmacological agents that influence the DA system via DAT have been the most effective treatments for ADHD (Zhu & Reith, 2008). DA is a

catecholamine involved in behavior and cognition; voluntary movement, motivation and reward (Jaber et al., 1996). The synthesis of DA takes place in DA neurons, starting with L-tyrosine which comes from the essential amino acid, phenylalanine, which is derived from food. L-Tyrosine is converted to L-dihydroxyphenylalanie (L-DOPA) via an enzyme located in the cytosol called tyrosine hydroxylase (TH), which is the rate limiting step in the synthesis of DA (Cooper et al., 2003). The co-factors for tyrosine hydroxylase include tetrahydrobiopterin, O^2, and Fe^{2+} (Cooper et al., 2003). Enzyme activity is regulated by phosphorylation at four different serine sites at the N-terminus and through the end-product inhibition and through competition for the required cofactors for the enzyme (Cooper et al., 2003). L-DOPA is then decarboxylated by aromatic L-amino acid decarboxylase (also located in the cytosol), which produces DA. The co-factor for this enzyme is pyridoxal 5-phosphate and is regulated by induction of synthesis of new protein rather than changes in activity (Squire LR, 1999). After DA is synthesized, it is stored in synaptic vesicles for future release from the terminal into the synapse. An action potential causes depolarization of the nerve terminal, which in turn causes the synaptic vesicles to fuse to the synaptic membrane and release DA into the synaptic cleft (Cooper et al., 2003; Squire LR, 1999). Specifically, Na^+ ions flow into the cell and K^+ channels open allowing the flow of K^+across the membrane causing the membrane to become depolarized. There is a rise in Ca^{2+} concentration that triggers the fusion of the vesicles with the plasma membrane and the release of their contents into the synaptic cleft. This is the physiological process known as exocytosis (Squire LR, 1999).

DA is inactivated by two enzymes, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) that are located in the postsynaptic neuron and outer mitochrondrial membrane, respectfully. The pathway for the metabolism of DA by MAO involves the conversion of DA to 3, 4-dihydroxyphenylacetaldehyde. Then, this product is converted to dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase. Subsequently, DOPAC is converted by COMT to form homovanillic acid (HVA). Another possible pathway is for DA to be converted to

3-methoxytyramine (3-MT) by COMT. 3-MT is then converted to 3-methoxy-4-hydroxyphenylacetaldehyde, which is then converted to homovanillic acid (HVA) by aldehyde dehydrogenase.

ii. DA receptors

In the synapse, DA has access to both presynaptic and postsynaptic DA receptors to which it binds. DA acts at both the D1-like (D1 and D5) and D2-like (D2, D3, D4) receptor families (Arnsten, 2006). The amino acid (AA) sequence for the entire family ranges from 387 AA (D4) – 477 AA (D5). The main structural differences between the D1-like and D2-like receptors are the intracellular loop between the 6^{th} and 7^{th} transmembrane domain (TMD) segments which are larger in the D2-like receptors. D2 receptors have a smaller C-terminal in the intracellular segments after the 7^{th} TMD segment. There are two isoforms of the D2 receptor, D2-long and D2-short. The D2-long receptor has 444 AA in rats and 443 AA in humans (Cooper et al., 2003). The D2-short has 415 AA in rats and 414 AA in humans. D1 receptors have a high density in striatum and nucleus accumbens and their activation stimulates adenylate cyclase to produce cyclic adenosine monophosphate (cAMP). D5 receptors are mostly located in hippocampus and hypothalamus, but are found also in striatum and nucleus accumbens to a lesser extent. The D5 receptor, which also stimulates adenylate cyclase, is only located in the hippocampus, thalamus, and hypothalamus. D5 receptors have a 10-fold higher affinity for DA compared to D1 receptors (Cooper et al., 2003).

The D2 receptor family includes the D2 subtype, which are both an autoreceptor located presynaptically and a postsynaptic receptor. The release of DA regulates the synthesis of DA by stimulating the DA receptors which can modulate the synthesis of DA via a negative feedback, such that when there is a decrease in DA, the DA receptors are stimulated to produce more DA (Cooper et al., 2003). A greater number of D2 receptors are located on the postsynaptic membranes, compared to presynaptic D2 receptors. Postsynaptic D2 receptors are located in striatum, nucleus accumbens, olfactory tubercle, and neuron cell bodies in substantia nigra and VTA D1 and D2 receptors have opposite effects

on adenylate cyclase activity. Adenylate cyclase catalyzes the conversion of ATP to c-AMP and pyrophosphate via a G-protein, which signals a downstream cascade of events, including the activation of protein kinase A (PKA). Recently,methylphenidate was suggested to modulate D2 receptors, thus indicating the involvement of this receptor in the mechanism of action of methlyphenidate (Volz et al., 2008). D3 receptors are localized in the nucleus accumbens, olfactory tubercle, and hypothalamus. The D4 receptors are found in frontal cortex, midbrain, and amygdala.

iii. DAT

After DA binds to its receptors, it is then transported back into the terminal by DAT and is transported into vesicles by the vesicular monoamine transporter (VMAT2) for repackaging and future exocytotic release (Hiongwa, 2004). DAT plays a vital role in the function of DA. DAT is among the family of 12 transmembrane domain (TMD; Figure 2) neurotransmitter transporter sodium symporter class (Saier, 1999), which also includes the norepinephrine transporter (NET) and the serotonin transporter (SERT). DAT is a Na^+/Cl^- dependent transmembrane transporter protein also called the neurotransmitter sodium symporter (NSS) that regulates the extracellular DA concentration (Amara & Sonders 1998; Krause et al., 2003). DAT contains 620 amino acid residues, and currently, there is no X-ray crystal structure of DAT. However, the recently published crystal structure of *Aquifex aedicus* leucine transporter ($LeuT_{Aa}$), allows researchers to have a suitable template for DAT because this prokaryotic organism processes a NSS that is homologous to the human NSS (Yamashita et al., 2005). $LeuT_{Aa}$ produced crystals which were determined by multi-wavelength anomalous dispersion (MAD), using crystals grown from selemethionine-labeled protein and diffraction data measured to Bragg spacings of 1.9A°. A more recent study employed a novel computational modeling approach, the Molecular Operating Environment program MOE 2005.06 and to two other modeling servers, using LeuT-$_{Aa}$ as a template, to expand upon the proposed molecular structure of DAT. As shown in Figure 2, the DAT model that was developed suggests, like the $LeuT_{Aa}$, that TMDs 3 and 8 combine with TMDs 1 and 6 to form

the substrate binding pocket. In addition, this model implies favorable interactions for substrate recognition between the 3rdTMD, and the valine amino acid, number 152 side chain and either the aromatic ring or the lipophilic hydrocarbon portion of both DA and amphetamine (Indarte et al., 2008).

DAT antibodies, which were all used for immunolocalization of DAT in rat brain at the light microscopic level, were characterized and developed by immunoblot analysis, immunoprecipitation, and immunocytochemistry to specifically detect DAT proteins. DAT is mainly expressed in the striatum and nucleus accumbens, but can also be found in the globus pallidus, cingulated cortex, olfactory tubercle, amygdala, and midbrain (Ciliax et al., 1995).

DAT-like immunoreactivity has been detected in striatum, nucleus accumbens, olfactory tuberacle, nigrostriatal bundle, which is a group of nerve fibers and lateral habenula (Hersch et al., 1997). The specific localization of DAT on the synaptic terminal has been studied using dual localization of DAT and TH with electron microscopic immunocytochemistry in the rat ventral pallidum (Pickel et al., 2004). Electron microscopy confirmed that the majority of DAT and TH were located in axonal profiles in the ventral pallidum. These findings were the first to provide structural evidence showing that DAT is localized to the axonal profiles with a significantly higher mean area density in the dorsolateral ventral pallidum than the ventromedial ventral pallidum. The pallidum is important because it is a major element of basal ganglia, which includes the striatum, which is also the region used in the studies of this dissertation research.

There has been a wealth of research performed on DAT knock-out (KO) mice as well (Fauchey et al., 2000; Gainetdinov & Caron, 2003; Giros et al., 1996; Hall et al., 2009; Jones et al., 1999; Jones et al., 1998a; Rocha et al., 1998). This research was performed in order to gain a better understanding of the function of DAT and to determine how the dopaminergic system acts in the absence of DAT. One report found that despite the decreased amount of DA in tissue, DAT KO mice have more than normal levels of extracellular DA and spontaneous hyperlocomotor activity (Rocha et al., 1998). One study reported

that DAT KO mice display a 300-fold increase in extracellular lifetime of DA and a 5-fold elevation in steady-state extracellular DA levels in striatum (Gainetdinov & Caron, 2003). In addition, these mice do not display an increase in locomotor activity typically seen upon administration of high doses of cocaine (Giros et al., 1996).

D1 and D2 receptor levels have also been investigated in DAT KO mice. Jones and colleagues found that in striatum, the D1 and D2 receptors were decreased by approximately 50%; TH levels were increased even though protein levels were down nearly 90%; and total tissue DA levels were only 5% of normal, whereas extracellular DA was increased by at least 5-fold in striatum (Jones et al., 1998a). Another study found that D1R mRNAs coding decreased for D1R and D2R by 34% and 36%, respectively in caudate putamen of DAT KO mice, which suggest that there are fewer D1R and D2R receptors in DAT KO mice (Fauchey et al., 2000). DAT KO mice have several characteristics found in individuals that have ADHD including hyperactivity, cognitive impairment, and a calming response to psychostimulants (Jones et al., 1999).

A more recent study further explored the effect of DAT KO by investigating the rewarding effects of cocaine and the ability of repeated cocaine administration to induce conditioned locomotion (Hall et al., 2009). Conditioned locomotion is the process by exposing animals to drug or stimuli over a period of time and then comparing those animals with control animals to determine if the drug or stimuli caused neurochemical changes, which are reflected by a change in locomotor activity. DAT KO mice were significantly more active compared to WT; however they did not have increased locomotion after acute cocaine administration. NET and SERT affect the ability of cocaine to produce conditioned locomotion. These results suggest that the ability of cocaine to produce conditioned locomotion is dependent on NET or SERT, but not DAT.

In summary, the research on DAT KO mice has illuminated the mechanisms by which DAT acts and its importance to the overall dopaminergic system. This work may also have implications for the development of certain

pharmacological agents for disorders that involve DAT, such as drug abuse and ADHD.

Numerous studies suggest that DAT is regulated by several mechanisms including, but not limited to internalization and recycling, also known as trafficking, which involves phosphorylation and protein-protein interactions (Kahlig & Galli, 2003; Loder & Melikian, 2003; Melikian, 2004; Torres et al., 2003; Zahniser & Doolen, 2001). Great efforts have been made towards elucidating the molecular and cellular mechanisms involved in the trafficking of DAT. Many different kinases, receptors and scaffolding proteins interact with DAT and regulate its activity or modulate its trafficking and degradation. Second messenger systems, such as protein kinase C (Boudanova et al., 2008), protein kinase A (PKA), and calcium-calmodulin kinase II alter DAT function, phosphorylation, and trafficking. Furthermore, DAT endocytosis is suggested to be the cause of the sustained DAT down-regulation in response to PKC activation (Eriksen, 2010). Another post-translational modification that may be involved in PKC activation is ubiquitination, a post-translational enzymatic modification that involves the ε-amino moiety of lysine residues in target cellular proteins (Miranda & Sorkin, 2007; Miranda et al., 2005; Sorkina et al., 2006). Although the underlying mechanisms of DAT trafficking are still not fully understood, new technical methodologies such as fluorescently tagged inhibitors and substrates have advanced our understanding of DAT trafficking. For example, fluorescence resonance energy transfer (FRET) experiments with cyan fluorescent protein-tagged DAT and yellow fluorescent protein tagged ubiquitin demonstrated that ubiquitination was most abundant in endosomes supporting that ubiquitination is a signal for endocytosis (Miranda et al., 2005). Other kinase pathways including downstream effectors of insulin signaling such as phosphatidylinositol-3-kinase and serine/threonine protein kinase, Akt have been shown to also affect DAT surface expression (Carvelli et al., 2002).

In addition, [^{3}H]DA uptake kinetics was assessed and the cellular localization profiles of the hDAT expressed in both Sf9 and COS-7 cells via immunofluorescent confocal microscopy following modulation of PKC and

protein PKA-dependent pathways were evaluated (Pristupa et al., 1998). Acute exposure of hDAT expressing Sf9 cells to the PKC activator PMA (1 μM), reduced the V_{max} (approximately 1 pmol/min/10^5 cells) for [^{3}H]DA uptake by approximately 40%, an effect which was blocked by the protein kinase inhibitor, staurosporine. V_{max} is defined as the maximal velocity of uptake. Pretreatment of cells with staurosporine (500 nM) alone, however, increased [^{3}H]DA uptake by approximately 30%, an effect mimicked by the potent PKA inhibitor Rp-cAMPS. Immunofluorescent confocal microscopy showed that PKC activation rapidly internalized the hDAT from plasmalemmal membrane, but PKC inhibition led to trafficking of hDAT to the cell surface. These results suggest that the differential regulation of DAT transport capacity by both PKC- and PKA-dependent pathways are not a result of modifications in DAT kinetics and that DAT function may be regulated by second messenger systems, possibly following activation of presynaptic DA receptors (Pristupa et al., 1998).

A recent report has also suggested that the PKC-induced DAT regulation may depend on the membrane localization of the transporter in reference to raft and non-raft DATs (Foster et al., 2008). Lipid rafts contain 3 to 5 times more cholesterol than the surrounding bilayer. They are heterogeneous, dynamic membrane microdomains enriched in cholesterol and glycosphingolipids, which are wider than non-raft regions and resistant to solubilization by detergents. Lipid rafts are associated with internalization and endocytic cargo delivery. DATs are located between raft and non-raft microdomains in rat striatal tissue and have the potential to effect dopaminergic neuronal activity (Foster et al., 2008). Decrease of surface DAT was only found in non-raft DAT populations. These results suggest that trafficking events regulate non-raft DATs and non-trafficking regulatory mechanisms occurs in raft DATs. These authors identified the presence of DAT in cholesterol-rich membrane raft domains, which could possibly serve as a platform for regulatory DAT activity, phosphorylation, and subcellular interactions. Thus, DAT is distributed between membrane raft and non-raft populations, where it is subject to specific regulatory controls that could provide distinct modulation of DA clearance and efflux. More recently, Sorkina

et al., (2009) found that the intracellular N-terminal tail of DAT has an inhibitory influence on internalization, by promoting the presence of DAT on the cell surface. In addition, DAT-mediated uptake activity is increased by replacing the first 65 amino acids of the N-terminal tail of DAT with a DAT mutant. This was shown by visual examination of human epithelial cervical cancer cells (HeLA) and porcine aortic endothelial cells (PAE). A significant accumulation of the DAT mutant was found in intracellular compartments (Sorkina et al., 2009). Another study used the fluorescent DAT substrate ASP^+ and live cell imaging techniques, such as bioluminescence resonance energy transfer (Brett) to identify the role of two D2R-linked signaling pathways, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phosphodinositide 3 kinase (P13K). These pathways mediate D2R activation and up-regulate DAT function based on the observations that ASP^+ rapidly accumulated in the cytoplasm of EM (embryonic) cells (Bolan et al., 2007). This accumulation was intensified by the D2R agonist quinpirole. In addition, eticlopride a D2R antagonist blocked quinpirole-evoked increase in ASP^+ accumulation. Furthermore, the MEK inhibitor PD98059 prevented quinopirole-evoked ERK1/2 phosphorylation, but the PI3K inhibitor LY294002 had no effect. These results suggest that D2SR regulation of DAT requires coupling to G_i/G_0 proteins and ERK1/2 activation.

DAT has also been reported to generate detectable currents during the process of substrate transport (DeFelice & Blakely, 1996; Lester et al., 1994; Sonders & Amara, 1996). One study used two-electrode clamp techniques with hDAT expressed in *Xenopus laevis* oocytes to examine the electrophysiological and pharmacological characteristics of DAT (Sonders et al., 1997). Oocytes expressing hDAT were voltage-clamped at -60 mV and were superfused with 20 μM DA, which produced a downward displacement in the current trace consistent with a net inward current. The inward current was suggested to be the result of the translocation of DA^+ and Na^+ ions. However, 10-μM cocaine produced an outward current, both on initial application and reapplication of cocaine after DA superfusion of the same oocyte. These findings suggest that

hDAT mediates at least two distinct steady-state ionic conductances that result from transport-associated currents (Sonders et al., 1997).

A more recent study combined confocal imaging, whole-cell steady-state and transient current recordings with HEK-293 cells transfected with a yellow fluorescent protein-tagged hDAT to monitor DAT cell surface expression and activity (Kahlig et al., 2004). At -160 mV, amphetamine decreased hDAT-mediated transient currents, and these currents were dependent upon extracellular Na^+. In addition, these currents corresponded to the amphetamine decrease in DAT expression at the cell surface, measured by cell-surface biotinylation. These findings suggest that DAT transient charge movements can be used to evaluate relative changes in DAT cell surface expression.

DAT is considered the main target for stimulant action, and stimulants interact directly with DAT (Gainetdinov & Caron, 2003; Krause et al., 2003; Volkow et al., 2007). A plethora of research has been conducted on the mechanisms by which DAT is regulated and expressed. The studies discussed here have shown how DAT may be regulated by a number of different pathways and signaling messengers. Some of these pathways include PKA and PKC, which are involved in the internalization of DAT. Others have investigated DAT structure and how its conformation plays a role in the regulation of DAT. In addition, studies were performed to determine how trafficking plays a role in DAT expression and function. Furthermore, others have investigated how ion currents involved in the electrophysiological and pharmacological characteristics of DAT. Collectively, these studies have provided a better understanding of how DAT is regulated and expressed. Hopefully the knowledge gained from this research will lead to pharmacological agents that can be used in the treatment of diseases that involve DAT, such as ADHD.

iv. NET

The norepinephrine transporter (NET) is the primary target for the first and only non-stimulant medication, atomoxetine, Strattera® approved by the U.S. Food and Drug Administration (FDA) to treat ADHD (Wilens, 2006). In addition, methylphenidate and amphetamine, the gold standard ADHD treatments, inhibit

NET function in addition to inhibiting DAT function (Bymaster et al., 2002; Han & Gu, 2006). The major function of NET is to translocate norepinephrine, a neurotransmitter involved in mood regulation, behavior, alertness and arousal, from the extracellular space to within the noradrenergic presynaptic terminals (Barker & Blakely, 1995; Pacholczyk et al., 1991; Zavosh et al., 1999; Zhou, 2004). NET is a member of the same family of transporters as DAT and is also a Na^+/Cl^- dependent transmembrane transporter protein (Hu et al., 2009; Zhou, 2004).

NET contains 617 amino acid residues (Torres et al., 2003). Human NET (hNET) has 10 cysteine residues, and two of these which are located in the second intracellular loop are linked by a disulfide bond (Sucic & Bryan-Lluka, 2005). TMD 2 and the first intracellular loop are important in determining cell surface expression of the transporter (Sucic & Bryan-Lluka, 2005). Residues 94-111 of NET appear to not be involved in substrate interactions; however, these residues are associated with interactions with various inhibitors (Sucic & Bryan-Lluka, 2005). The regulation of NET involves extracellular and intracellular signaling pathways including several associated proteins such as SNARE protein syntaxin 1A, protein phosphatase 2A (PP2A), catalytic sub unit (PP2A-C), and PP2A anchoring subunit (PP2A-Ar) (Miner et al., 2006; Sung & Blakely, 2007; Sung et al., 2005).

Xu and colleagues found that NET knockout mice have reduced body temperature and body weight and are supersensitive to psychostimulants; however, they have reduced intracellular norepinephrine, increased norepinephrine synthesis and elevated extracellular norepinephrine (Xu et al., 2000). These findings suggests that NET plays a role in regulating body temperature and body weight, and that the mechanism of action of the stimulant class of drugs involves NET, since in its absence supersensitivity to stimulants occurs. Focus on the development of selective NET inhibitors as a treatment for ADHD has increased, because of the potential abuse liability of stimulant medications, whereas NET inhibitors do not appear to have this side effect (Seu et al., 2009).

v. VMAT2

Another important transporter for DA function is VMAT2, which is the only transporter that translocates cytoplasmic DA from the cytosol into synaptic vesicles for storage, providing availability for future exocytotic release (Yelin & Schuldiner, 2002). VMAT2 and VMAT1 are members of the solute carrier (SLC) protein family encoded by separate genes VMAT1 (SLC18A1) located on chromosome 8p21 and VMAT2 (SLC18A2) located on chromosome 10q25 (Adam et al., 2008; Eiden et al., 2004). VMAT2 is an integral membrane protein with 12 putative transmembrane domains containing a large, hydrophobic, N-glycosylated loop between TMD1 and 2 facing the vesicle cytosol (Yao & Hersh, 2007; Yelin & Schuldiner, 2002). During embryonic development, both VMAT1 and VMAT2 are widely expressed in the CNS; however, by adulthood, VMAT2 predominates (Hansson et al., 1998). The decrease in VMAT1 expression has been suggested to be due to the absence of VMAT1 gene expression. In adulthood, VMAT1 is primarily expressed in neuroendocrine cells such as chromaffin cells found in the adrenal medulla and enterochromaffin cells located in the intestinal tract (Erickson et al., 1996; Peter et al., 1995; Weihe et al., 1994). VMAT2 is expressed in at least 2 endocrine cell populations, and moreover, in neurons. Adult mammalian monoaminergic neurons of the central nervous system and sympathetic postganglionic neurons express VMAT2, not VMAT1 (Erickson et al., 1996; Peter et al., 1995; Weihe et al., 1994).

The pharmacology of VMAT1 and VMAT2 is distinct. Although they both transport monoamines such as serotonin, DA, epinephrine and norepinephrine, VMAT1 transports histamine, whereas VMAT2 transports histamine (K_m~ 24 μM; (Erickson et al., 1996; Merickel & Edwards, 1995). K_m is defined as the concentration of substrate at half of maximal velocity. In addition, VMAT1 has a higher affinity for serotonin compared to VMAT2 (Brunk et al., 2006). VMAT2 is responsible for the transport of neurotransmitters such as DA, serotonin, norepinephrine, epinephrine, and histamine from the cytosol into synaptic vesicles (Schuldiner et al., 1995). VMAT2 is an essential protein as indicated by the finding that homozygous VMAT2 knockout mice do not survive after birth

(Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). Tetrabenazine, a benzoquinolizine compound (Zheng et al., 2006), and reserpine, an alkaloid, are considered classical VMAT2 inhibitors (Pletscher, 1977). Studies have shown that VMAT2 binding is altered by psychostimulants (Brown et al., 2001a; Fleckenstein & Hanson, 2003). For instance, methamphetamine administration (15 mg/kg; 4 ip injections at 2 hr intervals) decreases (22%) striatal VMAT2 binding of [^{3}H]dihydrotetrabenazine, assessed 14 days after treatment (Guilarte et al., 2003). Also, methylphenidate (40 mg/kg, sc) redistributes VMAT2 within the nerve terminals an hour after treatment, distributing VMAT2 between the cytoplasmic and membrane-associated vesicle fractions. In contrast, methylphenidate did not cause redistribution since a majority of DAT was present in just the membrane-associated vesicle fraction, not the cytoplasm (Volz et al., 2007). In addition, lobeline, a novel alkaloid, inhibits [^{3}H]DA uptake into rat striatal vesicle preparations with an $IC_{50}$of 0.88 ± 0.001 μM and displaces dihydrotetrabenazine binding with an IC_{50} of 0.90 ± 0.02 μM (Teng et al., 1998; Teng et al., 1997). VMAT2 plays an important role in protecting the neurons against damage from toxins, such as hydrogen peroxide, by maintaining a low cytoplasmic concentrations of neurotransmitter via translocating the neurotransmitters into synaptic storage vesicles (Liu & Edwards, 1997). In addition, GBR 12935, a potent DAT inhibitor, also blocks uptake into brain synaptic vesicles (IC_{50} between 34-45 μM) compared to synaptosomes (IC_{50}between1-6μM) (Reith et al, 1994) .

5. Pharmacotherapies for ADHD

Once the diagnosis of ADHD has been established, the next step is to choose the most appropriate treatment for the patient. Stimulants are considered the first-line pharmacological treatment option for ADHD. These include various dosage forms and formulations of methylphenidate and amphetamine, (Meijer et al., 2009; Spencer et al., 1996). However, there are potential side effects associated with these stimulants that can limit their use. These agents, along with their side effects are discussed below in more detail.

Pemoline, approved in 1975, was another stimulant used to treat ADHD that increases DA transmission in the central nervous system (Zaczek et al., 1989). However due to liver toxicity, pemoline was removed from the market in 2005 (Greydanus et al., 2007; Olfson, 2004). The most recent medication approved for ADHD is a long-acting form of guanfacine (Intuniv®; Figure 3), a non-stimulant, approved in September, 2009. Another non-stimulant approved to treat ADHD is atomoxetine (Strattera®; Figure 3). Atomoxetine is considered a second-line therapeutic agent since it is not as effective as stimulants (Michelson et al., 2003; Michelson et al., 2002; Michelson et al., 2001; Newcorn et al., 2008). These non-stimulant medications are a good alternative for parents with concerns about giving their children stimulants and for those patients that cannot tolerate or do not respond to stimulants. There are also drugs that are used off-label to treat ADHD for the reasons stated above. These agents include tricyclic antidepressants, bupropion, clonidine, and modafinil.

Stimulants produce pharmacological effects by inhibiting DAT and/or NET, which results in an increase in the amount of DA and norepinephrine in the synaptic cleft, which enhances neurotransmission in these systems (Greydanus et al., 2007). Additionally, stimulants are associated with untoward effects that include, but are not limited to insomnia, cardiac events, decreased appetite, abdominal pain, headache, weight loss, tics, depression, and growth delays (Bymaster et al., 2002; Cormier, 2008; Greydanus et al., 2007). Furthermore, the possibility of abuse and diversion have become major concerns with the use of stimulant medications (Bymaster et al., 2002; Cormier, 2008; Greydanus et al., 2007; Holman, 1994; Olfson, 2004), even though the AAP guidelines recommend a trial of at least three types or formulations of stimulant medication before considering different agents (Cormier, 2008). This recommendation is to ensure that each patient is treated as an individual, because there is such a high degree of variability of response to these medications. Thus, the issue with stimulants is apparent because they are considered the first line therapy for ADHD, even though the possibility of abuse is present.

a. Methylphenidate

i. Historical Background

Originally, methylphenidate was approved to treat chronic fatigue, lethargy, depressive states, disturbed senile behavior, psychosis associated with depression, and narcolepsy. In 1968, methylphenidate was approved to treat ADHD, and is a gold standard treatment for ADHD (Folsom et al., 1956; Leonard et al., 2004). The landmark study, conducted in the early 1990s, by the National Institute on Mental Health Multimodal Treatment study of children with ADHD (MTA), which found that methylphenidate treatment was superior to behavioral therapy for children with ADHD, which led to its widespread use (Heal et al., 2008; National Institute, 2001). Thus, the pharmacotherapeutic use of stimulants became the first line pharmacological therapy for ADHD.

ii. Formulations

Methylphenidate, a piperazine substituted phenylisopropylamine (Figure 3), was first synthesized in 1944 by Dr. Leandro Panizzon and marketed by Ciba-Geigy pharmaceutical company as Ritalin (Leonard et al., 2004). There are various trade names for methylphenidate depending on the formulation and dosage form. For instance, dl-threo-methylphenidate has the following trade names; Ritalin®, RitalinSR®, MetadateCD®, Concerta® and Daytrana®. The initial formulations were short-acting (3-4 hrs), which was a limitation for school-aged children, who would need medication administered multiple times per day (Greydanus et al., 2007). Ritalin SR® and Concerta® were developed to overcome this pharmacokinetics problem, since they have similar duration of actions, with Ritalin SR® being 8-9 hours and Concerta® being 10-12 hours (Biederman & Faraone, 2005). Daytrana®, which was approved in 2006, is a transdermal methylphenidate preparation and has a 9-12 hour duration of action (Greydanus et al, 2007). D-threo-Methylphenidate (dexmethylphenidate) has a higher affinity for DAT than l-threo-methylphenidate (Heal & Pierce, 2006; Patrick et al., 1987). Thus, dexmethylphenidate was developed as an immediate release formulation and as an extended release formulation (Focalin® and Focalin XR®).

iii. Pharmacokinetics and mechanism of action

The half-life of methylphenidate is 2-4 hours depending on the formulation. Its bioavailability is between 11-53% (Chan et al., 1983). Its onset of action is approximately 2 hours after administration (May & Kratochvil, 2010). Furthermore, a clinical study in which healthy subjects were given [^{11}C]methylphenidate intravenously (iv), found that the peak concentration of methylphenidate in the brain was achieved in 4-10 minutes and peak concentration of oral methylphenidate did not occur until 60 minutes after administration (Volkow et al., 1995). Methylphenidate is metabolized primarily through deesterification in humans to ritalinic acid, which is inactive.

The mechanism of action of methylphenidate involves binding to DAT and inhibiting its function, which leads to a greater concentration of DA in the synaptic cleft to bind to both postsynaptic and presynaptic DA receptors, thus augmenting dopaminergic neurotransmission. Surgical lesions of the medial forebrain bundle or intracerebroventricular administration of 6-hydroxydopamine (6-OHDA) were utilized to determine effects on [^{3}H]threo-(+/-)-methylphenidate and [^{3}H]DA uptake. After both of these procedures a reduction in the specific binding of [^{3}H]threo-(+/-)-methylphenidate to membranes of rat striatum was observed, which was highly correlated with the decrease in [^{3}H]DA uptake. However, intracerebroventricular administration of 5, 7-hydroxytryptamine, AF64A, or chronic parenteral administration of reserpine did not alter the number of [^{3}H]threo-(+/-)-methylphenidate binding sites. These results suggest that localization of the specific [^{3}H]threo-(+/-)-methylphenidate sites in striatum is on dopaminergic nerve terminals (Janowsky et al., 1985). Another report found that the highest specific [^{3}H]methylphenidate binding was in caudate putamen, olfactory tubercle, nucleus accumbens, bed nucleus of the stria terminalis, and median eminence; in contrast to [^{3}H]amphetamine, [^{3}H]methylphenidate binding was not high in brainstem; however, the Bmax was not included in this report (Unis et al., 1985). Although methylphenidate has high affinity for DAT (K_i = 160-340 nM), it also has high affinity for NET (K_i = 40-238 nM) and a lower affinity for serotonin transporter (SERT;K_i= 1000-22,000

nM) (Andersen, 1989; Easton et al., 2007; Kuczenski & Segal, 1997; Richelson & Pfenning, 1984).

Inhibition of DAT function leads to concerns regarding the use and diversion of methylphenidate for recreational purposes. In fact, methylphenidate and cocaine have similar affinity for DAT, with cocaine having a K_i of 555-640 nM for DAT (Gatley et al., 1996; Schweri et al.,1985; Ukairo et al., 2005). Methylphenidate is prescribed for ADHD and has a high potential for being diverted for recreational use (Klein-Schwartz, 2002; Kollins et al., 2001; McCabe et al., 2005; McCabe et al., 2006; Parran & Jasinski, 1991; Setlik et al., 2009; Sussman et al., 2006; Teter et al., 2003; Weyandt et al., 2009; Wilens et al., 2008). However, methylphenidate may have a lower abuse potential than cocaine due to its pharmacokinetics, such that methylphenidate has a longer half-life than cocaine, 90 minutes and 20 minutes, respectively (Volkow et al., 1999). Cocaine has a faster onset of action than methylphenidate, which likely attributes to the euphoric feeling of "high". An animal study using rats, (Izenwasser et al., 1990) examined the pharmacological effects of cocaine and methylphenidate and other monoamine uptake inhibitors on DA uptake. This study showed that methylphenidate and cocaine had similar Km values (100 nM) in striatum, nucleus accumbens, olfactory tubercle, and medial prefrontal cortex; however, cocaine inhibited [^{3}H]DA uptake to a lesser extent than methylphenidate. This suggests that there is a possible additional effect of cocaine that contributes to the potent reinforcing characteristics of this drug. Another study found that cocaine and methylphenidate have similar potency in the septum-caudate synaptosomes for inhibiting serotonin uptake based on the IC_{50} values of 70 μM and 118 μM, respectively. These results imply that the effect of cocaine on serotonin uptake may play a role in its increased abuse liability, compared to methlyphenidate (Taylor & Ho, 1978).

Positron emission tomography (PET) studies utilizing [^{11}C]methylphenidate in human subjects showed that occupation of 50% of DAT sites by [^{11}C]methylphenidate in striatum is required to elicit a therapeutic effect, and that the estimated oral dose of methylphenidate required to occupy 50% of

DAT sites corresponded to 0.25 mg/kg (Volkow et al., 1998). In other work, the temporal and spatial distribution of [^{11}C]methylphenidate was determined, and the result compared to those obtained previously with [^{11}C]cocaine (Volkow et al., 1999). DAT occupancies were measured with PET using [^{11}C]cocaine, as the DAT ligand, in 8 healthy subjects (average age of 32, 4 men and 4 women) for the methylphenidate study and 17 active cocaine abusers for the cocaine study (average age of 35, 12 men and 5 women). The 8 healthy subjects were injected with 4-8 mCi of [^{11}C]cocaine and then scanned four times over a 3 day period. The first scan was the placebo scan to establish a baseline and the other three scans were performed after intravenous methylphenidate doses (0.025, 0.1, 0.25, and 0.5 mg/kg). The subjects for the cocaine studies were scanned four times over a 2-day period, with the first scan being a placebo to achieve a baseline and the second scan was done 2 hours after the first, and the rest were performed after a range of intravenous doses of cocaine (0.05, 0.1,0.3, and 0.6 mg/kg). Cocaine was co-administered with [^{11}C]cocaine, while methylphenidate was given 5-8 minutes prior to [^{11}C]cocaine. The results from this study showed that methylphenidate has a slightly higher potency at DAT than cocaine in the human brain, based on the ED_{50} of 0.07 mg/kg and 0.13 mg/kg, respectively. Also, a double dose of cocaine was required to induce DA increases equivalent to those induced by methylphenidate utilizing PET and [^{11}C]raclopride, which is a D2R antagonist. The potencies of methylphenidate and cocaine were compared to other DAT blockers, including but not limited to norcocaine, mazindol, lidocaine, procaine, and WIN 35,065-3. These DAT blockers have 50-100 fold higher affinities than cocaine. These results show that the potencies of methylphenidate and cocaine are similar. Based on these observations, the difference in the abuse liability of these agents is not solely based on the pharmacological potencies at DAT. Pharmacokinetics may also play an important role in the abuse potential of these two drugs (Volkow et al., 1999). In another study by some of the same authors, PET was used to measure temporal and spacial distribution of [^{11}C]methylphenidate and [^{11}C]cocaine (Volkow et al., 1995). Eight healthy male subjects between the

ages of 20 and 51 years of age were scanned with [^{11}C]methylphenidate. The scans were done two hours apart. Three subjects underwent two repeated scans to test for re-test reproducibility. Four subjects underwent a baseline scan and a second scan 10 minutes after administration of methylphenidate (0.5 mg/kg, iv) to assess specific to nonspecific binding. One subject was scanned with both [^{11}C]methylphenidate and [^{11}C]cocaine to compare the distribution and kinetics of these two compounds in the same individual. The authors did not mention how they were able to tell the difference between the compounds, however based on their different pharmacokinetics; it may have been possible to distinguish the two compounds. In the same study, two baboons were scanned to evaluate if methylphenidate and cocaine compete for the same binding sites in brain. One animal underwent a baseline scan with [^{11}C]cocaine and a second scan with [^{11}C]cocaine 5 minutes after administration of cold methylphenidate (0.5 mg/kg, iv). The other animal underwent a baseline scan with [^{11}C]methylphenidate and a second scan with [^{11}C]methylphenidate 5 minutes after administration of cold cocaine (0.2 mg/kg, iv). In the human studies, the uptake of [^{11}C]methylphenidate into brain was 7.5 ± 1.5% (mean ± SD) of the injected dose, and was comparable to the uptake of cocaine, which was 7.5 ± 3.0% (mean ± SD) of the injected dose. Maximal concentrations of methylphenidate were observed in striatum, however low levels were detected in the cortex and cerebellum. Furthermore, pretreatment with cold methylphenidate 5 minutes prior to the administration of [^{11}C]cocaine and pretreatment with cold cocaine 5 minutes prior to administration of [^{11}C]methylphenidate significantly decreased binding of the corresponding tracer, but only in striatum, not in cerebellum. These results indicate that methylphenidate and cocaine compete for the same binding sites in the striatum.

The distribution of methylphenidate was very similar to that of cocaine. Although the brain regional distribution of [^{11}C]methylphenidate was identical to that of [^{11}C]cocaine, and these drugs competed for the same binding sites, they differed markedly in their pharmacokinetics. Clearance of [^{11}C]methylphenidate

from striatum (90 minutes) was significantly slower than clearance of [^{11}C]cocaine (20 minutes). For both drugs, fast uptake, 4-10 minutes for [^{11}C]methylphenidate and 2-4 minutes for [^{11}C]cocaine in striatum paralleled the experience of the "high" reported by the subjects. For methylphenidate, the "high" decreased rapidly despite that a significant amount of drug was still bound in striatum. In contrast, for cocaine, the decline in the feeling of "high" paralleled its fast rate of clearance from striatum. Therefore, the "high" appears to be associated with the fast uptake of methylphenidate and cocaine into brain, and the slow clearance of methylphenidate from brain may serve as a limiting factor in promoting its frequent self-administration (Gatley et al., 1996; Volkow et al., 1999). Thus, the pharmacokinetics alter the pharmacology, specifically the abuse liability of methylphenidate.

A recent study found that methylphenidate (0.3 mg/kg/infusion) is a relatively robust reinforcer for all strains, SHR, WKY, and SD based on the results that these strains acquired methylphenidate self-administration (Marusich et al., 2011b). Another study also conducted in the SHR strain found the same result in regards to methylphenidate self-administration, such that this strain acquired methylphenidate (0.25 mg/0.1ml infusion) self-administration as well (Pena et al., 2001). In addition, this study also showed that methylphenidate induced conditioned place preference (CPP) in SHR, however there was no difference found between the SHR and WKY in regards to CPP, suggesting that SHR may not be more sensitive to the rewarding effects of methylphenidate. These studies provide evidence that methylphenidate has the potential to be abused.

Evidence has been provided that methylphenidate also interacts with VMAT2, the only transporter that translocates cytoplasmic DA from the cytosol into synaptic vesicles for storage, providing availability for future exocytotic release (Yelin & Schuldiner, 2002). Pre-clinical studies have demonstrated that unlike amphetamine, the effects of methylphenidate on brain catecholamines were completely inhibited by reserpine, suggesting that methylphenidate interacts with a reserpine-sensitive pool of DA (Scheel-Kruger, 1971).

Methylphenidate rapidly and reversibly increases VMAT2 binding and vesicular uptake of DA (Sandoval et al., 2003). Rotating disk electrode (RDE) methods were utilized to measure the initial velocities of inwardly directed vesicular DA transport in vesicles purified from rat striata. VMAT2 immunoreactivity was used to measure the amount of VMAT2 protein. Methylphenidate (40 mg/kg, s.c.), which is considered a high dose for this animal model, was found to decrease VMAT2 immunoreactivity in membrane-associated vesicle fraction and increase VMAT2 immunoreactivity in the cytoplasmic vesicle fraction in nerve terminals by a 2-fold difference compared to control. In addition, membrane-associated vesicles were able to sequester 5-9 fold more DA than cytoplasmic vesicular associated vesicles (Volz et al., 2007). In another study using RDE, both eticlopride, the D2 receptor antagonist, and scopolamine, the muscarinic receptor antagonist, blocked methylphenidate-induced K+ stimulated DA release (Volz et al., 2008). These results suggest that effects of methylphenidate are mediated by both D2 and muscarinic receptors.

In addition to the effects of methylphenidate on the neurotransmitter transporters, PET studies using human subjects have investigated the effects of methylphenidate on DA release and how it relates to appetitive stimuli in the response. DA neurons fire in response to salient events. Salient events are defined as relevant events that require a response from the subject. Salient events have been hypothesized to contribute to the therapeutic effects of methylphenidate. Subjects were given methylphenidate (20 mg/kg, po) or placebo and then shown a salient stimuli (visual and olfactory presentation of food) or neutral stimuli, which was the description of family genealogy. No increase in DA was found in the placebo group shown the salient stimuli, demonstrating that the salient stimulus alone does not increase DA. Moreover, methylphenidate increased DA in striatum in response to the salient stimuli, but not to the neutral stimuli, suggesting that the methylphenidate-induced increase of DA in the striatum was dependent upon stimulus context (Volkow et al., 2005). This could impact therapy because the increase in the amount of DA in

the striatum is, in part, based on the stimulus a patient reacts to, which may be lead to an increase in the abuse liability of methylphenidate.

In conclusion, a wealth of research has been performed on methylphenidate and the mechanism by which it produces its pharmacological effect. Research, thus far, has found that methylphenidate inhibits DAT function, thereby causing an increase in extracellular DA. However, methylphenidate interacts with other systems within the brain, which may also contribute to its pharmacological effect. Additional work is needed to further study the effects methylphenidate to obtain a more comprehensive understanding of its underlying mechanisms.

b. Amphetamine

i. Historical background

Lazar Edeleano, a Romanian chemist studying in Germany, was the first to synthesize amphetamine (Figure 3) in 1887 (Edeleanu, 1887). In 1927, Gordon Alles first reported the stimulant effects of sympathomimetics. In 1937, Charles Bradley, reported the therapeutic effects of dl-amphetamine in children with neurological and behavioral problems (Alles, 1933; Bradley, 1937). The pharmaceutical company, Smith, Kline, and French, marketed amphetamine as Benzedrine® in 1932, and this drug was used for decongestion as an inhaled dosage form (Rasmussen, 2006). Benzedrine® was sold without a prescription, and over 50 million Benzedrine® tablets were sold during the initial 3 years of availability as an oral dosage form (Sulzer et al., 2005). The Spanish Civil War marked the beginning of the military using amphetamine to promote alertness in the troops (Sulzer et al., 2005). Also, the alerting properties of amphetamine were exploited by American troops during World War II, especially those in the air force during extended bombing missions. Today, amphetamine is still used by the air force in some cases in which prolonged attention is required (Caldwell et al., 2003).

The escalation in the use of amphetamine abuse during the early periods of its over the counter availability, led to the decision in 1939 to make it only available by prescription (Sulzer et al., 2005). During the subsequent period of

time, the recognized therapeutic uses of amphetamine increased, such that by 1946, it had more than 30 indications, including the treatment of schizophrenia, opiate addiction, sea sickness, and radiation sickness (Brett, 1946; Miller & Hughes, 1994). Even though amphetamine was considered a prescription-only drug, its diversion for recreational use continued. In 1972, in an attempt to discourage its diversion, the United States Justice Department enforced legal quotas of amphetamine production (Sulzer et al., 2005). D-amphetamine appears to have a similar dose-related profile of effects in humans to methamphetamine, which suggest their equivalence for abuse (Kirkpatrick et al., 2011).

ii. Formulations

There are various dosage forms of amphetamine available today. The most prescribed is Adderall®, which is a complex formulation of mixed-amphetamine salts consisting of ¼ dextroamphetamine saccharate, ¼ dextroamphetamine sulfate, ¼ racemic dextro/levo amphetamine aspartate monohydrate, and ¼ racemic dextro/levo amphetamine sulfate, resulting in a 3:1 ratio of d-amphetamine to l-amphetamine. Adderall®is available as immediate-release and extended-release formations (Adderall XR®). Dextroamphetamine (Dexedrine®) is an immediate release formulation and is marketed also in a capsule formulation for controlled release called Dexedrine SR®. The most recent development in the amphetamine series for the treatment of ADHD is lisdexamfetamine (Vyvanse®) and was FDA-approved in 2007 for the treatment of ADHD in children, and subsequently approved in 2008, for ADHD treatment in adults (Cowles, 2009). Lisdexamfetamine, a prodrug, is a novel agent designed to lower the potential for abuse, since it must be catabolized to the active compound dextroamphetamine which provides the pharmacological effect.

iii. Pharmacokinetics and mechanism of action

The pharmacokinetics of amphetamine is similar to that of methylphenidate. The onset of action is 30-60 minutes and the duration of action for immediate release is 4-6 hours and 10-12 hours for extended release

mixed-amphetamine salts (MAS-XR). An even longer duration of 16 hours is seen with the MAS-triple-bead formulation (Greydanus et al., 2007).

The mechanism of action of amphetamine is complex. Amphetamine produces a redistribution of DA from synaptic vesicles to the cytoplasm by reducing the vesicular pH gradient. VMAT2 mediated transport involves a vacuolar-type H+ pumping, which creates a pH gradient across the vesicle membrane. When amphetamine is transported into the vesicle, it reduces the synaptic vesicle pH gradient required for monoamine storage, thus reducing concentration of monoamines inside the vesicle (Sulzer & Rayport, 1990). In addition, amphetamine inhibits DA uptake at DAT and reverses the transport of DA causing its release into perisynaptic area and into the extracellular space, whereas cocaine and methylphenidate bind to DAT and only inhibit DA uptake at DAT, i.e., do not release DA (Bannon et al., 2000; Seiden et al., 1993; Solanto, 2002; Sonders et al., 1997). The mechanism of action of amphetamine is dependent on the amphetamine concentration, whereby at lower concentrations amphetamine is exchanged for DA via DAT, but at higher concentrations amphetamine can diffuse across the plasmalemmal membrane independently of DAT (Mack & Bonisch, 1979). Amphetamine is widely accepted to elicit its pharmacological effect by: 1) binding to neurotransmitter transporters and reversing the transport of neurotransmitters such as DA, norepinephrine, and serotonin from inside the presynaptic terminal to the extracellular space which facilitates their release and 2) inhibiting monoamine oxidase (MAO) (Seiden et al., 1993).

With respect to the effect on the plasmalemmal transporters, amphetamine has affinity (K_i) for NET (30 - 100 nM), followed by DAT (30 - 600 nM), and lastly SERT (1000 - 40,000 nM; (Easton et al., 2007; Han & Gu, 2006; Heal et al., 1998; Kula & Baldessarini, 1991; Richelson & Pfenning, 1984; Rothman et al., 2001). Extensive research has been conducted on the interaction of amphetamine with these transporters; however, most of the focus has been on the interaction with DAT. Amphetamine binds to the extracellular surface of DAT, competing with its substrate (e.g., DA), thus decreasing DA

uptake into the neuron (Seiden et al., 1993). Once inside the neuron, amphetamine is released from DAT, leaving DAT unoccupied. DA binds to the internally facing DAT protein and is reverse transported to the outside or extracellular space, where it is released, and the result is an increase in the extracellular concentration of DA (Levi & Raiteri, 1993; Sulzer et al., 1995).

A study by Cass and colleagues used *in vivo* electrochemistry to determine the effects of locally applied raclopride, a D2 receptor antagonist, and SCH-23390, a D1 receptor antagonist, on the clearance of locally applied DA in the striatum, nucleus accumbens, and medial prefrontal cortex of rats. Raclopride or SCH-23390 was applied locally prior to the pressure injection of DA. Raclopride increased the amplitude and time course of DA signals, suggesting significant inhibition of DAT. However, SCH-23390 had no effect of DA signals. These results were interpreted to indicate that D2, not D1 receptors, modulate the activity of DAT (Cass & Gerhardt, 1994). Another study confirmed these results by employing continuous amperometry and cyclic voltammetry and determined that amphetamine (10μM)-induced stimulation of DA overflow from striatal slices was inhibited (47%) by sulpiride, a D_2 receptor antagonist (Schmitz et al., 2001), suggesting that the response to amphetamine may be indirect and involve D_2 autoreceptor activation following DA release.

Amphetamine is a substrate for DAT. Amphetamine accumulation into striatal synaptosomes is saturable, temperature-dependent, and ouabain-sensitve, indicating that it is a substrate for transport (Zaczek et al., 1991). Similarly, a study investigating the effects of uptake blockers and substrates on transporter-associated ion currents found that amphetamine induced currents, whereas methylphenidate blocked transporter-associated current, indicating that amphetamine is a substrate for DAT (Sonders et al., 1997). More recently, studies using neuronal cultures and heterogeneous cells stably expressing hDAT showed that amphetamine produces DA efflux via two mechanisms that involve a rapid channel-like configuration with a millisecond firing rate of DA neurons and the other consisting of a slower, exchange-like mechanism of DA release (Kahlig

et al., 2005). Thus, results from these studies provide evidence that amphetamine is a substrate for DAT.

Amphetamine also modulates DAT cellular expression. The role of PKC in DAT modulation has been widely investigated because DAT is regulated by activation of protein kinase, which decreases DAT cell surface expression after amphetamine administration (Pristupa et al., 1998; Vaughan et al., 1997; Zhang et al., 1997). This stems from findings that phosphorylation of the N-terminus of DAT, which may be PKC dependent, causes an amphetamine-induced DA efflux (Khoshbouei et al., 2004). If phosphorylation is needed for internalization and PKC causes phosphorylation, then PKC may play a vital role in the regulation of DAT (Copeland et al., 1996; Huff et al., 1997; Vaughan et al., 1997; Zhang et al., 1997).

Specifically, amphetamine has been shown to increase striatal PKC and PKC activation stimulates DAT-mediated release of DA and triggers rapid internalization of DAT from the plasmalemmal membrane (Giambalvo, 1992; Kantor & Gnegy, 1998). For example, amphetamine acutely decreased cell surface expression of human DAT (hDAT) in cell lines, which was concomitant with a loss of DAT function (Saunders et al., 2000). In addition, HEK-293 cells transfected with a yellow fluorescent protein-tagged hDAT were employed to determine if loss of transporter activity was due to a modification in DAT function independent of cell surface redistribution or due to a reduction in the number of active transporters at the plasma membrane resulting from DAT trafficking (Kahlig et al., 2004). Confocal imaging combined with electrophysiology of the HEK cells revealed that after 1 hr exposure to 10 μM amphetamine, a reduction in hDAT function resulted and was directly related to the redistribution of hDAT from the plasma membrane. Thus, the decrease in DA uptake was associated with an increase in intracellular hDAT.

In other work, Gnegy and coworkers investigated the effects of amphetamine on DAT expression at very early time points using biotinylation in rat striatal synaptosomes. Within 3 seconds of application of 3 μM amphetamine, there was an increase in synaptosomal DAT surface expression was observed

lasting less than 2.5 min, which was prevented by cocaine pretreatment and associated with increased delivery of DAT to the plasmalemmal membrane (Johnson et al., 2005a).

The innovative work of Zahniser and colleagues showed that the hDAT oligomerizes (Sorkina et al., 2003). hDAT was fused with yellow or cyan fluorescent protein and transfected and expressed in PAE, human embryonic kidney (HEK) 293 cells, and an immortalized dopaminergic cell line $1RB_3AN_{27}$ to examine the oligomeric state and trafficking of DAT in different compartments of different types of living cells (Sorkina et al., 2003). Fluorescence resonance energy transfer (FRET) was used to determine the location of these specific compartments. FRET involves a donor chromophore in its electronic excited state that transfers energy to an acceptor chromophore through nonradiative dipole-dipole coupling in near field region. The excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore. The FRET signals were strongest in the endosomes, which provides evidence of the involvement of vesicles, where amphetamine caused the intracellular accumulation of hDAT on endosomal vesicles. Based on the results that a DAT mutant was retained in the endoplasmic reticulum after biosynthesis, suggests that DAT oligomers are formed in the endoplasmic reticulum and are maintained both at the cell surface and during trafficking between the plasma membrane and endosomes.

Another target for amphetamine action is VMAT2. An early study used isolated chromaffin granules and [^{3}H]reserpine-binding measurements to determine amphetamine interaction with VMAT2 (Rudnick & Wall, 1992). Amphetamine analogs such as 3,4-methylenedioxymethamphetamine (235 μM) and fenfluramine (30 μM) inhibited 50% of maximal binding; but parachloroamphetamine (800 μM) inhibited less than 10% of [^{3}H]reserpine binding. These results were interpreted to suggest that parachloroamphetamine effects on isolated chromaffin granules were only due to an alteration in pH, whereas methamphetamine and fenfluramine exerted effects both by altering the pH gradient and vesicular transport, which was also measured (Rudnick & Wall,

1992). This study demonstrates that there is a way to determine if a compound solely changes the pH versus directly interacts with the transporter.

Although, amphetamine interacts with VMAT2 (Brown et al., 2002; Gonzalez et al., 1994; Johnson, 1988; Mosharov et al., 2003); at this point in time, the focus in the literature has switched from amphetamine to methamphetamine, coinciding with the more wide spread abuse of the latter. One study found that 10-15 min of application of 10 μM amphetamine induced a 15-fold increase in cystosolic DA in synaptosomes, strongly suggesting redistribution of vesicular storage from the vesicle to the cytosol (Mosharov et al., 2003). Amphetamine displaced [^{3}H]tetrabenazine binding, a VMAT2 ligand with a nM affinity (Teng et al., 1998), which suggests amphetamine may increase cytoplasmic DA concentrations by inhibiting vesicular DA uptake (Ary & Komiskey, 1980; Gonzalez et al., 1994; Philippu & Beyer, 1973).

Yet, another target of amphetamine action is MAO. Thus, amphetamine also alters DA intracellular and extracellular concentrations by inhibiting MAO. MAO is one of the enzymes which metabolize catecholamines in brain. Thus, by inhibiting MAO, amphetamine increases the amount of cystolic DA available for reverse transport by DAT (Sulzer et al., 2005).

An alternative hypothesis regarding amphetamine-evoked DA release involves the physicochemical properties of amphetamine, as alluded to above. Amphetamine is a weak base, with apK_a of 9.9. This has been suggested also to play a role in the release of dopamine from the terminal into the extracellular space (Sulzer & Rayport, 1990). The weak base theory suggests that amphetamine enters the cell through both transport and diffusion, diffuses across the vesicular membrane, accumulates in vesicles, disrupts the proton gradient by binding to free protons, and thereby, increases the pH inside the vesicles, which is normally around 5.5 (Johnson, 1988). This disruption in pH decreases the driving force that provides energy for the accumulation of DA in the vesicle, causing the vesicle to release the stored DA. This DA release from the vesicle results in an increase of cytoplasmic DA available for reverse transport by DAT,

ultimately producing an increase in DA in the extracellular space (Sulzer et al., 1993; Sulzer & Rayport, 1990).

In summary, there has been a plethora of work conducted on amphetamine and its underlying multi-faceted mechanisms of action, which likely work in concert to ultimately increase the extracellular concentrations of DA and other neurotransmitters to produce its pharmacological effects including its therapeutic effects on ADHD. Despite the tremendous efforts to understand how amphetamine works in brain, further studies are needed to gain additional insight into the action of this very complicated pharmacotherapy. This dissertation work will compare the effects of amphetamine and methylphenidate, two gold standard treatments for ADHD, on DAT and VMAT2 function in rat striatum.

6. Alternative Therapies

Alternative therapies for ADHD include tricyclic antidepressants (TCA), such as desipramine and nortriptyline (Biederman & Spencer, 1999; Spencer et al., 1996; Wilens et al., 1996; Wood et al., 2007). These agents are somewhat effective in the treatment of ADHD, because they have affinity for NET, DAT and SERT (Wong et al., 1995). Desipramine has been reported to have a K_i of 3.8 nM for NET, 179 nM for SERT, but over 10,000 nM for DAT (Bymaster et al., 2002). Nortriptyline has been reported to have a K_i of 4.4 nM for NET, 18.5 nM for SERT, and 1140 nM for DAT (Owens et al., 1997). Unfortunately, these antidepressants have considerable side effects. For example, nortriptyline is associated with dry mouth (19% of subjects), constipation (11% of subjects), and headache (9% of subjects). Desipramine produces loss of appetite (25% of subjects), insomnia (19% of subjects), and dry mouth (10% of subjects), all of which have deterred the wide spread use of TCAs for ADHD (Prince et al., 2000; Spencer et al., 2002).

Another antidepressant, which is not a TCA, but is used in the treatment of ADHD is bupropion (Wellbutrin®). Bupropion is also used as smoking cessation agent (Zyban®).This is of interest because lobeline has been investigated as a smoking cessation aid. Both bupropion and lobeline interact with nicotinic systems (Damaj et al., 1997; Dwoskin & Crooks, 2002; Yamada et al., 1985).

Bupropion is an aminoketone antidepressant (Figure 3), that interacts with noradrenergic, dopaminergic, and nicotinic systems. Bupropion is thought to inhibit DA uptake in the mesolimbic DA system thereby, aiding in smoking cessation (Jorenby et al., 2006). However, bupropion is considered an adjunctive treatment for ADHD, because it is less effective than stimulants in eliminating symptoms of ADHD when used alone (Greydanus et al., 2007; Olfson, 2004; Wilens, 2006; Wilens et al., 2005; Wilens et al., 2001).

In addition, the antihypertensive medications, clonidine (Figure 3) and guanfacine (Figure 3), have been shown to be effective in the treatment of ADHD. A number of studies have shown that clonidine improved the hyperactivity and impulsivity symptoms, but not the inattention symptoms associated with ADHD (Connor et al., 1999; Heal et al., 2008; Nair & Mahadevan, 2009; Rains et al., 2006; Scahill et al., 2001). With respect to guanfacine, one study employing 25 children between the ages of 7-16 years with ADHD found that this therapeutic agent improved hyperactivity by 27%, improved teacher ratings on the hyperactivity/impulsivity scale by 36% and the teacher ratings on the ADHD scale by 32%, as well as the total tic severity scale by 39% (Boon-yasidhi et al 2005). Compared to clonidine, guanfacine may be more beneficial clinically because it has a longer duration of action (Greydanus et al., 2007). The major side effects of clonidine are sedation and hypotension (Greydanus et al., 2007; Olfson, 2004). In addition, comparisons of clonidine and guanfacine revealed that that guanfacine caused less somnolence than clonidine (21% vs. 35%, respectively; (Wilson et al., 1986).

With respect to the mechanism of action, clonidine acts centrally as an agonist at both α_1 and α_2 adrenergic receptors (Wilens, 2006). Clonidine is thought to improve neuropsychological function associated with the PFC by inhibiting norepinephrine release through stimulation of α_2 autoreceptors that are located presynaptically on noradrenergic neurons. This action is suggested to explain the effect of clonidine on impulsivity and cognition in ADHD (Arnsten & Dudley, 2005; Arnsten & Li, 2005).

Guanfacine is thought to act similar to clonidine, however, this drug is actually more selective for a subtype of α_2 adrenergic receptors. There are 2 main subtypes of adrenergic receptors, which are α and β. Both of these subtypes have several subtypes. Theα-receptors have the subtypes α_1 and α_2. The α_2-receptors have three highly homologous subtypes, which are α_{2A}, α_{2B} and α_{2C}-receptors. Theα_{2A}-receptors inhibit norepinephrine uptake in the PFC, which produces improvements in working memory, attention, and enhancement of impulse control. There is a high density of norepinephrine in PFC and locus coeruleus. Since guanfacine is a selective agonist of theα_{2A} subtype of norepinephrine receptor, with a reported wide range of K_d (0.1-100 nM), it effects the neural transmission of norepinephrine (Greydanus et al., 2007; Khalid et al., 2002; Wilens, 2006). Guanfacine inhibits norepinephrine release in this area, thereby increasing the blood flow to PFC. This effect is thought to improve the attention deficits associated with ADHD (Kolar et al., 2008). A preclinical study employing nonhuman primates revealed that guanfacine (0.2 mg/kg, im) increased blood flow in the PFC as determined by single photon emission computed tomography (Loo et al., 2003; Avery et al., 2000). Specifically, a significant 5.8% increase in the mid-dorsolateral PCF and an 8.5% increase in the caudal dorsolateral PFC were found. Cognitive function was evaluated using the delayed response task and three of the four subjects demonstrated 18% improvement in the task. Therefore, guanfacine may be an acceptable treatment for ADHD, without the adverse side effects associated with stimulants.

Another therapeutic agent that has been investigated for the treatment of ADHD is modafinil, an analeptic medication approved for the treatment of narcolepsy (Boellner et al., 2006; Greenhill et al., 2006; Hou et al., 2005; Swanson et al., 2006; Wilens, 2006). Modafinil is structurally different from methylphenidate and amphetamine (Figure 3). In a discrimination study using rhesus monkeys, modafinil dose-dependently substituted for cocaine in 6 out of 7 monkeys. These results suggest that modafinil shares discriminative stimulus effects with cocaine which alludes to the abuse potential of modafinil (Newman et al., 2010). One of the most recent clinical studies was a six week double-blind,

randomized trial that included 46 children between the ages of 6-15, who were given 200-300 mg/day of modafinil (depending on weight-200 if < 30kg and 300 if > 30kg) (Kahbazi et al., 2009). Modafinil significantly reduced parent ADHD rating scores, which was the primary outcome measure compared to baseline being -22 ± 8.9 (mean ± SD) and -8.2 ± 6.2 for modafinil and placebo, respectively. Modafinil significantly reduced the secondary outcome measure (teacher ADHD rating scores) compared to baseline being -23 ± 8.2 (mean ± SD) and -7.7 ± 5.0 for modafinil and placebo, respectively. No subjects discontinued treatment with modafinil during the study due to side effects. Side effects associated with modafinil include dry mouth (8.7%), insomnia (8.7%), and decreased appetite (15%) being most common. Limitations of this trial included a small n and no reasons were given as to why one subject dropped out of the modafinil group and 2 subjects dropped out of the placebo group.

With respect to mechanism of action, modafinil alters the balance of GABA and glutamate in brain, resulting in activation of hypothalamus, which is thought to improve the symptoms of narcolepsy (Ferraro et al., 1996; Kahbazi et al., 2009; Keating & Raffin, 2005; Lin et al., 1996; Wilens, 2006). The complex mechanism of action of modafinil has not been fully elucidated. Modafinil has at least four possible targets in the treatment of narcolepsy that are components of the wakefulness-promoting orexin-containing neurons of the lateral hypothalamic/perifornical area, the histamine-containing neurons of the tuberomammillary nucleus of the posterior hypothalamus, the noradrenergic neurons of the pontine locus coeruleus (LC), the mesencephalic dopaminergic neurons, and a group of sleep-promoting GABA and galanin-containing neurons of the ventrolateral preoptic nucleus of the hypothalamus (Hou et al., 2005). Cocaine and amphetamine-regulated transcript (CART) is also a potential modulator for alertness, which is found in the hypothalamus as well (Keating et al., 2010). In addition, it is localized in neurons of the nucleus accumbens, synaptic terminals of the ventral tegmental area and the substantia nigra, partially engaging the mesolimbic DA circuits. This involvement may have influence upon reward/motivation and locomotion. One of the targets of modafinil is the lateral

hypothalamic/perifornical area, where CART is expressed. This interaction between modafinil and CART suggests that modafinil may improve ADHD symptoms, especially those related to alertness and hyperactivity, which is the pathway that involves CART.

With regards to ADHD, modafinil elevates DA and norepinephrine levels in PFC and in rostromedial hypothalamus (de Saint Hilaire et al., 2001). Modafinil may activate noradrenergic neurons in the LC associated with the arousal without affecting the extra LC noradrenergic neurons involved in cardiovascular regulation (Hou et al., 2005). The papillary control of modafinil is of interesting because it is comparable with LC phasic responses to task relevant events (Beatty, 1982; Richer & Beatty, 1987), suggesting the potential for LC/norepinephrine system involvement in optimizing cognitive task performance (Aston-Jones & Cohen, 2005). Furthermore, a study demonstrated that a low dose of yohimbine, an α-$_2$ antagonist, potentiated the modafinil-induced wakefulness and activity (Lin et al., 1992). However, at high doses yohimbine attenuated the modafinil-mediated effects on activity (Duteil et al., 1979). These findings suggest that there is evidence that the adrenergic system may play a role in the mechanism of action of modafinil.

In addition, modafinil effects have been evaluated in ADHD. Contrasting results from various studies have been reported when investigating if modafinil improves the symptoms of ADHD. However, the variables of these studies such as dose, length of administration, age of subjects, and test measurements need to be taken into consideration. For example, a study including 20 adult ADHD subjects were given a single dose of 200 mg modafinil, which was associated with significant improvements in performance on digit span, visual recognition memory, spatial planning, and Stop-Signal Reaction Time (SSRT; (Turner et al., 2004). Conversely, a two-week study including 22 adult ADHD patients in which the modafinil-treated group was given a titrated dose over 4-7 days that averaged dose of 207 mg/day, found no treatment effects of modafinil on the Stroop or Digit Span tests (Taylor & Russo, 2000). Nevertheless, the majority of the modafinil studies have shown that modafinil is effective in treating the

symptoms of ADHD in children, adolescents and adults. In addition, modafinil is well tolerated at the dosages used (Biederman et al., 2006; Biederman et al., 2005; Greenhill et al., 2006;, Rugino & Copley, 2001; Rugino & Samsock, 2003).

The only FDA-approved (in 2002) non-stimulant medication for ADHD is the selective NET inhibitor, atomoxetine (Cormier, 2008; Heal et al., 2008; Olfson, 2004; Wilens, 2006). Atomoxetine has been shown to be effective in children, adolescents and adults with ADHD. Atomoxetine may not be as effective as the gold standard stimulants, as indicated by a study showing improvement of only 45% in ADHD ratings compared to a 56% improvement with methylphenidate (Michelson et al., 2003; Michelson et al., 2002; Michelson et al., 2001; Newcorn et al., 2008). Due to the untoward side effects of atomoxetine, the FDA requires a black box warning which states the potential for suicidal ideation (0.4%). In addition, the atomoxetine black box label was updated in 2004 to include information about the cases of serious liver injury. From January 2005 to March 2008, six post market cases of serious liver injury with atomoxetine were reported to the FDA (Diak & Senior, 2009). Furthermore, based on the FDA receiving six additional reports of serious liver injury in patients taking atomoxetine, the label was revised again in 2007. The Warnings and Precautions section of the label advises prescribers about the risk of severe liver injury with this drug (Diak & Senior, 2009; Lim et al., 2006). Thus, the use of atomoxetine has its own unique concerns to be aware of when employed as a treatment for ADHD that must be balanced with therapeutic benefit (Cormier, 2008; Greydanus et al., 2007). The most common side effects of atomoxetine are sedation (6% of subjects), abdominal pain (11% of subjects), decreased appetite (14% of subjects), and headache (18% of subjects; (Newcorn et al., 2008). Although, atomoxetine is not considered to be a first line agent, it is still a treatment option for ADHD, particularly in patients who do not tolerate or respond to stimulants. Between 2002 and 2007, 3.3 million patients received a prescription for atomoxetine in the US (Diak & Senior, 2009).

With regards to mechanism of action, atomoxetine has a high affinity for NET (K_i value of 2-5 nM) and lower affinity for other neurotransmitter transporters (Gehlert et al., 1995; Tatsumi et al., 1997; Wong et al., 1982). One study reported a K_i value of 1.9 nM for atomoxetine for NET (Wong et al., 1982). Another study extended that finding by determining that a tomoxetine inhibited binding of radioligands ([^{3}H]paroxetine, [^{3}H]nisoxetine, [^{3}H]WIN 35,428) to clonal cell lines transfected with human NET, SERT or DAT with K_i values of 5.0, 77 and 1450 nM, respectively, thus demonstrating over a 10-fold selectivity for NET over SERT and DAT (Bymaster et al., 2002). In the same study, microdialysis in male Sprague Dawley rats showed that local perfusion of 0.34 μM atomoxetine via dialysis probe into PFC significantly increased extracellular norepinephrine and DA to a maximum effect of 175 ± 33 and 190 ± 15% of basal concentration, respectively. In addition, atomoxetine (0.3, 1, 3 mg/kg, ip) produced a 3-fold increase in extracellular levels of norepinephrine in PFC, but did not alter extracellular serotonin levels. Atomoxetine also produced a 3-fold increase in extracellular DA in PFC, but no changes in striatum or nucleus accumbens (Bymaster et al., 2002), suggesting that it will not have drug abuse liability. In contrast, methylphenidate (3 mg/kg, ip) increased extracellular DA in striatum and nucleus accumbens to the same degree, whereas atomoxetine did not alter the amount of DA in these regions of the brain. However, methylphenidate increased extracellular norepinephrine and DA equally in PFC compared with atomoxetine. Furthermore, the latter study found that the expression of neuronal activity marker Fos was increased 3.7-fold in PFC by atomoxetine administration, but was not increased in striatum or nucleus accumbens, consistent with the regional distribution of increased extracellular DA. This study did not evaluate methylphenidate's effect on Fos expression. However, a previous study found that an oral administration of methylphenidate (2.5 mg/kg) given to cats increased Fos expression in striatum (Lin et al., 1996). The atomoxetine-induced increase of catecholamines in PFC, a region involved in attention and memory, may mediate the therapeutic effects of atomoxetine in ADHD and may be

associated with the improvements in executive function and other cognitive functions (Wilens, 2006).

7. Investigational Treatments

The current treatment options for ADHD have been discussed, however due to the adverse side effects of some of these medications, along with the lack of effectiveness of others, has spurred the development of novel therapeutic options for ADHD. According to the National Institute of Mental Health, there are clinical trials underway to investigate new candidate pharmacotherapies.

One particular clinical trial is entitled Betahistine: Novel Therapeutic in Attention Deficit Hyperactivity Disorder. Betahistine is an antivertigo drug, which was first registered in Europe in 1970 for the treatment of Méniére's disease. Betahistine has a high affinity for H_3 receptors and a low affinity for H_1 receptors. However, the mechanism of action of Betahistine is related to its ability to increase the levels of neurotransmitters in the brainstem (Barak, 2008). This study started in January of 2009 with an estimated completion date of December of 2009 (NIH, 2010).

Another ongoing clinical trial is an open-label multicenter sequential group, phase 1 study in 6-11 year old patients with ADHD (NIH, 2010). The agent being investigated is JNJ-310001074. However, there was no available literature on the pharmacology of this compound and how it relates to ADHD. An additional trial on this compound is ongoing in Wisconsin, where the investigators are evaluating the efficacy and safety/tolerability of 3 different doses of JNJ-310001074 compared to placebo.

There is one clinical trial investigating a medication that has already received approval from the FDA for another condition. This medication, called varenicline (Chantix®), was approved for the treatment of smoking cessation in May of 2006 (NIH, 2010). Varenicline interacts with specific nicotinic receptors. Nicotinic receptors are a complex system of different subunits that are a part of the super-family of ligand-gated ion channels (Xiong et al., 2007). These complexes are incorporated within the cell membrane and are composed of pentameric groups of α (α2-10) and β (β2-4) subunits. The subtypes contain only

one subunit type and are therefore called homomeric. Most of the subtypes are heteromeric, i.e., containing both an α (α2-10) and β (β2-4) subunit (Symons et al., 2010). The most common subtypes in the brain include the homo-oligomeric α7 and the heteromeric α4β2. The composition of the subtype can determine its sensitivity of a receptor for nicotine. Varenicline binds more potently to the α4β2 subtype of the nicotinic acetylcholine receptor compared to α7subunit>3500 fold; (Mihalak et al., 2006), without producing a full nicotinic effect on dopamine release. Therefore, it is considered a partial agonist of the α4β2 subtype. In addition, varenicline inhibits the ability of nicotine to stimulate the central nervous mesolimbic DA system. The purpose of this clinical trial is to determine if varenicline can improve the symptoms of ADHD and also decrease the amount of smoking in this population. A secondary outcome is to assess the tolerability and response to varenicline more fully in this population.

The most promising candidate for the treatment of ADHD is a new chemical entity, called AZT3480 (TC-1734; (NIH 2010). This compound, which is a partial agonist for the nicotinic acetylcholine receptor, is selective for the α4β2 subtype of the nicotinic acetylcholine receptor. As of May 2009, it has been evaluated in six Phase 2 clinical trials in various neurocognitive disorders, including ADHD. It has been evaluated in about 1,350 subjects from Phase 1 and Phase 2 trials and has exhibited consistently a favorable tolerability profile (Dunbar et al., 2007). Preliminary results show that AZT3480 met the primary outcome measure in a Phase 2 clinical trial in adult ADHD. The most common side effects that have been reported include dizziness and headache (Gatto et al., 2004). As of July of 2009, there were plans to conduct a vigorous drug development program for AZT3480.

One novel clinical candidate for the treatment of ADHD and the focus of our research is the alkaloid lobeline. The rationale for the potential utility of lobeline as a treatment option for ADHD is that it interacts with the same transporter proteins, DAT and VMAT2, as methylphenidate and amphetamine, which are the gold standard treatments for ADHD. In addition, it also has a high

affinity (Ki of 4-30 nM) for central nicotinic receptors as well (Broussolle et al., 1989; Damaj et al., 1997; Lippiello & Fernandes, 1986; Yamada et al., 1985).

8. Lobeline as a Candidate for ADHD Pharmacotherapy

a. Historical background and clinical uses:

Lobeline (Lob; Figure 3), an alkaloidal component of *Lobelia inflata*, is a novel compound that has been investigated in the treatment of drug abuse, including but not limited to, methamphetamine, nicotine, cocaine, opioid, and alcohol abuse (Dwoskin & Crooks, 2002; Farook et al., 2009; Miller et al., 2007; Polston et al., 2006). The first report of *Lobelia inflata* being used for medicinal purposes occurred in 1813, when Reverend D. Cutler referred to the alkaloid as a effective remedy for asthma (Millspaugh, 1974). In 1938, Proctor was the first to document the pharmacological effects of an alkaloid extract of the plant and reported on its use as an expectorant, asthma treatment, anti-spasmodic, emetic, diuretic, respiratory stimulant, and for narcotic overdose. The seeds of the plant contain the highest amount of lobeline, the principal alkaloid. Lobeline was named after Matthias de Lobel who was a French botanist and physician (Dwoskin & Crooks, 2002). Wieland identified the chemical structure of lobeline and subsequently synthesized it in 1925 (Wieland H, 1925).

Lobeline has also been considered as a therapeutic agent for smoking cessation dating back to 60 years ago (Dwoskin & Crooks, 2002). Lobeline was first reported by Dorsey, in 1936 as a smoking cessation agent (Dwoskin & Crooks, 2002). Although a review on the clinical studies using lobeline stated that lobeline had no effect on smoking (Stead & Hughes, 2001), others believe that if a new dosage form of lobeline was developed with improved bioavailability, lobeline would be an efficacious smoking cessation agent (Schneider & Olsson, 1996). Poor compliance due to the multiple dosing and side affects such as nausea, dizziness, vomiting, and hypertension could have contributed to the lack of efficacy. The evidence supporting the utility of lobeline as a smoking cessation agent may be inconclusive, but nevertheless, the interest in lobeline to reduce smoking is ongoing (Buchhalter et al., 2008). Due to this interest, a recent study was published in 2010 that had an objective of

evaluating the safety and efficacy of sublingual lobeline on smoking cessation (Glover et al., 2010). This was a multicenter Phase 3 trial involving 3 separate sites and a total of 750 smokers (250 from each site). The results of this study showed that the efficacy for lobeline was not significant (p= 0.62) compared to placebo. Based on these results, the authors concluded that lobeline does not appear to be an efficacious smoking cessation aid. It would be interesting to learn if another dosage form of lobeline would be more effective in smoking cessation by reducing the adverse effects, since the sublingual tablet does not seem to have any significant effect.

b. Pharmacokinetics and Mechanism of action

Research examining the pharmacokinetics of lobeline is very scarce. In a previous study in rats (Reavill et al., 1990), lobeline (4mg/kg, sc) had a reported lipophilicity of 1.68 at a ph of 7.4. The plasma concentration at this dose was 74.3 ng/ml and the brain concentration was 237 ng/ml. These results imply that lobeline has the ability to penetrate the blood-brain barrier by both carrier and lipid mediation. In addition, the authors suggest that based on the behavioral data demonstrating that lobeline did not produce a nicotine-like effect in doses as large as 6.4 mg/kg, that other receptors other than nAChRs may mediate the effects of lobeline (Reavill et al., 1990a). However, previous research has revealed similarities in the pharmacology of nicotine and lobeline, which resulted in lobeline being classified as a nicotinic agonist. Lobeline may have the similar effects of nicotine on cognition, based on studies utilizing radial-arm maze and spatial discrimination water maze, which showed that lobeline improved performance and learning in rats (Decker et al., 1993; Levin & Christopher, 2003). However, there is no common pharmacophore or apparent structural likeness of lobeline to nicotine (Dwoskin & Crooks, 2002). Some of the reasons why lobeline was considered a nicotinic receptor agonist were because lobeline caused tachycardia, hypertension, hyperalgesia, improvement of learning and memory, and in anesthetized rats it causes bradycardia and hypotension; which are all pharmacological effects of nicotine (Decker et al., 1993; Hamann & Martin, 1994; Olin et al., 1995; Sloan et al., 1988). In addition, studies have

shown that nicotine improves cognition. One study involved 62 male non-smokers, who were randomized into two groups, a low attention group and a high attention group (Poltavski & Petros, 2006). Based on the results from the Wisconsin Card Sorting Test, classic Stroop Test and the Conner's Continuous Performance Test (CPT), transdermal nicotine improved attention in the low attention group and decrease working memory in the high attention group. This suggests that nicotine optimizes rather than just improving cognitive function. Another study investigated transdermal nicotine in healthy non-smoking adults with no attentional deficits (Levin & Simon, 1998). Based on the results from the Profile of Mood States Test, nicotine significantly improved self-perceived vigor. Using the CPT, it was found that nicotine significantly reduced the number of errors of omission. Taken together, these results suggest that nicotine not only reduces attentional impairment, it also can improve attentiveness in normal adult non-smokers. A more recent study (Potter & Newhouse, 2008) used the Stop Signal Reaction Time to measure the effect of nicotine on cognition. The results showed that nicotine had a significant positive effect on this task without changes in the Go reaction time or accuracy. These data suggest that cholinergic agents like nicotine, may be potential pharmacotherapies for the cognitive deficits associated with ADHD.

However, lobeline also has effects that are different from nicotine. For instance, nicotine is reported to be self-administered by rats (Corrigall & Coen, 1989; Goldberg et al., 1981; Rasmussen & Swedberg, 1998; Sorge & Clarke, 2009); however lobeline is self-administered in mice and is not self-administered by rats (Harrod et al., 2003; Rasmussen & Swedberg, 1998). Chronic nicotine treatment results in an increase in locomotor activity (Clarke, 1990; Clarke & Kumar, 1983; Fung & Lau, 1988) and produces conditioned place preference (Fudala et al., 1985; Risinger & Oakes, 1995; Shoaib et al., 1994), but lobeline does not produce these effects (Dwoskin & Crooks, 2002). Even though one study reported that lobeline generalized to nicotine in a drug discrimination assay (Geller et al., 1971), additional studies have not been able to reproduce this finding (Reavill et al., 1990b; Romano & Goldstein, 1980; Schechter &

Rosecrans, 1972). Lobeline also produces transient, small cardiovascular effects, such as increase in heart rate, and with chronic use, it acts as a depressant of the sympathetic and parasympathetic ganglia as well as the adrenal medulla (Sloan et al., 1988), with side effects including decreased breathing rate and hypothermia.

Due to the complexity of the pharmacological actions of lobeline, further investigation is necessary to evaluate other potential effects of lobeline in addition to its nicotinic receptor agonist effects. Lobeline has high affinity for the nAChR (K_i = 4-30 nM; (Broussolle et al., 1989; Damaj et al., 1997; Lippiello & Fernandes, 1986; Reavill et al.,1990b; Yamada et al., 1985). Furthermore, in a study using in vitro [^{3}H]nicotine binding assays in rat brain, lobeline displaced binding of [^{3}H]nicotine with a K_i value of 4.4 ± 2.2 nM (Damaj et al., 1997). This finding confirms that lobeline has a high affinity for nicotinic receptors, but does not indicate if lobeline is an agonist or antagonist at these sites. However, using a frog oocyte expression system with a two-electrode voltage-clamp, 0.1 and 1 mM lobeline produced only a small current when applied for 10 sec to oocytes expressing the α4β2 subtype, suggesting that lobeline is not acting as an agonist at this subtype of nAChR (Damaj et al., 1997). Moreover, 10 μM lobeline antagonized 50% of the current-induced by 3 μM nicotine, although antagonism is not observed *in vivo* in mice (Damaj et al., 1997). Also, pretreatment with mecamylamine and dihydro-β-erythroidine at a dose of 10 μg/mouse, sc, 5 min before lobeline (40μg/mouse) did not decrease lobeline-induced motor impairment, but these compounds did inhibit effects of nicotine, including lethality, seizures, and cardiovascular effects. Taken together, the results of this study suggest that lobeline interacts with a different mechanism than nicotine. Furthermore, the interaction of ligands with their receptors depends on a number of factors including receptor localization, subtype specificity, and potency of antagonist (Damaj et al., 1997).

A series of studies investigated the action of lobeline as a nicotinic antagonist. The subsequent studies that investigated the action of lobeline, concluded that lobeline is a nicotinic antagonist (Clarke & Reuben, 1996;. Teng

et, al 1998; Teng et al., 1997; Terry et al., 1998). Specifically, lobeline evokes $^{86}Rb^{+}$ efflux from striatal synaptosomes with low efficacy (Terry et al., 1998). This $^{86}Rb^{+}$ efflux assay is a chemical method of investigating ion flux, whereby the $^{86}Rb^{+}$ acts like K^{+}. If there is an increase in $^{86}Rb^{+}$ efflux, then the compound in question acts as an agonist. In this case, lobeline produced low $^{86}Rb^{+}$ efflux, suggesting that it acts as an antagonist instead of an agonist.

In addition, lobeline-induced [^{3}H]DA release is independent of extracellular calcium concentration and is not sensitive to mecamylamine, which indicates that the lobeline-evoked DA release is not mediated by nAChRs, and that lobeline does not act as an agonist nAChRs (Clarke & Reuben, 1996; Teng et al., 1998; Teng et al., 1997). Importantly, a study using *in vivo* brain microdialysis was used to investigate the influence of lobeline on DA and DOPAC overflow in the core of nucleus accumbens in freely-moving rats pretreated with nicotine (0.4 mg/kg, sc) once daily for 5 days. Lobeline (4.0 and 10 mg/kg) inhibited nicotine-evoked [^{3}H]DA overflow when administered 10 minbefore nicotine, but not after 60 min (Benwell & Balfour, 1998). In addition, the low dose of lobeline had no effect at either time point in this study. The effect of lobeline on nicotine-evoked [^{3}H]DA overflow was investigated using striatal slices (Miller, 2000). Striatal slices were superfused with lobeline for 30 min and 1 of 4 concentrations of nicotine was added to the buffer containing lobeline and superfusion continued for an additional 60 minutes. The results showed that lobeline blocked nicotine-evoked [^{3}H]DA overflow from rat striatal slices (Benwell & Balfour, 1998; Miller et al., 2000), which suggests that lobeline is an nicotinic antagonist.

In addition, lobeline interacts with the same transporter proteins, DAT and VMAT2, at which psychostimulant drugs of abuse and treatments for ADHD interact (Miller et al., 2003). Lobeline inhibits [^{3}H]DA uptake into vesicles with an IC_{50} of 0.88 μM and inhibits the binding of [^{3}H]dihydrotetrabenazine, a VMAT2 ligand, to vesicular membranes with an IC_{50} of 0.90 μM (Teng et al., 1998; Teng et al., 1997). Lobeline inhibits [^{3}H]DA uptake into synaptosomes with an IC_{50} value of 80 μM (Teng et al., 1997). Results from this same study also demonstrated that lobeline increased DOPAC efflux, and did not increase

endogenous DA release (Teng et al., 1997). The increase in DOPAC suggests that lobeline does not inhibit MAO like amphetamine does. In addition, lobeline is more potent inhibiting DA uptake in vesicles (IC_{50}= 0.88 μM) than releasing DA, whereas amphetamine is equipotent in inhibiting and promoting DA release (EC_{50}~2.22 μM;(Teng et al., 1998). Both of these effects of lobeline may explain at least in part why lobeline is not self-administered.

Taken together, the evidence from the above studies indicates that the overall mechanism of action of lobeline is a combination of interactions with different targets, the outcome of which is a diminished extracellular concentration of DA. Specifically, lobeline inhibits nAChR, DAT and VMAT2 function. The outcome of the interaction with VMAT2 would be expected to decrease the vesicular DA pool and increases the cystosolic DA, although this has not been determined directly. The proposed increase in cryptozoic DA would be expected to be metabolized by MAO, leading to an increase in extracellular DOPAC, which has been observed (Teng et al., 1997).

9. Animal Models for ADHD

The development of an appropriate animal model to evaluate potential new agents for ADHD is based on the behavioral, neurochemical, and neuroanatomical profile of ADHD individuals. A valid ADHD animal model should: 1) mimic the clinical symptoms and presentation of individuals with ADHD (face validity), 2) show the underlying neurochemical changes in ADHD, thus confirming the theoretical underlying etiology for ADHD (construct validity) and 3) predict an appropriate response to the effective, currently available, ADHD treatments (van der Kooij & Glennon, 2007). Another factor to consider is the age of the rats in the animal models, since ADHD is primarily found in children and adolescents (American Psychiatric Association, 2000), but also persists into adulthood (Kessler et al., 2006). There are many ADHD animal models; however no one model satisfies all the criteria of an animal model for ADHD. Current models have been developed by either creating a lesion in the brain or by manipulation of the genome.

a. Neonatal 6-OHDA

The first studies to model ADHD used 6-hydroxydopamine (6-OHDA) administered to neonatal rats to produce hyperactivity, which was expressed temporarily from postnatal day (PND) 12-22 (Shaywitz et al., 1976). 6-OHDA (20 µg/µl; intracereoventricularly) is toxic to dopaminergic and noradrenergic neurons, decreasing DA and norepinephrine content and neurotransmission in brain. DA decreased in the frontal cortex by 76%, in striatum by 96%, and in nucleus accumbens by 84%. Norepinephrine levels decreased by 13% in frontal cortex and by 39% in nucleus accumbens (Archer et al., 1988). This model resulted in learning and memory deficits, which are consistent with ADHD symptoms (Archer et al., 1988). Autoradiographic studies have demon strated the involvement of the DA D4 receptor in the effects of neonatal 6-OHDA in this model of ADHD (Avale et al., 2004; Zhang et al., 2002; Zhang et al., 2001). These latter findings are consistent with clinical observations that there is a higher incidence of ADHD when a polymorphism, in which a direct repeat in the 48-base-pair sequence in the DA D4 receptor gene exists (Benjamin et al., 1996; Van Tol et al., 1992). Additional research has shown that the indolamine in addition to the noradrenergic neurotransmitter systems appear to be involved in the pathology of ADHD due to the observations that NET inhibitors, such as desipramine and nisoxetine and 5-HT inhibitors citalopram and fluvoxamine reduced hyperactivity in 6-OHDA (100 µg) lesioned juvenile male rats, but the DA inhibitors had no effect on hyperactivity in 6-OHDA (100 µg) lesioned juvenile male rats (Davids et al., 2002). These results suggest that the neonatal 6-OHDA rat is not a good model for ADHD. The neonatal 6-OHDA lesion model using rats also has excellent predictive validity because in one study methylphenidate (0.25 mg/kg, sc) reduced hyperactivity in 6-OHDA lesioned rats (Shaywitz et al., 1978). However, it takes more than predictive validity for an animal model to be considered a good model for ADHD. In another study, methylphenidate (1 and 4 mg/kg, sc) and amphetamine (0.25 and 1 mg/kg, sc) both decreased hyperactivity in neonatal 6-OHDA lesioned rats (Luthman et al., 1989). Moreover, a study evaluating the effect of atomoxetine (1 mg/kg) on neonatal 6-

OHDA lesioned rats found that this non-stimulant also was effective in reducing motor hyperactivity in this animal model (Moran-Gates et al., 2005). Taken together, these studies provide evidence that the neonatal 6-OHDA lesioned rat model is not the best animal model for ADHD. Furthermore, this model has limitations because there is a lack of evidence showing that ADHD medications improve learning and memory deficits, not just hyperactivity.

b. Neonatal alternative models

Alternative ADHD animal models include the neonatal hypoxia rat model and the neonatal bromodeoxyuridine (BrdU) rat model. The neonatal hypoxia rat model was developed by immersion of rats in 100% nitrogen for 25 min at various stages of development ranging from 30 min after birth (Davids et al., 2002), PND 2 (Dell'Anna et al., 1991), PND 4 (Shimomura & Ohta, 1988), or at PND 10 (Decker et al., 2003). This animal model also produces permanent deficits in learning and memory (Gramatte & Schmidt, 1986). Although amphetamine decreased hyperactivity, stimulants have not been thoroughly investigated for effects on improving learning and memory (Speiser et al., 1983). However, based on the limited research of this model, its predictive validity as a ADHD model is not clear (van der Kooij & Glennon, 2007).

The neonatal BrdU rat model incorporates BrdU administration (50 mg/kg body weight, ip, every 12 hours for 2.5 days) to dams during gestational days between 9 and 15 (van der Kooij & Glennon, 2007). This treatment produces behavioral problems, impaired sexual behavior, deficits in learning and memory, and hyperlocomotion in the male offspring of these dams (van der Kooij & Glennon, 2007). Nevertheless, no construct validity has been examined in this model and research has shown that methylphenidate (1 or 4 mg/kg, s.c.) has no effect in the BrdU rat model (Muneoka et al., 2006), which also questions the predictive validity of this model. In addition, this model also has a hyposexuality aspect that is not associated with ADHD. In conclusion, the overall validity of the neonatal BrdU rat model for ADHD remains questionable based on the above evidence.

c. Genetic models

There are also established genetic rat models of ADHD. These models include the spontaneously hypertensive rat (Schmitt et al., 2008), the Wistar-Kyoto derived hyperactive rat (WKHA), and the Naples high (and low) excitability rat (Krause et al) models. The SHR model, the most commonly studied animal model of ADHD, was developed in the 1960's by inbreeding individuals in the WKY strain that showed high systolic blood pressure, which resulted in hypertension in subsequent generations (Okamoto & Aoki, 1963). The SHR model satisfies many of the criteria for a valid model of ADHD because these rats display hyperactivity, impulsivity, poor performance stability, poor sustained attention, and spatial working memory deficits (De Bruin et al., 2003; Hernandez et al., 2003; Ueno et al., 2002) when compared to WKY (Tsai & Lin, 1988; van den Bergh et al., 2006).

However, hypertension is not associated with ADHD patients, leading to potentially confounding interpretations as to the results obtained with this model, i.e., the role that hypertension plays in contributing to the effects. In addition, the predictive validity of this model in identifying novel treatments is questionable because amphetamine and methylphenidate have been shown to increase hyperactivity in this rat model, which is the opposite result that is observed clinically (Amini et al., 2004; McCarty et al., 1980; Wultz et al., 1990). The age of these rats in this model may also be a concern to some investigators, because the rats between 10-12 months old, which is considered an adult rat (Spear, 2000), yet ADHD is diagnosed in adults and is an ongoing issue in the adult population as well. Conversely, a more current study found that methylphenidate (1.5 mg/kg, po) lowered the amount of errors in the attentional set-shifting task in the SHR rat model using 9 week old rats (Kantak et al., 2008). This same study sought to advance the SHR animal model of ADHD toward medication development by utilizing 3 behavioral tests to assess the validity of this model with respect to the deficiency in learning and memory associated with ADHD, using 9 week old rats versus adult rats (Kantak et al., 2008). Specifically, the function of the orbitofrontal cortex, the dorsal striatum, and the prelimbic cortex

were evaluated using the odor-delayed win-shift task (Di Pietro et al., 2004), the win-stay task (Kantak et al., 2001), and the attentional set-shift task (Birrell & Brown, 2000). The odor-delayed win-shift task training phase involves the rats discriminating among four odors in four arms and keeping online the memory of the odors for which the reinforcer was already received. The rats were given eight training trials per day for 4 days to learn to dig in an unscented sand cup for a hidden fruit loop reinforcer. The turn bias used a T-configuration for the maze and the rats were started from the less familiar stem arm and had to choose the correct strategy to advance to the extradimensional set-shifting trials conducted the following day. The food well of each choice arm was baited with a single pellet and a visual cue was randomly placed on the left wall of the one of the choice arms before each trial. If a rat made an incorrect choice, the discrimination trials continued until it was able to reach five consecutive correct reinforced choices.

In addition, Kantak and colleagues included a genetic control in this latter study, the WKHT strain, which is a Wistar-Kyoto-derived strain of rat inbred for hypertension, but not exhibiting the hyperactivity. This important aspect provided the first direct test of the impact of hypertension in these ADHD-relevant learning paradigms. Furthermore, in the experiments the authors used 9-week old rats, an age which is considered more comparable with adolescence. The results from the odor-delayed win-shift task revealed that the over the entire task there was no strain differences in the cumulative number of working memory errors or reference memory errors made over the 20 test phase sessions. However, the methylphenidate-treated SHR strain completed the test phase 78% faster than the vehicle-treated WKY strain. This suggests that methylphenidate elevates some of the working memory deficits. The SHR strain latencies to traverse the arm and recover a food pellet in the win-stay task were 42% faster than the WKY strain and the WKHT strain was 20% faster than the WKY strain. This indicates that the learning deficits of the SHR strain are not associated with a lower motivation level compared to the other two control strains. The model was validated by the findings that methylphenidate(1.5 mg/kg, po, 30 min

pretreatment) significantly improved latencies in the win-stay task, where the methylphenidate-treated SHR finished the task 66% faster than the vehicle-treated WKY strain. These results demonstrate that methylphenidate treatment was effective in enhancing the performance of the SHR, thus allowing them to complete the task faster than the vehicle-treated WKY. With the attentional set-shifting task, the vehicle-treated SHR strain had 23% more errors compared with the vehicle-treated WKY. Methylphenidate-treated WKY strain had 30% less errors compared to the methylphenidate-treated SHR strain. These findings demonstrate that methylphenidate eliminates strain differences in attention. The WKHT strain performed similarly to the WKY, suggesting that the hypertension is not associated with the hyperactivity found in the SHR rat model. The overall results of this study support the use of the SHR as a valid model for ADHD, particularly with respect to the neurocognitive deficits associated with this disease, and that the WKY is an appropriate control strain to compare with the SHR when neurocognitive endpoints are evaluated.

The SHR strain and the WKY strain were crossbred in order to develop a strain without the hypertension associated with the SHR model. This led to the development of the WKHT rat having hypertension but no hyperactivity and the WKHA rat model, which had no hypertension but had hyperactivity (Hendley & Ohlsson, 1991). In regards to face validity, in the absence of hypertension, this model has abnormal attentional processing, impulsivity, and learning deficits. The WKHA model also had a low predictive validity. This conclusion was demonstrated by the results of a study that administered methylphenidate (5 mg/kg) to WKHA rats and found that their locomotor activity increased by 200% (Drolet et al., 2002). Based on these results, the increase in locomotor activity decreases the predictive validity because methylphenidate should not increase locomotor activity, but decrease it. Furthermore, more research is needed to investigate the construct validity of this model because the role of the DA receptors and DAT is still unclear. In conclusion, these observations limit the usefulness of the WKHA model as an ADHD animal model.

The Napel High Excitability (NHE) rat model has been investigated as an ADHD model because of the different exploratory activity in the Làt maze, a commonly used method of testing locomotor activity and habituation in a novel situation. This model was based on reactivity to novelty, not baseline activity level (Sadile et al., 1993). NHE rat model has an advantage over the SHR model, because the NHE strain does not have hypertension (Cerbone et al., 1993), and does not have the limitation of evaluating the effect of hypertension. The face validity of this model needs to be improved because even though it demonstrates hyperactivity and attention deficits, no impulsivity has been observed. In addition, the predictive validity of this model still needs to be investigated. In reference to construct validity, one study evaluated mesencephalic TH expression in coronal sections as determined by immunohistochemistry in the NHE rat model (Viggiano et al., 2003). Results showed a larger neuron size in the VTA. These results suggest that the mesolimbic and nigrostriatal DA pathway are normal, but the mesocortical DA pathway is hyperfunctional and hyperinnervated. However, a larger VTA neuron size in ADHD has not been reported, thus limiting its construct validity. In addition, the size of the neuron can not be the only factor involved in the functional of the pathway. The NHE could be potential ADHD animal model because it demonstrates the aspects of ADHD including hyperactivity and deficits in tasks requiring visuospatial attention (Aspide et al., 1998; Papa et al., 2000). Nevertheless, further study is necessary to determine how ADHD medications affect this rat model and if this model demonstrates the other symptoms of ADHD, such as impulsivity. The age of the animal is not a limitation because this model used adult rats and as stated earlier, adults suffer with ADHD as well. In conclusion, this model should not be considered a suitable model for ADHD until additional research is performed such as investigating the construct validity in more detail in order to validate the model more appropriately.

There are also models of ADHD which employ mice, rather than rats. Some of these models include the hyperactive-wheel turning mice, the coloboma mutant mice, and the DAT-KO mice. The hyperactive wheel-running mouse only demonstrates hyperactivity in regards to face validity (Rhodes et al., 2001). As

far as construct validity, altered dopaminergic function regarding the D1 and DAT may be involved in producing the hyperactivity (Rhodes et al., 2001). The predictive validity is weak because only amphetamine has been shown to attenuate the hyperactivity (Rhodes et al., 2001). The coloboma mutant mice model was developed by neuron irradiation in mice (Searle, 1966). This model has been used as a ADHD model because it possesses a weak face validity in that it displays spontaneous hyperactivity (Hess et al., 1992) and delayed development (Heyser et al., 1995). However, impulsivity has not been observed. It is suggested that the neurochemical basis of this model is produced by elevated noradrenaline levels in striatum and nucleus accumbens of the coloboma mouse (Jones et al., 2001). The predictive validity is questionable because amphetamine blocks the hyperactivity, however methylphenidate increases hyperactivity (Hess et al., 1996). Based on the lack of validity of these two models, they are not recommended as suitable ADHD models. Due to the lack of research of these models, a greater focus has been placed on DAT-KO mice.

Since DAT is the major regulator of DA clearance and DAT-gene alterations have been associated with ADHD, DAT-KO mice were developed (Cook et al., 1995; Gill et al., 1997). In support of the validity of this model, DAT-KO mice have been found to display hyperactivity and spatial learning deficits (Gainetdinov & Caron, 2001; Gainetdinov et al., 1999). Methylphenidate and amphetamine attenuate the hyperactivity observed in the DAT-KO mice, also in support of the validity of the model. However, a disadvantage of this model is that the target of psychostimulants, DAT is missing, which makes it more difficult to assess the predictive validity. There is the possibility that not all the DAT has been knocked out which depends on the process used to develop the DAT-KO mice (NIH, 2010). The pups born from the embryos that had the altered embryonic stem cells injected into them do not have the DAT completely knocked out because there is also normal tissue present as well. In order to produce a homozygous line of knockout, crossbreeding is required where each copy from respective chromosomes is knocked out in the present tissue (NIH, 2010).

Nevertheless, a study utilizing fast scan cyclic voltammetry of extracellular striatal DA levels, show impaired DA clearance in DAT-KO mice x 300 times compared to controls. These results demonstrate that the DAT gene was knocked-out. A more logical explanation is that these psychostimulants are interacting with other neurotransporters, such as NET and 5-HT, to elicit their effects. This leaves open the question of the involvement of other targets for the action of effective pharmacotherapies for ADHD.

In order to eliminate some of the disadvantages associated with the DAT-KO mice mode, such as premature death, growth retardation, and the absence of DAT, the DAT-knock-down (DAT-KD) mouse model was created. This line was developed by breeding heterozygous mutants in a 129 Su/J genetic background (Zhuang et al., 2001). Both the DAT-KO/KD models respond to amphetamine and methylphenidate, thus supporting the predicative validity of the models for ADHD. However, more research is needed to find the construct validity marker such as D4 receptor involvement. In reference to the face validity, both models possess hyperactivity, impulsivity, and attention deficits. Another advantage of the DAT-KD model is that it expresses a small percentage of DAT, thus allowing it to have a more acceptable predictive validity because pharmacotherapies targeting DAT can be evaluated. The age of the mice used in these models is not clear, however the model suggest that the mice are adolescents since some do not live to adulthood.

In conclusion, there are several potential ADHD animal models from which to choose. Nonetheless, ADHD animal model needs to demonstrate the symptoms associated with ADHD (face validity), confirm an underlying theory of ADHD (construct validity), and possess the appropriate response to ADHD treatments (predictive validity). There is no perfect animal model for ADHD, but the most promising models include the neonatal 6-OHDA lesion model and the DAT/KO-KD mice model based on their ability to meet the aforementioned criteria.

10. Specific Aims and Hypotheses

The work discussed in this dissertation was based on the hypothesis that DAT and VMAT2 are the primary targets for the pharmacological effects of ADHD psychostimulants, methylphenidate, and amphetamine. DAT is generally thought to be a major target for these agents; however, it may not be the only target. In addition, these pharmacotherapies have untoward effects such as abuse liability, which limits their use and demonstrates the need for additional ADHD treatment options. Lobeline may be a potential treatment option for ADHD because it interacts with the same transporters as methylphenidate and amphetamine, but does not have abuse liability.

The **specific hypotheses** for this project were:

1) Lobeline will decrease DAT function after acute and repeated *in vivo* administration, as shown by a decrease in Vmax, based on results from previous *in vitro* studies performed in our lab

2) Lobeline will have a greater effect on VMAT2 function after acute and repeated *in vivo* administration as shown by the parameters of Vmax and K_m, based on results from previous *in vitro* studies

3) DAT trafficking is the underlying mechanism behind the modulation of DAT function as shown by an increase of DAT to the neuronal cell surface of the striatum.

a. Specific Aims

Specific Aim 1) Determine the effect of lobeline after acute and repeated *in vivo* administration on DAT function using [^{3}H]DA uptake assay.

Specific Aim 2) Determine the effect of lobeline after acute and repeated *in vivo* administration on VMAT2 function using [^{3}H]DA uptake assay.

Specific Aim 3) Determine if the underlying mechanism of modulation of DAT function is due to DAT trafficking, using biotinylation and western blot analysis.

The effects of lobeline were compared to the effects of methylphenidate and amphetamine for all experiments.

Figure 1. Schematic of a DA nerve terminal.

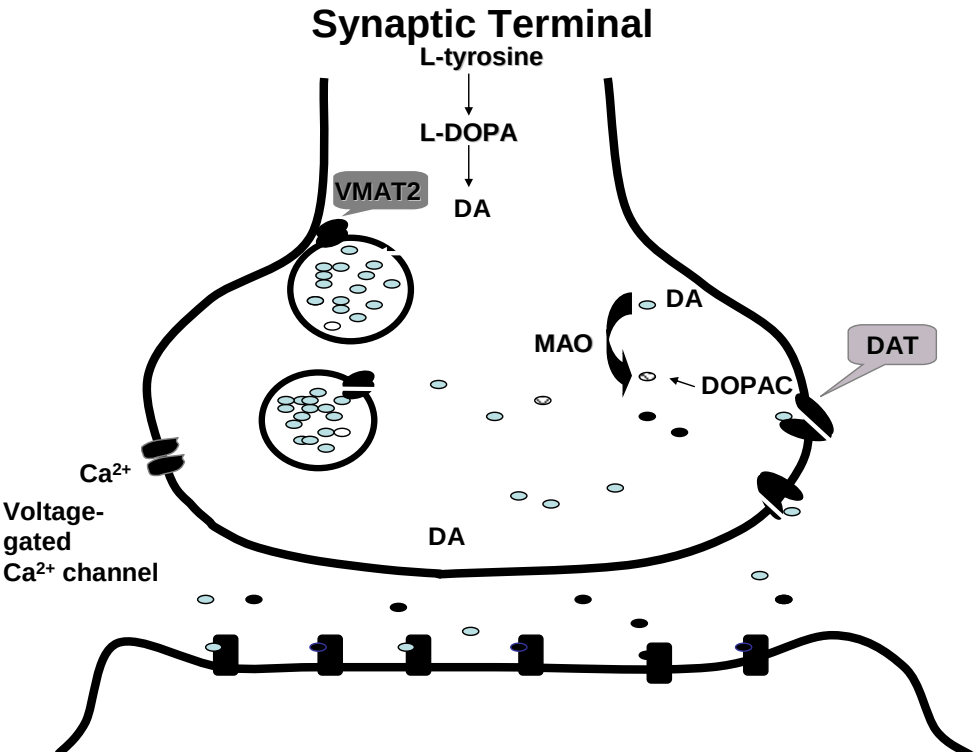


Figure 2. 3D-Image of a DA transporter. Used with permission by Dr. Zhan.

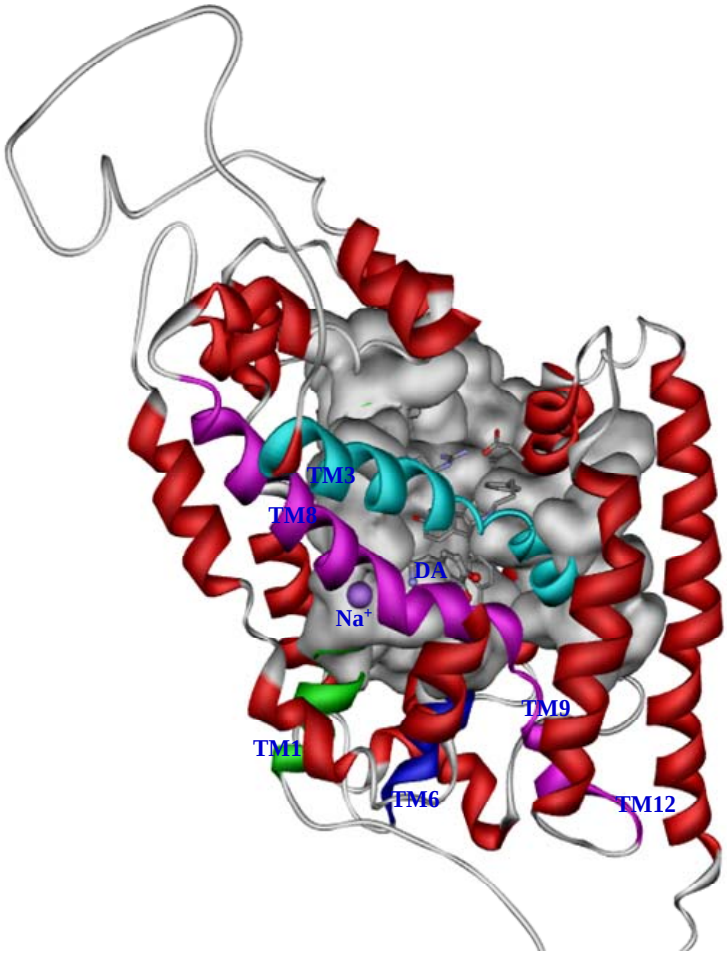
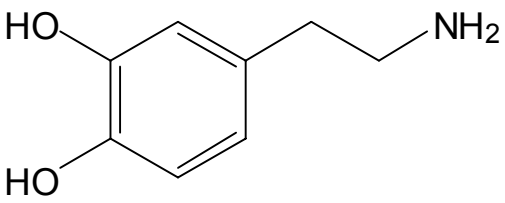
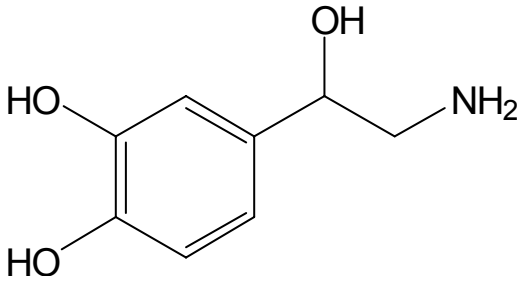


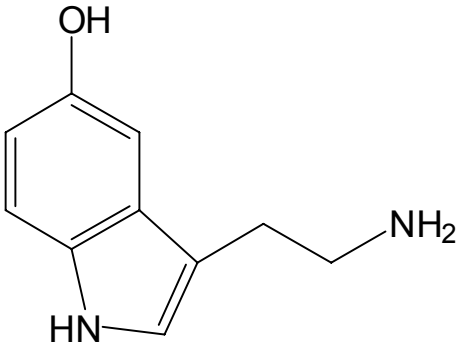
Figure 3. Structures



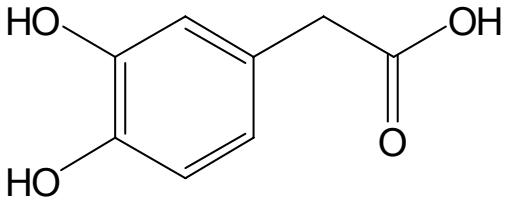
Dopamine



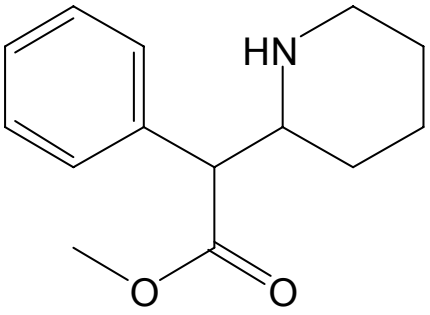
Norepinephrine



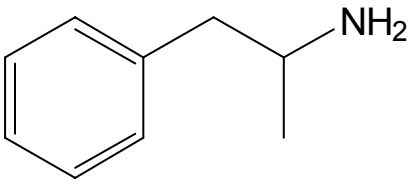
Serotonin



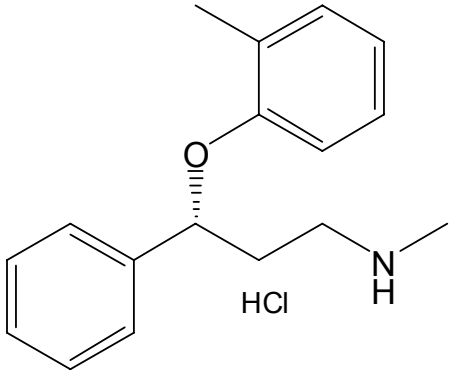
Dihydroxyphenylacetic Acid (DOPAC)



Methylphenidate

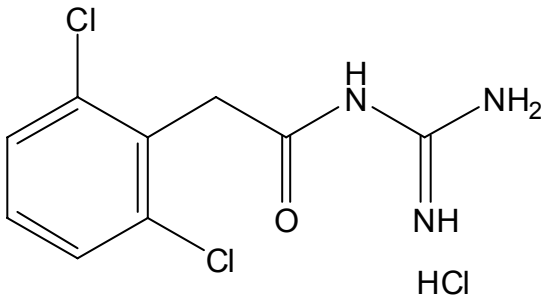


Amphetamine

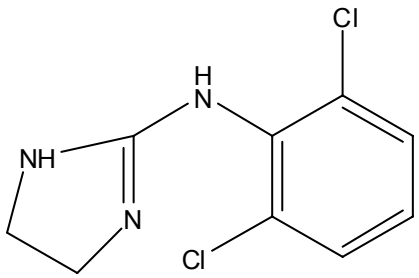


Atomoxetine

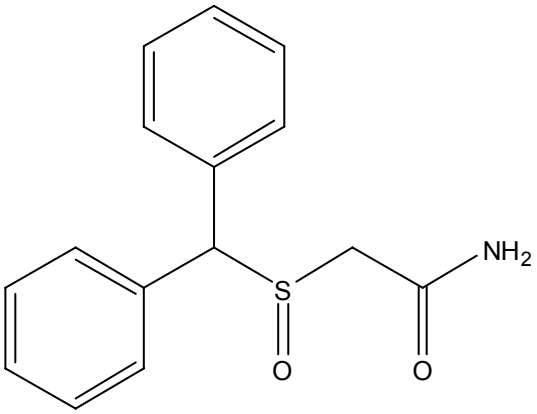
Figure 3. Structures (Continued)



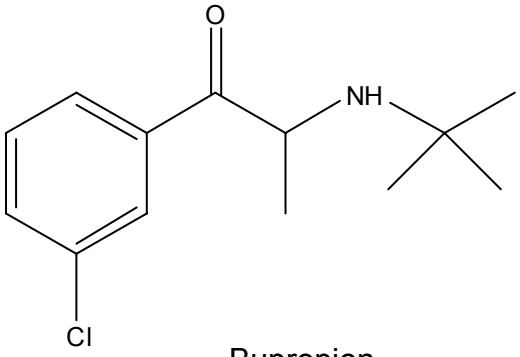
Guanfacine and Intuniv®



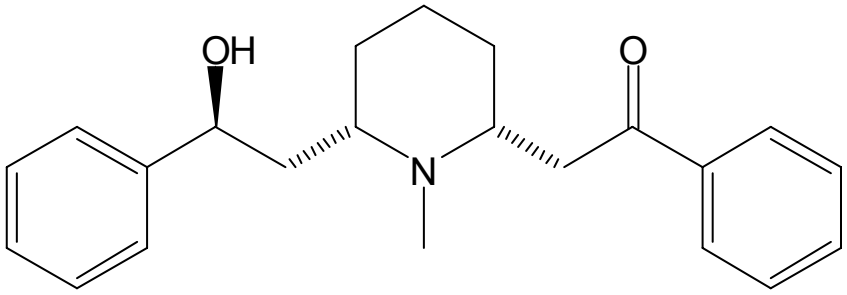
Clonidine



Modafinil



Bupropion



Lobeline

Chapter Two

Effect of Acute and Repeated *In Vivo* Administration of Lobeline, Methylphenidate, and Amphetamine on Dopamine Transporter Function

A. Introduction

ADHD is a childhood disorder that can, in some cases, persist into adulthood (Madras et al., 2005) and is associated with behavioral dysfunctions including three major components, hyperactivity, impulsivity, and inattention. Estimations are that ~15% of school age children (Scahill et al., 1999) and 5-10% of adults have ADHD, which translates into at least 8 million adults having ADHD. (Swanson et al., 1998a). This suggests that ADHD is not just a childhood disorder, but a disorder of all ages. The striatum, which contains high concentrations of DA, is suggested to be involved in executive function and motor response associated with ADHD (Dinn et al., 2001; Volkow et al., 2001; Wilens, 2008).

The etiology of ADHD is unknown; however DAT, which is located on the DA nerve cell membrane, is believed to play a major role in the pathophysiology of ADHD (Easton et al., 2007). DAT is the primary regulator of extracellular DA concentration (Gainetdinov & Caron, 2003). DAT is thought to be involved in the mechanism of action of stimulants, which are the gold standard options to treat ADHD (i.e., methylphenidate and amphetamine). This is supported by work that shows that stimulants that block DAT are effective treatments for ADHD (Volkow et al., 2002); thus, DAT is a pharmacological target for ADHD therapeutic agents.

Methylphenidate inhibits the reuptake of DA into the synaptic terminal, thus increasing the amount of DA in the synaptic cleft (See Introduction; (Greydanus et al., 2007). Amphetamine increases the amount of DA via a different mechanism, whereby it releases DA from the terminals by causing a reversal of DAT (Jones et al., 1998b). Due to the potential for abuse associated with these stimulants, there are concerns about the use of these agents. Additional concerns include cardiovascular effects, abnormal growth, and suppressed appetite (Gibson et al., 2006).

Lobeline may be a suitable candidate for the treatment of ADHD. When lobeline was evaluated in behavioral studies, lobeline was not self-administered by rats, nor did it substitute for *d*-methamphetamine, which suggest that it has no abuse liability (Harrod et al., 2003). Lobeline is the major alkaloidal component of the Indian tobacco plant, *Lobelia inflata,* and is similar to nicotine in some ways. For example, both have a high affinity for the nicotinic receptor despite the structural differences of nicotine and lobeline (Dwoskin & Crooks, 2002). Based on *in vivo* studies, lobeline does not stimulate locomotor activity (Miller et al., 2003) and acts as a nicotinic antagonist, i*n vitro*, (Miller et al., 2001) and i*n vivo* (Miller et al., 2003). Lobeline binds to and inhibits (IC_{50} value ≈ 40-100 μM) DAT, inhibiting DA uptake *in vitro* (Miller et al., 2004; 1998, Teng et al., 1997).

The concerns of the use of stimulant medications clearly indicates that more effective treatment options with less side effects are needed for ADHD. Based on *in vitro* experiments where lobeline inhibited DAT function, lobeline may be an option (1998, Teng et al., 1997). However, the effect of *in vivo* administration of lobeline needs to be determined. In order to determine if the mechanism of action of lobeline on DAT function is similar to either methylphenidate or amphetamine, the effects of methylphenidate and amphetamine on DAT function were also investigated after *in vivo* administration. Therefore, the goal of the present study was to elucidate the effect of acute and repeated *in vivo* administration of lobeline and to determine if this effect was observed in a dose-dependent manner. In addition, the effect of lobeline on DAT function was compared to the effect of methylphenidate and amphetamine administered *in vivo*. Such experiments are helpful in providing more insight into the mechanism of action of all these agents.

B. Methods

Materials.

[^{3}H]DA (3,4-ethyl-2[N-[^{3}H]dihydroxyphenylethylamine Specific Activity 28 Ci/mmol) was purchased from PerkinElmer Life Sciences Inc. (Boston, MA). L-Ascorbic acid, bovine serum albumin (BSA), catechol, α-D-glucose, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), nomifensine

maleate, pargyline hydrochloride, sucrose, methylphenidate hydrochloride, amphetamine hydrochloride, and 3-hydroxytyramine hydrochloride DA were obtained from Sigma Chemical Co. (St. Louis, MO). Lobeline was purchased from ICN Biomedicals (Costa Mesa, CA). All other chemicals used in the *in vitro* assay buffers were purchased from Fisher Scientific (Pittsburgh, PA).

Subjects.

Adult male Sprague-Dawley rats (200-220g body weight upon arrival) were obtained from Harlan Inc. (Indianapolis, IN). Adult male rats were used because as stated earlier, ADHD is found in adult humans as well as adolescents (Swanson et al., 1998b). Animals were housed two per cage with free access to food and water in the Division of Lab Animal Resources, University of Kentucky. Experimental protocols were in concordance with the NIH 1996 *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.

Drug Administration.

Lobeline, methylphenidate, and amphetamine were administered by subcutaneous (sc) injection in a volume of 1 ml/kg body weight and expressed as the salt weights. In order to remain consistent with previous studies, the subcutaneous route was selected. Groups of rats were injected acutely with either saline (control), lobeline (1, 3, and 10 mg/kg), methylphenidate (2.5, 5, 20, and 40 mg/kg), or amphetamine (0.1, 0.25, 1, and 5 mg/kg). The striatum was obtained 20 min after the last injection for the lobeline group, 60 min for the methylphenidate group and 30 min for the amphetamine group. Separate groups of rats were injected repeatedly for either 7 or 14 days once daily with saline, lobeline (3.0 mg/kg), methylphenidate (2.5, 5, and 20 mg/kg), or amphetamine (0.25 and 5 mg/kg). The doses for the repeated experiments were chosen based on the effect observed during the acute experiments. If no effect was observed in the acute experiments, doses were chosen based on results of previously reported behavioral studies (Harrod, 2003; Miller, 2001).

Lobeline dose range and time point after injection was chosen based on previous behavioral studies in which the effect of lobeline (1, 3, and 10 mg/kg) on methamphetamine self-administration was determined. Results showed that lobeline (3 mg/kg) inhibited methamphetamine self-administration 20 min after administration (Harrod et al., 2001). Methylphenidate dose range and time point were chosen based on experiments assessing [^{3}H]DA uptake, 60 min after administration and behavioral studies which concluded that doses of 2.5 and 5.0 of methylphenidate were more clinically relevant (Kuczenski & Segal, 2001; Wooters et al., 2006). Amphetamine dose range and time point were based on behavioral studies showing that low doses of amphetamine (0.25-0.75 mg/kg) improved stimulus detection performance in rats 30 min after injection (Grilly, 2000).

Striatal Synaptosomal Preparation and [^{3}H]DA Uptake Assay (DAT Function).

[^{3}H]DA uptake assays were conducted using previously published methods (Zhu et al., 2004). Briefly, striata were dissected and homogenized with 10-12 passes of a Teflon pestle homogenizer in 20 mL of ice cold 0.32 M sucrose buffer containing 5 mM sodium bicarbonate (pH 7.4). Homogenates were centrifuged at 2000 g for 10 min (4°C). Supernatants were centrifuged at 20,000 g for 15 min (4°C). Pellets were resuspended in 2.4 ml of ice-cold assay buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.5 mM KH_2PO_4, 10 mM glucose, 25 mM HEPES, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM pargyline and 0.1 mM L-ascorbic acid, saturated with 95% O_2/5% CO_2, pH 7.4). Protein concentrations were determined using the Bradford assay (Bradford, 1976). Striatal synaptosomes (20 µg protein in 50 µl) were incubated in an oxygenated environment for 5 min at 34°C. [^{3}H]DA (1 nM - 5µM) was added to the samples. Total assay volume was 500 µl. Nonspecific uptake was determined in the presence of 10 µM nomifensine. Incubation continued for 10 min at 34°C and was terminated by the addition of 3 ml ice-cold assay buffer containing 1 mM pyrocatechol. Samples were filtered through Whatman GF/B glass fiber filters (presoaked with 1 mM pyrocatechol for at least 3 h) and

washed three times with ice-cold assay buffer containing 1 mM pyrocatechol, using a Brandel cell harvester (Model MP-43RS; Biochemical Research and Development Laboratories Inc., Gaithersburg, MD). Radioactivity was determined using a liquid scintillation spectrometer (Model B1600TR, Perkin-Elmer Life Sciences, Downers Grove, IL).

C. Data Analysis.

A one-way ANOVA with each dose of drug serving as an independent group revealed that [^{3}H]DA uptake was not different among the saline groups across the dose range for each drug (Table 1, 2, and 3). Therefore, the data for the saline-control groups were pooled for statistical analysis and graphical presentation, which also explains the wide range of n.

Kinetic parameters V_{max}(pmol/min/mg) and K_m(μM)) for [^{3}H]DA uptake were determined using GraphPad Prism software (GraphPad Prism, version 5.02; GraphPad Software, San Diego, CA). V_{max} is the maximum velocity at which the substrate is 100% bound and K_m is the concentration of the substrate at half of the $V_{max.}$ Data are expressed as mean values ± S.E.M. To analyze the kinetic parameters, separate unpaired Student t-tests were performed on the V_{max} and K_m for [^{3}H]DA uptake of the drug-treated group for each drug at each dose and saline-control groups. Log transformed K_m values were used for statistical analyses using the SPSS (Statistical Packages for the Social Sciences; standard version 17, SPSS Inc., Chicago, IL). To determine the methylphenidate (20 mg/kg) response on DAT cell surface expression, separate student t-tests were conducted on DAT immunoreactivity from each of the cell fractions (total, non-biotinylated and biotinylated) between methylphenidate-treated and saline control groups. Differences were considered significant at $p< 0.05$.

D. Results

Effects of Acute and Repeated *In Vivo* Methylphenidate Administration on DA Transporter Function

Kinetic analysis of [^{3}H]DA uptake was performed on synaptosomes after acute *in vivo* administration of methylphenidate. Specific [^{3}H]DA uptake for the saline-control groups for methylphenidate 2.5, 5.0, 20.0, and 40.0 mg/kg doses

were 16.7 ± 1.5, 12.8 ± 2.1, 17.4 ± 3.9, and 10.8 ± 2.5 pmol/mg/min, respectively. Methylphenidate (5.0, and 20 mg/kg) increased (~60%) V_{max} (23.5 ± 1.6 and 23.3 ± 2.7 pmol/mg/min) for [^{3}H]DA uptake compared to pooled saline-control (14.5 ± 1.3 pmol/mg/min, Table 1), respectively. The current results show an increase (~60%) of DAT function, with acute methylphenidate (5.0 and 20 mg/kg) having a significantly higher V_{max} compared to control (Fig. 4A). Acute *in vivo* administration of methylphenidate had no effect on K_m values (Table 6).

Repeated methylphenidate administration for 7 days at 2.5 and 20 mg/kg doses had a V_{max} of 25.1 ± 0.9 and 27.3 ± 2.0 pmol/mg/min, respectively, which increased (36% and 48%, respectively) DAT function compared to pooled saline-control with a V_{max} of 18.5 ± 1.3 pmol/mg/min (Fig. 5A). No significant effect was observed for the 5.0 mg/kg dose after 7 days or after 14 days (Fig. 5A). Repeated methylphenidate *in vivo* administration had no effect on K_m values (Table 6).

Effects of Acute and Repeated *In Vivo* Amphetamine Administration on DA Transporter Function

Acute *in vivo* administration of amphetamine had no effect on DAT function (Fig. 7). Seven-day repeated *in vivo* administration of amphetamine had no effect on DAT function (Fig. 8). Fourteen-day-repeated *in vivo* administration of amphetamine had no effect on DAT function (Fig. 9). Specific [^{3}H]DA uptake for the saline-control groups for acute amphetamine 0.1, 0.25, 1.0, and 5.0 mg/kg doses were 18.6 ± 1.0, 27.0 ± 4.9, 15.3 ± 1.1, and 14.6 ± 0.7 pmol/mg/min, respectively, with no effect onK_m (Table 7). Specific [^{3}H]DA uptake for repeated amphetamine 0.1, 0.25, 1.0, and 5.0 mg/kg doses were 15.9 ± 2.2, 17.7 ± 1.4, 14.3 ± 1.1, and 15.9 ± 0.9 pmol/mg/min, respectively, compared to the pooled saline-control group with a V_{max} of 19.1 ± 1.7 pmol/mg/min (Table 2).

Effects of Acute and Repeated *In Vivo* Lobeline Administration on DA Transporter Function

Kinetic analysis of synaptosomal [^{3}H]DA uptake after acute and repeated (7 or 14 days) *in vivo* administration of lobeline showed no effect on V_{max} or K_m values (Fig. 10-12;Table 8). Specific [^{3}H]DA uptake for the saline-control groups

for lobeline 1.0, 3.0, and 10.0 mg/kg doses were 16.4 ± 2.3, 20.8 ± 2.3, and 13.1 ± 1.8 pmol/mg/min, respectively. Specific [^{3}H]DA uptake for acute lobeline 1.0, 3.0, and 10.0 mg/kg doses were 13.3 ± 0.9, 20.3 ± 1.5, and 13.3 ± 1.7 pmol/mg/min, respectively, compared to16.3 ± 1.3 pmol/mg/min for the pooled saline-control group (Table 3). The 3 mg/kg dose had the highest effect of all the doses with a V_{max} of20.3 ± 1.5 pmol/mg/min; however, this effect was not significant.

E. Discussion

In the present study, the effects of *in vivo* administration of lobeline were determined and compared to the effects of methylphenidate and amphetamine using kinetic analysis of [^{3}H]DA uptake into striatal synaptosomes. This work investigating *in vivo* drug administration, extends previous work in our laboratory demonstrating that *in vitro* lobeline inhibits DAT function (Teng et al., 1997). However, the results from these studies show that lobeline (1, 3, and 10 mg/kg) has no effect on DAT function 20 min after *in vivo* administration. There are a number of possible explanations for this observation.

First, the concentration of lobeline after *in vitro* administration may be higher than the final concentration after *in vivo* administration. *In vitro* experiments involve the direct application of the drug in question to the striatal slice of tissue or synaptosomes; however with *in vivo* administration that is not the case because the drug is injected into the whole animal. Furthermore, when a drug is given *in vivo*, the drug is exposed to various processes that could decrease the amount of drug available to elicit its pharmacological effect such as absorption, distribution, metabolism, and elimination. Previous research has provided evidence that the ability of any compound to alter monoamine uptake *in vitro* does not necessarily predict its ability to modulate monoamine transporters after *in vivo* administration (Fleckenstein et al., 1999).

Another plausible explanation for the lack of effect with lobeline could be that the time point may not have been appropriate. The time point of 20 minutes post injection, was based on behavioral effects, such as the lobeline-induced decrease in methamphetamine self administration in rats, observed after *in vivo*

administration. A way to address this issue would be to conduct a time-course curve to determine if the effect of lobeline is seen at any other time point besides 20 min post injection, such as every 5-10 minutes.

A third possible explanation for not observing an effect with lobeline, may be that the brain region examined was not the region where the effect was taking place. Even though the striatum is very dense in DA, the PFC is an important region also involved in ADHD, where DA is present to a lesser degree (Arnsten, 2006; Casey et al., 2007; Kieling et al., 2008).

Acute *in vivo* administration of methylphenidate showed an increase in DAT function at 5 and 20 mg/kg, but showed no effect with the 40 mg/kg dose. While surprising, this result is consistent with previous results showing that a single 40 mg/kg s.c. injection of methylphenidate caused little or no change in synaptosomal DA uptake (Fleckenstein et al., 1999; Sandoval et al., 2002). This present study also extends the work of Fleckenstein by including a dose-response curve as well as 7 and 14 day repeated daily injections. The 7-day response curve showed an increase of 36% by the 2.5 mg/kg methylphenidate dose and the 20 mg/kg dose showed an increase of 48%. It has been shown that methylphenidate indirectly acts as a DA agonist (Wilens, 2008). This may explain the increase of DAT function because if methylphenidate acts as a DA agonist, it could activate both presynaptic and postsynaptic D2 DA receptors. This action could indirectly cause an increase in DAT function that removes the DA in the synaptic cleft, which would decrease the activation of the D2 DA receptors. Furthermore, methylphenidate has a more direct and less complicated mechanism of action when compared to amphetamine and lobeline, which could also explain the increase of DAT function compared to lobeline and amphetamine, whereas both have a more complex mechanism of action.

After 7 days of repeated *in vivo* methylphenidate administration, the 2.5 and 20 mg/kg doses showed a significant increase in V_{max}, while the 5.0 mg/kg dose had no effect. However, after 14-days of repeated *in vivo* administration of methylphenidate (2.5, 5.0 or 20 mg/kg) the effect was no longer present. Methylphenidate administration did not alter the K_m, which suggests that

methylphenidate competes with DA at DAT in a noncompetitive manner. It could also be possible that methylphenidate increased the number of receptors in order to enhance DAT function.

Acute and repeated *in vivo* administration of amphetamine did not alter DAT function in these studies. Previous investigations have shown that amphetamine causes a biphasic effect on DAT trafficking and acts rapidly to control DAT in the plasmalemmal membrane (Johnson et al., 2005a). Intracellular amphetamine produces DAT trafficking (Kahlig et al., 2003). Since amphetamine causes DAT trafficking, one would expect to see a difference in DAT function after *in vivo* administration of amphetamine. However, consistent with our results, Johnson et al., found that amphetamine did not alter [^{3}H]DA uptake following a 1-min incubation (Johnson et al., 2005b). This suggests that the chosen time point plays a critical role when determining if an effect is present. It is possible that the DAT response was not observed with the time point that was chosen. In addition, DAT may not be the primary target responsible for pharmacological actions of amphetamine based on its complex mechanism of action, which includes acting as a DAT substrate. The results also showed the amphetamine did not alter K_m, which may imply that amphetamine also competes with DA at DAT in a noncompetitive manner.

The current results suggest that lobeline acts more similar to amphetamine compared to methylphenidate, with respect to the effects observed on DAT function. Since there was no effect of amphetamine at DAT, we reevaluated the doses used in these experiments by searching the literature. Amphetamine administered at 0.25 mg/kg appears to be an adequate dose to observe a behavioral effect since it increased choice accuracy in rats (Grilly et al., 1998). Another study of amphetamine administration (0.25, 0.75, and 1.25 mg/kg, s.c.) prior to behavioral testing, observed an increase in accuracy at 0.25 mg/kg however, a decrease in accuracy at 1.25 mg/kg (Grilly et al., 1989). Based on a recent review by Grilly and Loveland, 0.1-0.4 mg/kg of amphetamine was considered a low dose, 0.4-1.0 mg/kg a moderate dose, 1.0-3.0 mg/kg a high dose, and ≤3.0 mg/kg was considered a very high dose (Grilly & Loveland,

2001). Furthermore, Kuczenski and colleagues have done extensive research with amphetamine and found using microdialysis, the maximal concentration of amphetamine was achieved 30 minutes after administration (Kuczenski et al., 1997). Thus, the dose range of 0.1-5.0 mg/kg of amphetamine, which was used in the current studies, offers a complete dose-response.

The results of this study suggest that amphetamine, lobeline and methylphenidate influence DAT differently at the time points and doses used in this study, since the drugs have been shown to target DAT (Greydanus et al., 2007; Thanos et al., 2007). However, there are limitations to this current study that need to be addressed. The first limitation is the choice of the time point. The time points were based on behavioral observations, and may not be the optimal time points to observe the pharmacological effects ex vivo. A complete time-course of the pharmacological effect is necessary to determine the best time point for each drug after *in vivo* administration. However, the data in these studies could be a result of the difference in their mechanisms of action. The interaction of methylphenidate at DAT may depend on the release of DA in the synaptic cleft, whereas amphetamine induces release of vesicular DA from the synaptic terminal (Schiffer et al., 2006). This could possibly explain our observations of an increase in V_{max} by methylphenidate and no effect by amphetamine. Another limitation is the fact that we are evaluating the effect on DAT using synaptosomes. This limits the findings to only effects found in the synaptosomes, while other effects may be present in other areas, such as in vesicles as well.

The common lack of effect with *in vivo* administration of lobeline and amphetamine on DAT function is consistent with the fact that both are weak bases and very lipophilic compounds (Teng et al., 1997). The IC_{50} value determined in previous studies suggest that the synaptic vesicular DA transporter is significantly more sensitive to lobeline versus the plasma membrane DA transporter, which provides additional evidence as to why *in vivo* VMAT2 experiments need to be conducted (Teng et al., 1997).

Overall, the results of the present study, present evidence that methylphenidate effects DAT function after *in vivo* administration in a different manner than that of lobeline and amphetamine, suggesting that more research is necessary to further understand the mechanisms of actions of these agents.

In conclusion, there is evidence to warrant more investigation of lobeline to determine its effects on VMAT2, due to the lack of effect on DAT function after *in vivo* administration of amphetamine and lobeline. This may propose that another target may be involved in the mechanisms of actions of these drugs, such as VMAT2. Lobeline binds to and inhibits (IC_{50} value ≈ 1 μM) the function of the VMAT2, which is the only transporter protein that transports DA from the cytoplasm into the vesicle (Eyerman & Yamamoto, 2005; Teng et al., 1998; Wilhelm et al., 2004; Zheng et al., 2006). Therefore, additional experiments need to be conducted to determine the effects of *in vivo* administration of lobeline, methylphenidate, and amphetamine on VMAT2 function. Ultimately, studies of this nature may aid in the discovery of new treatment options for ADHD that improve behavioral symptoms while reducing detrimental drug side-effects by providing more information as to how certain compounds interact with the targets in question.

TABLE 1. Acute saline-treated control groups did not differ between MPD doses for DAT experiments. Acute independent saline-treated controls did not vary between MPD doses during DAT experiments based on results of a one-way ANOVA ($F_{3,21}$ = 1.110, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 4-8 rats/group.

ACUTE MPD (mg/kg)	Vmax pmol/min/mg
MPD 2.5 (n= 7)	17 ± 1.5
MPD 5 (n= 8)	13 ± 2.1
MPD 20 (n= 6)	17 ± 3.9
MPD 40 (n= 4)	11 ± 2.5
POOLED MEAN	15 ± 1.3[a]

TABLE 2. Acute saline-treated control groups did not differ between AMPH doses for DAT experiments. Acute independent saline-treated controls did not vary between AMPH doses during DAT experiments based on results of a one-way ANOVA ($F_{3,12}$ = 3.010, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 4-5 rats/group.

ACUTE AMPH (mg/kg)	Vmax pmol/min/mg
AMPH 0.1 (n= 5)	19 ± 1.0
AMPH 0.25 (n= 5)	27 ± 5.0
AMPH 1.0 (n= 5)	15 ± 1.1
AMPH 5.0 (n= 4)	15 ± 0.7
POOLED MEAN	19 ± 1.7[a]

TABLE 3. Acute saline-treated control groups did not differ between LOB doses for DAT experiments. Acute independent saline-treated controls did not vary between LOB doses during DAT experiments based on results of a one-way ANOVA ($F_{2,16}$ = 2.221, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 3-8 rats/group.

ACUTE LOB (mg/kg)	Vmax pmol/min/mg
LOB 1 (n= 8)	16 ± 2.3
LOB 3 (n= 3)	21 ± 2.3
LOB 10 (n= 8)	13 ± 1.8
POOLED MEAN	16 ± 1.9[a]

TABLE 4. Repeated 7-day saline-treated control groups did not differ between drug treatments for DAT experiments. Repeated 7-day independent saline-treated controls did not vary between drug treatments during DAT experiments based on results of a one-way ANOVA ($F_{3,17}$ = 0.5413, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n =4-6 rats/group.

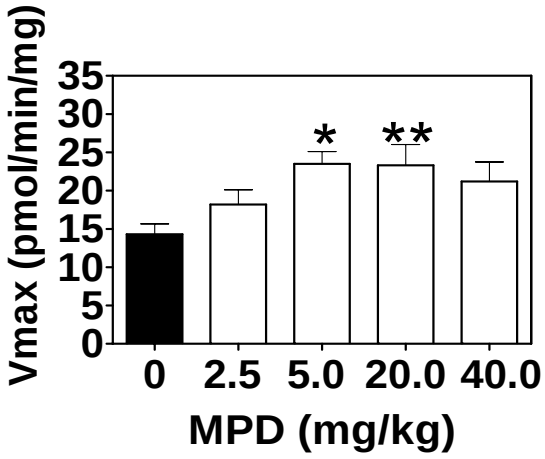
7-Day Drug Treatment	Vmax pmol/min/mg
MPD (n=4-6)	18 ± 1.9
AMPH (n=6)	20 ± 2.3
LOB (n=5)	18 ± 1.8
POOLED MEAN	18 ± 1.8[a]

TABLE 5. Repeated 14-day saline-treated control groups did not differ between drug treatments for DAT experiments. Repeated 14-day independent saline-treated controls did not vary between drug treatments during DAT experiments based on results of a one-way ANOVA ($F_{2,10}$ = 3.021, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ between treatment groups. [a]Data are presented as mean ± S.E.M for n = 5-6 rats/group.

14-Day MPD (mg/kg)	Vmax pmol/min/mg
MPD (n=6)	22 ± 3.3
AMPH (n=6)	19± 3.0
LOB (n=5)	12 ± 0.7
POOLED MEAN	23 ± 3.3[a]

Figure 4. A. Acute MPD increased DAT function in striatal synaptosomes. Rats were injected acutely once daily with MPD (0 (n = 24), 2.5 (n = 7), 5.0 (n = 7), 20 (n = 8), and 40 (n = 6) mg/kg, s.c.; open bars). Synaptosomes were prepared 60 min post-injection. Control (0; black bar) represents saline-treated control group. Acute MPD (5 and 20 mg/kg) increased striatal [^{3}H]DA uptake 60 min after administration (*p<0.05 for the 5 mg/kg group and **p<0.01 for the 20 mg/kg group compared to control).Vmax is represented in pmol/min/mg. Data are presented as mean ±S.E.M for n = 6-25 rats/group. Data were pooled for control groups, **Figure 4**. **B. Comparison of saturation analysis of control and acute MPD (5 and 20 mg/kg).** Specific uptake for MPD (0, 5 and 20 mg/kg) increased by ~60% compared to control.

A.



B.

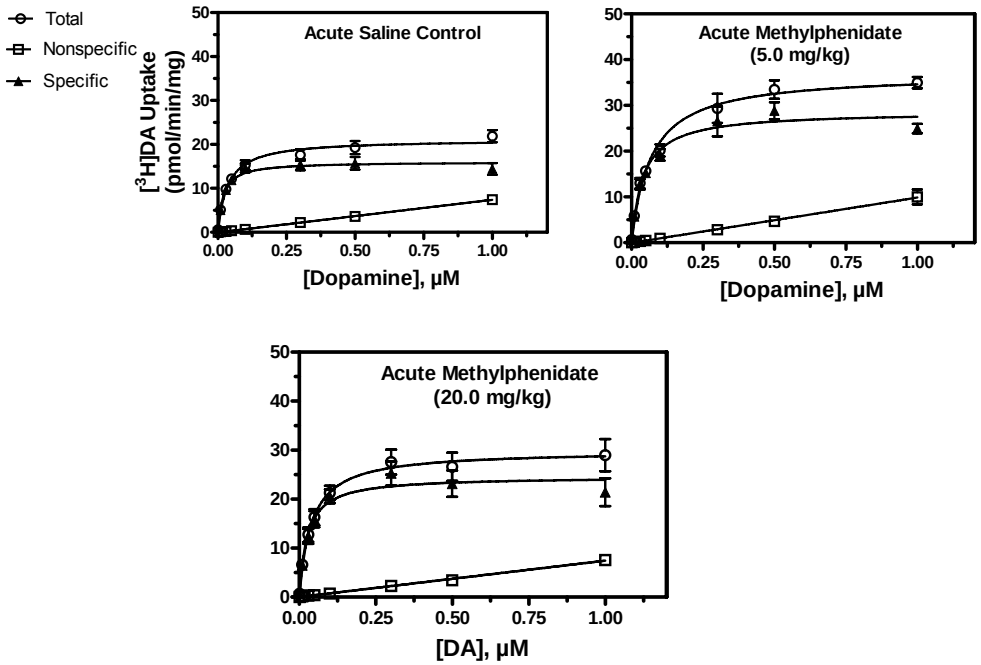
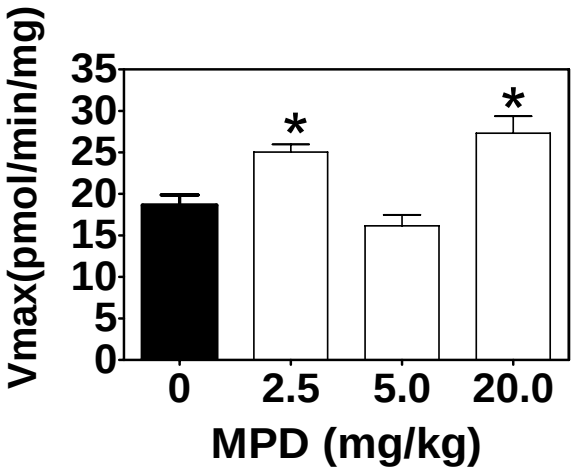


Figure 5. A. Repeated 7-day MPD increased DAT function. Rats were injected repeatedly once daily for 7 days MPD (0 (n = 10), 2.5 (n = 4), 5.0 (n = 5), and 20 (n = 5) mg/kg, s.c.; open bars). Synaptosomes were prepared 60 min post-injection. Control (0; black bar) represents saline-treated control group. Repeated MPD (2.5 and 20 mg/kg for 7 days) increased [^{3}H]DA uptake (*p<0.05 for 5 and 20.0 mg/kg group compared to control).Vmax is represented in pmol/min/mg. Data are presented as mean ±S.E.M for n = 4-10 rats/group. Data were pooled for control group, **Figure 5. B. Comparison of saturation analysis of control and acute MPD (2.5 and 20 mg/kg).** Specific uptake for MPD (0, 2.5 and 20 mg/kg) increased by 36% and 48%, respectively, compared to control.

A.



B.

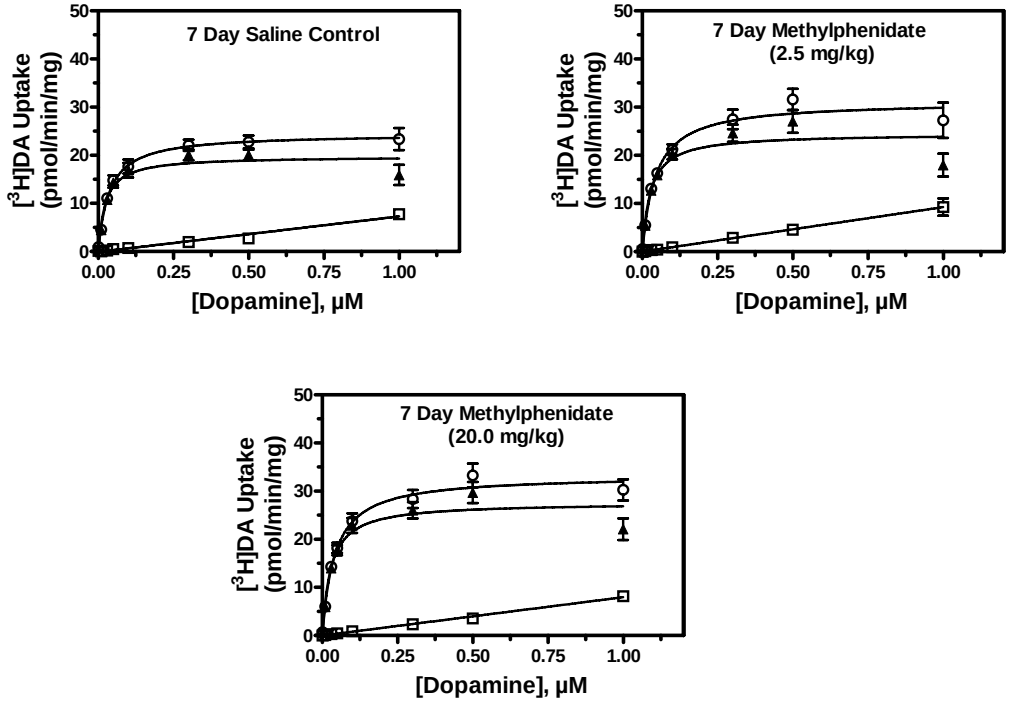


Figure 6. Repeated 14-day MPD did not alter DAT function. Rats were injected repeatedly once daily for 14 days MPD (0 (n = 9), 2.5 (n = 3), 5.0 (n =3), and 20 (n = 5) mg/kg, s.c.; open bars). Synaptosomes were prepared 60 min post-injection. Control (0; black bar) represents saline-treated control group. Repeated MPD (2.5, 5.0, or 20 mg/kg for 14 days) did not alter [^{3}H]DA uptake. Vmax is represented in pmol/min/mg. Data are presented as mean ±S.E.M for n = 3-9 rats/group. Data were pooled for control group, Table 5.

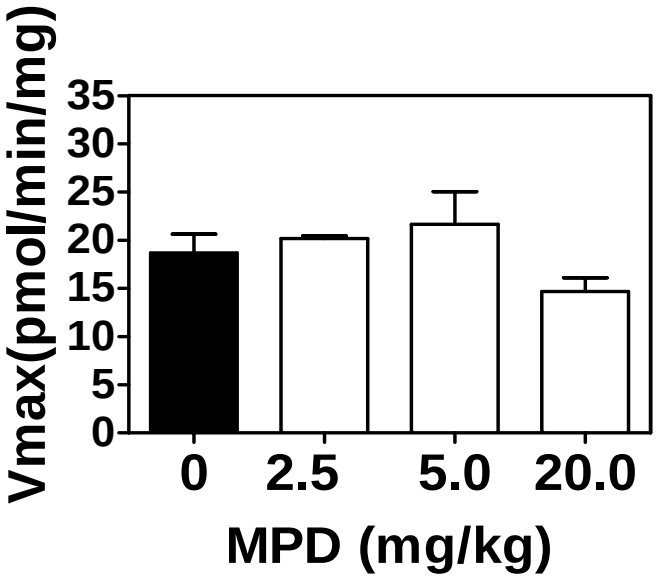


Figure 7. Acute AMPH did not alter DAT function. Rats were injected acutely once daily with AMPH (0 (n = 19), 0.1 (n = 4), 0.25 (n = 5), 1.0 (n = 5), 5.0 (n = 5) mg/kg, s.c.; open bars). Synaptosomes were prepared 30 min post-injection. Control (0; black bar) represents saline-treated control group. Acute AMPH had no effect on striatal [^{3}H]DA uptake 30 min after administration. Vmax is represented in pmol/min/mg. Data are presented as mean ±S.E.M for n = 5-19 rats/group. Data were pooled for control group, Table 2.

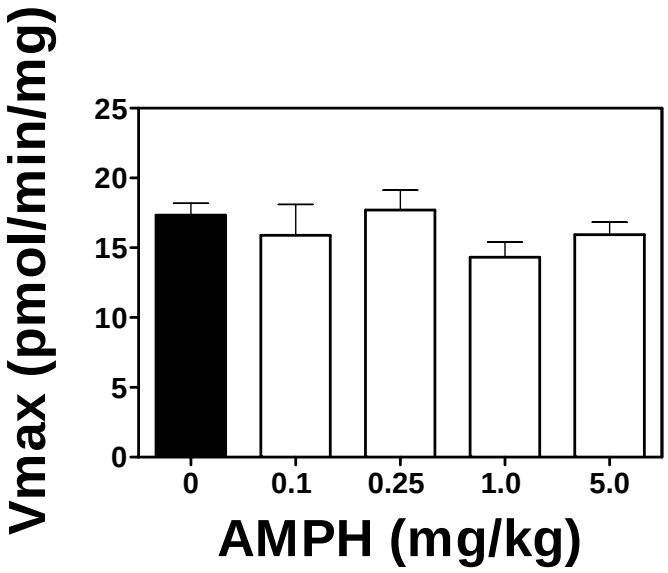


Figure 8. Repeated 7-day AMPH did not alter DAT function. Rats were injected repeatedly once daily for 7 days AMPH (0 (n = 6), 0.25 (n = 5) and 5.0 (n = 5) mg/kg, s.c.; open bars). Control (0; black bar) represents saline-treated control group. Repeated AMPH (7 days) did not alter striatal [^{3}H]DA uptake. Vmax is represented in pmol/min/mg. Data are presented as mean ±S.E.M for n = 5-6 rats/group. Data were pooled for control group, Table 4).

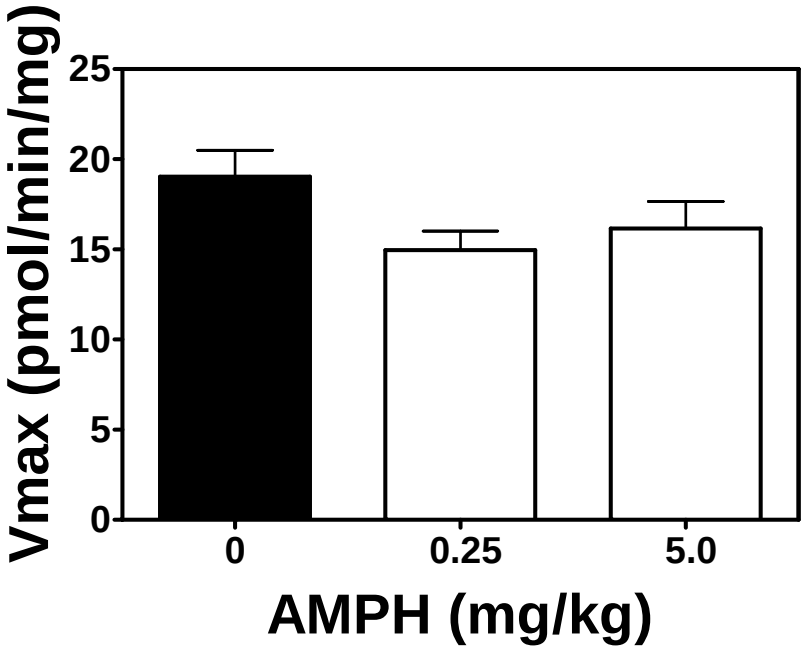


Figure 9. Repeated 14-day AMPH did not alter DAT function. Rats were injected repeatedly once daily for 14 days AMPH (0 (n = 6), 0.25 (n = 6) and 5.0 (n =4) mg/kg, s.c.; open bars). Control (0; black bar) represents saline-treated control group. Repeated AMPH (14 days) did not alter striatal [^{3}H]DA uptake. Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n = 4-6 rats/group. Data were pooled for control group, Table 5.

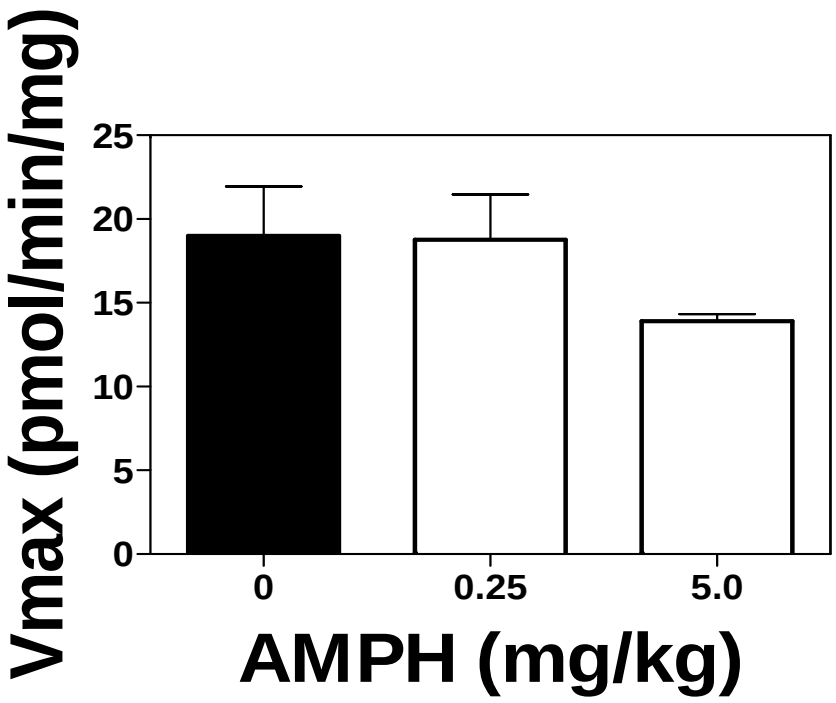


Figure 10. Acute LOB did not alter DAT function. Rats were injected acutely once daily with LOB (0 (n = 19), 1.0 (n = 7), 3.0 (n = 3), and 10.0 (n = 6) mg/kg, s.c.; open bars). Synaptosomes were prepared 20 min post-injection. Control (0; black bar) represents saline-treated control group. Acute LOB did not alter [^{3}H]DA uptake 20 min after injection. Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n=3-19 rats/group. Data were pooled for control group, Table 3.

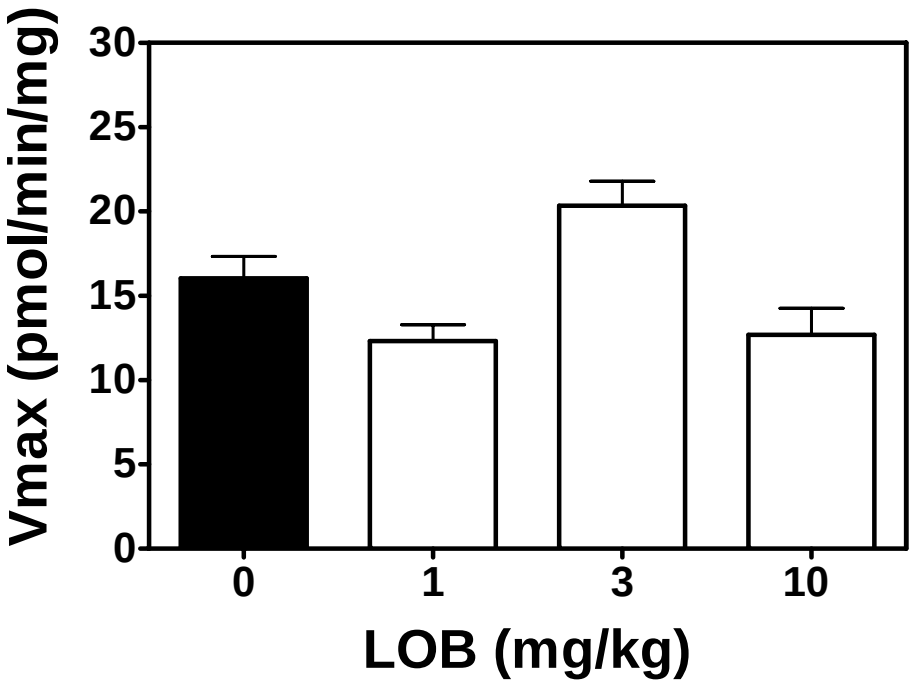


Figure 11. Repeated 7-day LOB did not alter DAT function. Rats were injected repeatedly once daily for 7 days with LOB (0 (n = 5), 3.0 (n = 5) mg/kg, s.c.; open bars) Synaptosomes were prepared 20 min post-injection. Control (0; black bar) represents saline-treated control group. Repeated LOB for 7 days did not alter [^{3}H]DA uptake 20 min after injection. Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n = 5 rats/group. Data were pooled for control group, Table 4.

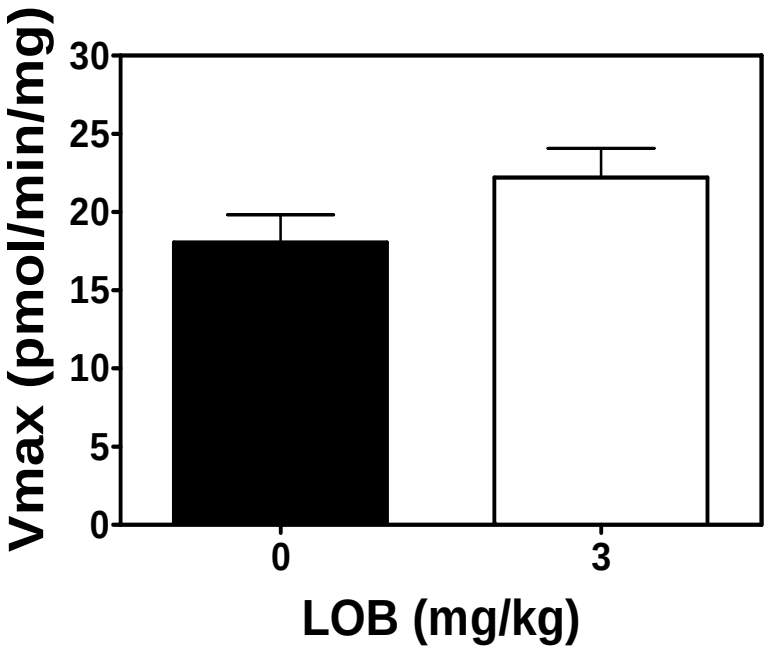


Figure 12. Repeated 14-day LOB did not alter DAT function. Rats were injected repeatedly once daily for 14 days with LOB (0 (n = 5), 3.0 (n = 5) mg/kg, s.c.; open bars). Synaptosomes were prepared 20 min post-injection. Control (0; black bar) represents saline-treated control group. Repeated LOB for 14 days did not alter [^{3}H]DA uptake. Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n = 5 rats/group. Data were pooled for control group, Table 5.

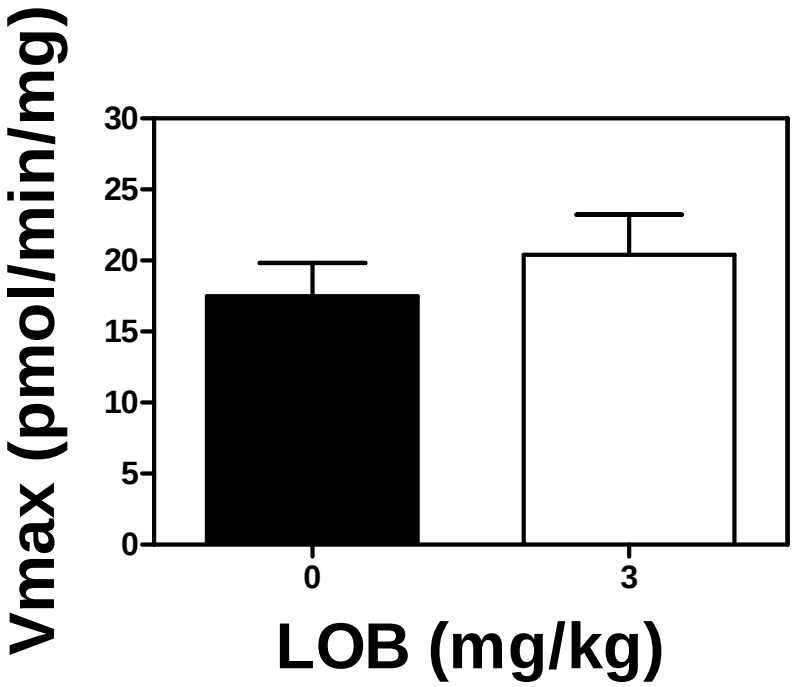


Table 6. K_m values for [^{3}H]DA uptake at DAT were not altered by acute, 7-day, or 14-day MPD treatment regimens.

ACUTE MPD (mg/kg)	Km (Mick et al)	7-DAY MPD (mg/kg)	Km (Mick et al)	14-DAY MPD (mg/kg)	Km (Mick et al)
0	16±3.7	0	21±1.8	0	14±1.0[a]
2.5	14±2.1	2.5	24±1.1	2.5	11±2.4
5	25±3.5	5	12±2.4	5	14±5.4
20	22±2.9	20	27±1.2	20	11±2.1
40	22±4.8				

[a]Data are presented as mean ± S.E.M for n= 4-25 rats/treatment group. ANOVA ($F_{4,47}$ = 2.67, p>0.05) revealed that K_m values were not different among independent control groups across the given treatments, and thus, these data for the saline-treated groups for each treatment group were pooled for statistical analysis.

Table 7. K_m values for [^{3}H]DA uptake at DAT were not altered by the acute, 7-day, or 14-day AMPH treatment regimens.

ACUTE AMPH (mg/kg)	Km (Mick et al)	7-DAY AMPH (mg/kg)	Km (Mick et al)	14-DAY AMPH (mg/kg)	Km (Mick et al)
0	18±2.9	0	16±1.4	0	13±1.9[a]
0.1	16±3.7	0.25	10±1.7	0.25	12±2.3
0.25	10± 0.9	5	14±1.9	5	10±2.3
1	9.0±1.2				
5	16±2.2				

[a]Data are presented as mean ± S.E.M for n= 4-19 rats/treatment group. ANOVA ($F_{4,32}$ = 1.67,p>0.05 revealed that K_m values were not different among independent control groups across the given treatments, and thus, these data for the saline-treated groups for each treatment group were pooled for statistical analysis.

Table 8. K_m values for [^{3}H]DA uptake at DAT were not altered by the acute, 7-day, or 14-day LOB treatment regimens.

ACUTE Lobeline (mg/kg)	Km (Mick et al)	7-DAY Lobeline (mg/kg)	Km (Mick et al)	14-DAY Lobeline (mg/kg)	Km (Mick et al)
0	19±2.5	0	19±1.9	0	20±3.4[a]
1	16±4.6	3	20±1.5	3	22±4.7
3	24±2.8				
10	12±3.7				

[a]Data are presented as mean ± S.E.M for n= 3-19 rats/treatment group. ANOVA ($F_{3,32}$ = 1.80, $p>0.05$) revealed that K_m values were not different among independent control groups across the given treatments, and thus, these data for the saline-treated groups for each treatment group were pooled for statistical analysis.

III. Chapter Three

Effect of Acute and Repeated *In Vivo* Administration of Methylphenidate on Dopamine Transporter Trafficking

A. Introduction

DAT plays a vital role in regulating the dopaminergic signaling in the synaptic cleft and in maintaining a releasable storage of DA (Eriksen et al., 2010; O'Malley et al., 2010). Numerous studies have shown that various factors such as kinase activators, phosphatase inhibitors, and transported substrates are involved in regulating DAT trafficking between the plasma membrane and endosomal compartments (Eriksen et al., 2010; Kahlig & Galli, 2003; Loder & Melikian, 2003; Merickel & Edwards, 1995; O'Malley et al., 2010; Torres et al., 2003). DAT is the primary target for certain psychostimulants which are commonly abused (Thomsen et al., 2009). Second messenger systems and psychostimulants can alter function, phosphorylation, and trafficking of DAT. For example, protein kinase C beta, which is a kinase important for DAT trafficking, was found to co-localize with DAT in mesencephalic neurons (O'Malley et al., 2010). Methylphenidate which is a psychostimulant as well, was the only drug to alter DAT function after acute and repeated *in vivo* administration in the present studies. Based on these results, we investigated if the effect of methylphenidate involved DAT trafficking.

B. Methods

Materials

Methylphenidate hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in the *in vitro* assay buffers were purchased from Fisher Scientific (Pittsburgh, PA).

Subjects

Adult male Sprague-Dawley rats (200-220g body weight upon arrival) were obtained from Harlan Inc. (Indianapolis, IN). Adult male rats were used because as stated earlier, ADHD is found in adult humans as well as adolescents (Swanson et al., 1998b). Animals were housed two per cage with free access to

food and water in the Division of Lab Animal Resources, University of Kentucky. Experimental protocols were in concordance with the NIH 1996 *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.

Drug Administration

Methylphenidate (20 mg/kg) was administered by subcutaneous (sc) injection in a volume of 1 ml/kg body weight and expressed as the salt weights. Groups of rats were injected acutely with either saline (control) or methylphenidate (20 mg/kg). The striatum was obtained 60 min for the methylphenidate group. Separate groups of rats were injected repeatedly 7 days once daily with saline or methylphenidate (20 mg/kg).

Biotinylation and Western Blot Assay (DAT Cellular Localization)

Cell surface biotinylation experiments were performed as described previously (Zhu et al., 2005). Synaptosomes from striatum (500 µg protein/sample) were incubated for 1 h at 4°C with sulfo-NHS-biotin, a biotinylation reagent, and continual shaking 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 7.3). After incubation, samples were centrifuged at 8,000 *g* for 4 min at 4°C. To remove the free biotinylation reagent, the resulting pellet was resuspended 3 times with 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer, and centrifuged at 8,000 *g* for 4 min at 4°C. Resuspension and centrifugation steps were repeated using the same parameters. Final pellets were resuspended in 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and incubated with continual shaking for 30 min at 4°C. Subsequently, samples were centrifuged at 8,000 *g* for 4 min at 4°C, and the resulting pellets were resuspended in 1 ml ice-cold PBS/Ca/Mg buffer and centrifuged again. Resuspension and centrifugation steps were repeated twice more using the latter parameters. Final pellet was lysed by sonication for 2-4 s in 300 µl Triton X-100 buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 1 µg/ml aprotinin (protease inhibitor), 1 µg/ml leupeptin (protease inhibitor), 1 µM pepstatin (protease inhibitor), 250 µM phenylmethysulfonyl

fluoride (serine protease inhibitor), pH 7.4) followed by incubation and continual shaking for 20 min at 4°C. Lysates (300 µl) were centrifuged at 21,000 *g* for 20 min at 4°C. Pellets were discarded, and 100 µl of the supernatants were stored at -20°C for determination of total immunoreactive DAT. Remaining supernatant was incubated with continuous shaking in the presence of monomeric avidin beads (Pierce Biotechnology, Inc, Rockford, IL) in Triton-X100 buffer (100 µl/tube) for 1 h at room temperature. Subsequently, samples were centrifuged at 17,000 *g* for 4 min at 4°C, and supernatants (containing non-biotinylated, intracellular protein) were stored at -20°C. Resulting pellets containing the avidin-absorbed biotinylated proteins (cell-surface) were resuspended in 1 ml of 1.0% Triton X-100 buffer and centrifuged at 17,000 g for 4 min at 4°C, and the pellet was resuspended and centrifuged twice. Final pellets consisted of the biotinylated proteins adsorbed to monomeric avidin beads. The biotinylated proteins were eluted by incubating with 50 µl Laemmli buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% β-mercaptoethanol and 0.05% bromophenol blue (Bio-Rad Hercules, CA), pH 6.8) for 20 min at room temperature. The samples were stored at -20°C.

To obtain the immunoreactive DAT protein in the three separate fractions: total, intracellular and cell surface, samples were thawed and subjected to gel electrophoresis and Western blotting. Briefly, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 150 V, and subsequently, transferred to Immobilon-P transfer membranes (Cat # IPVH00010, 0.45 µm pore size; Millipore Co., Bedford, MA) in transfer buffer (50 mM Tris, 250 mM glycine, 3.5 mM SDS) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd., Hercules, CA) for 110 min at 72 V. The transfer membranes were incubated with blocking buffer (5% dry milk powder in PBS containing 0.5% Tween 20) for 1 h at room temperature, followed by incubation with goat polyclonal DAT antibody (sc-1433; 1:1000 dilution in blocking buffer) overnight at 4 ºC. All specific antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfer membranes were washed 5 times with washing buffer (PBS containing 0.5% Tween 20) at room

temperature, and then incubated with rabbit anti-goat DAT antibody (sc-7210; 1:4000 dilution in blocking buffer) for 1 h at 22°C. Bands were detected using enhanced chemiluminescence and developed on Hyperfilm (ECL-plus; Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). After detection and quantification of DAT protein, each membrane was stripped in 10% of Re-blot plus mild antibody stripping solution (Chemicon, Temecula, CA) for 20 min at room temperature and reprobed for detection of PP2A (FL-309) and β-actin. PP2A and β-actin were used as control proteins for monitoring biotinylation and protein loading between methylphenidate- and saline-treated samples.

C. Data Analysis

Multiple autoradiographs were obtained using different exposure times, and immunoreactive bands within the linear range of detection were quantified by densitometric scanning using Kodak Image Station software (Carestream Health, New Haven, CT). Band density measurements, expressed as relative optical density, were used to calculate levels of DAT in total, non-biotinylated and biotinylated fractions. The net density measurements were calculated by substracting the background from the raw density value. Specifically, total DAT levels in the biotinylated and non-biotinylated fractions were calculated based on the density of DAT-immunoreactive bands in an aliquot of synaptosomal extract multiplied by total volume of extract and divided by the total volume of synaptosomal extract subjected to SDS-PAGE. To determine the methylphenidate (20 mg/kg) response on DAT cell surface expression, separate student t-tests were conducted on DAT immunoreactivity from each of the cell fractions (total, non-biotinylated and biotinylated) between methylphenidate-treated and saline control groups. Differences were considered significant at $p < 0.05$.

D. Results

Effects of Acute and Repeated *In Vivo* Methylphenidate Administration on DA Transporter Trafficking

The biotinylation and western blot assay revealed that methylphenidate (20 mg/kg)given acutely, decreased intracellular DAT expression with no change

in total or surface DAT expression (Fig. 13B), although not significantly. However, *in vivo* administration of methylphenidate (20 mg/kg) after 7 days significantly decreased intracellular DAT expression, leaving only 20% of intracellular DAT (19.8 ± 3.3%) compared to control without changing the total or surface DAT expression (Fig. 14B). PP2A, an intracellular protein, was the control for monitoring biotinylation during experiments. One would expect to find more PP2A in the intracellular section, which is the non-biotinylated section, than in the biotinylated section since the biotinylated section is the section on the surface of the membrane. If more PP2A was found in the biotinylated section, this would suggest that cell leakage has occurred, thus implying that the biotinylation inefficient. However, these present results show that there is more PP2A in the non-biotinylated section, therefore indicating that there was very little cell leakage during the biotinylation experiment. β-actin was the control for protein loading for the samples used in the biotinylation experiments. The level of β-actin should remain consistent through all the samples to ensure that the same amount of protein was added to each sample. Our results showed that the levels of β-actin were constant throughout the samples, thus confirming that the level of protein used in the biotinylation experiments did not vary between samples.

E. Discussion

The results from this experiment suggest that the methylphenidate induced-increase in DAT function was not independent of trafficking, since a significant decrease in intracellular DAT was observed. However, this phenomenon where methylphenidate did not alter total DAT expression has been observed previously with other compounds. For example, one report found that nicotine increased V_{max} for [^{3}H]DA uptake, however no increase in cell surface DAT expression was seen (Middleton et al., 2007). Furthermore, another study reported that insulin increased NET function without a change in transport cellular localization (Apparsundaram et al., 2001). Methylphenidate may have caused the phosphorylation of DAT, thus making DAT unrecognizable to the DAT antibodies used in the western blot analysis. Ubiquitination, another enzymatic process that involves the ε-amino moiety of lysine residues in target cellular proteins, has the

ability to affect DAT cell surface expression and membrane trafficking of transporters (Schmitt and Reith, 2010).

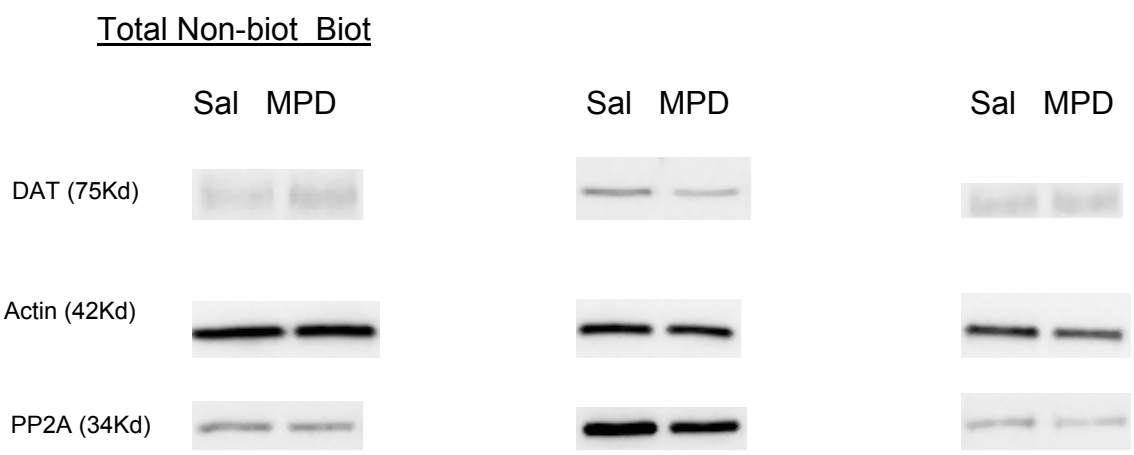
Another possible explanation could be the involvement of PKA and PKC. Activation or inhibition of PKC, PI3K, and members of the MAPK family can cause transport membrane redistribution, although specific kinases responsible for direct phosphorylation are still unknown. One report found that methylphenidate had no effect on PKC activator-induced down-regulation of DAT function (Gorentla & Vaughan, 2005), however results from a previous study showed that methylphenidate decreased PKA levels when given repeatedly for five days (Crawford et al., 1998). These results demonstrate that the mechanism behind the regulation of DAT by methylphenidate is still under debate and further investigation is needed to gain a clearer picture of how methylphenidate regulates DAT. In addition, Wagner et al observed a significant increase in V_{max} in rats with a controlled cortical impact (CCI) that received a daily injection of methylphenidate (5 mg/kg) for 14 days, but there was no change in total tissue or membrane bound DAT expression compared to the CCI rats that received saline (Wagner et al., 2009). These findings demonstrate that methylphenidate has the ability to increase the V_{max} without altering total of surface DAT expression, which is consistent with the results of this current study.

To our knowledge, no work has been conducted on how methylphenidate alters DAT expression. However, Fleckenstein's group examined the effect of methylphenidate on VMAT2 immunoreactivity (Sandoval et al., 2003). They found that a single injection of methylphenidate (40 mg/kg, sc) redistributes VMAT2 immunoreactivity. Specifically, methylphenidate increased VMAT2 expression in the vesicular subcellular fraction, but a decrease was found in the plasmelemmal membrane fraction, with no change in the whole synaptosomal fraction, which included the vesicular subcellular fraction and the plasmelemmal membrane fraction. Our current findings demonstrating that methylphenidate did not alter total surface DAT coincides with this report where methylphenidate had no effect on the whole synaptosomal fraction, but altered the vesicular subcellular fraction and the plasmalemmal membrane fraction. Even though this

study was done with VMAT2, it provides support that methylphenidate can redistribute transporter expression. In summary, there is evidence that collectively suggests that multiple pathways exist to regulate neurotransmitter transport function (Jayanthi et al., 2005) and our results indicate that methylphenidate may regulate transporter function by altering transporter expression.

Figure 13. Acute MPD did not alter intracellular DAT expression. Rats were injected acutely with MPD (20 mg/kg; open bar) and fractions of striatal synaptosomes were obtained 60 min after MPD (Clarke & Kumar, 1983). Control (Sal; black bar) represents saline-treated control group. **A)** Representative immunoblots of total synaptosomal fraction (Total), intracellular fraction (non-biotinylated, Non-biot), and cell surface fraction (biotinylated, Biot) for DA, β-actin and PP2A. β –actin was used as a control for protein loading and PP2A was used to assess efficiency of biotinylation. **B)** DAT immunoreactivity is presented as percentage of the saline-treated control. DAT immunoactivity for saline controls for total, non-biot, and biot were 3.5 ± 1.04, 6.3 ± 2.79, and 73.0 ± 61.53, respectively. Data expressed as mean ±S.E.M of densitometry values of DAT immunoreactivity plotted as arbitrary units for n = 5 rats/group. Acute MPD 20 mg/kg did not alter DAT expression.

A



B

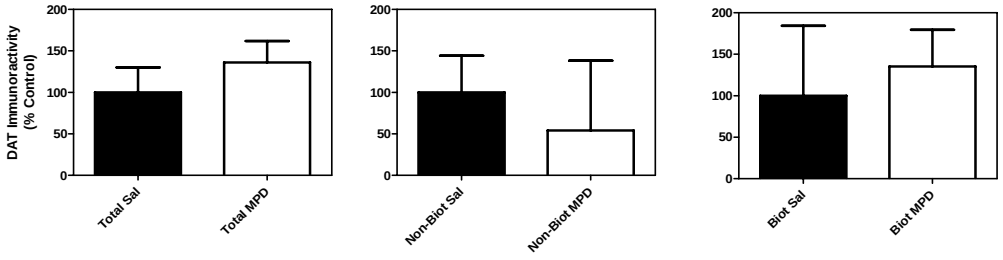
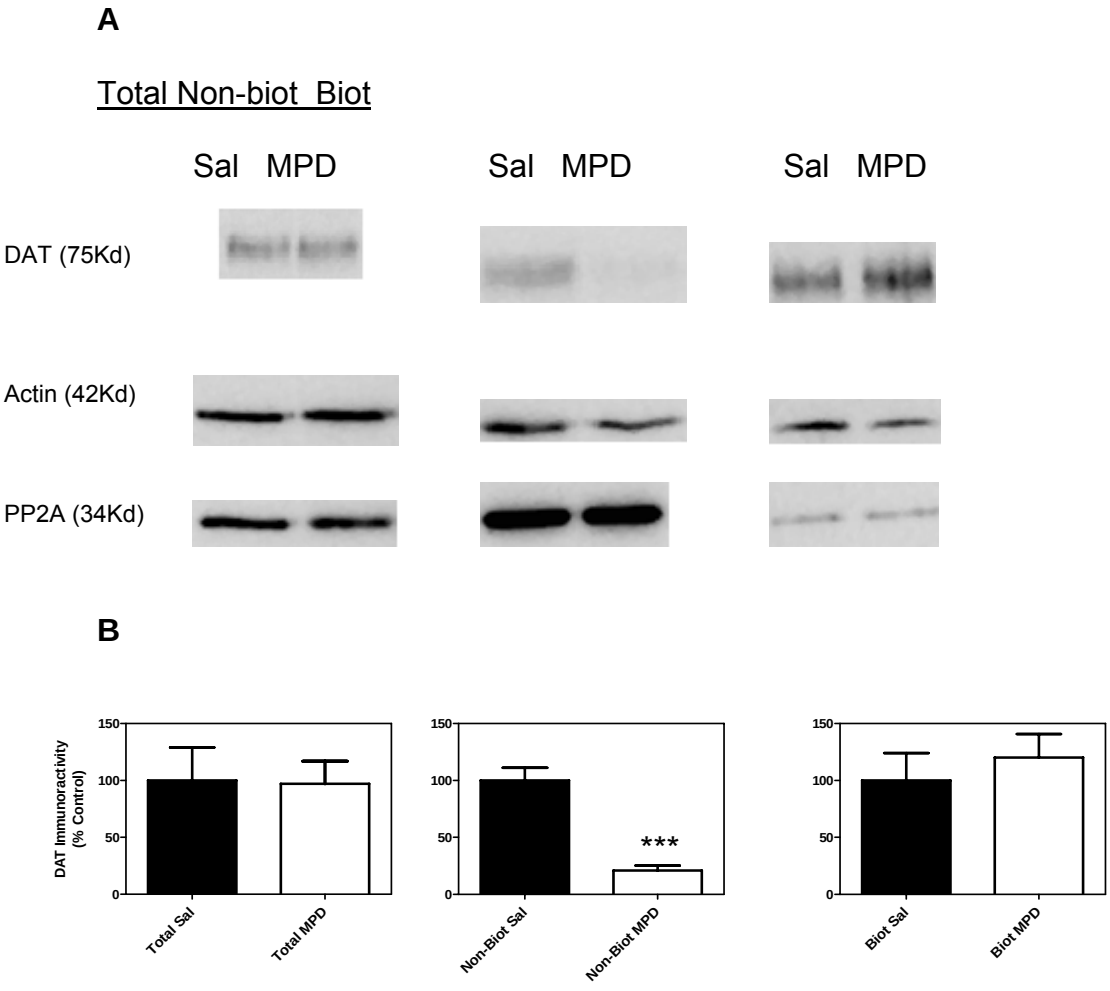


Figure 14. Repeated 7-day MPD decreased intracellular DAT expression. Rats were injected once daily with MPD (20 mg/kg; open bar) for 7 days and fractions of striatal synaptosomes were obtained 60 min after MPD (20 mg/kg) injection (s.c.). Control (Sal; black bar) represents saline-treated control group. **A)** Representative immunoblots of total synaptosomal fraction (Total), intracellular fraction (non-biotinylated, Non-biot), and cell surface fraction (biotinylated, Biot) for DAT and β-actin. β –actin was used as a control for protein loading and PP2A was used to assess efficiency of biotinylation. **B)** DAT immunoreactivity is presented as percentage of the saline-treated control. DAT immunoactivity for saline controls for total, non-biot, and biot were 3.2 ± 0.91, 16.0 ± 1.79, and 24.2 ± 5.87, respectively. Data expressed as mean ±S.E.M of densitometry values of DAT immunoreactivity plotted as arbitrary units for n = 5 rats/group. Repeated MPD 20 mg/kg for 7 days decreased intracellular fraction (non-biotinylated). * indicates difference from saline-treated control group, $p<0.001$.



IV. Chapter Four

Effect of Acute and Repeated *In Vivo* Administration of Lobeline, Methylphenidate, and Amphetamine on Vesicular Monoamine Transporter Function

A. Introduction

Even though the etiology of ADHD is unknown, DAT has become the major target of interest for pharmacotherapies to treat ADHD (Easton et al., 2007). The striatum, which contains high dopamine concentrations, is suggested to be involved in executive function and motor response associated with ADHD (Dinn et al, 2001; Volkow et al., 2001; Wilens et al., 2008). Stimulants used to treat ADHD (i.e., methylphenidate and amphetamine) interact with DAT (Volkow et al., 2002). But they also interact with VMAT2, which is also an important transporter protein that is responsible for transporting cytoplasmic DA into vesicles for storage and release into the synaptic cleft (Schuldiner, 1994). VMAT2 plays a vital role in protecting the cells against damage from toxins, such as hydrogen peroxide, by maintaining the low cytoplasmic concentrations of neurotransmitters via initiating their reuptake into storage vesicles (Liu & Edwards, 1997). Recent studies have shown that VMAT2 function is altered by psychostimulants (Brown et al., 2001a; Fleckenstein & Hanson, 2003).

Methylphenidate inhibits the reuptake of dopamine (DA) into the synaptic terminal, thus increasing the amount of DA in the synaptic cleft (Greydanus et al., 2007). Recent studies have found that methylphenidate alters VMAT2 transport and rapidly and reversibly increases VMAT2 binding and vesicular uptake of dopamine (Sandoval et al., 2003; Sandoval et al., 2001). Amphetamine elicits its pharmacological effect in three ways: 1) by binding to and reversing the transport of neurotransmitters such as dopamine, norepinephrine, and serotonin, 2) by facilitating release of neurotransmitters of these same neurotransmitters, and 3) by inhibiting MAO (Jones et al., 1998b; Seiden et al., 1993). One study found that 10 μM amphetamine induced a 15-fold increase in cystosolic dopamine with 10-

15 min of application, strongly suggesting redistribution of vesicular catecholamine (Mosharov et al., 2003). An experiment by Gonzalez and others, revealed that amphetamine displaced the VMAT2 ligand tetrabenazine with a relatively low (μM) affinity for VMAT2, which suggest amphetamine may be a substrate of VMAT2 as well (Gonzalez et al., 1994). Another study suggests that the response to amphetamine is indirect and the apparent inhibition of VMAT2 involves D2 autoreceptor activation following dopamine release (Brown et al., 2002). Due to the potential for abuse (Bymaster et al., 2002; Cormier, 2008; Greydanus et al., 2007; Holman, 1994; Olfson, 2004) and other additional side effects including cardiovascular effects, abnormal growth, and suppressed appetite (Gibson et al., 2006) associated with these stimulants, there are concerns about the use of these agents. There is an obvious need for additional treatment options that possess a less unfavorable side effect profile.

However, lobeline, evaluated in behavioral studies, was not self-administered by rats, nor did it substitute for *d*-methamphetamine, which suggest that it has no abuse liability, (Harrod et al., 2003). Lobeline is the major alkaloidal component of the Indian tobacco plant, *Lobelia inflata* and is similar to nicotine in some ways. For example, both have a high affinity for the nicotinic receptor despite their structural differences (Dwoskin & Crooks, 2002). Lobeline does not stimulate locomotor activity (Miller et al., 2000) and acts as a nicotinic antagonist (Miller et al., 2001). Lobeline, in addition to, methylphenidate and amphetamine also interacts with DAT. Lobeline also binds to and inhibits (IC_{50} value ≈ 40-100 μM) DAT, inhibiting DA uptake (Miller et al., 2004; Teng et al., 1997). *In vitro* studies, have found that lobeline inhibits [^{3}H]DA uptake into vesicles with an IC_{50} value of 0.88 μM and inhibits the binding of [^{3}H]dihydrotetrabenazine, a VMAT2 ligand, to the vesicular membrane with an IC_{50} value of 0.90 μM (Teng et al., 1998; Teng et al., 1997).

The concern of the use of stimulant medications clearly indicates that more treatment options are necessary for ADHD. Based on *in vitro* experiments, lobeline may be an option (Teng et al., 1998; Teng et al., 1997). However, the effect of *in vivo* administration of lobeline on VMAT2 function needs to be

examined. In order to determine if the mechanism of lobeline on VMAT2 function is similar to methylphenidate and amphetamine, the effects of methylphenidate and amphetamine on VMAT2 function were also investigated. Previous research in our lab found that after *in vivo* administration of lobeline, methylphenidate, and amphetamine, only methylphenidate altered DAT function. This led us to believe that VMAT2 may possibly be the primary target involved in the mechanism of action of these agents. Therefore, the goal of the present study was to elucidate the effect of acute and repeated *in vivo* administration of lobeline on VMAT2 function and compare its effect to the effects of methylphenidate and amphetamine. Such experiments are helpful in providing more insight into the mechanism of action of all these agents.

B. Methods

Materials.

[^{3}H]DA (3,4-ethyl-2[N-[^{3}H]dihydroxyphenylethylamine; Specific Activity 28 Ci/mmol) was purchased from PerkinElmer Life Sciences Inc. (Boston, MA). L-Ascorbic acid, bovine serum albumin (BSA), catechol, α-D-glucose, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), nomifensine maleate, pargyline hydrochloride, sucrose, methylphenidate hydrochloride, amphetamine hydrochloride, and 3-hydroxytyramine hydrochloride DA were obtained from Sigma Chemical Co. (St. Louis, MO). Lobeline was purchased from ICN Biomedicals (Costa Mesa, CA). All other chemicals used in the *in vitro* assay buffers were purchased from Fisher Scientific (Pittsburgh, PA).

Subjects.

Adult male Sprague-Dawley rats (200-220g body weight upon arrival) were obtained from Harlan Inc. (Indianapolis, IN). Animals were housed two per cage with free access to food and water in the Division of Lab Animal Resources, University of Kentucky. Experimental protocols were in concordance with the NIH 1996 *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.

Drug Administration.

In the current study, rats were randomly assigned to 3 treatment groups (lobeline, methylphenidate, and amphetamine) for acute and repeated administration. Lobeline, methylphenidate, and amphetamine were administered by subcutaneous (sc) injection in a volume of 1 ml/kg body weight and expressed as the salt weights. The rats in the lobeline treatment group were injected acutely with lobeline (1, 3, and 10 mg/kg) and repeatedly for 7 days with lobeline (3 mg/kg). The rats in the methylphenidate treatment group were injected acutely (n = 4-18) with methylphenidate (2.5, 10, and 20 mg/kg) and repeatedly for 7 days with methylphenidate (2.5 and 10 mg/kg). The rats in the amphetamine treatment group were injected acutely with amphetamine (0.25, 1.0, and 5.0 mg/kg) and repeatedly for 7 days with amphetamine (0.25 and 5 mg/kg). Control groups for the lobeline treatment group were administered saline contemporaneously for all doses and the methylphenidate and amphetamine treatment groups had independent saline groups for the individual doses.

Lobeline dose range and time point after injection were chosen based on previous behavioral studies in which lobeline (1, 3, and 10 mg/kg) were used and lobeline (3 mg/kg) significantly inhibited methamphetamine self-administration 20 min after injection (Harrod et al., 2001). Methylphenidate dose range and time point were chosen based on experiments assessing [^{3}H]DA uptake, 60 min after administration and behavioral studies which concluded that doses of 2.5 and 5.0 of methylphenidate were more clinically relevant (Kuczenski & Segal, 2001; Wooters et al., 2006). Amphetamine doses range and time point were based on behavioral studies showing that the low doses of amphetamine (0.25-0.75 mg/kg) improved stimulus detection performance in rats 30 minutes after injection. (Grilly, 2000). The doses for the repeated experiments were chosen based on the effect observed during the acute experiments. If no effect was observed during the acute experiments, doses were chosen based on previous behavioral and *in vitro* studies.

Vesicle Preparation and [^{3}H]DA Uptake Assay.

The uptake of [^{3}H]DA into striatal synaptic vesicles were determined by using a previously published method (Erickson et al., 1990). Striata from the rats were homogenized over a 2-min period in 14ml of 0.32 M sucrose (pH 7.5) with 10 up and down strokes of a Teflon pestle (clearance ~0.009 inches). The homogenate was centrifuged at 2000 g for 10 min at 4°C, and the resulting supernatant will be centrifuged at 10,000 g for 30 min at 4°C. Synaptosomes (buffy coat) were separated from the underlying mitochondria and cellular debris (reddish pellet) by gentle swirling in 2 ml of 0.32 M sucrose. The enriched synaptosome fraction (2.0 ml) was subjected to osmotic shock by the addition of 7 ml distilled H_2O and homogenized with 5 up and down strokes of the Teflon pestle. The osmolarity was restored by the addition of 900 µl of 0.25 M HEPES and 900 µl of 1.0 M neutral potassium-tartrate buffer (pH 7.5) followed by a 20-min centrifugation (20,000 g at 4°C). The supernatant was centrifuged for 60 min (55,000 g at 4°C). Then 1 ml of solution containing 10 mM $MgSO_4$, 0.25 M HEPES and 1.0 M potassium-tartrate buffer was added to the supernatant and the suspension was centrifuged (100,000 g for 45 min at 4°C). Immediately before use, the final pellet was resuspended in the assay buffer (in mM: 25 HEPES, 100 potassium tartrate, 0.05 EGTA, 0.10 EDTA, 2 ATP-Mg^{++} and 1.7 ascorbic acid, pH 7.4). Aliquots (160 µl containing 8-10 µg protein) of the resuspension were incubated with 20 µl of drug (final concentrations: nicotine, 0.001-100 µM; lobeline, 0.001-100 µM; tetrabenazine, 0.001-100 µM) and 20 µl of [^{3}H]DA (final concentration 0.3 µM) for 8 min at 37°C in a total volume of 200 µl. The reaction was terminated by the addition of 2.5 ml of ice-cold assay buffer containing 2 mM $MgSO_4$. Samples were rapidly filtered through Whatman GF/F filters using the Brandel cell harvester (Model MP-43RS; Biochemical Research and Development Laboratories Inc., Gaithersburg, MD). The filters were washed three times with 4 ml of ice-cold assay buffer containing 2 mM $MgSO_4$. Filters were previously soaked in 0.5% polyethylenimine (Speiser et al., 1983) solution for 2 hr at 4 °C. Nonspecific uptake was determined by incubation of duplicate samples in the presence of R0412-84 (10 µM), a VMAT2 inhibitor. Filters were

placed into scintillation vials, 10 ml scintillation cocktail were added, and radioactivity was determined by scintillation spectrometry (Model B1600TR, Perkin-Elmer Life Sciences, Downers Grove, IL).

C. Data Analysis.

Kinetic parameters (V_{max} and K_m) for [^{3}H]DA uptake were determined using GraphPad Prism software (GraphPad Prism, version 5.02; GraphPad Software, San Diego, CA). Data are represented as mean values ± S.E.M. To analyze the kinetic parameters, V_{max} and K_m for [^{3}H]DA uptake in drug-treated for each drug at each dose and saline-control groups, separate unpaired Student t-tests were performed. Log transformed K_m values were used for statistical analyses using the Statistical Packages for the Social Sciences (SPSS; standard version 17, SPSS Inc., Chicago, IL). Differences were considered significant at $p< 0.05$. An individual one-way ANOVA with each dose of methylphenidate, amphetamine and lobeline serving as an independent group revealed that [^{3}H]DA uptake was not different among the saline groups across the dose range for each drug (Tables 9-11). Therefore, the data for the saline-control groups were pooled for statistical analysis and graphical presentation, which also explains the wide range of n.Specific [^{3}H]DA uptake for the saline-control groups for acute methylphenidate 2.5, 10, and 20 mg/kg doses were 19.1 ± 3.6, 23.0 ± 2.7, and 28.0 ± 4.1 pmol/mg/min, respectively, with a pooled mean of 23.8 ± 2.0 pmol/mg/min. Specific [^{3}H]DA uptake for the saline-control groups for acute amphetamine 0.25, 1.0, and 5.0 mg/kg doses were 35.3 ± 3.0, 43.1 ± 5.8, and 31.0 ± 3.2 pmol/mg/min, respectively with a pooled mean of 36.0 ± 3.3 pmol/mg/min.

D. Results

Effects of Acute and Repeated *In Vivo* Methylphenidate Administration on Vesicular Monoamine Transporter Function

Kinetic analysis of [^{3}H]DA uptake was performed on synaptosomes after acute *in vivo* administration of methylphenidate. Methylphenidate (10, and 20 mg/kg) increased V_{max} (53.4 ± 5.0 and 43.4 ± 4.7 pmol/mg/min) for [^{3}H]DA uptake compared to pooled saline-control (23.8 ± 2.0 pmol/mg/min), respectively. The

current results show an increase (84-124%) of VMAT2 function, with acute methylphenidate (10 and 20 mg/kg) having a significantly higher V_{max} compared to control (Fig. 15). Repeated methylphenidate *in vivo* administration for 7 days at 2.5 and 10 mg/kg doses had a V_{max} of 33.0 ± 1.1 and 52.7 ± 4.0 pmol/mg/min, respectively, which increased (53% and 145%) VMAT2 function compared to pooled saline-control with a V_{max} of 21.5 ± 1.4 pmol/mg/min (Fig.16). Neither acute nor repeated methylphenidate *in vivo* administration had an effect on K_m values (Table 15).

Effects of Acute and Repeated *In Vivo* Amphetamine Administration on Vesicular Monoamine Transporter Function

Acute, as well as 7 day-repeated *in vivo* administration of amphetamine had no effect on VMAT2 function, (Figs. 17, 18). Specific [^{3}H]DA uptake for amphetamine 0.25, 1.0, and 5.0 mg/kg doses were 35.3 ± 3.0, 43.1 ± 5.8, and 31.0 ± 3.2 pmol/mg/min, respectively, compared to the pooled saline-control group with a V_{max} of 36.0 ± 3.3 pmol/mg/min. Amphetamine had no effect on K_m values following acute *in vivo* administration (Table 15); however, repeated *in vivo* administration significantly decreased the K_m after 7 days (Fig. 19;Table 15).

Effects of Acute and Repeated *In Vivo* Lobeline Administration on Vesicular Monoamine Transporter Function

Kinetic analysis of vesicular [^{3}H]DA uptake after acute (Fig. 20) and repeated (Fig. 20) *in vivo* administration of lobeline for 7 days showed no effect on V_{max} or K_m values (Tables 11 and 15). Specific [^{3}H]DA uptake at VMAT2 for acute lobeline 1.0, 3.0, and 10.0 mg/kg doses were 29.7 ± 4.5, 33.9 ± 6.9, and 33.7 ± 7.4 pmol/mg/min, respectively, compared to 29.7 ± 5.0 pmol/mg/min for the pooled saline-control group.

E. Discussion

In the present study, the effects of acute and repeated *in vivo* administration of lobeline on VMAT2 function were determined and compared to the effects of methylphenidate and amphetamine using kinetic analysis of [^{3}H]DA uptake into striatal vesicles. This work investigating *in vivo* drug administration, extends previous work in our laboratory demonstrating that *in vitro,* lobeline

inhibits VMAT2 function (Teng et al., 1997). However, the results from these studies show that lobeline (1, 3, and 10 mg/kg) has no effect on VMAT2 function 20 min after *in vivo* administration. Previous research using striatal vesicles has provided evidence that the ability of a compound to alter monoamine uptake *in vitro* does not necessarily predict its ability to modulate monoamine transporters after *in vivo* administration (Fleckenstein et al., 1999). This may be the case with in vesicles as well. Thus, it is necessary to conduct *in vivo* experiments as well as *in vitro* experiments.

Another plausible explanation for the lack of effect with lobeline could be that the time of the effect was not in the timeframe that was used in this study. The time point was based on behavioral effects observed after *in vivo* administration. The pharmacological effect may not coincide with the behavioral effect, thus no pharmacological effect was seen at the time point in question.

Acute *in vivo* administration of methylphenidate showed an increase in VMAT2 function at 10 and 20 mg/kg, but showed no effect with the 2.5 mg/kg dose. These results suggest that there is a specific range of doses of methylphenidate that elicit an effect on VMAT2 function. If the dose is too high or too low, the effect could be lost. This result is consistent with previous results showing that a single high dose (40 mg/kg s.c.) of methylphenidate increased vesicular DA uptake accessed 60 minutes after *in vivo* administration (Fleckenstein et al., 1999; Sandoval et al., 2002). After 7 days of repeated *in vivo* methylphenidate administration, the 2.5 and 20 mg/kg doses showed a significant increase in V_{max}. No tolerance developed with repeated administration of methylphenidate after 7 days. Methylphenidate administration did not alter the K_m, which suggests that methylphenidate competes with dopamine at VMAT2 in a noncompetitive manner. Very little is known about VMAT2 regulation and only the Fleckstein lab to our knowledge has conducted research on how methylphenidate interacts with VMAT2. However, the interest in VMAT2 as a target for drugs of abuse is growing based on a current review by Eden and Weihe (Eiden, 2011).

Acute and repeated *in vivo* administration of amphetamine did not alter VMAT2 function in these studies. The time points chosen in this current study were based on behavioral observations of amphetamine and the time course of the neurochemical effects may not correspond with the behavioral effects of amphetamine on VMAT2 function. A complete time course would allow more insight as to what the optimal time point should be in order to observe the maximal pharmacological effect of amphetamine. However, Fon and colleagues found that VMAT2 was not required for amphetamine to cause DA release in an *in vitro* measure of amphetamine in VMAT2 knock-out mice with an absence of vesicular monoamine stores (Fon et al., 1997). This finding may be difficult to relate to methylphenidate, since methylphenidate does not release DA. Based on this evidence and what is already known about amphetamine, the current results of this study showing amphetamine did not alter VMAT2 function are intriguing to say the least.

The current results suggest that methylphenidate acts differently than lobeline and amphetamine, in reference to the effects observed on VMAT2 function. The observation that amphetamine and methylphenidate influence VMAT2 differently at the time point and doses used in this study may be rather surprising since both drugs have been shown to target VMAT2 (Greydanus et al., 2007; Thanos et al., 2007). However, the data in these studies could be a result of the difference in their mechanisms of action.

Overall, the results of the present study, have demonstrated that methylphenidate affects VMAT2 function differently than amphetamine and lobeline after *in vivo* administration. These changes in VMAT2 function are interesting considering that little is known as to how methylphenidate interacts with VMAT2. These findings suggest that methylphenidate increases VMAT2 function, which decreases the amount of DA in the cytosol. These results also imply the VMAT2 is a target of methylphenidate and that VMAT2 could be involved in the etiology of ADHD.

Although lobeline did not alter VMAT2 function in the present study, additional evidence suggests that lobeline could still be evaluated as a potential treatment option for ADHD. Lobeline has been found to improve retention performance 24 hours after injection in spatial discrimination water maze (Decker et al., 1993). Furthermore, Levin et al. showed lobeline enhanced learning on the post-acquisition working memory performance in the radial-arm maze (Levin et al., 2003). Previous work has also demonstrated that lobeline may be an effective smoking cessation agent by showing that lobeline acts as a nicotinic antagonist by inhibiting nicotine-evoked DA release and nicotine-evoked $^{86}Rb^{+}$ efflux (Miller et al., 2000). Lobeline has been used as an over-the-counter smoking cessation agent in the past in the US (Nunn-Thompson & Simon, 1989; Prignot, 1989) and numerous human studies have been conducted to determine efficacy of lobeline as a smoking cessation agent, although its use is still debatable (Dwoskin & Crooks, 2002). This characteristic gives lobeline an added benefit to the currently used ADHD treatments because it has been shown that there is a higher rate of smoking in people who have ADHD compared to those who do not (Milberger et al., 1997). Furthermore, lobeline does not have typical stimulant side-effects such as abuse liability.

In conclusion, there is evidence to warrant more investigation into the mechanism of action of these agents because it is still unclear as to how they elicit their pharmacological effects. Therefore, additional experiments need to be conducted to elucidate the pharmacological effects of lobeline, methylphenidate, and amphetamine. Ultimately, studies of this nature may help in the discovery of new treatment options for ADHD that improve behavioral symptoms while reducing detrimental drug side-effects.

TABLE 9. Acute saline-treated control groups did not differ between MPD doses for VMAT2 experiments. Acute independent saline-treated controls did not vary between MPD doses during VMAT2 experiments based on results of a one-way ANOVA ($F_{2,15}$ = 1.428, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 4-8 rats/group.

ACUTE MPD (mg/kg)	Vmax pmol/min/mg
MPD 2.5 (n= 4)	19 ± 3.6
MPD 10 (n= 8)	23 ± 2.7
MPD 20 (n= 6)	28 ± 4.1
POOLED MEAN	24 ± 1.8[a]

TABLE 10. Acute saline-treated control groups did not differ between AMPH doses for VMAT2 experiments. Acute independent saline-treated controls did not vary between AMPH doses during VMAT2 experiments based on results of a one-way ANOVA ($F_{2,13}$ = 3.076, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 5-6 rats/group.

ACUTE AMPH (mg/kg)	Vmax pmol/min/mg
AMPH 0.25 (n= 5)	35 ± 3.0
AMPH 1.0 (n= 5)	43 ± 5.8
AMPH 5.0 (n= 6)	29 ± 3.3
POOLED MEAN	36 ± 3.3[a]

TABLE 11. Acute saline-treated control groups did not differ between LOB doses for VMAT2 experiments. Acute independent saline-treated controls did not vary between LOB doses (1, 3, and 10 mg/kg) during VMAT2 experiments based on results of a one-way ANOVA ($F_{3,27}$ = 0.1494, p>0.05), with the Vmax as the dependent variable. This demonstrates that the saline-treated control groups did not differ over time or between treatment groups. [a]Data are presented as mean ± S.E.M for n = 7 rats/group.

ACUTE LOB (mg/kg)	Vmax pmol/min/mg
POOLED MEAN	30 ± 5.0[a]

TABLE 12. Repeated 7-day saline-treated control groups did not differ between MPD doses for VMAT2 experiments. The 7-day independent saline-treated controls were used for both MPD doses during VMAT2 experiments. Therefore, no ANOVA was performed. [a]Data are presented as mean ± S.E.M for n = 8 rats/group.

7-day MPD (mg/kg)	Vmax pmol/min/mg
POOLED MEAN	22 ± 1.4[a]

TABLE 13. Repeated 7-day saline-treated control groups did not differ between AMPH doses for VMAT2 experiments. The 7-day independent saline-treated controls were used for both AMPH doses during VMAT2 experiments. Therefore, no ANOVA was performed. [a]Data are presented as mean ± S.E.M for n = 5 rats/group.

7-day AMPH (mg/kg)	Vmax pmol/min/mg
POOLED MEAN	30 ± 5.3[a]

TABLE 14. Repeated 7-day saline-treated control groups did not differ between LOB doses for VMAT2 experiments. The 7-day independent saline-treated controls were used for both LOB doses during VMAT2 experiments. Therefore, no ANOVA was performed. [a]Data are presented as mean ± S.E.M for n = 5 rats/group.

7-day LOB (mg/kg)	Vmax pmol/min/mg
POOLED MEAN	24 ± 2.5[a]

Figure 15. Acute MPD increased VMAT2 function. Rats were injected acutely once daily with MPD (0, 2.5, 10.0, and 20 mg/kg; open bars). Control (0; black bar) represents saline-treated control group. An independent control group was administered saline contemporaneously with each MPD dose (open bars) for the acute treatment group. Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n = 4 -18 rats/group (Data were pooled for control groups).

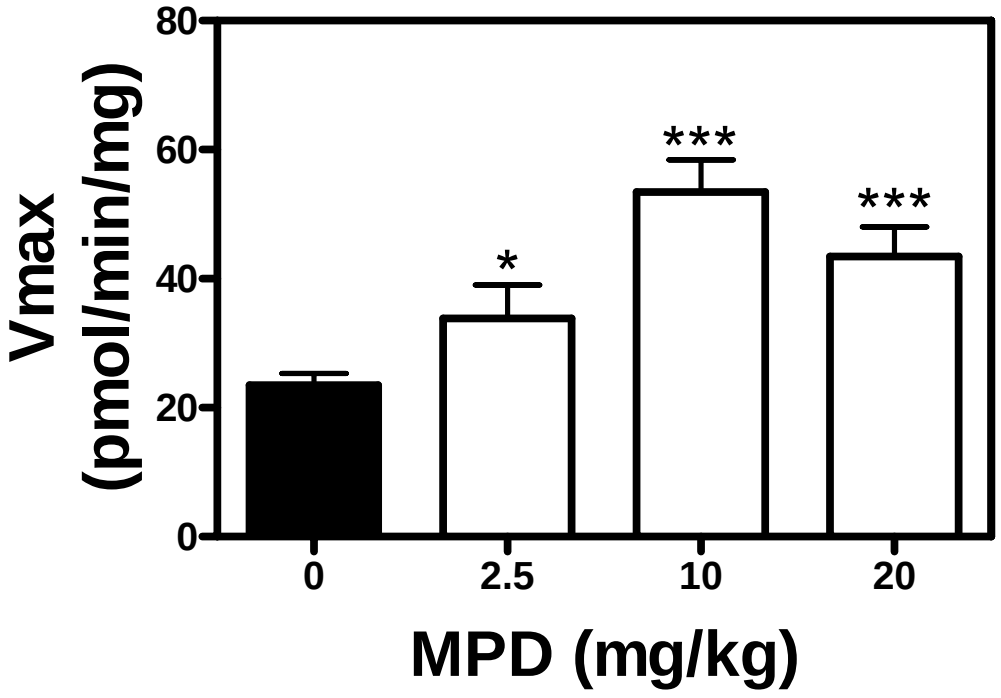


Figure 16. Repeated 7-day MPD increased VMAT2 function. Rats were injected repeatedly once daily with MPD (0; black bar; 2.5, and 10.0 mg/kg; open bars). Repeated MPD (2.5and 10 mg/kg for 7 days) increased [^{3}H]DA uptake (***p<0.001 for 2.5 and 10.0 mg/kg group compared to control). Vmax is represented in pmol/min/mg. Data are presented as mean ± S.E.M for n = 6-8 rats/group.

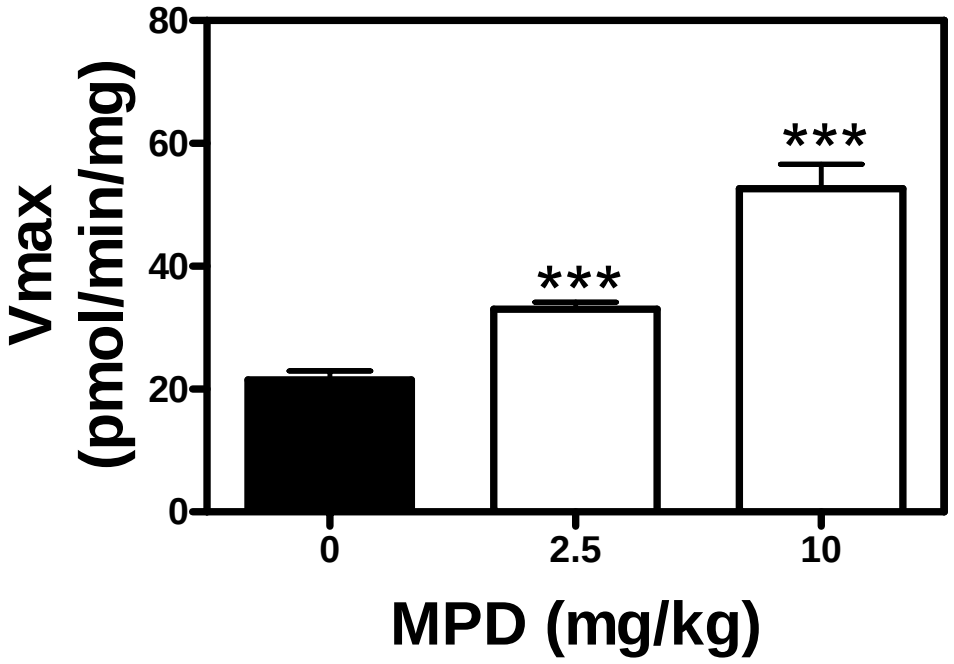


Figure 17. Acute AMPH did not alter VMAT2 function. Rats were injected acutely once daily with AMPH (0, 0.25, 1.0, and 5.0 mg/kg). Control (0; black bar) represents saline-treated control group. An independent control group was administered saline contemporaneously with each AMPH dose (open bars) for the acute treatment group. Vmax is represented in pmol/min/mg. Acute AMPH had no effect on striatal [^{3}H]DA uptake 30 min after administration. Data are presented as mean ± S.E.M for n=4-12 rats/group (Data were pooled for control groups).

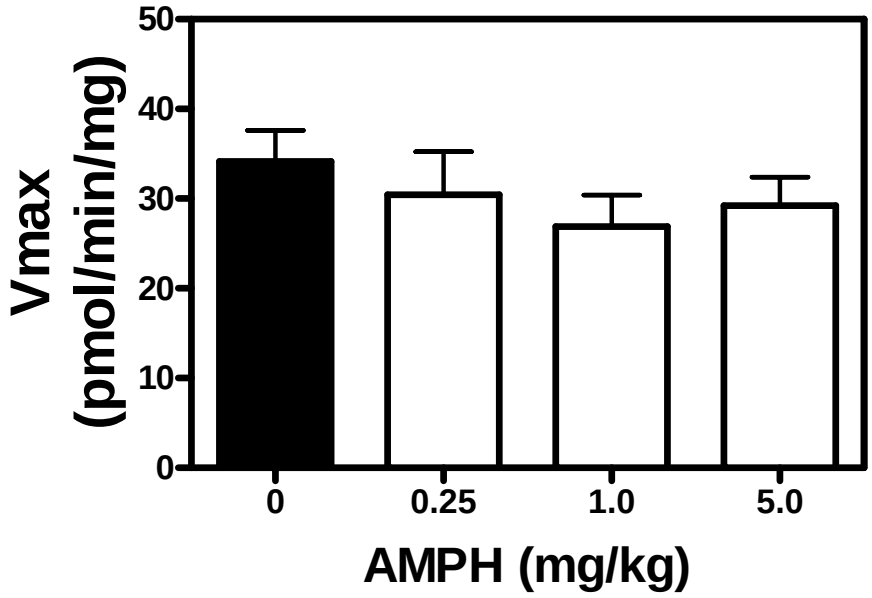


Figure 18. Repeated 7-day AMPH did not alter VMAT2 function. Rats were injected repeatedly once daily for 7 days AMPH (0, 0.25, and 5.0 mg/kg; open bar). Control (0; black bar) represents saline-treated control group. Vmax is represented in pmol/min/mg. Repeated AMPH (7 days) did not alter striatal [^{3}H]DA uptake. Data are presented as mean ± S.E.M for n = 5 rats/group.

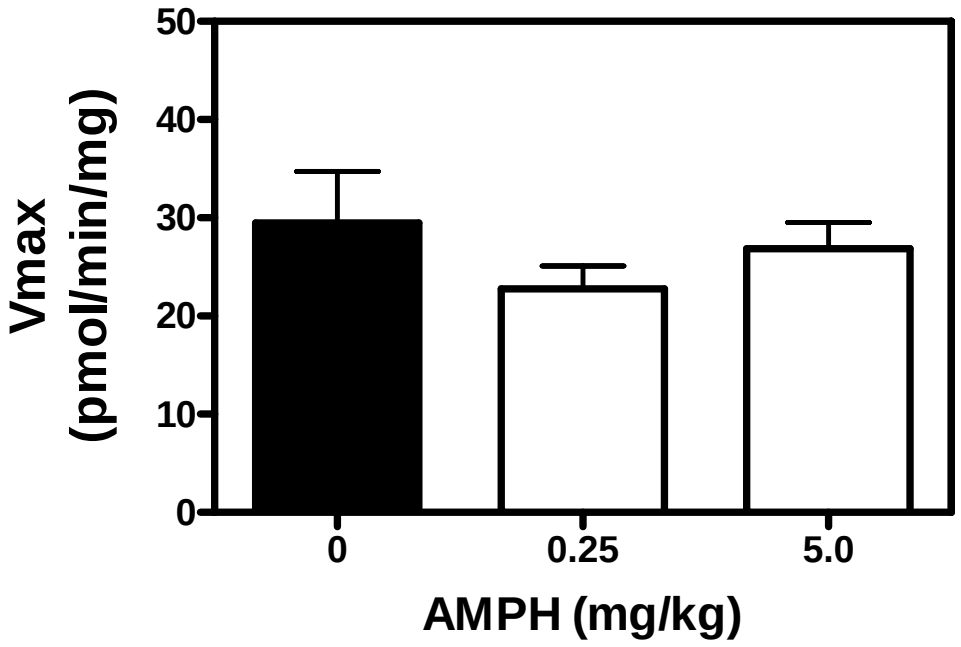


Figure 19. Repeated 7-day AMPH decreased K_m for VMAT2. Rats were injected repeatedly once daily with AMPH (0, 0.25, and 5.0 mg/kg; open bars) for 7 days. Control (0; black bar) represents saline-treated control group. Repeated AMPH (5 mg/kg) decreased Km (0.12 ± 0.01) compared to control (0.24 ± 0.05). Data are presented as mean ± S.E.M of the log transform of K_m for n = 5 rats/group.

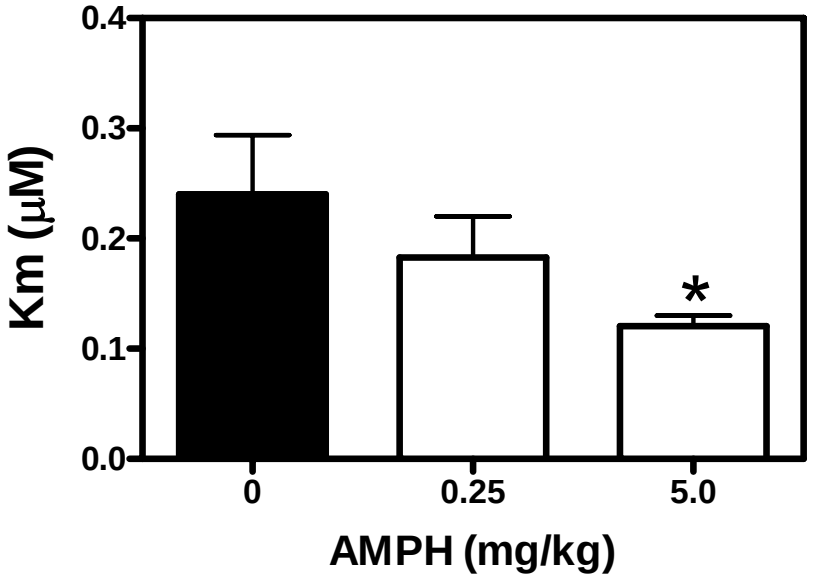


Figure 20. Acute LOB did not alter VMAT2 function. Rats were injected acutely once daily with LOB (0, 1.0, 3.0, and 10.0 mg/kg) Control (0; black bar) represents saline-treated control group. A control group was administered saline contemporaneously for all LOB doses (open bars) for the acute treatment group. Vmax is represented in pmol/min/mg. Acute LOB did not alter striatal [^{3}H]DA uptake 20 min after injection. Data are presented as mean ± S.E.M for n = 7-8 rats/group.).

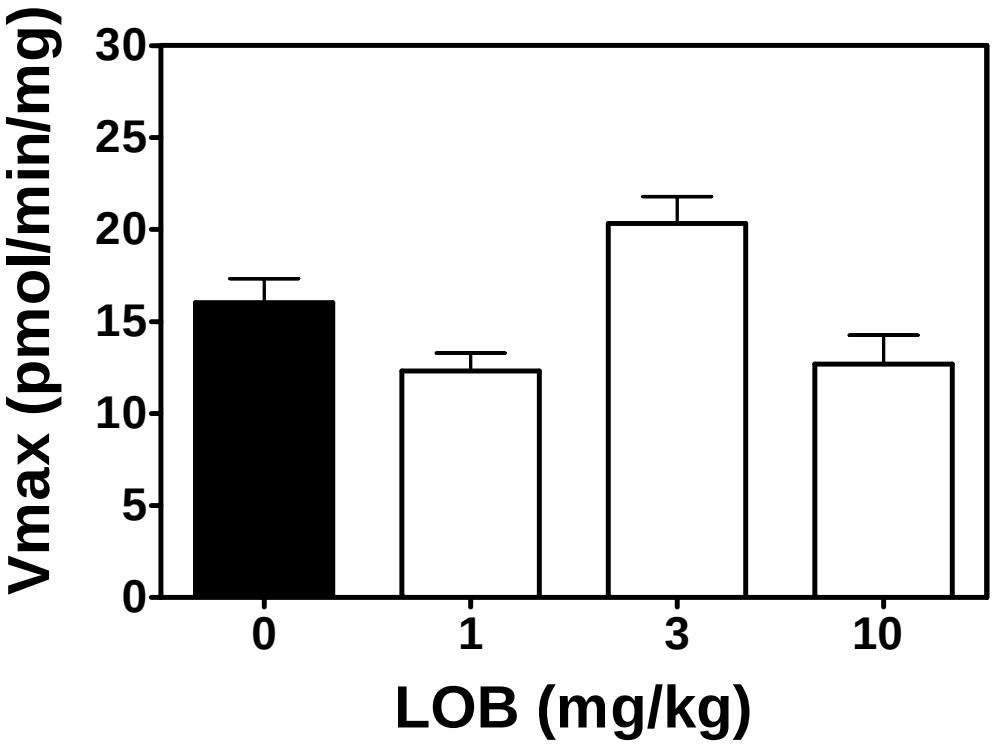


Figure 21. Repeated LOB did not alter VMAT2 function. Rats were injected repeatedly once daily for 7 days LOB (0 and 3.0 mg/kg). Vesicles were prepared 20 min post-injection. Control (0; black bar) represents saline-treated control group. A control group was administered saline contemporaneously for LOB 3 mg/kg dose (open bar) for the7-day treatment group. Vmax is represented in pmol/min/mg. Repeated LOB for 7 days did not alter [^{3}H]DA uptake. Data are presented as mean ± S.E.M for n=5-7 rats/group.).

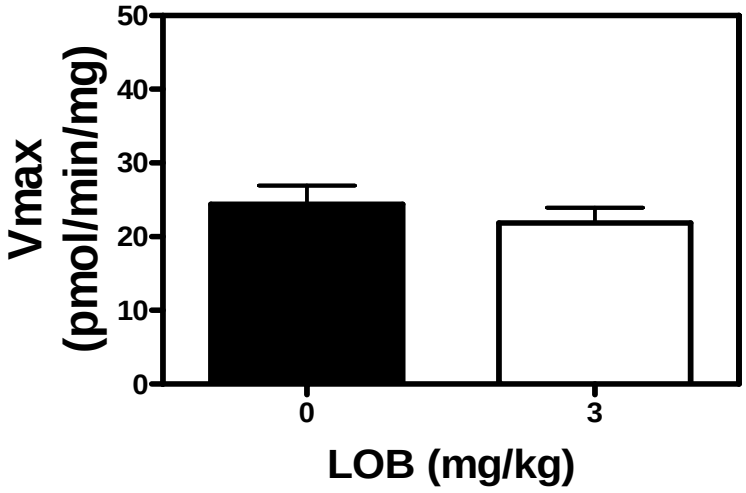


Table 15. K_m values for [^{3}H]DA uptake at VMAT2 were only altered by the AMPH (7-day treatment) regimen.

ACUTE MPD (mg/kg)	Km (Mick et al)	7-DAY MPD (mg/kg)	Km ((Mick et al)
0	11 ± 1.0	0	13 ± 1.0
2.5	12 ± 3.0	2.5	10 ± 1.0
10	12 ± 3.0	10	12 ± 1.0
20	9.0 ± 0.4		
ACUTE AMPH (mg/kg)	**Km (Mick et al)**	**7-DAY AMPH (mg/kg)**	**Km (Mick et al)**
0	11 ± 3.0	0	24 ± 5.0
0.25	9.0 ± 2.0	0.25	18 ± 4.0
1	10 ± 1.0	***5***	***12 ± 1.0***
5	10 ± 2.0		
ACUTE Lobeline (mg/kg)	**Km (Mick et al)**	**7-DAY Lobeline (mg/kg)**	**Km (Mick et al)**
0	20 ± 4.0	0	15 ± 3.0
1	21 ± 4.0	3	11 ± 2.0
3	13 ± 1.0		
10	13 ± 3.0		

[a]Data are presented as mean ± S.E.M for n = 4-18 rats/treatment group. ANOVA ($F(6,48) = 3.21$, $p > 0.05$ revealed that K_m values were not different among independent control groups across the given treatments, and thus, these data for the saline-treated groups for each treatment group were pooled for statistical analysis.

V. Chapter Five

Discussion and Conclusions

A. Summary

ADHD is a psychiatric disorder that has a worldwide impact. ADHD has become more of a focus in research due to the increasing number of children and adults being diagnosed with ADHD and there is still much to be learned about the safety issues of ADHD medications (Meijer et al., 2009). This increase in ADHD research, has also led to a need to develop novel agents without the harmful side effects associated with the current therapies used to treat ADHD. The first line therapy for ADHD consists of stimulants, namely methylphenidate and amphetamine. Amphetamine is a DAT substrate, which competes with DA for DAT and also reverses the transport of dopamine causing release of dopamine, where as methylphenidate binds to DAT and inhibits DAT function, without releasing DA (Bannon et al., 2000; Seiden et al., 1993; Solanto, 2002; Sonders et al., 1997). These agents also interact with VMAT2, which is the transporter responsible for moving cytoplasmic dopamine into the synaptic vesicles for storage and future release (Yelin & Schuldiner, 2002). Methylphenidate has been shown to rapidly and reversibly increase VMAT2 binding and vesicular uptake of dopamine (Sandoval et al., 2001). Amphetamine has been found to displace the VMAT2 ligand tetrabenazine with a relatively low (μM) affinity for VMAT2, which suggest amphetamine may be a substrate of VMAT2 (Gonzalez et al., 1994).

Lobeline, a major alkaloidal component of the Indian tobacco plant, has been shown in previous *in vitro* studies performed in our laboratory, to interact with DAT and VMAT2. For instance, lobeline binds to and inhibits (IC_{50} value ≈ 40-100 μM) DAT, inhibiting DA uptake (Miller et al., 2004; Teng et al., 1997). Lobeline also inhibits [^{3}H]DA uptake into vesicles with an IC_{50} value of 0.88 μM and inhibits the binding of [^{3}H]dihydrotetrabenazine, a VMAT2 ligand, to the vesicular membrane with an IC_{50} value of 0.90 μM (Teng et al., 1998; Teng et al., 1997). However, lobeline may not have any abuse liability based on results from

behavioral studies, that showed lobeline was not self-administered by rats, nor did it substitute for *d*-methamphetamine (Harrod et al., 2003).

The purpose of this dissertation research was to examine the effects of acute and repeated *in vivo* administration of a compound called lobeline on dopamine and vesicular monoamine transporter functions and compare those effects with the effects of methylphenidate and amphetamine to warrant further investigation of lobeline as a potential treatment option for ADHD. The experiments for this dissertation research were conducted in the striatum, a region of the brain rich in dopamine and is also suggested to be involved in executive function and motor response associated with ADHD (Dinn et al., 2001; Volkow et al., 2001; Wilens, 2008).

The first aim of this dissertation research was to determine the effect of lobeline on DAT function after acute and repeated *in vivo* administration using [^{3}H]DA uptake assay and compare its effects with the effects of methylphenidate and amphetamine. The novel findings from these experiments showed an increase of DAT function (~60%), with acute methylphenidate (5 and 20 mg/kg) having a significantly higher V_{max} compared to control. Repeated methylphenidate administration for 7 days at 2.5 and 20 mg/kg doses also increased DAT function (36% and 48%) compared to pooled saline-control. Neither acute nor repeated methylphenidate *in vivo* administration had an effect on K_m values. Acute, as well as repeated (7 or 14 days) *in vivo* administration of amphetamine and lobeline had no effect on DAT function. Amphetamine and lobeline had no effect on K_m values following acute or repeated *in vivo* administration. The results of these experiments do not support the hypothesis that lobeline would decrease DAT function *in vivo* as observed in the *in vitro* experiments. To our knowledge, this is the first report of the effects of DAT and VMAT2 function after acute and repeated *in vivo* administration of methylphenidate, amphetamine, and lobeline.

The second aim was to determine the effect of lobeline on VMAT2 function after acute and repeated *in vivo* administration and compared its effects to methylphenidate and amphetamine. Acute *in vivo* administration of

methylphenidate showed an increase in VMAT2 function at 10 and 20 mg/kg. After 7 days of repeated *in vivo* methylphenidate administration, the 2.5 and 20 mg/kg doses showed a significant increase in V_{max}. Methylphenidate administration did not alter the K_m. Acute and repeated *in vivo* administration of amphetamine did not alter VMAT2 function in these studies. Amphetamine had no effect on K_m values following acute *in vivo* administration; however, repeated *in vivo* administration significantly decreased the K_m after 7 days. Acute and repeated *in vivo* administration of lobeline did not alter VMAT2 function or K_m in these studies. In summary, all three compounds act differently at this site. The results of these experiments did not support the hypothesis that lobeline would decrease VMAT2 function as seen in the previous *in vitro* studies.

The third aim was to determine if the underlying mechanism of the methylphenidate-induced increase of DAT function was due to DAT trafficking using biotinylation and western blot analysis. The biotinylation and western blot assay revealed that methylphenidate given acutely, slightly but not significantly decreased intracellular DAT expression with no change in total or surface DAT expression. However, *in vivo* administration of methylphenidate after 7 days significantly decreased intracellular DAT expression, where only ~ 20 % of intracellular DAT was found compared to control, without a change in the total or surface DAT expression. These results agree with the hypothesis that DAT trafficking was responsible for the methylphenidate-induced increase in DAT function, but in a different manner since there was no increase in surface DAT expression. Nevertheless, the significant decrease in intracellular DAT caused by methylphenidate administration for 7 days does suggest that methylphenidate redistributes DAT, which means that some type of trafficking is involved and that this effect is time-dependent.

B. Potential Mechanism for the Methylphenidate-Induced Increase in DAT Function

Methylphenidate is a potent DAT inhibitor, which results in an increase in extracellular dopamine (Easton et al., 2007; Ferris et al., 1972; Richelson & Pfenning, 1984). Other studies have investigated the effect methylphenidate has

on DAT density and occupancy (Muneoka et al., 2006; Spencer et al., 2006; Volkow et al., 1998; Volkow et al., 2007; Wilcox et al., 2008). The results from the current work revealed that methylphenidate increased DAT function rather than inhibited DAT function. Specifically, acute methylphenidate (5.0 and 20 mg/kg) increased DAT function by ~60% and repeated methylphenidate (2.5 and 20 mg/kg) increased DAT function by 36% and 48% respectively. However, repeated *in vivo* administration of methylphenidate (2.5, 5.0, or 20 mg/kg) after 14 days did not alter DAT function. These findings suggest that the effect of methylphenidate is lost between 7 and 14 days and is time-dependent. This observation may be due to the compensatory effect of the potent inhibition of methylphenidate on DAT function. As methylphenidate inhibits DAT function, the system may respond by upregulating the existing DAT in order to compensate for the lack of DAT function. Overtime the compensatory effect could become less, thus causing methylphenidate to have no effect on DAT function, which was observed in the 14-day experiments.

Another potential explanation could be the involvement dopamine transporter phosphorylation and protein-protein interactions. A study investigating the effect of methylphenidate on dopamine-and cAMP-regulated phosphoprotein, M_r 32kDa (DARPP-32) phosphorylation using neostriatal slices found that methylphenidate increased (DARPP-32) phosphorylation in adult (6-8-week old) slices but not young (14-15-and 21-22-day old) slices (Fukui, 2003). The phosphorylation state of DARPP-32 is involved in the mechanism for integrating information coming from dopamine neurons, multiple brain regions, utilizing various neurotransmitters, neuromodulators, neuropides and steroid hormones (Svenningsson et al., 2004). This finding suggests that age is an important factor to consider when determining the effect of methylphenidate on phosphorylation. Vaughan's group conducted a more recent study to determine if certain DAT blockers, including methylphenidate, had an effect on basal and phorbol 12 myristate 13 acetate (PMA) (Li et al)-stimulated DAT phosphorylation (Gorentla & Vaughan, 2005). PMA is a PKC activator and PKC regulates DAT activity and phosphorylation. The authors exposed rDAT LLC-PK1 cells to methylphenidate

(10 μM) for 30 minutes and found that methylphenidate had no effect on basal or PMA-stimulated phosphorylation. This evidence suggests that methylphenidate does not cause DAT phosphorylation, thus no decrease in DAT function with methylphenidate was observed. Taken together, these data imply that DAT function can be regulated by various mechanisms and more work is required to elucidate the intricacies of these mechanisms.

1. Methylphenidate increase in DAT Function is trafficking dependent

Several lines of evidence suggest that psychostimulants are involved in the regulation of DAT at the level of surface expression (Zhu & Reith, 2008). In the current study, methylphenidate decreased intracellular DAT without altering total or surface DAT. DAT function is regulated by numerous cellular signaling pathways, including PKC, tyrosine kinases, phosphatases, calcium and calmodulin-dependent kinases, and protein kinase A (PKA), which strongly modulate DAT expression on the plasma membrane (Melikian, 2004; Robinson, 2002; Vaughan, 2004; Zahniser & Doolen, 2001). G protein-coupled receptors (GPCRs), enzymatic modification such as phosphorylation or ubiquitination, and protein-protein interactions between DAT and other scaffolding proteins have also been found to be involved in the regulation of DAT (Schmitt et al., 2010). Methylphenidate may have caused the phosphorylation of DAT, thus making DAT unrecognizable to the DAT antibodies used in the western blot analysis. Ubiquitination is the covalent attachment of the small soluble protein ubiquitin to the ε-amino moiety of lysine residues in target cellular proteins. This process affects DAT cell surface expression and membrane trafficking of transporter (Schmitt et al., 2010). A possible mechanism mediating the methylphenidate-induced decrease in intracellular DAT could be D3 receptor activation. Zapata et al conducted an experiment using hEK and neuro2A cells, found a significant increase in cell surface DAT parallel to a significant increase in DAT function after brief D3 receptor stimulation (Zapata et al., 2007). This result suggests D3 activation increases DAT redistribution from the intracellular compartment to the cell surface. This finding is consistent with our results where methylphenidate

caused the same DAT redistribution from the intracellular compartment based on the significant decrease in intracellular DAT that was observed. However, prolonged D3 receptor activation had the opposite effect, causing a decrease in cell surface DAT. In addition, this study also found that the D3 activation requires mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) activation. This suggests that there are multiple mechanisms and pathways that may be involved in the methylphenidate-induced decrease of intracellular DAT.

Another possible explanation could be the involvement of PKA and PKC. Activation or inhibition of PKC, PI3K, and members of the MAPK family can cause transport membrane redistribution, although specific kinases responsible for direct phosphorylation are still unknown. One report found that methylphenidate had no effect on PKC activator-induced down-regulation of DAT function (Gorentla & Vaughan, 2005), however results from a previous study showed that methylphenidate decreased PKA levels when given repeatedly for five days (Crawford et al., 1998). These results demonstrate that the mechanism behind the regulation of DAT by methylphenidate is still under debate and further investigation is needed to gain a clearer picture of how methylphenidate regulates DAT. In addition, Wagner et al., observed a significant increase in V_{max} in rats with a controlled cortical impact (CCI) that received a daily injection of methylphenidate (5 mg/kg) for 14 days, but there was no change in total tissue or membrane bound DAT expression compared to the CCI rats that received saline (Wagner et al., 2009). These findings demonstrate that methylphenidate has the ability to increase the V_{max} without altering total of surface DAT expression, which is consistent with the results of this current study. A more recent study examined the long-term effects on striatal DAT density of a 2-week methylphenidate treatment given to SHR rats and WKY rats (Roessner et al., 2010). Methylphenidate was given through drinking water and was adjusted to about 2 mg/kg/day based on daily monitoring of amounts consumed by the two rats per cage and their body weight. Methylphenidate significantly decreased [^{3}H]GBR binding in SHR and WKY models at post-natal day 90 when compared to controls. This study demonstrates that long-term administration of

methylphenidate reduced the striatal DAT density in both rat strains. However, this finding reveals that methylphenidate caused a decrease in the amount of DAT, but the effect on DAT function after *in vivo* administration of methylphenidate was not assessed. Due to a time constraint, the 14-day experiment on DAT trafficking could not be performed, however based on the results from the 7-day experiment, it is speculated that methylphenidate may cause a decrease in surface DAT in regards to DAT trafficking because the effect of methylphenidate on DAT function was no longer observed after 14 days. One study found that prolonged D3 receptor stimulation can cause a decrease in DAT function and a decrease in DAT cell surface expression (Zapata et al., 2007). In addition, a study has shown that methylphenidate interacts with the D3 receptors (Andersen et al., 2008), adding credence to the theorized results for the 14-day experiment.

This current work, to our knowledge, is the first to report the effect of acute and repeated *in vivo* administration of methylphenidate on DAT function. In summary, with DAT being a part of such a dynamic system, there is evidence that collectively suggests that multiple pathways exist to regulate neurotransmitter transport function (Samuel et al., 2005).

There have also been other examples where there was an increase in DAT function, but no change in DAT expression. For instance, one report found that nicotine increased V_{max} for [^{3}H]DA uptake, however no increase in cell surface DAT expression was seen (Middleton et al., 2007). In addition, subfractionation experiments revealed no difference in dopamine transporter levels in total and plasma membrane fractions. Furthermore, another study reported that insulin increased NET function without a change in transport cellular localization (Apparsundaram et al., 2001). Taken together, these results suggest that it is possible to have an increase in DAT function with no change in total DAT expression, which is what the present study found.

C. Potential Mechanism for the Methylphenidate-Induced Increase in VMAT2 Function

The results of the VMAT2 experiments with methylphenidate are consistent with previous results showing that a single 40 mg/kg s.c. injection of methylphenidate increased vesicular DA uptake (Fleckenstein et al., 1999; Sandoval et al., 2002). The latter study also revealed that D1 and D2 receptor activity may also by involved in the increase in VMAT2 function by methylphenidate, since pretreatment with SCH23390, a D1 receptor antagonist and eticlopride, a D2 receptor antagonist both, completely inhibited methylphenidate-induced increases in VMAT2 function. Furthermore, methylphenidate may indirectly act as a DA agonist (Wilens, 2008). This may also explain the increase of VMAT2 function because if methylphenidate acts as a DA agonist, it could activate both pre and post synaptic D2 DA receptors, thus indirectly causing an increase in VMAT2 function in order to remove the DA in the cytosol, which would increase the activation of the D2 DA receptors.

Previous reports have shown that methylphenidate (40 mg/kg) can increase VMAT2 function accessed 60 minutes after *in vivo* administration (Sandoval et al., 2002). Based on the present results, it is very likely that methylphenidate may be interacting with vesicles in the same way as its interactions with synaptosomes, since methylphenidate increased both VMAT2 and DAT function. Methylphenidate administration did not alter the K_m, which suggests that methylphenidate competes with dopamine at VMAT2 in a noncompetitive manner. The current results suggest that methylphenidate acts differently than lobeline and amphetamine, in reference to the effects observed on VMAT2 function. The observation that amphetamine and methylphenidate influence VMAT2 differently at the time point and doses used in this study maybe rather surprising since both drugs have been shown to target VMAT2 (Greydanus et al., 2007; Thanos et al., 2007). However, the data in these studies could be a result of the difference in their mechanisms of action. This could possibly explain our observations of an increase in DAT and VMAT2 function by methylphenidate and no effect by amphetamine. The data produced

from this dissertation research are the first to show that *in vivo* administration of methylphenidate increased DAT and VMAT2 function. A recent review on VMAT2 regulation suggest that the altered sensitivity to the locomotor activity effects of cocaine, amphetamine, and alcohol in VMAT2-knockout mice is tied closely to the expression of VMAT2 (Takahashi et al., 1997). This review also states that by gaining a better understanding of VMAT2 dynamics may open the door to improved pharmacotherapies used to treat psychiatric disorders and addiction. In addition, methylphenidate was shown to decrease the intracellular DAT expression, implying that methylphenidate does redistribute DAT. Initially, methylphenidate was thought to have a more direct and less complicated mechanism of action based on previous studies. However, the novel results from this research demonstrate that the mechanism of action of methylphenidate may be more complicated than previously thought.

D. Implications

The results of this dissertation work imply that methylphenidate interacts with DAT in a different manner than amphetamine and lobeline. Based on the current results showing that lobeline had no effect on DAT and VMAT2 function, lobeline does not appear to be a plausible treatment option for ADHD. The lack of effect on DAT and VMAT2 function observed with lobeline could be that the behavioral effect, which was the decreased self-administration of methamphetamine observed in the behavioral study, may involve an interaction with the α4β2 nAChR and not due to DAT or VMAT2 involvement. However, lobeline, an analogue of lobeline, causes this same behavioral effect and has little or no affinity for α4β2* or α7* nAChRs (Miller et al., 2004; Zheng et al., 2005). Therefore, the cause of the behavioral effect of lobeline requires additional research to determine what receptors and transporter proteins are involved in eliciting the behavioral effect of lobeline.

However, amphetamine, an effective treatment for ADHD, had no effect either. Therefore, additional information is needed to determine if lobeline is actually a potential treatment option for ADHD. Furthermore, Levin et al. showed lobeline enhanced learning on the post-acquisition working memory performance

in the radial-arm maze (Levin et al., 2003). In addition, lobeline has been found to improve retention performance 24 hours after injection in spatial discrimination water maze (Decker et al., 1993). Previous work has also demonstrated that lobeline may be an effective smoking cessation agent by showing that lobeline acts as a nicotinic antagonist by inhibiting nicotine-evoked DA release and nicotine-evoked $^{86}Rb^{+}$ efflux (Miller et al., 2000). Lobeline has been used as an over-the-counter smoking cessation agent in the past in the US (Nunn-Thompson & Simon, 1989; Prignot, 1989) and numerous human studies have been conducted to determine efficacy of lobeline as a smoking cessation agent, although its use is still debatable (Dwoskin & Crooks, 2002). This characteristic gives lobeline an additional advantage to the currently used ADHD treatments because it has been shown that there is a higher rate of smoking in people who have ADHD compared to those who do not (Milberger et al., 1997). Another benefit of lobeline is that it does not have typical stimulant side-effects such as abuse liability (Harrod et al., 2003).

In addition, this study unexpectedly found that amphetamine had no effect on DAT function. However, there have been behavioral reports that have demonstrated a difference in effect between methylphenidate and amphetamine. For example, a previous study discovered that 5 mg/kg of amphetamine produced more sniffing over a 2-hour observation period than rats receiving either saline or 30 mg/kg of methylphenidate (Roffman & Raskin, 1997). In addition, methylphenidate-treated rats exhibited significantly higher total gnawing than saline or amphetamine-treated rats. Moreover, another study found that amphetamine and methylphenidate had differential effects on enriched environmental condition (EC) and impoverished condition (IC) rats. Amphetamine increased impulsivity choice in EC rats, however methylphenidate had no significant effect in EC rats (Perry et al., 2008). The authors hypothesized that the differences in DAT function in mPFC may be associated with these results because EC rats show a decreased DAT function in mPFC, but not in striatum or nucleus accumbens, implying that EC rats could have higher levels of extracellular DA in the mPFC than IC rats (Zhu et al., 2004; Zhu et al., 2005).

Biochemical studies have also obtained similar results. For instance, one study found that methylphenidate may interact with a domain on DAT that is distinct from that of amphetamine due to their different affinities observed with mutant DAT (Dar et al., 2005). In addition, Schiffer et al., found that amphetamine increased extracellular DA more than methylphenidate using microdialysis, suggesting that the differences between these agents in modulating extracellular DA may be based on their differences in their molecular mechanisms, i.e., amphetamine also causes release of DA and methylphenidate does not (Schiffer et al., 2006). The differential effects of amphetamine and methylphenidate have been recorded in the clinical setting as well. Borcherding conducted a study with 45 hyperactive boys and found that subjects taking amphetamine had increased cleaning and checking behaviors similar to childhood-onset Obsessive-Compulsive Disorder (OCD). On the other hand, the subjects that were given methylphenidate displayed perfectionistic and detail-oriented behavior, as well as abnormal movements and compulsive behavior (Borcherding et al., 1990). Taken together, the results of the preclinical behavioral and biochemical studies, as well as the clinical findings, suggest that methylphenidate and amphetamine may interact at DAT in different manners to cause these differential effects.

This research has also led to clinical implications. Based on the preclinical data of lobeline, a clinical trial was conducted using lobeline. The clinical trial was a single small-N pilot study to determine the ability of lobeline to decrease ADHD symptoms in adults. The study was a double-blind, double-dummy, placebo-controlled, within-subject design. Inclusion criteria for the study included being healthy individuals between 21 and 45 yrs old, with childhood histories and current symptoms of ADHD (meet diagnostic criteria on the clinical interview and CAARS) and able to stop current medications for 7 days prior to the clinical trial. Females were either nonchildbearing (tubal ligation or total hysterectomy) or of childbearing potential using one or more of the following barrier methods of contraception: male or female condoms (with spermicide), diaphragm (with spermicide) and/or intrauterine device (with/without spermicide). No other

contraceptives were acceptable. Subjects must have a body mass index (BMI) between 18 and 30 and be willing and able to give written consent. Subjects must have no medical contraindications determined by the following: an adequate medical history, a physical examination including vital signs, 12-lead ECG, CBC with differential and liver functions and urinalysis and a negative drug test (barbiturates, benzodiazepines, amphetamines, opiates, cocaine, cannabinoids, ethanol) at screening and at the time of each admission to the GCRC and CO ≤ 8. Salivary cotinine was collected and assessed at baseline to determine whether or not the subject was using smokeless tobacco included being a non-smoker between the ages of 21-45, in good general health, no current drug use, and having a history of childhood ADHD symptoms and current ADHD symptoms. Medical and psychiatric evaluations were conducted. The medical evaluation consisted of a physical examination (conducted by the PI) and an EKG reviewed by an Internist/pediatrician (Dr. C. Feddock) with particular attention to arrhythmias, prolonged QT or heart block. Subjects provided a urine sample for drug screening, standard urinalysis and βHCG pregnancy tests (for female subjects). A blood sample was drawn for a complete blood count with differential and liver function tests. Subjects will undergo breath sample analysis on-site with an Alco-Sensor Intoximeter and an Innovative Medical CO Monitor. To qualify for the study, subjects must be non-smokers and CO must be ≤ 8 ppm.

The psychiatric assessment included a structured clinical interview based on the DSM-IV (SCID) supplemented with ADHD symptoms from the KSADS-E (Wilens et al, 2003). In addition, the Conners Adult Rating Scale (CAARS) will be completed. These measures assessed the presence of ADHD and degree of symptomatology. The Shipley Institute of Living Test was obtained to determine an estimate of intellectual functioning.

Exclusion criteria included regular use of drugs of abuse, current use of psychiatric medications other than short acting medications for ADHD, having an abnormal EKG or hypertension (blood pressure over 150/90 on two consecutive measures over 15 min when the subject is at rest), being pregnancy, having current medical difficulties or legal problems, regular use of prescription medicine

that cannot be discontinued and current use of nicotine and active suicidal behaviors.

The acute effects of LOB on indices of attention, impulsivity and working memory were the primary outcomes measures to be assessed; outcomes will be compared to results obtained with placebo and with the positive control comparator, methylphenidate, a medication with efficacy for this disorder. Secondary outcome measures will include physiological and subjective drug effect reports to provide additional information on safety, abuse liability and tolerability (e.g., appetite, sleep, side effects).

The first subject was consented on July 30, 2008 and the final subject consented on October 8, 2009. A total of 42 subjects were enrolled in the study and screened at intake (18 females and 24 males). Out of the 42 subjects, only 13 were randomized into the protocol because 29 did not meet the inclusion criteria. Some of the screening failures included elevated BMI, elevated scores on the Beck Depression Inventory, a CAARS score of >65, 2 positive drug screens, failure to meet the demands of the protocol (i.e., scheduling conflicts), and family history of early cardiac death. Out of the 13 subjects that were randomized 4 withdrew, leaving a total of 9 subjects that completed the study. In regards to demographics of the 13 subjects, 1 was an African American female, 5 were White females, and 7 were White males. The subjects that withdrew were the African American female, 1 White female, and 2 White males.

The results of the study showed that lobeline did not have a significant effect on reducing ADHD symptoms using the outcomes assessed in this trial. However, it was also difficult to observe a significant effect with methylphenidate, suggesting that the testing parameters may not have been most appropriate to use. In addition, various subjects complained of a bitter taste and a burning sensation with the administration of lobeline sublingual tablet. This adverse effect could have played a role in the lack of effect observed with lobeline, causing the subjects to become distracted by the bitter taste or burning sensation. Therefore, if the subjects were distracted, they might have performed worse on the tests used to assess for improved attention after administration of lobeline. To

determine if the adverse effects affected the results of the study, another study should be conducted with a different dosage form of lobeline, such as a transdermal patch to alleviate the adverse effect of the bitter taste and burning sensation. This additional study would confirm if lobeline is able to reduce ADHD symptoms and provide an overall perspective of the clinical implications of lobeline as a treatment option for ADHD.

E. Limitations

One limitation was the choice of using adult rats for the study rather than adolescent rats, since ADHD is known to be a childhood disorder. However, adult rats were used to obtain preliminary results as to how lobeline interacts with DAT and VMAT2 in normal adult rats. Just as clinical studies use healthy subjects to investigate the characteristics of an investigational drug, such as pharmacokinetics and side effects, we used normal adult rats to gain knowledge as to how lobeline interacts with DAT and VMAT2. In fact, using adults rats may not be an actual limitation since ADHD has been shown to persist into adulthood and more cases of adult ADHD are being diagnosed (American Psychiatric Association, 2000; de Graaf et al., 2008).

Another limitation was only examining one region of the brain. The striatum was initially chosen based on the support that DAT is highly expressed in the striatum (Ciliax et al., 1999). However, recent research focused on the PFC and reports that it is an essential region of the brain that governs and maintains attention (Arnsten et al., 2009). For example, improved PFC cognitive function, i.e., spatial working memory, in monkeys was observed with the optimal dose of methylphenidate and amphetamine (Gamo et al., 2010). This same study also stated that PFC function is vastly controlled by DA and NE. In addition, the authors found that the regulation of the PFC is altered in many ADHD patients. Based on this increasing amount of evidence, it is necessary to conduct studies in the PFC to gain a better understanding of the mechanisms of actions of lobeline, methylphenidate, and amphetamine in this brain region.

The use of individual doses for the chronic studies could be a limitation as well. The chronic doses were to be based on the effective acute doses. However,

amphetamine and lobeline did not have an effect in the actue experiments. Therefore, the doses chosen for the chronic studies for these compounds were based on behavioral studies. Since a complete dose-response was used for acute studies, it would be more beneficial to also conduct a complete dose-response with the chronic studies as well. This would ensure a more complete picture as to how the compounds interact with the various targets. Determining the dose-response relationship is imperative when studying different compounds and their effects on certain targets because it provides vital information as to how the dose affects the primary outcome measure. Therefore, to address this limitation it would be prudent to include a dose-response for the chronic studies as well.

F. Future Directions

The experiments completed in this dissertation examined the effects of acute and repeated *in vivo* administration of lobeline, methylphenidate, and amphetamine on DAT function in the striatum. Further studies are needed to investigate the effects of acute and repeated *in vivo* administration of lobeline, methylphenidate, and amphetamine on DAT function in the PFC since the PFC is also widely studied because of its involvement in the regulation of behavior, attention, affect, motor and cognitive control (Arnsten, 2006; Kieling et al., 2008). Numerous studies have considered the PFC as a region of interest in examining the pathophysiology of ADHD (Barkley et al., 1992; Goldman-Rakic, 1996; Robbins, 1996).

Due to the recent addition of atomoxetine, a selective NET inhibitor, as a treatment option for ADHD, it is important to determine the effects lobeline has on this transporter function as well. Impaired regulation of NE neurotransmission is suggested to contribute to ADHD (Beane & Marrocco, 2004). Studies have shown, using microdialysis that methylphenidate and amphetamine substantially increased NE and DA efflux within the PFC to enhance cognitive function (Berridge et al., 2006; Berridge & Stalnaker, 2002). Methylphenidate also has an affinity for NET (Andersen, 1989; Easton et al., 2007; Kuczenski & Segal, 1997; Richelson & Pfenning, 1984). One report even suggests that methylphenidate

has a higher affinity for NET than DAT (Eshleman et al., 1999). Moreover, a preclinical study found that methylphenidate-treated rats (0.5 mg/kg, ip) produced a maximal increase in NE levels significantly larger than the increase of DA in the PFC (Berridge et al., 2006). Furthermore, the first *in vivo* study in humans showed that clinically relevant doses of methylphenidate occupy significant levels of NET. The results from this study imply that the therapeutic effect of methylphenidate in ADHD may be modulated via NET inhibition as well as DAT (Hannestad et al., 2010). A recent review also stated that the majority of drugs shown to be effective in treating ADHD have important effects on NE transmission (Del Campo et al., 2011). Thus, there is overwhelming evidence that NET is an important target that needs to be investigated to determine the effect lobeline may have on it.

Another suggested future study involves investigating the effects of acute and repeated *in vivo* administration of lobeline, methylphenidate, and amphetamine on DAT and VMAT2 function in adolescent rats as well. Studies have indicated that age plays a major role in response to ADHD medications due to the fact that the adolescent brain is still undergoing development, which makes it more sensitive to the effects of these medications (Canese et al., 2009; Yang et al., 2003; Yang et al., 2006).

Furthermore, in order to validate the use of lobeline for the treatment of ADHD, it would be useful to determine if lobeline is effective in reducing the symptoms of ADHD found in an ADHD rat model. Based on the review of the literature, convergent evidence suggests that the SHR is one of the best animal ADHD models (Heal et al., 2008; McCarty et al., 1980; Sagvolden, 2000). Thus, it would be worthwhile to investigate the effect lobeline has on SHR rats and to compare its effect to the effects of methylphenidate and amphetamine.

In conclusion, this dissertation research has provided new insights into the mechanism of action of lobeline and how it interacts with DAT and VMAT2. This work also extended previous findings as to how methylphenidate interacts with DAT and VMAT2. Further studies are needed to gain a better understanding of the exact mechanism responsible for the increase in DAT function caused by

methylphenidate. Based on the current results showing that lobeline had no effect on DAT and VMAT2 function, lobeline does not appear to be a plausible treatment option for ADHD. However, amphetamine, an effective treatment for ADHD, had no effect either. Therefore, more experiments are warranted in order to elucidate the mechanism of lobeline and the other compounds. Ultimately, these additional studies may offer leads for developing related novel and more effective neuropharmacologic therapeutic agents for ADHD and other psychiatric disorders. Furthermore, we did find exciting new data that showed that acute and repeated methylphenidate increased DAT and VMAT2 function after *in vivo* administration. These data provide evidence that VMAT2 is also an important target worthy of further research for additional novel therapeutic compounds.

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VITA

YOLANDA D. WILLIAMS

PERSONAL INFORMATION
Date of Birth: December 18, 1972
Birthplace: Washington, DC

EDUCATION
University of Kentucky
Lexington, Kentucky
Clinical Pharmaceutical Sciences, 2003 - 2011

Hampton University,
Hampton, Virginia
Doctor of Pharmacy, May 2003

Spelman College,
Atlanta, Georgia
Bachelor of Science in Chemistry, December 1995

RESEARCH EXPERIENCE
University of Kentucky, Department of Clinical Pharmaceutical Sciences (2003-present)
Thesis Title: Preclinical Evaluation of Lobeline for the Treatment of ADHD: Comparison with Psychostimulant Therapies
Advisor: Dr. Linda Dwoskin

TEACHING EXPERIENCE

Teaching Assistant for the following courses:

Patient Care Laboratory V (PHR 959 (2006),

Patient Care Laboratory V (PHR 959, 966/967 (2005),

Patient Care Laboratory VI (PHR 969, 956/957 (2004),

Patient Care Laboratory III (PHR 939 (2003)

Responsibilities included distributing lecture materials, grading of quizzes and examinations offered in the course including case based questions and pharmacokinetic homework. Posting information and grades on Blackboard® course shell and excel sheets, leading small group discussions, demonstrating clinical techniques, and proctoring examinations.

WORK EXPERIENCE

Licensure: North Carolina (2003-present) and Kentucky (2003-2011)
Certification: Immunizations, March 18, 2006

***Part-time Pharmacist,* Target Pharmacy,** Louisville, Kentucky
February 2010-May 2011

***Part-time Pharmacist,* Rite Aid Pharmacy,** Lexington, Kentucky
April 2007-September 2009

***Part-time Pharmacist,* Toyota Family Pharmacy,** Georgetown, Kentucky
September 2007-December 2008

***Pfizer Intern,* Pfizer, Inc.,** La Jolla, CA
June-August 2007

***Part-time Pharmacist*, Kroger Pharmacy**, Lexington, Kentucky
February 2004- December 2004

***Pharmacy Intern,* Wal-Mart Pharmacy,** Newport News, Virginia
May 2002- June 2003

***Pharmacy Intern,* Eckerd Pharmacy,** Hampton, Virginia
September 2000- May 2002

***Pharmacy Intern*, Target Pharmacy,** Newport News, Virginia
May 2000

***Pharmacy Intern,* Big K-Mart**, Hampton, Virginia
October 1999- March 2000
Responsibilities included assisting pharmacist in prescription processing preparation, distribution, maintenance of physical inventory, performance of register operations and providing customer service to customers in the pharmacy and on the floor.

***Forensic Chemist*, Office of the Chief Medical Examiner,** Chapel Hill, North Carolina
September 1997- July 1999
Responsibilities included using a variety of wet laboratory techniques to extract drugs and other substances from biological specimens such as blood, urine, and liver to determine the cause of death.

GRANTS AND FELLOWSHIPS

NIH-funded Career Training in Therapeutics and Translational Research (K-30)Seed Grant Recipient, University of Kentucky (7/2006)
NIDA Training Grant (Training in Drug Abuse Related Research (5 T32 DA016176)) Recipient (2004-2006)
Petite Grant Recipient from the Center on Drug and Alcohol Research, University of Kentucky (2004)

PUBLICATIONS

Douglas YA, Guilford A, Bleidt B, Coleman CA, Jenkins TM. "Evaluation Of Blood Pressure on the Hampton University Campus," Clinical Research and Regulatory Affairs, Vol. 20(1):27-33, 2003.

Williams YD, Ansong MA, and Landers MW. "Forteo (Teriparatide)". Advance for Physicians Assistants. Vol. 11(9):18, 2003.

Bardo MT, **Williams Y**, Dwoskin LP, Moynahan SE, Perry IB, and Martin CA. "The Sensation Seeking Trait and Substance Use: Research Findings and Clinical Implications". Current Psychiatry Review, Vol. 3(1): 3-13, 2007.

Williams YD, Darna M, Dwoskin LP. Effect of acute and repeated in vivo administration of methylphenidate on DAT and VMAT2 function. (In progress)

ABSTRACTS

Williams, Y.D., Dwoskin, L.P.Effect of acute and repeated administration of lobeline, methylphenidate or amphetamine on dopamine and vesicular monoamine transporter function. University of Kentucky,Center for Clinical and Translational Science Spring Conference, Lexington, KY, April 23, 2009.

Williams, Y.D., Dwoskin, L.P.Effect of acute and repeated administration of lobeline, methylphenidate or amphetamine on dopamine and vesicular monoamine transporter function. 38th Annual Society for Neuroscience meeting,Washington, DC, November 17, 2008.

Williams, Y.D., Dwoskin, L.P.Effect of acute and repeated administration of lobeline, methylphenidate or amphetamine on dopamine and vesicular monoamine transporter function. Frontiers in Addiction Research: NIDA Mini-Convention, Washington, DC, November 14, 2008.

Williams, Y.D., Dwoskin, L.P.Effect of acute and repeated administration of lobeline, methylphenidate or amphetamine on dopamine and vesicular monoamine transporter function. University of Kentucky 2nd Annual Graduate Student Interdisciplinary Conference, Lexington, KY, March 28, 2008.

Williams, Y.D., Dwoskin, L.P.Effect of acute and repeated administration of lobeline, methylphenidate or amphetamine on dopamine and vesicular monoamine transporter function. Bluegrass Neuroscience Chapter, Spring Neuroscience Day, Lexington, KY, March 12, 2008.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline or methylphenidate on dopamine transporter function. 37th Annual Society for Neuroscience meeting, San Diego, CA, November 6, 2007.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline, methylphenidate or amphetamine on dopamine transporter function. Rho Chi Society Research Day, Lexington, KY, April 12, 2007. Received a 3rd place award.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline, methylphenidate or amphetamine on dopamine transporter function. Bluegrass Neuroscience Chapter, Spring Neuroscience Day, Lexington, KY, March 12, 2007.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline or methylphenidate on dopamine transporter function. Center for Clinical and Translational Science Spring Conference, Lexington, KY, June 6, 2006.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline or methylphenidate on dopamine transporter function. 67th Annual CPDD meeting, Orlando, FL, June 22, 2005.

Williams, Y.D., Dwoskin, L.P.Effect of acute and chronically administered lobeline or methylphenidate on dopamine transporter function. Bluegrass Neuroscience Chapter, Spring Neuroscience Day, Lexington, KY, March 15, 2005.

Douglas, Y., Guilford, A., Bleidt, B. Coleman, C., & Jenkins, T.Evaluation of Blood Pressure Measurements on The Hampton University Campus**.** 36th Annual American Society of Health-System Pharmacists (ASHP) Midyear Clinical Meeting, New Orleans, LA, December 1- 4, 2001.

Douglas, Y., Jenkins, T, MS, Pharm.D, & Bleidt, B. PhD., Pharm.D. Expanding the Possibilities: The Dr. James A. Ferguson Emerging Infectious Diseases Fellowship Program. 35th Annual American Society of Health-System Pharmacists (ASHP) Midyear Clinical Meeting, Las Vegas, NV, December 3-7, 2000.

Douglas, Y. In Vitro Testing of Candidate Microbicides for Toxicity and Antiviral Activity Against Human Immunodefiency Virus Type-1.Dr. James A. Ferguson Infectious Diseases Fellowship Program, Sponsored by the Minority Health Professions Foundation, Altanta, GA, July 28, 2000.

Douglas, Y,Gonzales, M., & George, A.How Pharmacists Can Eliminate Racial and Ethnic Disparities. The 22nd Annual Black Family Conference, Hampton University, Hampton, VA, March 15-17, 2000.

ORAL PRESENTATIONS

"**Effect of Acute and Repeated *in vivo* Administration of Lobeline, Methylphenidate, and Amphetamine on Striatal Dopamine Transporter and Vesicular Monoamine Transporter Function**". University of Kentucky, CDART Research Forum, Lexington, KY, February 16, 2009.

"**Effect of Acute and Repeated *in vivo* Administration of Lobeline, Methylphenidate, and Amphetamine on Striatal Dopamine Transporter and Vesicular Monoamine Transporter Function**". University of Kentucky, School of Pharmacy, Department of Pharmaceutical Sciences Seminar, KY, December 19, 2008.

"**Effect of Acute and Repeated *in vivo* Administration of Lobeline, Methylphenidate, and Amphetamine on Striatal Dopamine Transporter and Vesicular Monoamine Transporter Function**". University of Kentucky, CCTS Spring Conference Lexington, KY, June 3, 2008.

"**Effect of Acute and Chronically Administered Lobeline or Methylphenidate on Dopamine Transporter Function**". University of Kentucky, NIDA Training Grant Symposium. Lexington, KY, October 6, 2005.

"**Effect of Acute and Chronically Administered Lobeline or Methylphenidate on Dopamine Transporter Function**". University of Kentucky, School of Pharmacy, Department of Pharmaceutical Sciences. AGS Seminar. Lexington, KY, February 8, 2005.

"**Lobeline as a Potential Treatment for ADHD**". University of Kentucky, School of Pharmacy, Department of Pharmaceutical Sciences. AGS Seminar. Lexington, KY, April 20, 2004.

"Advances in the Treatment of Trachoma". Hampton University, School of Pharmacy, Hampton, VA, January 2003.

"Comparison of Cycle Control with a Combined Contraceptive Ring and Oral Levonorgestrel/Ethinyl Estradiol". Hampton University, School of Pharmacy, Hampton, VA, January 2003.

"Assessing the Cost-Effectiveness of Schizophrenia Treatment". Hampton University, School of Pharmacy, Hampton, VA, September 2002.

"Sertraline and Fluoxetine Treatment of Obsessive Compulsive Disorder: Results of a Double-Blind, 6-Month Trial."Journal Club Presentation. Hampton University, School of Pharmacy, Hampton, VA, September 2002.

"Gout" Hampton University, School of Pharmacy, Hampton, Virginia, August 2002.

"Comparison of Salmeterol and Formoterol in Patients with Severe Asthma" Journal Club Presentation. Hampton University, School of Pharmacy, Hampton, VA, July 2002.

"Top Drug Interactions" Virginia Beach Health Clinic, Virginia Beach, VA, July 2002.

"Nexium", Wal-Mart Pharmacy, Hampton, VA, July 2001. Wal-Mart Pharmacy

"GlucoWatch", Hampton University, Hampton, VA, June 2001.

"Crohn's Disease", DePaul Hospital, Norfolk, VA, May 2001.

HONORS AND AWARDS

Clinical Translational Science Program Scholar (2009)
University of Kentucky, Graduate Student Travel Award Recipient (11/2008)
Peter G. Glavinos Jr., Ph.D., Fall Travel Award Recipient (10/2008)
Frontiers in Addiction Research:2008 NIDA Mini-Convention Travel Award Recipient (4/2008)
Member, Alpha Xi Chapter of Rho Chi Honor Society, University of Kentucky (2008)
Poster Presentation for Rho Chi Research Day (Placed 3rd) (4/2007)
S. Elizabeth Helton Spring Graduate Student Travel Award Recipient (4/2007)
Pfizer Summer Intern in La Jolla, CA. (6/2007 thru 8/2007): 370 applicants 2 accepted
Lyman T. Johnson Award Recipient, University of Kentucky (2003-2006)
Research Challenge Trust Fund Scholar, University of Kentucky (2003, 2005, 2006)
Kappa Alpha Mu Honor Society (1999-2003)
Kellogg Scholarship Recipient (1999-2000, 2000-2001)
Kroger Scholarship Recipient (2001)
UNCF Rite Aid Scholarship Recipient (2000-2001, 2001-2002)

Yolanda D. Williams

October 25, 2011