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NEUROCHEMICAL STUDIES OF ATTENTION-DEFICIT/ HYPERACTIVITY DISORDER MEDICATIONS IN THE STRIATUM AND NUCLEUS ACCUMBENS OF THE FISCHER 344 RAT

Barry Matthew Joyce
University of Kentucky, mattjoyce@uky.edu

ABSTRACT OF DISSERTATION

Barry Matthew Joyce

The Graduate School

University of Kentucky

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ABSTRACT OF DISSERTATION

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

By
Barry Matthew Joyce

Lexington, Kentucky

Director: Dr. Greg Gerhardt, Professor of Anatomy and Neurobiology

Lexington, Kentucky

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NEUROCHEMICAL STUDIES OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER MEDICATIONS IN THE STRIATUM AND NUCLEUS ACCUMBENS OF THE FISCHER 344 RAT

Stimulant medications such as D-amphetamine, mixed-salts (75% D- and 25% L-) amphetamine; Adderall®, and methylphenidate are first-line treatments for Attention-Deficit/Hyperactivity Disorder (ADHD). *In vivo* studies have predominantly focused on these stimulants in the context of drug abuse, and their therapeutic mechanistic properties are only theoretical. Previously, *in vivo* techniques have been limited by poor temporal and spatial resolution, and characterizations of these medications in rodent models have not been possible at low, clinically relevant levels. In order to address these issues, our laboratory used *in vivo* high speed chronoamperometric microelectrodes to characterize the effects of local applications of D-amphetamine, L-amphetamine, D,L-amphetamine, and Adderall® at low levels in the striatum and nucleus accumbens of 3-6 month old, male Fischer 344 (F344) rats. Our results showed significant differences between the faster kinetics of dopamine (DA) release signals caused by D,L-amphetamine and the slower kinetics resulting from D-amphetamine. These data support that resulting DA concentrations evoked by D- and D,L-amphetamine are correlated with the amount of D-amphetamine in the drug and only the time courses of the signals are affected by L-amphetamine. Additionally, locally applied D- and L-amphetamine caused DA release signals with similar amplitudes or concentrations of evoked DA; however, the signals were significantly faster for L-amphetamine. Adderall® caused significantly greater DA release that lasted over a longer time course compared to DA release caused by D- or D,L-amphetamine. These data support the hypothesis that amphetamine isomers, alone or in combination, interact differently with the DA transporter (DAT) to subsequently cause reversal of transport of DA out of presynaptic membranes of DA neuronal projections. Finally, reverse microdialysis studies were carried out to assess low levels of D-amphetamine, Adderall® (75% D-, 25% L-amphetamine), methylphenidate, and a new mixed-salts amphetamine that we referred to as Reverse Adderall (75% L-, 25% D-

amphetamine) in the striatum of F344 rats. These data reveal a stimulant concentration-response curve for DA with double plateaus that may be explained by dual mechanisms of reverse transport of DA through the DAT. In addition, reverse microdialysis of methylphenidate caused DA overflow similar to the effects of the other stimulants.

KEYWORDS: ADHD, Amphetamine, Dopamine, Voltammetry, Microdialysis

Barry Matthew Joyce

November 29, 2006

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By

Barry Matthew Joyce

Greg A. Gerhardt, Ph.D.
Director of Dissertation

Jennifer K. Brueckner, Ph.D.
Director of Graduate Studies

November 29, 2006

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DISSERTATION

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The Graduate School
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In memory of my grandparents, Billie and Harold Alsup
For beginning this journey with me and believing that I would see it to completion

Dedicated to my wife Kimberly, my parents Barry, Jan, Mike, and Donna, my brother Christopher, my sister Gretchen, my brother-in-law Jon, my niece Kaitlyn, nephew Ethan, and my grandparents Walton and Elizabeth

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"For everyone to whom much is given, of him shall much be required." -- Luke 12:48

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Chapter 1: Introduction

Attention-Deficit/Hyperactivity Disorder

This dissertation describes work completed to investigate the neurochemical properties of medications prescribed for Attention-Deficit/Hyperactivity Disorder (ADHD). Concerns are often raised about the validity of such a disorder, due in part to the increasing use of stimulant medications in children over the last decade. However, a growing body of data exists regarding the neurobiological basis of ADHD symptoms with increasing evidence that the disorder is continual from childhood to adulthood. ADHD is not a unique disorder to the United States and has a cross-cultural prevalence in the estimated range of 3-17% with an estimated prevalence of 5-10% in elementary school age children (Lahey et al. 1999). Likewise, adult ADHD prevalence is accurately estimated to be 2-4% due to improved recognition and diagnosis in adults (Kessler et al. 2005).

Although stimulants used to treat ADHD are very safe medications with a long track record of clinical use, they are under increasing scrutiny due to some isolated rare cardiac deaths while using the medications. The news media have been buzzing about a proposed "black-box" warning for stimulant medications, which would alert physicians and patients that a drug carries rare but significant risks (Vendantam 2006). Proposals are under review to require the warning by the Food and Drug Administration on medications such as Ritalin®, Adderall®, and Dexedrine®, all discussed in the text of this dissertation. Fewer than 10% of prescription drugs carry such a warning (Vedantam 2006). As of 2004, sales of all ADHD drugs totaled $3.1 billion, and an estimated 2.5 million children and 1.5 million adults were using these medications (Vedantam 2006; Mathews 2006).

George Still first described the condition clinically termed now as ADHD in 1901 by noticing the symptoms of "over-activity, inattention, poor inhibitory volition, aggressiveness, defiance, resistance to discipline, lawlessness, spitefulness, and dishonesty" that were observed in over 20 children (Still 1902). In an early Diagnostic and Statistical Manual of Mental Disorders (1968) the

condition was given the name of Hyperkinetic Disorder of Childhood. Today, the current DSM-IV-TR (2000) classifies the disorder as ADHD with three distinct subtypes: 1) predominantly inattentive 2) predominantly hyperactive-impulsive and 3) combined. Each subtype requires the fulfillment of at least six of nine symptoms present prior to age seven. Symptoms must persist in two or more settings, and clearly demonstrate clinically significant impairment in social, academic, or occupation functioning (DSM-IV-TR, 2000).

ADHD Etiology

Many studies have investigated the etiology of ADHD including genetic, neuroimaging, and electrophysiological pursuits. Familial studies have found a greater risk for siblings and parents of children with ADHD (Faraone and Biederman 1994). The risk for the disorder was found to be higher in biological than adopted children of ADHD individuals (Morrison and Stewart 1973). Twin studies have been carried out to investigate heritability, shared environment, and non-shared environments. Conclusive evidence supported that deficient parenting and family adversity did not lead to the manifestation of ADHD (Levy et al. 1997; Goodman and Stevenson 1989). Finally, studies that investigated correlations with lower socioeconomic status, higher psychosocial adversity, and increased parental conflict found no direct links (Scahill et al. 1999; Biederman et al. 1995a, 1995b). Conclusive evidence points to common genetic vulnerability of probands and first-degree relatives.

To date, components of the dopaminergic system have been studied because of the consensus that dysregulation of dopaminergic neurotransmission is central to the disorder. The dopamine transporter (DAT) and the allelic variation in the DAT gene, DAT1, have been associated with ADHD and remain functionally unexplained (Cook et al. 1995). The dopamine (DA) D4 receptor (D_4R), having seven 48 base pair repeats in exon 3, is slightly less effective than other variants in the inhibition of the second messenger cAMP. Activated cAMP has been shown to impair function in the prefrontal cortex. (Asghari et al. 1995).

DAT1 and D_4R genes are modestly accepted to be associated with susceptibility to ADHD.

Neuroimaging studies have investigated the anatomical substrates of ADHD and support dysfunction and dysregulation in multiple brain regions. Data support anomalies in cerebellar-striatal/adrenergic-prefrontal circuitry which can be improved by use of stimulant medications (Castellanos et al. 1996; Solanto et al. 2001). It is widely accepted that dysfunction within this distributed circuit underlies the symptomatology of ADHD. These circuits specifically include multiple brain regions such as right prefrontal cortex, the caudate nucleus, globus pallidus, and cerebellar vermis, all reported to have reduced volumes in ADHD individuals consistent with the possibility of hypofunctionality of normal brain function (Castellanos et al. 1996). However, these findings are given with caution due to normal variability in anatomical brain measures and the inclusion of treated and untreated individuals (Solanto et al. 2001).

Dopamine Function in the Normal Mammalian Central Nervous System

An extensive review of DA neurotransmission, including interactions with other neurotransmitters, is beyond the scope of this thesis; however, the following outline provides a general explanation of the system we investigated. In the next section, the effects of stimulants will be described in the context of DA neurotransmission. DA synthesis is initiated in the soma of DA neurons by the precursor, L-tyrosine, being converted to 3,4-dihydroxyphenylalanine (DOPA) via the enzyme tyrosine hydroxylase (TH) (Cooper et al. 1996; Seiden and Dykstra 1977). The decarboxylation of DOPA into DA by L-aromatic amino acid decarboxylase is the final synthetic step in neurons that use DA as their primary neurotransmitter (Fig. 1.1). TH activity is determined via end-product inhibition and phosphorylation (Cooper et al. 1996). During times of increased need for DA neuronal release, new TH protein can be synthesized or TH activity will be increased to meet the demands of the releasing neuron. TH is described to be

the rate limiting step in the synthesis of DA based on known saturation levels of TH (Squire et al. 2003)(Fig 1.1).

The majority of cell bodies that synthesize DA in the brain lie in the zona compacta of the substantia nigra (SN) and project via the nigrostriatal pathway to the striatum, including the caudate nucleus, putamen, and the amygdala (Solanto et al. 2001; Cooper et al. 1996). The mesolimbic pathway originates from DA synthesis in the cell bodies located in the ventral medial tegmentum. These DA projections run lateral to the nigrostriatal pathway and innervate the nucleus accumbens, olfactory tubercle, and selective regions of the prefrontal cortex forming the mesocortical pathway. Projections exist as well from the midbrain to the anterior cingulated cortex (mesocortical pathway), and finally the tuberoinfundibular tract that forms from the arcuate nucleus projecting to the pituitary gland (Dahlstrom and Fuxe 1964; Lindvall and Bjorklund 1974; Seiden et al. 1993). DA levels are highest in the striatum (10 μg/g), nucleus accumbens (5 μg/g), olfactory tubercle (6 μg/g), but much less in the cortex (0.1 μg/g) (Cooper et al. 1996).

Following synthesis, DA is released through two mechanisms: impulse dependent release and transporter-mediated release. Cytoplasmic DA is packaged into the vesicles via the Mg^{2+}-dependent vesicular monoamine transporter, VMAT2, predominantly found in brain catecholamine neurons (Solanto et al. 2001; Cooper et al. 1996). Vesicles concentrate near the presynaptic terminal where they remain primed for cellular membrane fusion and subsequent exocytosis thereby releasing the stored DA into the synaptic cleft. DA release from vesicles predominantly occurs via a Ca^{2+}-dependent exocytotic process whereby Ca^{2+} influx leads to ATP hydrolysis and depolarization of the membrane (Winkler 1988; Seiden and Sabol 1993). Transporter-mediated release of DA occurs mainly as a result of pharmacologic manipulation of the DAT in the presence of uptake inhibitors such as amphetamine (Blaszkowski and Bogdanski 1972; Paton 1973) (Fig 1.2).

Following release of DA, multiple synaptic targets determine the downstream effects on future DA release. For example, DA stimulates

presynaptic DA receptors (autoreceptors) that exist on most parts of the DA neuron and are a part of the D2 receptor family (Civelli et al. 1993; Cooper et al. 1996; Squire et al. 2003). The D2 family contains D_2, D_3, and D_4 subtypes of receptors, with DA receptor 2 (D_2R) primarily found as an autoreceptor that regulates future DA synthesis and release. Release-modulating autoreceptors function to provide feedback inhibition of further transmitter release and can be found on neurons that release other neurotransmitters. Synthesis-modulating autoreceptors are suggested to regulate DA synthesis based on increases in DA synthesis in the presence of DA receptor antagonists (Squire et al. 2003). The D_2Rs are localized to the striatum, nucleus accumbens, olfactory tubercle, and neuron cell bodies in the substantia nigra and ventral tegmental area. They most often serve as autorceptors that inhibit future DA synthesis and release. D_3 receptors are mainly found in the nucleus accumbens, olfactory tubercle, and hypothalamus. They are sparsely found in the caudate, cortex and DA neurons originating in the substantia nigra. These receptors, primarily autoreceptors, are less abundant than D_2Rs and are thought to inhibit Ca^{2+} entry into the presynaptic cell. D_4Rs are predominantly located in the frontal cortex, midbrain, and amygdala, are less in number than D_2Rs, and are proposed to be highly variable in humans. Impulse-modulating autoreceptors located to the soma and dendrites of DA neurons regulate overall cellular firing rate (Cooper et al. 1996).

The D1 type DA receptor family classifies two DA receptor subtypes, D_1 and D_5. The D_1 receptors are most highly concentrated in the striatum and nucleus accumbens and typically lead to the stimulation of adenylate cyclase and increase IP_3 turnover. D_5 receptors are most often found in the hippocampus and hypothalamus, but can be found in the striatum and nucleus accumbens and have been shown to stimulate adenylate cyclase (Cooper et al. 1996).

The termination of DA neurotransmission is accomplished via three distinct mechanisms. The first is diffusion away from a receptor followed by dilution in extracellular (EC) fluid to subphysiological levels. Second, enzymes can inactivate EC and intracellular (IC) DA such as monoamine oxidase (MAO) and catechol-O-methyl-transferase (COMT). Finally, the NaCl-dependent

neuronal DAT, having 12 transmembrane domains, can accumulate both IC DA and NE. DAT function is Na^+- and temperature-dependent and requires 2 Na^+ ions and 1 Cl^- ion for activation (Harris and Baldessarini 1973; Holz and Coyle 1974; Krueger 1990).

Stimulant Treatments of ADHD

Amphetamine Isomers

In 1887, synthetic amphetamine was invented by Lazar Edeleano, a student at the University of Berlin studying under A. W. Hofmann (Edeleano 1887). It received this generic name from a contraction of α-methyl-phenethylamine and today boasts greater than 20 trade names as described by the Merck Index listing (Sulzer et al. 2005). Amphetamine became available commercially in 1932 as Benzedrine® (50% D-, 50% L- amphetamine), available primarily in inhaler form for treatment of narcolepsy. Over time, Benzedrine® was made available in tablet form as an over-the-counter medication (Prinzmetal and Bloomberg 1935). Within the first three years of availability, greater than 50 million Benzedrine® tablets were sold (Sulzer et al. 2005). In the 1970s, 10 billion tablets were produced annually leading to the imposition of legal quotas brought forth by the United States Justice Department (Sulzer et al. 2005). Amphetamine has been administered in all major United States wars and military conflicts to promote alertness among the troops, in particular the air forces for flights and bombing missions lasting many hours (Caldwell et al. 2003). While amphetamine derivatives have more than 30 known uses, its use as a treatment for ADHD surpasses them all. The National Institute of Mental Health (NIMH) estimates an occurrence of ADHD in 3-5% of the population; and two million prescriptions are written per month for stimulant medications to treat ADHD (Vendantam 2006).

In general, it is believed that amphetamine interacts with the uptake process of released catecholamines. Amphetamine is thought to mimic DA and

is taken up into the synaptic terminal via the DAT in a competitive relationship with DA. Amphetamine then interferes with the normal uptake process of DA and causes a reversal of normal DAT function (Kuczenski 1983; Reith et al. 1986; Heikkila et al. 1975). Catecholamine release is thought to be independent of spike activity supported by amphetamine-induced release of DA that was inhibited by drugs that interfere with catecholamine synthesis such as α-methyl-para-tyrosine (Solanto et al. 2001). Likewise, the majority of released catecholamines in the presence of amphetamine undergo reverse transport through the DAT rather than release after Ca^{2+}-dependent spike activity (Cooper et al. 1996). Amphetamine will also accumulate catecholamines released due to spike activity as the drug is thought to block normal reuptake of DAT or the norepinephrine transporter (NET) (Solanto et al. 2001) (Fig 1.2).

The following effects of amphetamine have been described at the monoaminergic synapse: a) inhibition of monoamine oxidase (MAO), b) blockade of reuptake by the DAT, and c) promotion of monoamine release into the synaptic cleft (Seiden et al. 1993) (Fig. 1.2). After amphetamine gains presynaptic access by way of the monoamine transporter, it is thought to cause direct inhibition of IC vesicular monoamine transporters (VMAT) that package DA into vesicles. This leads to an increase in IC levels of monoamine neurotransmitters which cause reverse transport of the amines via cell membrane transporters (Sulzer et al. 1993,1995). Increases in synaptic and EC DA result in a dose-dependent manner in the presence of amphetamine (Solanto et al. 2001). Amphetamine directly inhibits MAO, the enzyme that metabolizes DA into 3,4-dihydroxy-O-phenylacetic acid (DOPAC) and homovanillic acid (HVA). This leads to decreased formation of the oxidized metabolites of DA, NE, and (serotonin) 5-HT. This occurs even at low doses leading to an increase in DA release and a decrease in DOPAC production (Bowers and Hoffman 1984; Elchisak et al. 1976; Imperato and Di Chiara 1984; Karoum et al. 1994; Zetterstrom et al. 1988, 1983). There are no known interactions between amphetamine and COMT, another enzyme that metabolizes DA into 3-methoxytyramine (3-MT). After the increases in EC and synaptic DA, elevated 3-

MT levels result (Karoum et al. 1994). The physiological effect of increased levels of EC DA from nigrostriatal DA neurons is feedback inhibition of DA neuronal firing via stimulation of autoreceptors and second messenger systems decreasing phasic DA release (Solanto et al. 2001) (Fig 1.2).

Studies have been done to investigate the cellular targets of amphetamine and most data support interactions of amphetamine with catecholamine transporters. Combinations of reserpine and amphetamine have been used to evaluate the role of vesicles in causing the amphetamine-evoked release of DA. The VMAT inhibitor, reserpine, was administered prior to amphetamine yielding mixed results; some *in vivo* experiments supported no effect on DA release and others reported reserpine blockade (Arbuthnott et al. 1990, Parker and Cebeddu 1986, Sabol et al. 1993). Some evidence support that vesicular stores of DA contributed to increased synaptic DA levels after amphetamine administration due to data indicating that TH is upregulated in the presence of reserpine or amphetamine. It is also likely that most cellular DA is vesicular and must be released to concentrate the cytoplasm in preparation for reverse transport through the DAT (Fon et al. 1997; Mosharov et al. 2003). Cellular studies have linked the roles of the DAT and the VMAT in causing amphetamine-induced DA release. COS cells that expressed both VMATs and/or DATs were exposed to amphetamine. These studies indicated that amphetamine caused DA release in cells with the DAT, but significantly more release occurred in the presence of cells containing VMAT or both VMAT/DAT. Finally, these data supported that no DA release occurred from cells without VMAT or DAT (Pifl et al. 1995). Jones et al. (1998) stimulated DA terminals to cause a stable baseline of DA release after which amphetamine was added and a subsequent increase in DA resulted. These data support that vesicular storage was altered in the presence of amphetamine. Discussion is often raised due to information that supports *de novo* synthesis of DA in the presence of amphetamine caused by upregulation of TH activity (Kuczenski 1975). Other data indicate an interaction between MAO and amphetamine that would decrease DA metabolite levels such as DOPAC.

The DAT functions normally to maintain cytosolic DA, likely dependent on a gating mechanism (Jardetzky 1966). While a number of theoretical explanations exist, prevailing ideas propose an asymmetric operation of the DAT to more efficiently take up EC DA in efforts to concentrate cytosolic DA. Alternatively, gradients of ions coupled with substrate and membrane potential may cause the DAT to favor DA uptake (Sulzer et al. 2005). Interference of these mechanisms by amphetamine leads to reverse transport of DA through the DAT. Previous literature discussed facilitated exchange diffusion as the predominant model of amphetamine-induced reverse transport of the DAT based on structural properties and their inclination to cause asymmetric substrate flux in a one-for-one exchange pattern (Sulzer et al. 2005). Facilitated exchange diffusion was first studied in other systems looking at glucose transport and other molecules (Stein 1967); however, more recent investigations have directly attempted to measure the exchange properties of the DAT in the presence of various substrates. Jones et al. (1999) used reserpine-like compounds to displace vesicular DA stores leading to increased levels of DA in the cytosol. Increasing cytosolic levels of DA alone was not enough to cause efflux of DA, and DAT uptake of amphetamine was necessary for reverse transport of the DAT. However, some reports have noted inconsistencies and support DA release independent of amphetamine uptake. For example, amphetamine injected into large dopamine neurons of the pond snail caused reverse transport of DA without transport of amphetamine (Sulzer et al. 1995). Therefore, other conformational states of the DAT have been noted that would account for all sources of amphetamine-dependent DA efflux. Patch-clamp recordings of cells expressing the DAT have supported rapid, relatively increased current events that indicate ion channel-like conductance of DA (Galli et al. 1996; Galli et al. 1998). A channel-like state of the DAT could allow a greater net flux of DA that exceeds the amounts of DA released via reverse transport. Recently, recordings from dopamine neurons and cells transfected with DATs supported channel-like events that caused relatively transient (milliseconds), robust release of DA molecules (Kahlig et al. 2005).

Dextroamphetamine sulfate is commercially available for ADHD treatment and has compared favorably with stimulants such as methylphenidate (Pelham et al. 1990; Elia et al. 1993). Racemic amphetamine, the first available treatment for children with behavioral disorders, is no longer commercially available. However, Adderall® (75% D- 25% L-amphetamine) is a leading prescription stimulant for children with ADHD. Data have supported that both enantiomers can be more effective in treating ADHD than the other, dependent on different subtypes of ADHD patients (Arnold et al. 1972, 1976). Other data support that the dextro- isomer caused greater presynaptic release of DA than the levo- isomer (Taylor and Snyder 1970, 1971; Arnold et al. 1972, 1973, 1976; Phillips and Fibiger 1973; Segal 1975) while the levo- isomer has shown greater effects on behavioral measures in rats in comparison to D-amphetamine (Segal 1975). Further discussion of amphetamine enantiomer-dependent effects will occur in Chapters 3, 4, and 5.

Methylphenidate

From the mid 1980's until now, methylphenidate (Ritalin®) topped market sales as one of the most prescribed stimulants for ADHD pharmacotherapy (Patrick and Markowitz 1997). Due to increased incidence of side effects with D-amphetamine and a dosing schedule that corresponded with school hours, methylphenidate became a mainstay for ADHD treatment. The use of methylphenidate increased five-fold in the early 1990s due in part to clinical diagnostic guideline revisions but not without growing concerns regarding its recreational abuse (Diller 1996).

The majority of methylphenidate used clinically contains a racemic form of 50:50 *threo*-R,R(+)- and *threo*-S,S(-)-isomers, and data support that the catecholaminergic effects of racemic methylphenidate are attributed to the active *threo*-R,R (+)-stereoisomer (Patrick et al. 1987; Srinivas et al. 1992). Studies to assess the necessity for the costly removal of the (-) isomer have provided data to support that both the therapeutic and adverse side effects are attributable to

the active (+)-isomer. (-)Methylphenidate has been found to penetrate the brain following systemic metabolism and may interact with ethanol to form the metabolite ethylphenidate (Patrick et al. 2005). While ethylphenidate is known to be active in the CNS, its pharmacological effects are uncertain (Markowitz et al. 2000). Due in part to this and other factors, a new separation of the methylphenidate isomers has recently been approved in an immediate release formulation and can be administered at one-half the dose of the racemic mixture (Patrick et al. 2005).

The attributed decrease of ADHD symptoms when using methylphenidate appears to be dependent on the facilitation of catecholaminergic neurotransmission similar in some ways to amphetamine. Methylphenidate binds selectively to the DAT or NET with high affinity (Schweri et al. 1985; Gatley et al. 1996) and blocks the synaptic clearance of impulse-released DA and NE leading to EC neurotransmitter accumulation (Fig. 1.2). In a study of the striatum in baboons, methylphenidate was shown to bind to the DAT, and (+)-isomer caused the most potent DA accumulation in the rat striatum (Ding et al. 1994; Aoyama 1994, 1996). Similarities to cocaine have been noted as methylphenidate competes with cocaine for DAT binding in the striatum to accumulate EC DA (Volkow et al. 1995; Gatley et al. 1996).

Methylphenidate is primarily thought to interrupt normal DA function by blocking the DAT from uptake of impulse-dependent release of DA differing from the effects of amphetamine that have been shown to trigger DA release through the DAT (Fig 1.2). Studies were done to determine the source of accumulating DA after methylphenidate. Reserpine, an agent that disrupts vesicular membranes, attenuated DA release following treatment with methylphenidate (Braestrup 1977; Patrick and Markowitz 1997). Reduction of a newly-synthesized pool of DA via inhibition with α-para-methyl-tyrosine decreased DA release caused by amphetamine but not methylphenidate (Patrick and Markowitz 1997). Taken together, most of the literature support postsynaptic dopaminergic agonistic activity as a key component of eliciting a response to methylphenidate.

The Dopamine Transporter: Regulation of Stimulant Effects

Although some debate exists, immunohistochemical studies of the DAT have supported that this catecholamine transporter protein can exist outside of the synapse (Nirenberg et al. 1996, 1997a, 1997b; Hoffman et al. 1998; Squire et al. 2003; Cragg and Rice 2004). Conclusions from these studies have discussed that the predominant role of the DAT is to accumulate DA which has diffused from the synaptic cleft. Additionally, studies of DAT knockout mice have supported decreased DA clearance and a complete loss of autoreceptor-mediated tone (Jones et al. 1999). Without the DAT, intraneuronal DA levels are decreased leading to attenuated feedback inhibition of TH and subsequent synthesis and release of DA (Gainetdinov et al. 1998).

The stimulants that we tested and their effects in the striatum are primarily regulated by the DAT. Amphetamine analogues: methylphenidate and cocaine are thought to block the DAT to increase synaptic and extracellular levels of DA. The DAT constitutively cycles from the cell membrane to the intracellular milieu where it is ultimately degraded or undergoes recycling and returns to the cell membrane (Daniels and Amara 1999; Loder and Melikian 2003). In addition, recent evidence has supported substrate induced effects on DAT function and/or trafficking (Melikian and Buckley 1999; Fleckenstein et al. 1999; Kahlig et al. 2005; Gulley and Zahniser 2003; Saunders et al. 2000; Johnson et al. 2005; Little et al. 2002; Daws et al. 2002; Cass et al. 1993a). Pharmacological interactions with DA release, DAT function, and/or trafficking are theoretically believed to be the underlying basis for addictive properties and likely therapeutic activity. Table 1.1 provides a summary of the effects of DAT substrates on subsequent DAT function and availability after acute and chronic exposure.

Pharmacological interactions of stimulants and the DAT have been shown to be substrate dependent. The endogenous substrate for the DAT, DA, has been shown to cause changes in surface expression and/or DAT function. Data have supported decreased DAT clearance abilities after repeated, rapid DA applications (Gulley et al. 2002) and increased localization of the DAT to the

cytosol (Saunders et al. 2000). Finally, DA has been shown to indirectly cause upregulation of surface DAT by stimulating D_2R autoreceptors that upregulate DA clearance (Parsons et al. 1993; Cass and Gerhardt 1994; Rothblat and Schneider 1997; Dickinson 1999; Hoffman et al. 1999; Mayfield and Zahniser 2001). Acute amphetamine administration caused a reduction of transport capacity thought to be due to intracellular DAT trafficking (Fleckenstein et al. 1999; Saunders et al. 2000); however, some reports support that amphetamine initially caused upregulation to the cell surface prior to causing internalization (Johnson et al. 2005). Other studies support substrate-dependent differences in DAT conformation that differentially allow DA to pass through via formation of a pore versus the constitutive state, an alternating access gate (Kahlig et al. 2005). Alternately, drugs thought to predominantly inhibit reuptake of DA by the DAT, such as cocaine and methylphenidate, have opposite effects on DAT levels and upregulation of the DAT to the cell surface has been supported (Daws et al. 2002; Little et al. 2002). Additionally, studies of DAT surface expression have supported inconsistencies between the effects of chronic and acute exposure of amphetamine isomers, methylphenidate, and cocaine. For example, while acute cocaine exposure caused upregulation of DATs to the cell surface, chronic cocaine has been shown to cause decreased levels of functional plasma membrane DATs (Benmansour et al. 1992; Cass et al. 1993a; Jones et al. 1996; Fleckenstein et al. 1999).

We hypothesized that differential effects of the stimulants we tested could be attributed to differential regulation of the DAT caused by the amounts of DA released and/or interactions with the DAT. The methodologies that we employed for our studies are predominantly thought to provide a means for *in vivo* monitoring of the function of cellular machinery involved in DA release and uptake such as the DAT. The DA release signals we present in Chapters 3 and 4 represent the effects of amphetamine isomers as they cause reverse transport of DA release (measures of rise time) and then blockade and/or down regulation of DAT function and location (measures of 80% decay) (See Chapter 2 Figure 2.1).

Rationale of Specific Aims

Upon initial review of the body of literature describing differences between D- and L- amphetamine, it is evident that most clinical and scientific studies were carried out in the 1970s. These studies were inconclusive and, in some cases, the extension was made that either enantiomer could be helpful in decreasing ADHD symptoms. Since the 1970s, a majority of the literature has focused on methylphenidate in terms of its clinical use, and amphetamine in terms of abuse. Due to the historical nature of safety and efficacy of amphetamine, it has largely been ignored in terms of mechanistic interactions with catecholaminergic signaling. As the number of stimulants prescribed per month continues to increase, it is important to build upon the data collected over two decades ago using sensitive techniques with high resolution to examine target effects and more efficient ways of causing a clinical effect. Additionally, the neurochemical effects of the D- and L-amphetamine isomers have remained difficult to identify and replicate (Popper 1994).

The dextro- isomer has been estimated to be at least two times more potent *in vivo* in causing stereotypies in rats than the levo- isomer (Taylor and Snyder 1970). Harris and Baldessarini (1973) present data that supported a four-fold difference in potency of D-amphetamine versus L-amphetamine as measured using *in vitro* DA uptake studies. Sprague-Dawley rats trained to discriminate between levers in response to drug-induced physiological states, self-administered both amphetamine enantiomers in two different studies (Jones et al. 1974; Yokel and Pickens 1973). However, data from these studies support that it took greater amounts of L-amphetamine to equal the behavioral responses induced by a smaller dose of D-amphetamine. Studies completed in mice assessing L-amphetamine and D-amphetamine potentiation of locomotor activity support increased potency of D-amphetamine (Stromberg and Svensson 1975). Additionally, the L-amphetamine dose-response curve displayed biphasic properties that support stimulation of locomotor activity at high doses while

depression of activity occurred at low doses (Stromberg and Svensson 1975). The most recent study conducted investigated differences in D- and L-derivatives and their effects on a canine model of narcolepsy (Kanbayashi et al. 2000). The data from the Kanbayashi et al. (2000) study indicate that D-amphetamine was two times more potent in increasing wakefulness, while the enantiomers were similar in effects on rapid eye movement. Microdialysis studies revealed similarities in the overall effect of maximum DA overflow with L-amphetamine, reaching a DA overflow plateau over a longer time period (Kanbayashi 2000). Finally, the distribution kinetics of D- and L- amphetamine were studied after intravenous administration of racemic amphetamine to Sprague-Dawley rats and these data support similar isomer terminal half-lives (Hutchaleelaha et al. 1994).

Clinical studies have provided evidence that D-amphetamine was superior to racemic amphetamine in terms of improvement in ratings of target symptoms (Gross 1976). Smith and Davis (1977) suggested that L-amphetamine was only half as potent as D-amphetamine in causing feelings of euphoria. Taken together, these data support enantiomer-dependent differences in molecular, behavioral, and clinical effects. Conversely, other studies support similarities between isomers of amphetamine. Arnold et al. (1972) administered L-amphetamine and D-amphetamine to hyperkinetic children (the clinical diagnosis for ADHD at the time) and determined that both were significantly more effective than placebo in influencing parent-teacher ratings. Data from this study indicate that D- and L-amphetamine targeted symptoms of hyperactivity and aggressiveness, while D-amphetamine also decreased inattentiveness (Arnold et al. 1972). Arnold et al. (1976) suggested in a later study that L-amphetamine was a useful drug option for treatment of minimal brain dysfunction (the clinical diagnosis for ADHD at that time), and the behavioral outcomes of D- and L-isomers were similar. Prior to these studies, a clinical study assessed and found no clinical differences between Benzedrine® (50:50 D,L- amphetamine) and Dexedrine® (D-amphetamine) (Bradley 1950). These clinical studies, conducted to examine stimulant medications with different amphetamine isomers, have

provided information to support the use of drugs containing L-amphetamine while others have provided equivocal results. These data were collected at a time when the symptoms of the disorder were not well characterized. Likewise, participant exclusion/inclusion criteria were not always similar, and the number of participants included for statistical analysis was not always optimal.

Based on the rise and fall of treatment alternatives for ADHD, the market has seen racemic amphetamine (Benzedrine®) come and go, replaced by a separated D-amphetamine (Dexedrine®), paralleled in the most recent years with the return of a mixture containing both enantiomers (Adderall®, mixed salts amphetamine). Even though Adderall® currently leads the ADHD prescription market, no studies have been published addressing the components and the chosen ratio of D- and L- amphetamine. Originally marketed to be longer-lasting and the only ADHD drug that could eliminate the need for "in-school" dosing, Adderall® maintains a sparse collection of data that would support or reject this hypothetical conjecture (Popper 1994). Unpublished data from the Richwood Pharmaceutical Company (1997) supported a differential rate of absorption and an associated increased efficacy due the activity of the four Adderall® salts not found in other stimulants. While no animal studies exist, a few clinical studies sponsored by Shire Pharmaceuticals have addressed the claims of Adderall's® longer lasting activity. An initial study by Swanson et al. (1998) found data to support rapid improvements in teacher ratings that occurred within 1.5 hours after administration that lasted throughout the day. The peak time of effects and duration of action increased dependent on dose (Swanson et al. 1998). In a comparison of Adderall® versus Ritalin® (methylphenidate), Adderall® continued to improve measures taken at time points of the day when the effects of a single dose of Ritalin had dissipated (Pelham et al. 1999). This study collected data that consistently supported the 1:2 dosing ratio between D-amphetamine and methylphenidate, and suggested that Adderall® is at least twice as potent in acutely improving the behavior and academic productivity of children with ADHD (Pelham et al. 1999). An independent study of high relevance to the data we present is a recent clinical study that compared Adderall® versus extended-

release and immediate-release D-amphetamine sulfate in a double-blind, placebo-controlled paradigm. James et al. (2001) took dependent measures of classroom behavior, recreational activity, parent observation, locomotor activity, and adverse effects. These data revealed similarities between immediate-release D-amphetamine and Adderall® while extended-release D-amphetamine displayed more sustained effects on most measures. Specifically, Adderall® significantly reduced locomotor activity relative to D-amphetamine (immediate- and extended-release) with effects that lasted longer than the other drugs. Weight loss occurred with all three drugs tested in this study; however, Adderall® did not cause decreased sleep duration (James et al. 2001).

Basic science studies with increased temporal and spatial resolution, and techniques with high sensitivity are necessary to further examine the properties of amphetamine enantiomers *in vivo*. The studies completed for this dissertation are the first studies to characterize the effects of locally applied, clinically relevant, commercially available stimulants and their components on DA neurotransmission. These studies made use of local drug applications to eliminate drug pharmacokinetic issues from the study. Due to this, drugs were applied in low levels to approximate clinically relevant levels of ADHD medications based on theoretical tissue dilution (voltammetry) and percent recovery (microdialysis) (Gerhardt and Palmer 1987; Shader et al. 1999; Solanto et al. 2001). At this date, there are no *in vivo* animal studies of Adderall® regarding measures of neurotransmitters or behavior. In general, we hypothesize that amphetamine analogs differ in their ability to cause DA release and differentially alter DA uptake. This hypothesis was studied using the following specific aims:

Specific Aim 1: Is it possible to reliably measure DA release signals evoked by amphetamine isomers at maximally effective concentrations related to therapeutic levels using high speed chronoamperometry? Do locally applied amphetamine isomers differentially affect DA release signals as measured by *in vivo* voltammetry? Since amphetamine isomer associated differences have been

noted in the clinic, it is possible that *in vivo* differences will be visible in DA neurotransmission in the striatum and nucleus accumbens of the rat brain, regions implicated in human studies of ADHD.

Specific Aim 2: Adderall® has been noted by clinicians to be a long-acting stimulant treatment for ADHD and data have supported that Adderall® decreases symptoms of hyperactivity over a longer time period than D-amphetamine. Therefore, do local applications of Adderall® cause differential effects on DA release signals compared to D-amphetamine at lower, clinically relevant levels of drug?

Specific Aim 3: Using techniques (microdialysis and HPLC) that allow for increased sensitivity to measure lower levels of analytes *in vivo*, will comparisons of stimulant concentration-response curves support differences in efficacy and potency?

Summary of Experiments

Single isomer compounds such as dextroamphetamine (Dexedrine), dexmethylphenidate (Focalin®), and racemic compounds such as methylphenidate (Ritalin®) and mixed-salts amphetamine (Adderall®) are the most current medications available for treating the symptoms of ADHD. The drug Adderall® is made of an approximate combination of 25% L-amphetamine and 75% D-amphetamine and has been marketed since the 1990s as a first-line stimulant treatment for ADHD. Following clinical use of Adderall®, Benzedrine® (D,L-amphetamine), and D-amphetamine, differences have been noted in regards to their individual effects in decreasing symptoms. Potential differences at the level of neurotransmission have not been characterized between these treatment options. To investigate any differences in amphetamine isomers, high speed chronoamperometry was used allowing for measures of real-time DA release signals in Chapter 3. Using Nafion®-coated, single carbon fiber

microelectrodes, amphetamine-induced DA levels and signal time courses were measured in the striatum and nucleus accumbens core of anesthetized male Fischer 344 rats. To study the individual effects of amphetamine isomers on DA neurotransmission, low concentrations of drugs were administered locally via pressure ejection through a micropipette.

For these studies, the L-amphetamine in a 50:50 D,L-amphetamine solution did not cause increased release of DA; however, it did seem to affect DA release signal kinetics. Signals evoked by D,L-amphetamine had significantly faster rise and decay times in both the striatum and nucleus accumbens core. After local applications of L-amphetamine alone, evoked DA signals were not significantly different in measures of amplitude from D-amphetamine evoked DA release signals; however, these L-amphetamine-evoked DA release signals displayed the rapid signal kinetics seen with D,L-amphetamine. The results of these studies supported the hypothesis that amphetamine isomers differentially cause release of DA in the striatum and nucleus accumbens core. These data support the possibility that L-amphetamine may have unique actions on the DAT, and the way in which reverse transport of DA occurs following administration of amphetamine.

Following the studies discussed in Chapter 3, we then tested Adderall® in the 75% D: 25% L-amphetamine combination making use of all four amphetamine salts to make comparisons with D-amphetamine and D,L-amphetamine at lower, more clinically relevant concentrations in Chapter 4. During the time in which these studies were carried out, prescriptions and sales of Adderall®, for the first time, were similar to those of methylphenidate. Additionally, these are the first data generated from *in vivo* measures of Adderall® since its introduction to the market. The technique of high speed chronoamperometry using Nafion®-coated, single carbon fiber microelectrodes was used to study amphetamine-evoked DA release produced by Adderall®, D-amphetamine, or D,L-amphetamine in the striatum of anesthetized male Fischer 344 rats. The amphetamine solutions were locally applied from micropipettes by pressure ejection. Local applications of Adderall® resulted in significantly greater

DA release signal amplitudes and prolonged time courses compared to D-amphetamine and D,L-amphetamine. These are the first *in vivo* data to support the hypothesis that the combination of amphetamine enantiomers and salts in Adderall® have effects on DA release, which result in increased and prolonged DA release compared to D- and D,L-amphetamine.

While the results discussed in Chapter 3 and 4 made use of second-by-second recording methods to determine the effects of acute applications of stimulants on DA release, Chapter 5 describes studies completed using a technique having greater sensitivity to detect lower levels of analyte. We tested the hypothesis that differential stimulant concentration-response curves of DA and metabolites would result dependent on the amount of amphetamine isomer present. In addition, we predicted that DA overflow caused by methylphenidate would differ from amphetamine. For these studies, complete Adderall®, D-amphetamine, and methylphenidate concentration-response curves were determined across theoretical subtherapeutic to abuse levels of drug. Finally, comparisons were made with "Reverse Adderall" containing 25% D-amphetamine and 75% L-amphetamine. The technique of reverse microdialysis was used to study local drug-evoked DA release accumulation in the striatum of anesthetized male Fischer 344 rats. These data support a D-amphetamine concentration-response curve of DA with double plateaus. These resulting concentration-response curves provide insight into functional properties of the DAT and/or specific release of DA stores. Additionally, DA levels after local applications of methylphenidate were similar to those caused by amphetamine. These methylphenidate data likely resulted due to reuptake blockade of a small amount of impulse-dependent DA being released under anesthesia. However, these data may also support a DA releasing effect of methylphenidate.

Table 1.1 DAT and Substrate Interactions

DAT Substrate	Effects on the DAT (Acute unless otherwise noted)
Dopamine	• Decreased transporter currents *in vitro* (Gulley and Zahniser 2003) • Rapid applications of DA decreased DAT clearance abilities *in vivo* (Gulley et al. 2002) • Increased localization of DATs to cytosol *in vitro* (Saunders et al. 2000) • Inhibited channel mode of the DAT *in vitro* (Kahlig et al. 2005)
Amphetamine	• Rapidly decreased DAT function and caused intracellular accumulation and internalization of an active DAT *in vitro, in vivo* (Kahlig et al. 2004; Saunders et al. 2000; Fleckenstein et al. 1999) • Induced a channel-like DAT *in vitro* (Kahlig et al. 2005) • Initially recruited DATs to the plasma membrane to cause DA efflux followed by internalization after continued exposure *in vitro* (Johnson et al. 2005)
Methylphenidate	• Increased DAT density and caused upregulation of DATs to the plasma membrane *in vitro* (Little et al. 2002) • Chronic use was followed by decreased levels of DATs density in the rat striatum but not nucleus accumbens (Izenwasser et al. 1999; Moll et al. 2001)

Table 1.1 Continued Next Page

Table 1.1 Continued

Cocaine	• Increased DAT density and caused mobilization of the DAT to the cell surface *in vitro* (Daws et al. 2002; Little et al. 2002) • Functional upregulation and increased clearance of the DAT *in vitro, in vivo* (Cass et al. 1993, Sabeti et al. 2002; David et al. 1998; Zahniser et al. 1999; Zahniser and Sorkin 2004) • Chronic exposure caused down regulation of DAT function and substrate binding *in vivo, in vitro* (Benmansour et al. 1992; Cass et al. 1993; Jones et al. 1996; Fleckenstein et al. 1999) • Following cessation of chronic use, increased DAT levels (Malison et al. 1998)

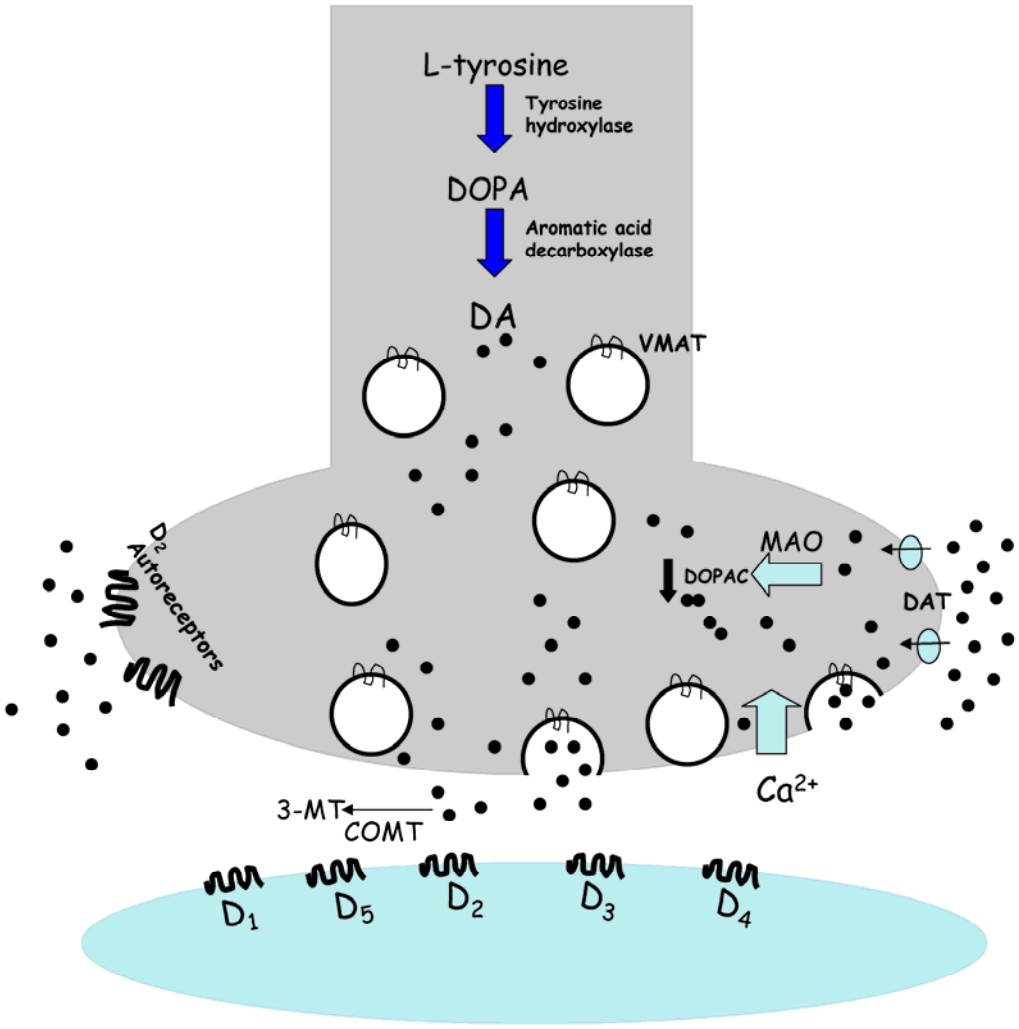

Figure 1.1 Dopamine Synthesis and Clearance

The precursor L-tyrosine is converted to dihydroxyphenylalanine (DOPA) which reacts with aromatic acid decarboxylase to form DA. DA can be packaged in vesicles for storage until depolarization leads to vesicular fusion with the plasma membrane and DA is released. DA clearance results from **(a)** metabolism via COMT into 3-MT **(b)** uptake by the DAT and **(c)** intracellular degradation via enzymes such as MAO into DOPAC. Extracellular levels of DA interact with DA receptors (autoreceptors and postsynaptic receptors) resulting in presynaptic effects and postsynaptic down regulation of further DA release.

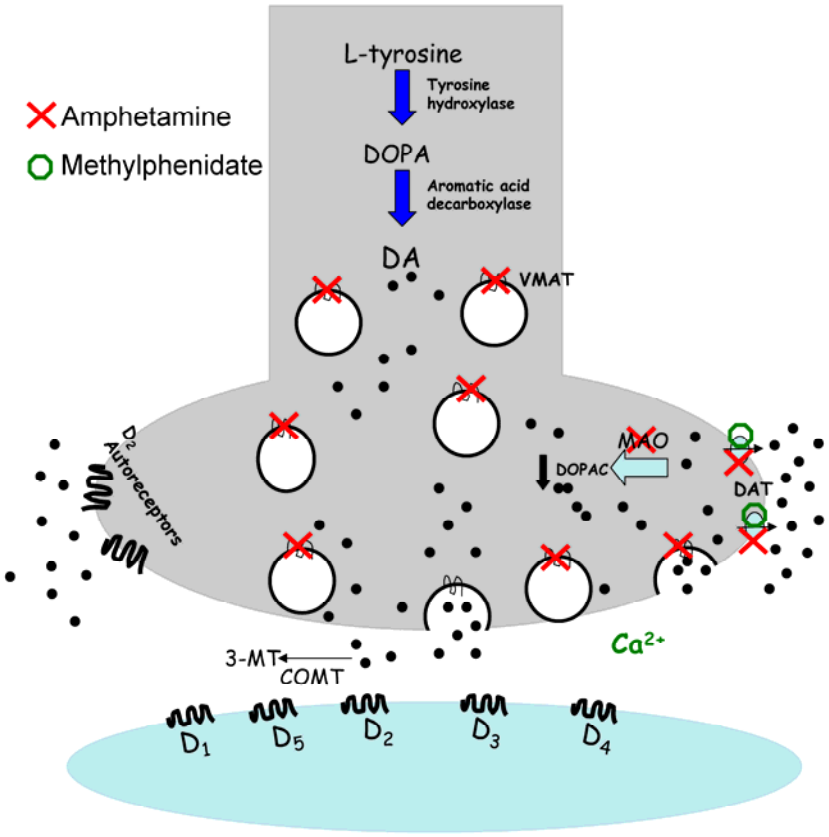

Figure 1.2 Theoretical Targets of Amphetamine Activity

Amphetamine (**X**) theoretically interacts with multiple cellular targets. Data support that DA gains presynaptic access through the DAT where it can block VMATs and cause emptying of vesicles to increase cytoplasmic levels of DA. Interactions with MAO have been noted leading to decreased levels of DOPAC. Finally, due to increased cytoplasmic levels of DA and/or interactions with amphetamine, the DAT begins to work in reverse fashion to allow DA to exit the cell. Amphetamine subsequently blocks future reuptake or normal function of the DAT and undergoes conformation changes and/or internalization. Methylphenidate (○) is thought to block the reuptake of Ca^{2+}-dependent DA released through the DAT, causing DA release different from the activity of amphetamine.

Chapter 2: Materials and Methods

The Fischer 344 Rat as an Animal Model of Normal Dopaminergic Function

Since the primary purpose of these studies was to assess mechanistic properties using low levels of stimulants, we chose normal male Fischer 344 (F344) rats as a model of the mammalian central nervous system. The F344 is an inbred strain of rat lending to increased genetic homogeneity. While some variations still exist between rats within this strain, the central nervous system lacks the heterogeneity of other outbred strains (Masoro 1990). Additionally, any variations between these rats are thought to mimic the variations present in the normal human population. While F344 rats, in general, show a constant increase in body weight and weight fluctuations between individual rats do not occur when fed ad libitum, they are also known to be a rat strain most related to human aging (Austad 1997; Stanford et al. 2001). This rat strain was also chosen due to the cost effective nature of their inclusion in these studies and availability. Data have supported that stimulants work to improve attention via maintenance of alertness in normal, healthy subjects (Syed et al. 2005), and the success of stimulant treatments for ADHD to decrease hyperactivity and increase attention is known to be highly variable from one patient to another (Rapoport et al. 1978; Elia et al. 1991). Therefore, in characterizing the effects of stimulants on dopaminergic neurotransmission, it was important to choose a non-diseased rat model. Finally, 3-6 month old rats were chosen for these studies to mimic developmental similarities of a young human population inclusive of the majority of patients receiving stimulants for ADHD. Additionally, this age group of the F344 rat is considered to be developmentally mature which would decrease variations in brain physiology. Finally, the F344 rat has been extensively used in our laboratory due in part to small variations in brain size across age levels allowing for more accurate targeting of stereotaxic coordinates.

Urethane as Anesthesia for Electrochemical Recordings and Microdialysis in the Living Fischer 344 Rat Brain

Intraperitoneal (i.p.) injections of 25% urethane prepared in 0.9% saline and administered at 1.25 g/kg were given to each rat. Each awake rat was restrained and administered 0.7 ml of 25% urethane for an initial injection. After 15 minutes, the weight of the rat was determined and the final dosing amount was calculated. The remainder of dosing was divided evenly among the next two injections both 15 minutes apart. After 45 minutes from initial dosing, a response to a toe pinch was determined. If no response resulted from the toe pinch, the experiment was carried out as described below. Sabeti et al. (2003) recently studied the effects of different anesthesias on measures of dopamine (DA) signals recorded in awake, behaving animals until they were completely anesthetized. The results of these studies support that urethane only minimally affected DA release and signal decay in comparison to chloral hydrate and ethanol (Sabeti et al. 2003).

In Vivo Chronoamperometric Recordings

Animal Preparation for Acute Electrochemical Recordings

The F344 rats were anesthetized as described above and placed into a stereotaxic frame (Kopf, Tujunga, CA) with the incisor bar set at -2.3 mm. Body temperature was maintained at 37º C as indicated by a rectal thermometer while rats rested on a heating pad (Braintree Scientific, Braintree, MA). A cross-type incision was made over the medial portion of the skin between the rat's ears and retracted in place using bulldog clips to anchor the movement of the skin during surgery. The skull overlying the medial cortex was removed bilaterally for recordings in striatum and nucleus accumbens where noted. Working microelectrodes were inserted bilaterally into the striatum (AP + 1.0 mm, ML +/- 2.3 mm, DV -4.0 to -6.0 mm; 0.5 mm increments). Reference electrodes (200µm

o.d., Ag/AgCl) were placed 8-10 mm posterior to *bregma* and 0.5 to 1.0 mm lateral to midline and were cemented in place using dental acrylic. All stereotaxic coordinates were determined with respect to bregma using the atlas of Paxinos and Watson (1998). All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and use of Mammals in Neuroscience and Behavioral Research (2003) and were approved by the Animal Care and Use Committee of the University of Kentucky. Figure 2.4 shows a F344 rat prepared for *in vivo* electrochemical recordings.

Electrochemical Microelectrodes

Single carbon-fiber type electrochemical working microelectrodes (30 µm o.d.) were used to measure DA release signals (Fig. 2.6). The microelectrodes (Quanteon, L.L.C., Nicholasville, KY) were pre-dried at 200 ºC prior to coating with Nafion® (5% solution, 1-3 coats, Aldrich Chemical Co., Milwaukee, WI). They were dipped in Nafion® solution and dried at 200º C for 5 minutes between each coat (Fig. 2.7). Prior to use, all microelectrodes were calibrated *in vitro* to determine their selectivity, sensitivity, and reduction/oxidation current responses to DA. For use in these studies, microelectrodes had an average selectivity of >900:1 for DA over 3,4-dihydroxyphenylacetic acid (DOPAC) or ascorbic acid. Each microelectrode displayed linear responses to DA additions up to 8 µM (Hoffman and Gerhardt 1999; Gerhardt and Hoffman 2001). Reduction/oxidation current ratios averaging between 0.5 to 0.7 were exhibited, indicating that the microelectrode was detecting DA and not serotonin (5-HT) or ascorbic acid (Gerhardt and Hoffman 2001; Gerhardt and Burmeister 2000). The limit of detection for DA was typically 25 nM. Refer to Figure 2.1 for further detail regarding the reduction/oxidation current ratios and performance of the microelectrodes.

Electrochemical Instrumentation

The microelectrodes were connected to a headstage (Quanteon, L.L.C. Nicholasville, KY) with a gold-plated amphenol (wire-crimp, Mill-Max®, Oyster Bay, New York). The headstage was connected to a high-speed Pentium-IV microcomputer- controlled instrument, the FAST-12 (Fast Analytic Sensing Technology, Quanteon, L.L.C. Nicholasville, KY) and chronoamperometric measurements (5 Hz) were performed. An oxidation potential was applied (+0.55 V; 0.0V resting versus Ag/AgCl reference) to the working microelectrode. The potential of the working microelectrode was changed relative to a stable, Teflon™ coated, silver wire electroplated in 1- M HCl saturated in NaCl (Ag/AgCl Reference Electrode). The resulting oxidation current and subsequent reduction current from the microelectrodes were integrated during the final 80% of each 100-ms pulse. Both oxidation and reduction currents were continually recorded and averaged to 1 Hz. In all recordings, reverse current ratios (redox ratios) were used to further confirm the detection of DA by the microelectrode measures (Gerhardt and Hoffman 2001; Gerhardt and Burmeister 2000).

Drugs for Use with *In Vivo* Electrochemical Recordings

Urethane, D-amphetamine and D,L-amphetamine were obtained from Sigma (St. Louis, MO). The L-amphetamine isomer was obtained from the NIH-NIDA/Division of Neuroscience and Behavioral Research (Bethesda, MD). The four components of Adderall®, D-amphetamine saccharate, D,L-amphetamine aspartate, D-amphetamine sulfate, and D,L-amphetamine sulfate, were obtained from Shire Pharmaceuticals (Hampshire, Chineham, England). DA and DOPAC were obtained from Aldrich (Milwaukee, WI). All drug solutions were prepared in 0.9% saline and adjusted to a final pH of 7.4

Pressure Ejection Coupled to *In Vivo* Electrochemical Recordings

To circumvent the use of parental drugs to alter monoaminergic function and to directly study the extracellular regulation of DA, local application of drugs coupled with *in vivo* electrochemical recordings was used to study drug-induced release of DA (Hebert and Gerhardt 1999; Hoffman and Gerhardt 1999). Single-barrel glass micropipettes (Kopf Puller, Tujunga, CA) (1 mm outer diameter, 0.58 mm inner diameter, AM systems, Inc., Everett, WA) with an inner tip diameter of 7-11µm were attached to Nafion®-coated carbon-fiber microelectrodes with sticky wax (Kerr, Orange, CA) so that the tip of the electrode and micropipette were in the same plane and measured 250 µm apart.

The volume of applied drug was kept constant at 500 nl and was measured using a dissection microscope fitted with a calibrated reticule (1 mm change=25 nl of fluid) (Cass et al. 1992, 1993a; Friedemann and Gerhardt 1992). The amounts of drugs applied in Chapter 3 were determined as: 2 nanomoles D-amphetamine, 4 nanomoles D,L-amphetamine, 2 nanomoles D,L-amphetamine and 2 nanomoles L-amphetamine. For Chapter 4, Adderall®, D,L-amphetamine or D-amphtamine solutions were applied in the following amounts (0.68 nanomoles Adderall®, 1 nanomole D,L-amphetamine, and 0.5 nanomoles D-amhetamine). For the data presented in Appendix 1, high concentrations of D-amphetamine and Adderall were selected for use and were applied in the following amounts 2 nanomoles D-amphetamine and 2.72 nanomoles Adderall® (West et al. 1999; Shader et al. 1999; Kuczenski and Segal 2001; Solanto et al. 2001). The data from Chapter 3 support that L-amphetamine only regulates the time-course of D-amphetamine and does not contribute to the potency of an amphetamine solution. Therefore a constant 0.5 nanomoles of the D-amphetamine isomer were applied in all drug treatments in Chapter 4 in order to investigate the effects of differing amounts of L-amphetamine.

The stimulant concentrations used for pressure ejection were chosen based on the necessary amount of stimulus needed to give consistent and comparable DA responses. It is difficult to accurately predict the dilution factor of

a locally applied drug to the brain tissue at varying depths. We estimate that our drug solutions used in Chapter 3 (40-400 μM) were in the upper range of effective concentrations of plasma levels determined after clinical use of stimulants (10-50 μM)(West et al. 1999; Shader et al. 1999; Gerhardt and Palmer 1987; Solanto et al. 2001). The concentrations were then decreased for the studies described in Chapter 4 and were estimated to be in the therapeutic range for ADHD.

Stimulant-Evoked Release of DA

Electrochemical measurements were performed at 5 Hz to establish a baseline response. After achieving a steady state signal (usually in 5 minutes), the effects of local applications of the amphetamine solutions were studied (Gerhardt et al. 1986,1987; Gerhardt and Palmer 1987; Cass et al. 1993b; Luthman et al. 1993). The drug solutions were applied over a 20 second period to minimize any local dilution of DA release signals by the drug solutions. A typical amphetamine-induced DA release signal is shown in Figure 2.1. After returning to baseline, the electrode assembly was lowered an additional 0.5 mm, and the drug solution was ejected at the next depth. Typically, six- eight signals were recorded in the striatum (three signals in the nucleus accumbens) bilaterally in the individual animals.

Histology After *In Vivo* Electrochemical Recordings

Following the recording sessions, rats were perfused with saline, followed by 4% paraformaldehyde. The brains were subsequently removed, frozen with dry ice, sliced into 50-μm coronal sections, and stained with Cresyl Violet stain for histological evaluation of probe placement and recording tracks. Data from histologically confirmed correct placement of microelectrodes into the striatum (and nucleus accumbens) were used for data analysis. No data were rejected based on incorrect placement of microelectrode assembly in these studies.

Figure 2.2 shows a typical slice from the striatum of a F344 rat following after an *in vivo* chronoamperometric recording session. This technique is noted to be minimally invasive relative to other *in vivo* recording techniques, and greater spatial resolution can be resolved due to the 30 μm outer diameter of the microelectrode (Fig. 2.2).

Analysis of Data Collected Using *In Vivo* Voltammetry

We chose recordings with redox ratios indicative of predominant DA signals (0.5-0.7) with signal amplitudes ≥0.05 μM DA for analysis of temporal dynamics of DA release: rise times (T_R) and 80% decay times (T_{80}) (Fig. 2.1) (Gerhardt and Hoffman 2001). The numbers of animals and recording signals for each drug group in Chapter 3 for striatum were: D-amphetamine (n=7 animals, 28 recordings), D,L-amphetamine (n=7 animals, 34 recordings), 2 x D,L-amphetamine (n=7 animals, 40 recordings) and nucleus accumbens: D-amphetamne (n=7 animals and 17 recordings), 2 x D,L-amphetamine (n=7 animals and 16 recordings), and D,L-amphetamine (7 animals, 21 recordings). The number of animals used for the comparison of D- versus L-amphetamine were n=9, 34 recordings and n=7 rats, 17 recordings in the striatum and n=16 and 4 rats in the nucleus accumbens respectively. For the studies completed for Chapter 4, the following animals and signals were used for data analysis: D,L-amphetamine (n=10 animals, n=30 signals), Adderall® (n=10 animals, n=54 signals), and D-amphetamine (n=10 animals, n=42 signals). DA signals were heterogeneous in nature, which is the normal distribution of DA signals in the rat striatum (Friedemann and Gerhardt 1992). Multiple recordings were taken bilaterally at various striatal (and nucleus accumbens) depths in each animal and each recording depth. Therefore, to avoid artificially increasing degrees of freedom and pseudoreplication by treating each recording independently, a nested between-groups analysis of variance (MANOVA) was used (Hurlbert 1984; Salvatore et al. 2004). SYSTAT's Multivariate General Linear Model (SYSTAT Software, Richmond, CA, USA) was used for statistical analysis while

all analyses were followed by Tukeys post-hoc comparisons. Statistical significance was defined as $p<0.05$.

Intracerebral Reverse Microdialysis Measures of Dopamine and Metabolites

Animal Preparation

The animals were prepared for study similar to the descriptions provided earlier in this chapter. Differences in surgical procedures are described here. After the retraction of the skin and tissue and exposure of the skull overlying the striatum, a small craniotomy was placed in the right hemisphere only (coordinates with respect to bregma: +1.0 mm AP, ±2.2 mm ML) (Paxinos and Watson 1998). A microdialysis probe with a 2-mm membrane (CMA/11, CMA Microdialysis, Stockholm, Sweden) (Fig. 2.9) was lowered into the striatum (6 mm below the cortical surface) and remained at this depth for the duration of the experiment Fig. 2.5). 1000 μl gastight syringes (1001 LTN, Hamilton USA, Reno, NV) containing dialyzing fluids were positioned in a syringe pump (KDS230, KD Scientific, Holliston, MA) which was set at flow rate of 1 μl/min, chosen based on desired percent recovery for striatum and the microdialysis probe membrane length employed (Fig. 2.10). Syringes were connected to a liquid switch (CMA/110, CMA Microdialysis, Stockholm, Sweden) that allowed for alternation between treatments: artificial cerebral spinal fluid (aCSF) and aCSF + drug (Fig. 2.8). Teflon tubing (FEP tubing, 0.12 mm inner diameter, CMA Microdialysis, Stockholm, Sweden) and tubing adapters (CMA Microdialysis, Stockholm, Sweden) were used to establish all connections.

In Vivo Intracerebral Reverse Microdialysis

Following probe insertion, perfusion with aCSF (in mM: NaCl 123, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 25, NaH_2PO_4 1, and glucose 5.9) at a pH of 7.4 was initiated. Samples were then collected at twenty minute intervals into a 0.2 ml

microcentrifuge tube and injected into the HPLC-EC system. The order of administration for each of the drug solutions tested was as follows: samples 1-6 (aCSF), sample 7 (aCSF + amphetamine solution), samples 8-12 (aCSF).

Probe recoveries were collected using a standard solution with known concentrations of DA, norepinephrine (NE), serotonin (5-HT), 3,4-dihydroxphenylacetic acid (DOPAC), Homovanillic Acid (HVA) and 5-Hydroxyindole Acetic Acid (5-HIAA). In order for a probe to be used in these studies, an *in vitro* probe recovery of 10% ± 1 was required based on methodological standards. Based on this exchange rate of 10-20%, seen for molecules similar in size to amphetamine such as DA, NE, and 5-HT, we determined the effective concentrations of stimulant drugs across a range of starting concentrations. Stimulant concentrations were chosen to represent a range of concentrations that included clinically relevant levels. Based on the approximate exchange rate, solutions of aCSF only, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 400 µM (for D-amphetamine, L-amphetamine, methylphenidate, D,L-amphetamine, and cocaine), 533 µM (for Reverse Adderall only), and 539 µM (for Adderall® only) were prepared via serial dilutions at a pH of 7.4. Solutions were prepared for study and consisted of normal aCSF and drug. Prior to each experiment, 20 mM ascorbic acid was added to each solution and solutions were aerated with 95% O_2/5% CO_2. Solutions were immediately added to individual 1000 µl gastight syringes.

Histology of the Striatum Following Intracerebral Reverse Microdialysis

Following each experiment, rats were intracardially perfused with 0.9% NaCl solution followed by a 4% paraformaldehyde solution. They were then decapitated, and their brains were frozen, sliced on a cryostat, and stained with Cresyl Violet. Probe placements were confirmed histologically. An example of a brain section after a microdialysis experiment is shown in Figure 2.3.

Analysis of Microdialysis Samples

HPLC analysis followed the methods described by Hall et al. (1989). The low level detections of DOPAC, DA, 5-HT, NE, 5-HIAA, and HVA were performed using an isocratic HPLC system (Beckman, Inc., Fullerton, CA) coupled to a dual-channel electrochemical array detector (model 5300A, ESA, Inc., Chelmsford, MA), E_1 = +0.35 mV and E_2 = -0.25 mV, with an ESA model 5011A dual analytical cell. The compounds of interest were separated with reverse-phase chromatography, using a C18 column (4.6 mm x 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan) with a pH 4.1 citrate-acetate mobile phase, containing 4% methanol and 0.34 mM 1-octane-sulfonic acid and delivered at a flow rate of 2.0 ml/min. Peaks for the analytes were identified by retention times from known standards.

Statistical Analysis of Analyte Levels in Microdialysis Samples

Data were collected from 5-6 animals per 10 drug concentrations (for Adderall®, D-amphetamine, Reverse Adderall, and methylphenidate). Data were collected for 5-6 animals for the highest drug concentration only for L-amphetamine, D,L-amphetamine, and cocaine. The raw microdialysis values were expressed as nM based on a 1 x 10^{-7} M mixed standard of known analytes and concentration used to determine a percent recovery *in vitro* prior to use of each probe. Outliers were excluded based on data falling outside of 2 standard deviations from the mean. Concentration-response curves were constructed based on the mean peak DA overflow concentration following the twenty minute reverse microdialysis of each drug concentration. GraphPad Prism statistical analysis software, version 4.0 (Prism, San Diego, CA, USA), was used to determine the appropriate nonlinear curve fit and Log half maximal effective concentration (EC_{50}) of each drug. An initial one-way analysis of variance was used to determine significance of DA overflow from the aCSF control. A second one-way analysis of variance was used followed by post-hoc *t*-tests with

Bonferroni's corrections to compare DA following reverse microdialysis of clinically relevant drug concentrations and maximum concentrations. Statistical significance was defined as $p<0.05$.

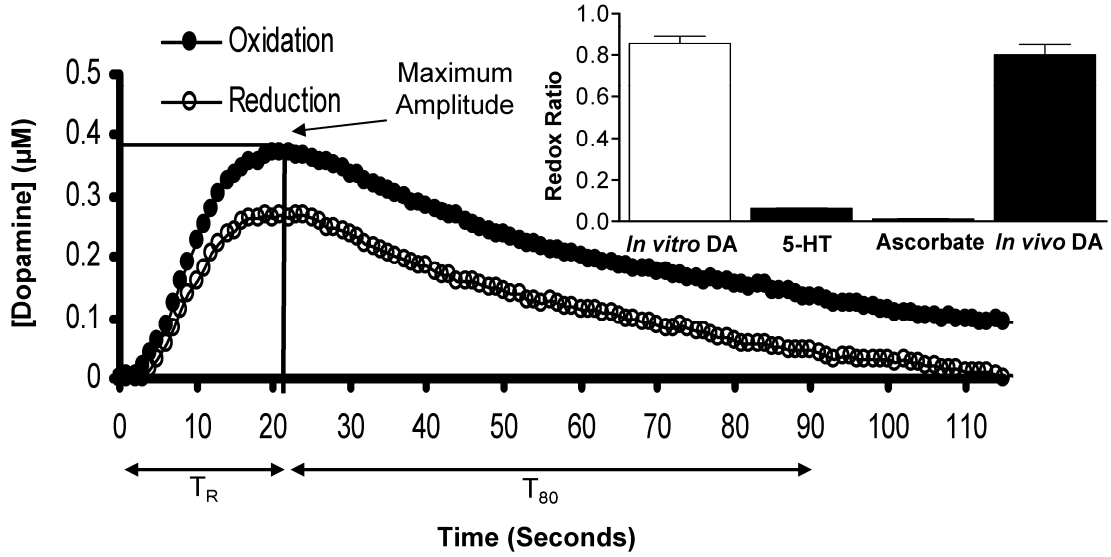

Figure 2.1 Typical Amphetamine-Evoked DA Release Signal

A representation of typical tracings of D-amphetamine-induced DA release measured by high-speed (5 Hz) chronoamperometry is shown. The top signal indicates the oxidation of DA and the bottom signal indicates the reduction current. The measured reduction/oxidation (redox) ratio of this DA release signal is characteristic of DA detection by the microelectrode. Rise time (T_R) is indicated and defined by the total time required for the DA signal to reach the indicated maximum amplitude. 80% decay time (T_{80}) is a measure of the total time required for the DA release signal to decay 80%. **Inset:** Measured redox ratios of carbon fiber microelectrodes used for experiments. Prior to use of each microelectrode, *in vitro* calibrations were completed to determine redox ratios before insertion into the rat brain. The mean *in vivo* redox ratio was determined by averaging ratios from amphetamine-induced DA recordings in multiple rat brains. The *in vivo* and *in vitro* redox ratios are similar to those expected for DA and notably different from recordings of 5-HT and ascorbate *in vitro*.

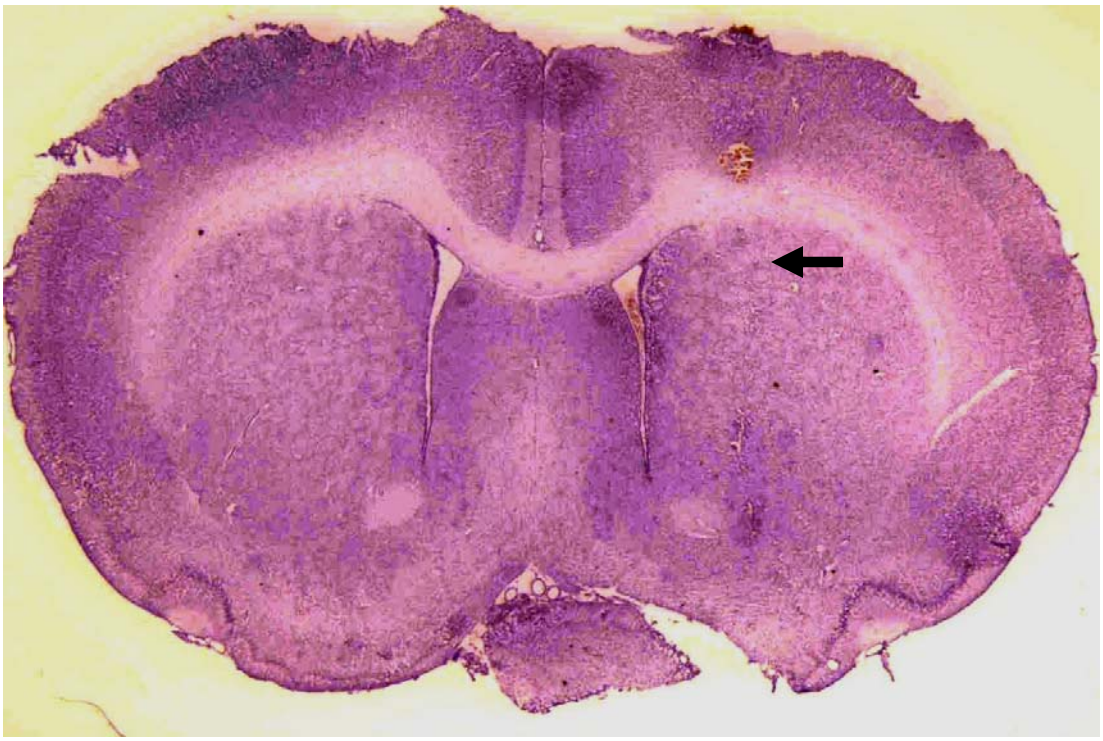

Figure 2.2 Histological Preparation of a Rat Brain After Acute Electrochemical Recordings

Following the recording session and perfusion of the rat, brains were removed, frozen, sectioned, and stained with Cresyl Violet to check accuracy of probe placement. Arrow denotes probe track of a carbon fiber microelectrode coupled to a micropipette in the striatum (Chapters 3 and 4). The tip of the microelectrode, with an outer diameter of 30 μm, was waxed 250 μm from the tip of a micropipette (inner diameter of 10 μm). The histology above shows little damage after a complete experiment.

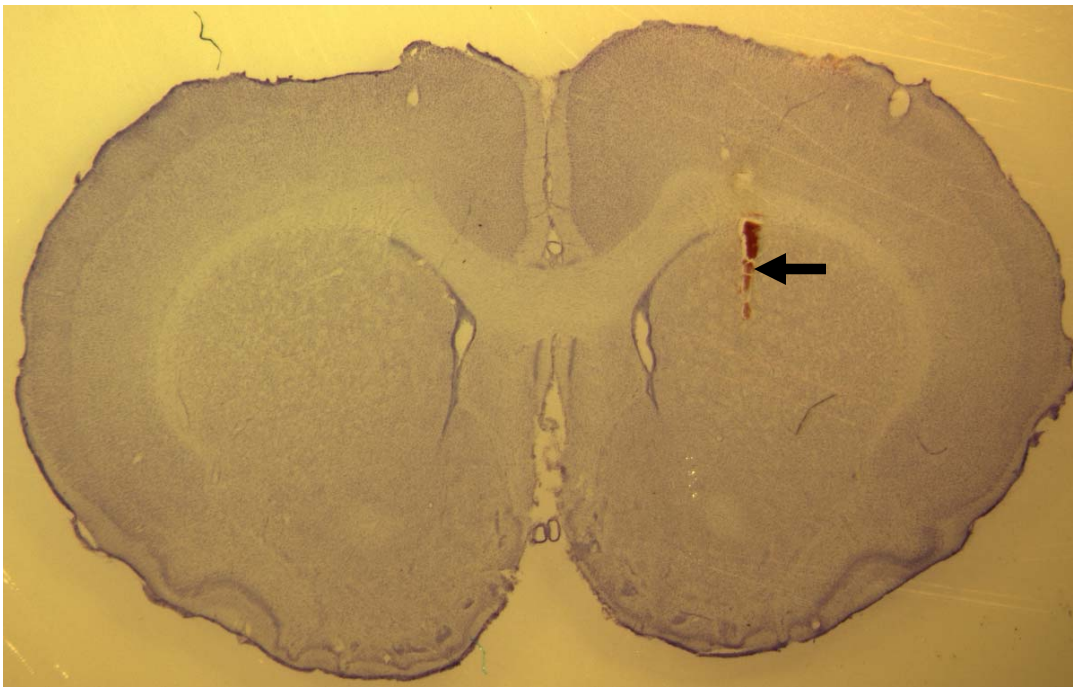

Figure 2.3 Histological Preparation of Rat Brain Striatum After a Microdialysis Experiment

Following the microdialysis session and perfusion of the rat, brains were removed, frozen, and sectioned on a cryostat and subsequently stained with Cresyl Violet to check accuracy of probe placement. Arrow denotes probe track of the microdialysis probe in the striatum (Chapter 5). The membrane of the microdialysis probe had an outer diameter of 240 μm and was 2 mm in length.

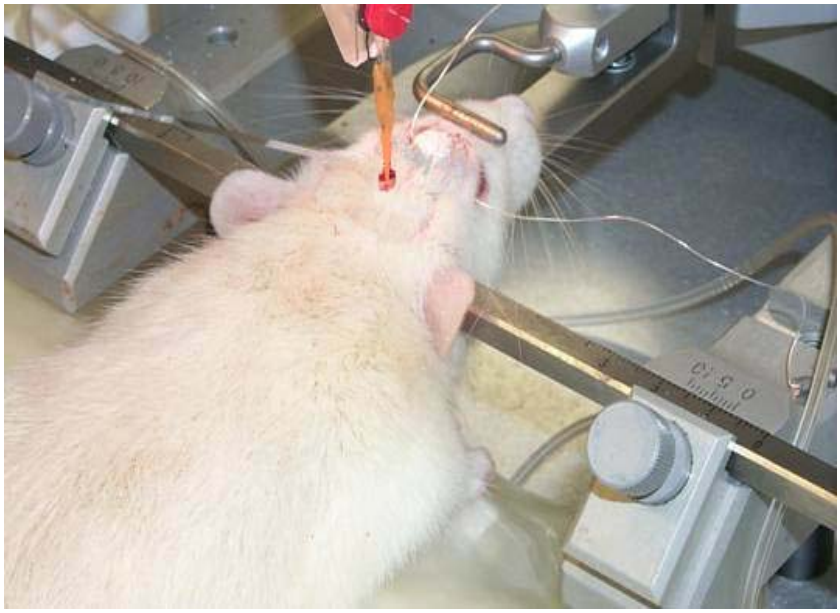

Figure 2.4 Fischer 344 Rat Prepared for *In Vivo* Voltammetric Recordings

3-6 month old F344 rats were anesthetized with 25% urethane and carbon fiber microelectrodes were lowered into the striatum for recordings of DA release signals.

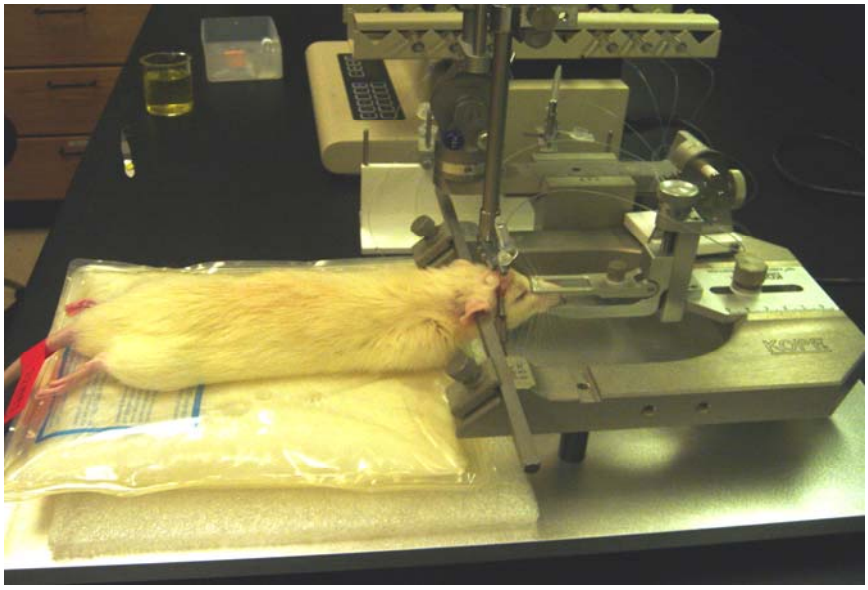

Figure 2.5 Fischer 344 Rat Prepared for Intracerebral Reverse Microdialysis

3-6 month old F344 rats were anesthetized with 25% urethane and CMA microdialysis probes were lowered into the striatum for microdialysis of aCSF.

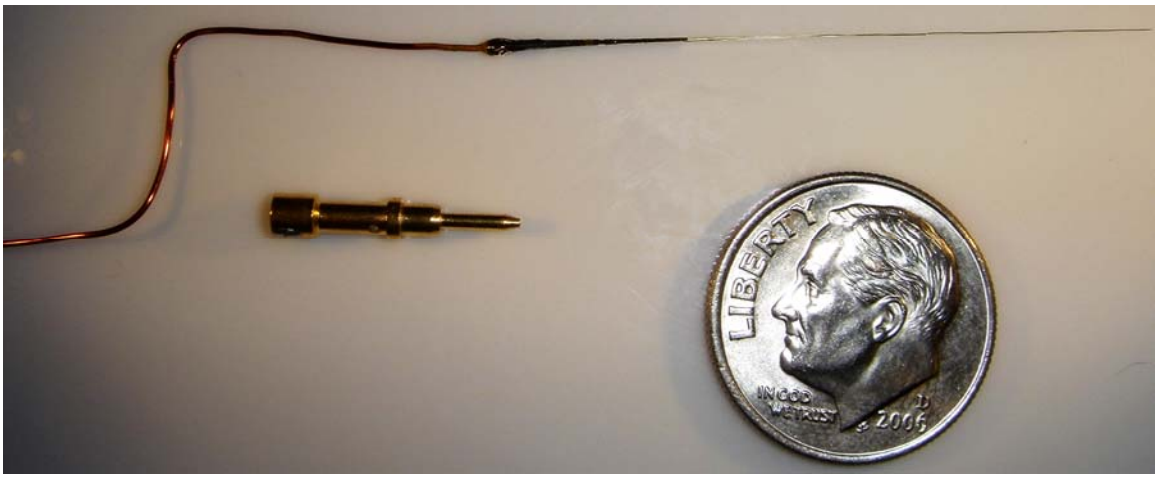

Figure 2.6 Carbon Fiber Microelectrode

Carbon fiber microelectrode (above) and the gold-plated amphenol (below) used to connect the probe to the recording system head stage.

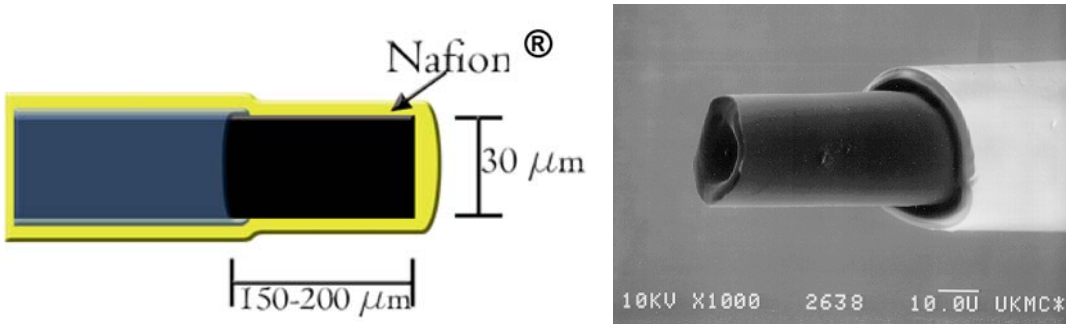

Figure 2.7 Schematic of a Carbon Fiber Microelectrode

The single channel recording tip of a carbon fiber microelectrode coated with Nafion® (yellow)(left) to increase selectivity for DA, and an electromicrograph of a carbon fiber microelectrode tip (Right).

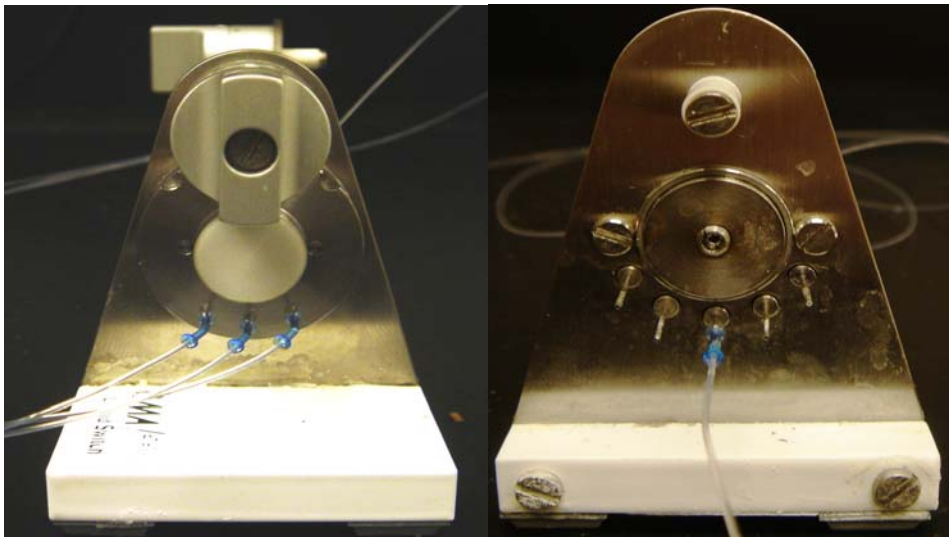

Figure 2.8 CMA/110 Liquid Switch

The CMA/110 Liquid Switch with 3 syringe capacity, blue tubing adapters, and tubing (left) and the reverse side (right) with central outlet tube that connects to the microdialysis probe.

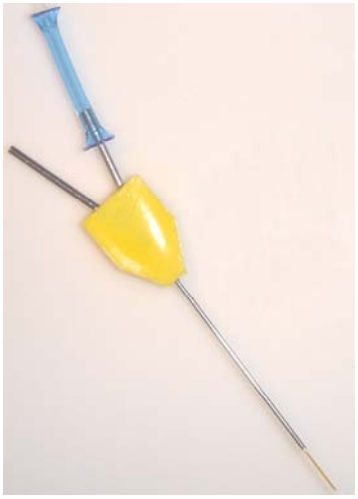

Figure 2.9 CMA/11 Microdialysis Probe (2 mm membrane)

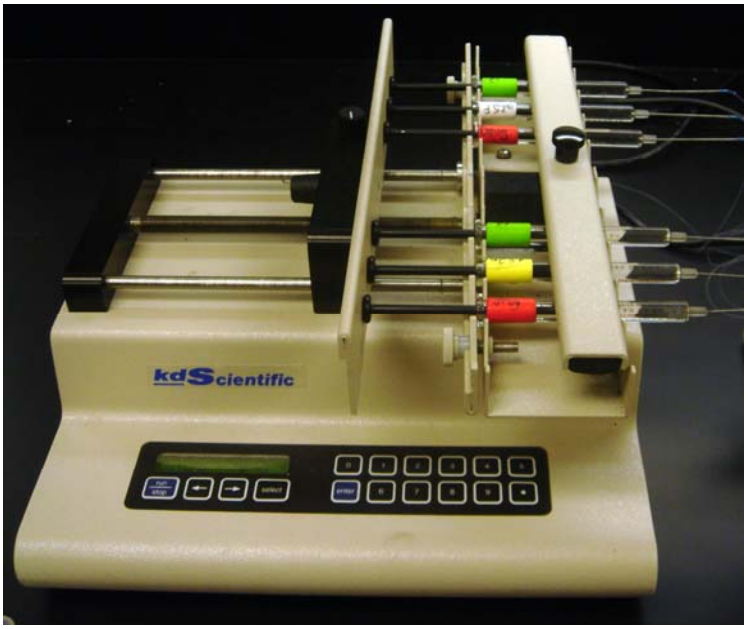

Figure 2.10 Microsyringe Pump and1000µl Hamilton Syringes Containing aCSF and Stimulant

Figure 2.11 ESA Coulochem III High Performance Liquid Chromatography Electrochemical Detector and Auto-Sampler

Chapter 3: Differential Effects of Amphetamine Isomers on Dopamine Release in the Rat Striatum and Nucleus Accumbens Core

Introduction

Since their initial discovery in the mid 1900s, stimulants have been used as first-line agents in treating ADHD. Recently, development of sustained-release delivery systems, racemic compounds, and single-isomers of already approved stimulants have led to an increase in the number of marketed stimulants available for treatment options. In the 1990s Adderall® was marketed as a stimulant with an increased half-life of efficacy that attained approximately 29% of the market share in stimulant prescriptions by 2000 (Goodman and Nachman 2000). The drug Adderall® is made of an equal-weight composition of four amphetamine salts (D-amphetamine saccharate, D,L-amphetamine aspartate, D-amphetamine sulfate and D,L-amphetamine sulfate) yielding a combination of 75% D-amphetamine and 25% L-amphetamine. The longer efficacy of Adderall® has generally been attributed to differential absorption of component salts (Hampshire, Chineham, England), however, data supporting this has never been shown. James et al. (2001) recently published the first randomized, double-blind, cross-over comparison of Adderall® versus immediate and timed-release D-amphetamine. This study demonstrated the faster onset and longer duration of action of Adderall® when compared to D-amphetamine on target symptoms of hyperactivity, however potencies between the two are similar. Benzedrine® (D,L-amphetamine) had been used as a treatment for ADHD until removed from the market in the 1970s due to the increased abuse potential associated with this medication. A clinical study completed around this same time indicated that individuals respond differently in regards to efficacy and side effects of Benzedrine® versus D-amphetamine (Gross, 1976).

For several decades, stimulants such as methylphenidate and D-amphetamine have been instrumental in treating the symptoms of ADHD and are thought to be safe and effective. Given their widespread use, their mechanisms

of therapeutic action in the context of ADHD remain unclear (Solanto et al. 2001; Goodman et al. 2001). In the presence of D-amphetamine, dose-dependent increases in pre-synaptic dopamine (DA) and norepinephrine (NE) release occur via a calcium-independent mechanism (Carboni et al. 1989; Kahlig and Galli 2003). Amphetamine is likely to be active at multiple cellular targets including the vesicular monoamine transporter (VMAT), the DA transporter (DAT), and monoamine oxidase (MAO). The therapeutic nature of stimulants and their known interactions with DA support the current consensus hypothesis that ADHD symptomatology is likely due to dysregulation of catecholaminergic neurotransmission. Interruptions in dopaminergic neurotransmission in the prefrontal cortex, striatum, and nucleus accumbens have been implicated (Solanto et al. 2001; Biederman and Faraone 2002).

In regards to increasing extracellular DA in the brain, L-amphetamine is often considered to be inactive (Goodman et al. 2001) while differential pharmacokinetic properties have been shown in comparison with D-amphetamine. L-amphetamine has displayed paradoxical effects such as decreased locomotion in mice at low doses and increased locomotion in mice at high doses (Stromberg and Svensson 1975). Rats will self-administer L-amphetamine, and children with ADHD have been successfully treated with L-amphetamine (Yokel and Pickens 1973; Arnold et al. 1976). Hippocampal data have shown that the enantiomers initiate differential release of DA (Kuczenski et al. 1995). Kanbayashi et al. (2000) showed that D-amphetamine treats different symptoms than L-amphetamine in a canine model of narcolepsy. D-and L-amphetamine administered alone versus in combination and the relevance of the enantiomer interactions have not been shown in the context of therapeutic doses.

For the studies described in this chapter we investigated the potential differences between the dynamics of DA release produced by racemic amphetamine (D,L-amphetamine) compared to D-amphetamine. In addition, we directly compared the effects of the single D- and L-amphetamine enantiomers. We tested the hypothesis that L-amphetamine has differential effects on DAT regulation in comparison to D-amphetamine. We used the technique of high

speed chronoamperometry coupled with Nafion®-coated single carbon-fiber microelectrodes to measure locally-applied (D,L-, D-, and L-) amphetamine-evoked DA release on a second-by-second basis in the striatum and nucleus accumbens core of anesthetized Fischer 344 (F344) rats. Local applications of drugs were used to avoid pharmacokinetic issues and interference with the kinetics of the direct actions of amphetamine isomers.

Methods

Drug Concentrations used for *In Vivo* Electrochemical Recordings

The volume of applied drug was kept constant at 500 nl and was measured using a dissection microscope fitted with a calibrated reticule (1 mm change=25 nl of fluid) (Cass et al. 1992, 1993a; Friedemann and Gerhardt 1992). Drugs were dissolved in 0.9% physiological saline and final drug solutions were brought to a pH of 7.4. The amounts of drugs applied were determined as: 2 nanomoles D-amphetamine, 4 nanomoles D,L-amphetamine, 2 nanomoles D,L-amphetamine and 2 nanomoles L-amphetamine. **For additional methodological details, see Chapter 2.**

Results

Amphetamine-Induced Release of DA

All amphetamine solutions applied locally yielded increases in extracellular levels of DA and elongated signals similar to the slow releasing properties associated with amphetamine and in comparison to the faster signals produced via potassium induced depolarization release of DA (Hoffman and Gerhardt 1999). Figure 2.1 (Chapter 2) displays the oxidation and reduction current signals recorded following local application of approximately 500 nl of an amphetamine solution. Carbon fiber microelectrodes that primarily measure DA

were used according to *in vitro* calibration data; the average *in vitro* redox ratios are shown as the inset of Figure 2.1. These ratios were compared to those observed *in vivo* during the recordings in rat brain and indicated no significant difference. However, these ratios were significantly different than *in vitro* redox ratio measurements of 5-HT and the interferent ascorbic acid. These high-temperature treated, Nafion®-coated microelectrodes reliably measured amphetamine evoked DA signals that yielded the chemical fingerprint redox ratio for DA (~0.78) (Gerhardt and Hoffman 2001).

Comparisons of D-amphetamine and D,L-amphetamine-Induced DA Release in the Rat Striatum

At equivalent concentration and volume, D-amphetamine, D,L-amphetamine and 2x D,L-amphetamine (double the concentration of the D,L-amphetamine to match the concentration of D-amphetamine used in the first solution) were locally applied to the striatum. A total of 28, 34, and 40 DA release recordings for D-amphetamine, D,L-amphetamine and 2x D,L-amphetamine respectively were recorded. Figure 3.1 displays representative signals from 2 x D,L- and D-amphetamine-evoked DA, indicating the similar amplitudes and significantly different time course of DA release signals: the inset displays the differences in time course. When considering the amplitudes of the recorded DA signals from multiple sites in the rat striatum, D,L-amphetamine evoked significantly lower DA amplitudes that were approximately one-half of those evoked by both D-amphetamine and 2x D,L-amphetamine ($p<0.01$, Fig. 3.2 a). Amplitudes of the DA signals produced by D-amphetamine and 2x D,L-amphetamine were not significantly different. In Figure 3.2 b the data were expressed by the amplitudes of the DA signals in respect to the nanomoles of the D-amphetamine isomer that was locally applied. In this format, the DA release amplitudes were normalized and there was no significant difference between the three amphetamine solutions. The amplitude of the DA signal was related to the

concentration of the D-amphetamine isomer in the applied solution, and the L-amphetamine had little or no effect on the absolute amplitude of DA release.

The temporal properties of the evoked DA release signals were significantly different between the D,L-amphetamine and D-amphetamine solutions. Rise time (T_R) comparisons indicated that both D,L-amphetamine solutions resulted in DA signals with significantly faster T_R than D-amphetamine (2 x D,L-amphetamine $p<0.01$; D,L-amphetamine $p<0.05$, Fig. 3.3 a). Finally, D,L-amphetamine solutions yielded DA signals with a significantly faster T_{80} ($p<0.001$, Fig. 3.3 b).

Comparisons of D-, D,L-, and 2 x D,L-amphetamine- Evoked DA Release in the Nucleus Accumbens Core

These studies were carried out to examine the effects of local applications of D-amphetamine and D,L-amphetamine on DA release signals in the nucleus accumbens core. A total of 17, 16, and 21 DA release signals, collected from seven rats for each group, were used to make comparisons between D-amphetamine, D,L-amphetamine, and 2x D,L-amphetamine respectively. Amplitudes of DA release signals caused by D,L-amphetamine were significantly lower than D-amphetamine and 2 x D,L-amphetamine-evoked DA release signal amplitudes, while the latter two were not significantly different from each other ($p<0.05$; Fig. 3.4 a). Figure 3.4 b shows the amplitude data normalized to the nanomoles of the D-amphetamine isomer applied. Similar to data collected in the striatum, there were no differences when viewing the amplitudes of DA release in respect to the amount of D-amphetamine applied. These data support that the overall DA release corresponded to the amount of D-amphetamine isomer applied and was independent of the amount of L-amphetamine.

DA signal kinetic differences resulted in the nucleus accumbens core analogous to data collected in the striatum. D,L-amphetamine solutions produced DA signals with faster T_R than D-amphetamine-evoked DA release ($p<0.01$; Fig 3.5 a). While T_{80} decay times were not significantly different when

compared via repeated-measures ANOVA, significance was shown using a pair wise comparison followed by Bonferroni corrections for multiple comparisons. T_{80} decay times were significantly faster with D,L-amphetamine evoked DA signals ($p<0.001$; Fig 3.5 b).

D-amphetamine Versus L-amphetamine Induced DA Release in the Striatum and Nucleus Accumbens Core

A comparison of the properties of single isomer drug solutions were carried out to investigate the effects of locally applied D-amphetamine and L-amphetamine on DA release in the striatum and nucleus accumbens core. A major finding supported by these data was that D-amphetamine evoked DA signal amplitudes were not significantly different from L-amphetamine evoked DA signal amplitudes (Fig 3.6 a). DA release signals were recorded from a total of 9 rats, 34 signals for D-amphetamine and 7 rats, 17 signals for L-amphetamine. Significantly faster T_R and T_{80} were observed in the presence of L-amphetamine when compared to D-amphetamine ($p<0.001$; Fig 3.6 b,c) similar to the differences found between D-amphetamine and D,L-amphetamine. Similar to the differences between D- versus L- amphetamine in the striatum, differences in the nucleus accumbens were supported by data from a total of 4 rats with L-amphetamine and 16 rats with D-amphetamine (data not shown).

Discussion

The studies described here explain the faster onset and offset of DA release in the presence of D-amphetamine versus D,L-amphetamine when the drugs were applied locally to the striatum and core of the nucleus accumbens of anesthetized rats. The amplitudes of DA release were related to the amount of D-amphetamine present in the solution, and the presence of L-amphetamine only seems to regulate the time course of DA release. When comparing local applications of the D- and L-isomers, we saw that the overall DA signal

amplitudes were slightly smaller in the presence of L-amphetamine indicating its potency in causing DA release in the CNS. These findings are similar to previous literature that support differential potency and efficacy reported in clinical studies (Arnold et al. 1976). Comparatively, previous animal studies have shown differences in the effects on behavior between D- and L-amphetamine (Yokel and Pickens 1973; Jones et al. 1974; Smith and Davis 1977). In HEK 293 cells transfected with the human DAT (hDAT), L-amphetamine has been shown to serve as a potential substrate for the transporter (Sitte et al. 1998). In regards to measurements of transporter currents, the potency associated with L-amphetamine was three to six times lower than that reported with D-amphetamine, while both D- and L- amphetamine produced similar currents. This is the first time that the high temporal resolution of voltammetry has been used to examine *in* vivo differences in the presence of rapid, first-time exposure to stimulants as opposed to alternate systemic routes of administration. The data presented in this chapter differ from data collected supporting greater differences in potency between the amphetamine isomers and a lack of kinetic differences that have been supported by data from alternative methodologies (Kuczenski et al. 1995; Kanbayashi et al. 2000).

For these studies, L-amphetamine was given in combination with D-amphetamine and the results support a theoretical mechanism describing the role of L-amphetamine in altering D-amphetamine-evoked DA release. One possibility involves effects not previously described of L-amphetamine interactions with the DAT. DA neurotransmission is rapidly regulated by the DAT, and a plausible explanation is a novel relationship between the DAT and L-amphetamine (Gulley and Zahniser 2003). On the order of seconds (acute amphetamine exposure), previous information support the down regulation of DAT activity in two ways: by direct inhibition of D-amphetamine or via the internalization of the DAT from the membrane to intracellular locations regulated by protein kinase C. L-amphetamine could block the rapid down regulation of membrane DAT activity caused by D-amphetamine. This would allow the rapid efflux of DA through the DAT and a faster influx of DA via DAT reuptake after the

DA signal amplitude approaches its peak measurement. Potential L-amphetamine competitive inhibition may block D-amphetamine for the D_2R autoreceptors that would phosphorylate the DAT downstream via PKC. A second explanation could involve a target site on the DAT where L-amphetamine may inhibit rapid down regulation caused by D-amphetamine. This hypothesis could be studied in cells transfected with the hDAT, while measuring the V_{max} of D-amphetamine evoked DA efflux with or without L-amphetamine. The study of second messenger regulation involved in this process would be valuable for determining its role in DAT membrane expression and/or activity. While these are potential mechanisms of activity, it is possible that these differential effects are being determined via other cellular targets such as MAO inhibition, V-MAT effects, or direct regulation of the DAT and/or DA receptors.

These studies were designed to measure the effects of amphetamines used for the treatment of ADHD on DA neurotransmission however some caveats should be noted. While a racemic mixture of D- and L-amphetamine was used and compared to D-amphetamine, this combination of amphetamine is not commercially available except in the form of Adderall®, 25% L-amphetamine: 75% D-amphetamine. In Chapters 4 and 5, we present data describing the effects of Adderall similar to what is commercially available. Therapeutic levels of amphetamine are projected to be in the range of 10-50 μM (Shader er al. 1999; Solanto et al. 2001) or even lower, however, the concentrations used for these studies may have been higher. Concentrations were used that would have yielded effective tissue concentrations in the range of 40-400 μM based on local dilution of the applied drug after ejection from the micropipette (Gerhardt and Palmer 1987). Data collected at lower drug concentrations will be presented in Chapters 4 and 5. The F344 rats that were used for analysis are not animal models of ADHD; therefore the information gained from these studies explain the normal biology of the system and not a diseased model. The recordings completed for this study were taken from the striatum and nucleus accumbens core of rats allowing for predominant measurement of DA versus other neurotransmitters that may be important to the effects of amphetamine on the

CNS. The drug solutions were locally applied to the brain region of interest circumventing issues of systemic nature that would be caused by oral or intraperitoneal dosing of amphetamine (Gerasimov et al. 2000). It is necessary to carry out future studies that will use lower doses/concentrations of stimulant medications to mimic the therapeutic effects (Kuczenski and Segal 2001).

The data support that D,L-amphetamine and L-amphetamine locally applied to the striatum and nucleus accumbens result in more rapid release of DA in comparison to evoked DA release caused by D-amphetamine. The data shown here indicates that L-amphetamine does not significantly increase DA release evoked by D,L-amphetamine, yet it evokes a similar DA response when applied alone. In correlation with clinical and experiment data, our findings suggest that the presence of L-amphetamine given alone, or in combination with D-amphetamine can cause differential behavioral effects relative to D-amphetamine. Future studies are needed to create a better explanation of the mechanisms of drugs that are used in the treatment of ADHD.

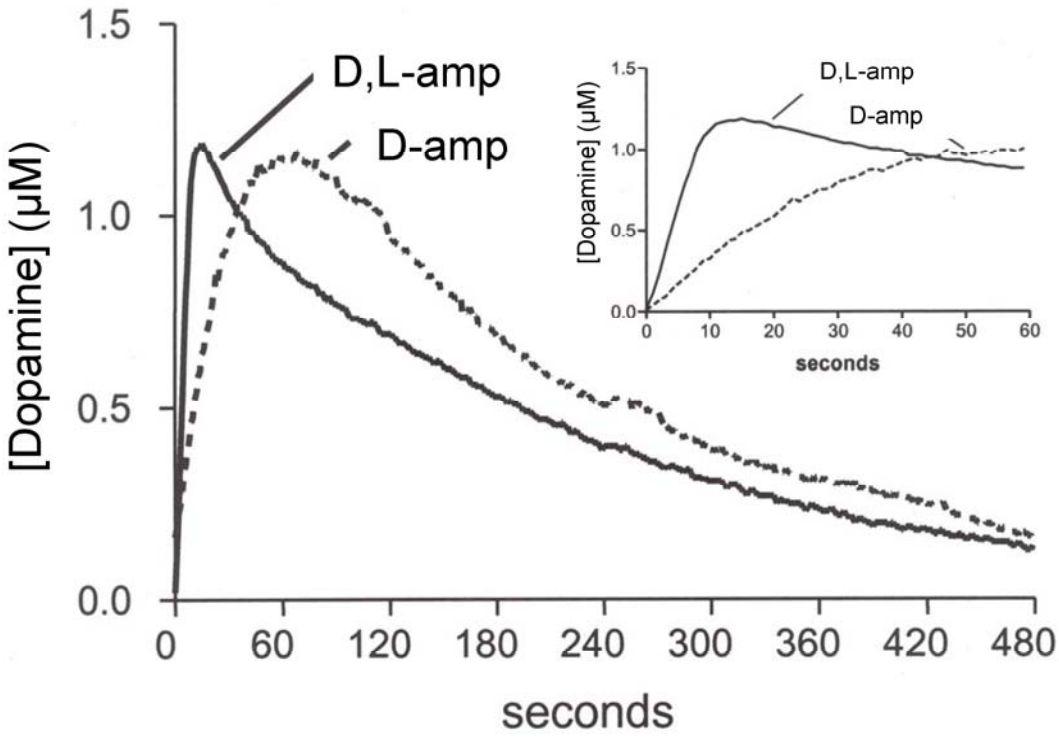

Figure 3.1 Representative DA Release Signals Evoked by D- and 2 x D,L-amphetamine

Representative DA release signals from the F344 rat striatum indicating the time course of DA release. The faster T_R and T_{80} of 2x D,L-amphetamine is indicated by the solid line and the longer time kinetics of D-amphetamine is shown by the dashed line. **Inset:** The first minute of DA release of these representative signals and their significant differences.

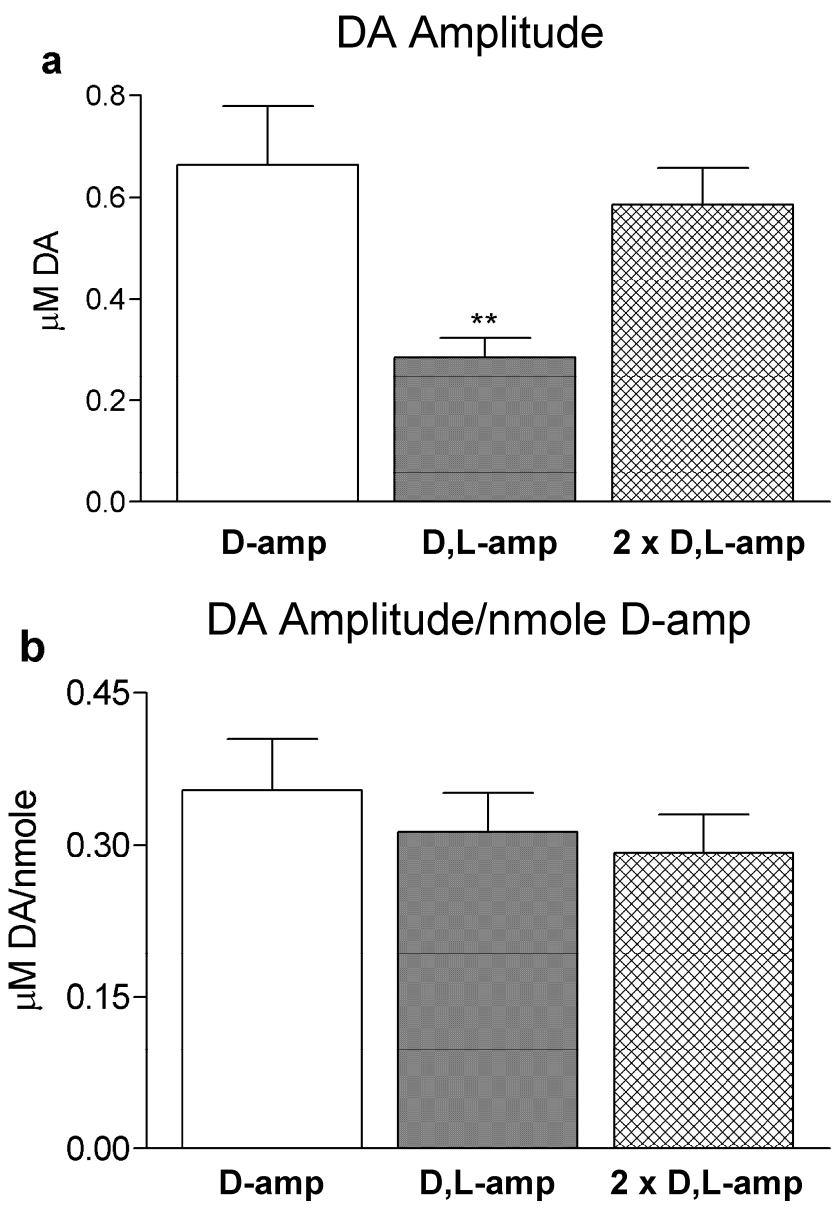

Figure 3.2 Amplitudes of Amphetamine-Evoked DA Signals in the Striatum

a. Amplitudes of DA release recorded in the striatum following local application of D-amphetamine, D,L-amphetamine, and 2 x D,L-amphetamine. 2 x D,L-, D-amphetamine versus D,L -amphetamine (**p<0.01). **b.** Amplitudes of DA release normalized with respect to nanomoles D-amphetamine in the drug solution. Redox ratios of predominantly DA confirmed signal identities.

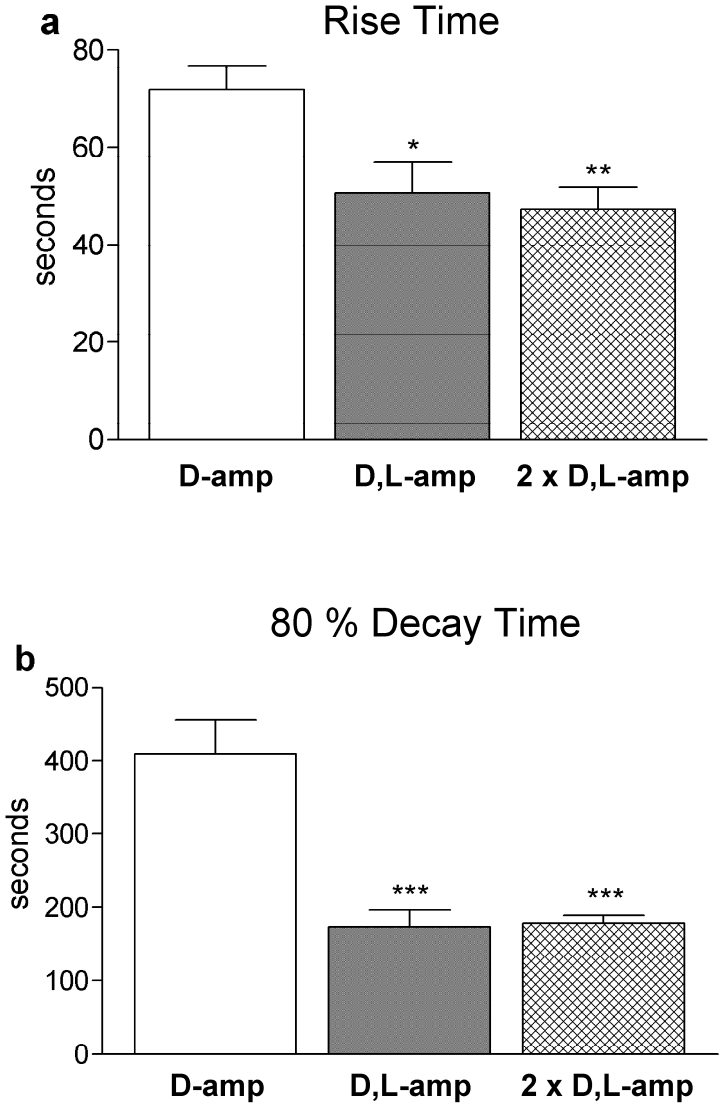

Figure 3.3 Kinetics of Amphetamine-Evoked DA Release Signals in the Striatum

a. Rise times of DA release signals evoked by local applications of amphetamine in the rat striatum. D,L-amphetamine and 2x D,L-amphetamine versus D-amphetamine; *p<0.05 and **p<0.01, respectively. Redox ratios of predominantly DA confirmed signal identities. **b.** T_{80} decay times of DA release signals evoked by local applications of amphetamine in the rat striatum. D,L-amphetamine and 2x D,L-amphetamine versus D-amphetamine; ***p<0.001. Redox ratios of predominantly DA confirmed signal identities.

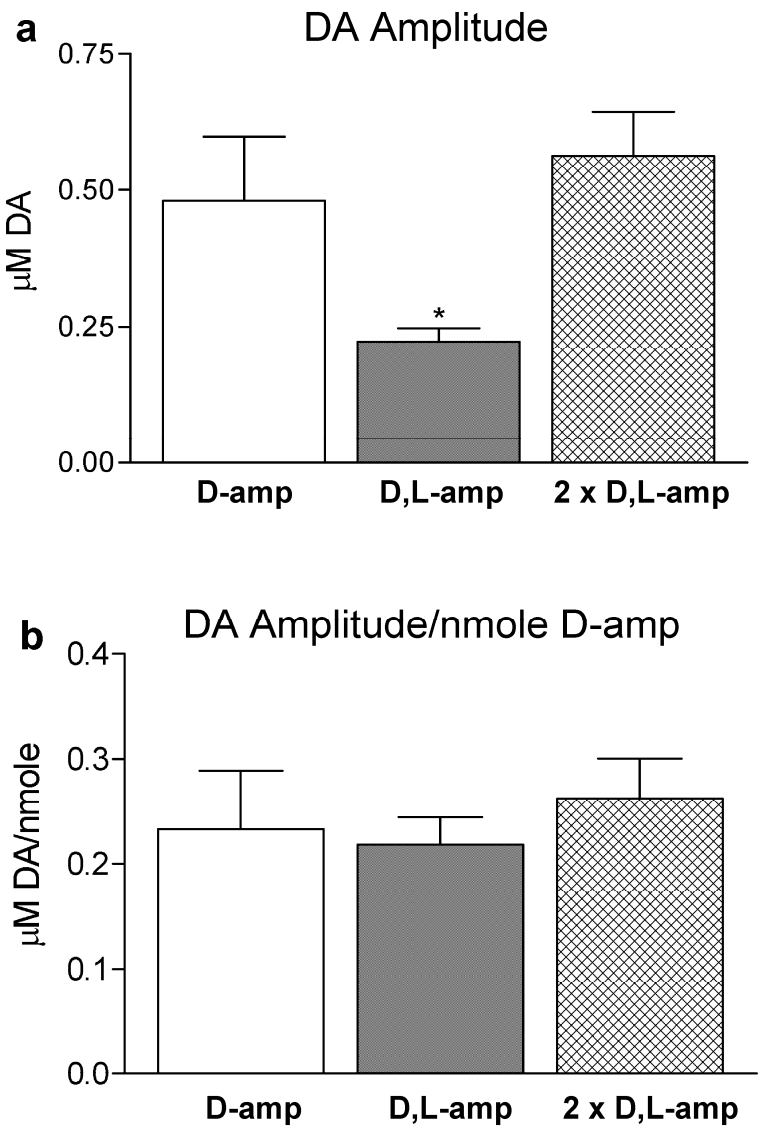

Figure 3.4 Amplitudes of Amphetamine-Evoked DA Signals in the Nucleus Accumbens Core

a. Amplitudes of DA release recorded in the nucleus accumbens core following local application of D-amphetamine, D,L-amphetamine, and 2 x D,L-amphetamine. D,L-amphetamine versus D-amphetamine (*$p<0.05$). **b.** Amplitudes of DA release normalized with respect to nanomoles D-amphetamine in the drug solution. Redox ratios of predominantly DA confirmed signal identities.

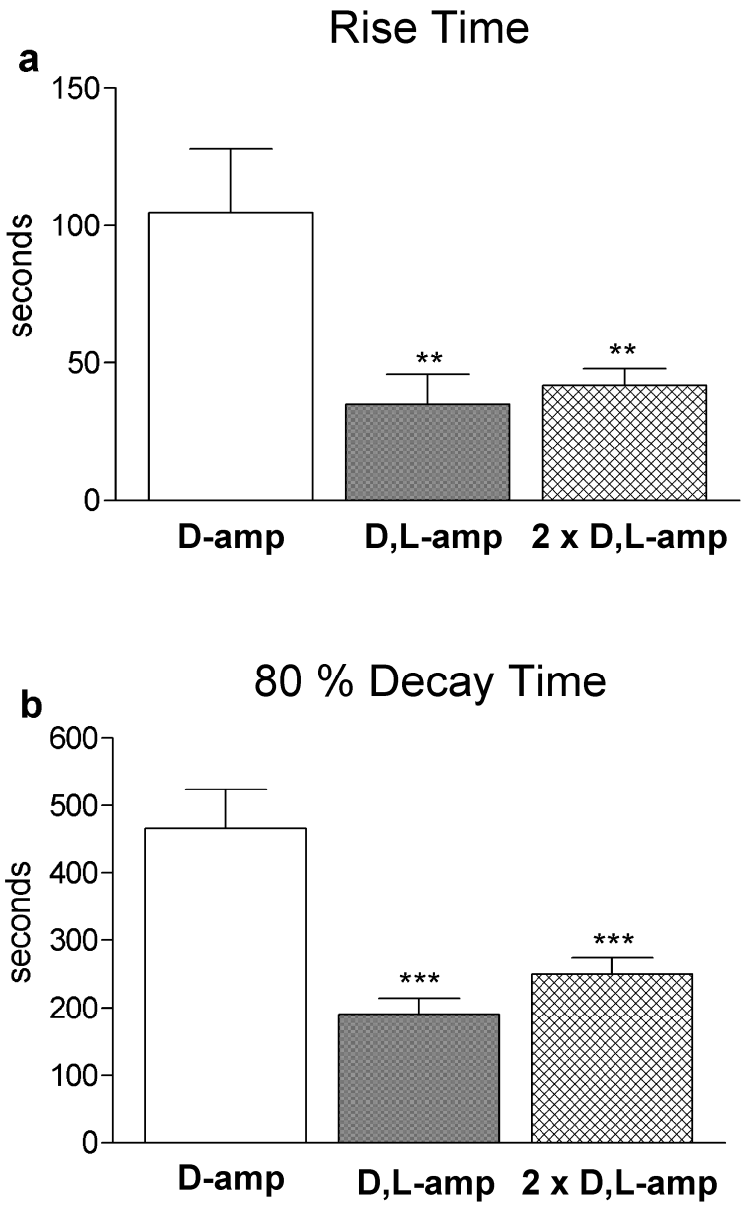

Figure 3.5 Kinetics of Amphetamine-Evoked DA Release Signals in the Nucleus Accumbens Core

a. Rise times were significantly longer for D-amphetamine versus D,L-, and 2 x D,L- amphetamine (**p<0.01) **b.** T_{80} decay times were significantly longer for D-amphetamine versus D,L-, and 2 x D,L- amphetamine (***p<0.001).

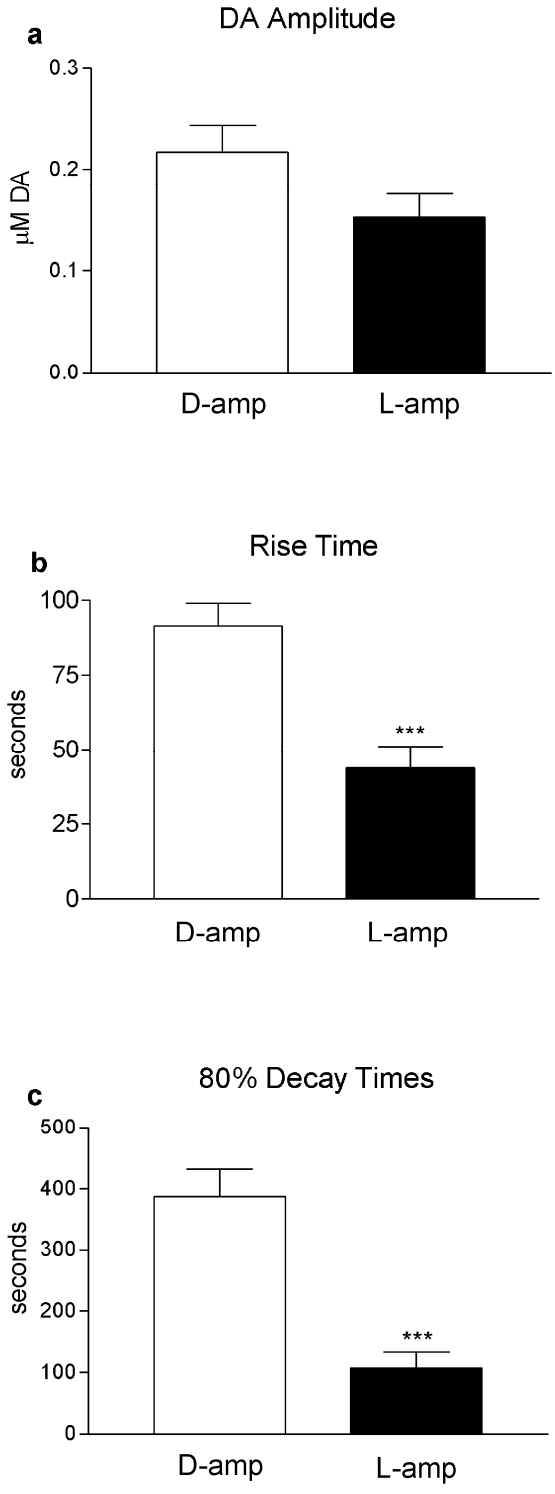

Figure 3.6 Amplitude and Kinetics of D- and L-amphetamine Evoked DA Release in the Striatum (Continued on next page)

Figure 3.6 Continued a. DA release signal amplitudes comparing the effects of single enantiomer applications of D- and L-amphetamine in the striatum revealed no significant differences. **b.** L-amphetamine rise times were significantly faster than D-amphetamine rise times (***$p<0.001$). **c.** T_{80} decay times of L-amphetamine were significantly faster than D-amphetamine (***$p<0.001$).

Portions of this work have been published in Psychopharmacology:

Glaser P.E.A., Thomas T.C., Joyce B.M., Castellanos F.X., Gerhardt G.A. (2005) Differential effects of amphetamine isomers on dopamine release in the rat striatum and nucleus accumbens core. Psychopharmacology (Berl) 178: 250-258.

Chapter 4: Adderall® Produces Increased Striatal Dopamine Release and A Prolonged Time Course Compared to Amphetamine Isomers

Introduction

Adderall® has been available for clinical use since the 1990s and was initially advertised as a long-acting amphetamine that would eliminate the need for multiple administrations during the day (Popper 1994). The drug made a prominent entry into the market and by the turn of the century constituted 25% of the prescriptions written for ADHD (Goodman and Nachman 2000). Recent data indicate that Adderall® in its extended release form (Adderall XR®) continues to increase in yearly sales that now outsell the main methylphenidate sustained release form, Concerta® (Mathews 2006). Adderall® consists of a combination of four amphetamine salts: D-amphetamine saccharate, D,L-amphetamine aspartate, D-amphetamine sulfate, and D,L-amphetamine sulfate at equal weights. This mixture gives an approximate amount of D- versus L-amphetamine of 75-80% and 20-25% in a 10 mg tablet (Patrick and Markowitz 1997).

The prolonged activity of this drug in its immediate release form has been suggested by the pharmaceutical company (Hampshire, Chineham, England) to be due to the differential absorption of the component salts, however these data were never published (Popper 1994). The first randomized, double-blind, crossover comparison of Adderall® versus D-amphetamine supports that Adderall® worked slightly faster and lasted longer than D-amphetamine (at the same total doses) in decreasing hyperactivity, while the two drugs displayed similar potencies (James et al. 2001). There are few published studies reporting the *in vivo* DA releasing properties of clinically used stimulants such as Adderall® and Dexedrine® (100% D-amphetamine).

D-amphetamine and related isomers are believed to promote presynaptic release of dopamine (DA) in the striatum by inducing reverse transport and blocking the reuptake capabilities of the dopamine transporter (DAT) (Glowinski

et al. 1966; Carboni et al. 1989; Sulzer et al. 1993, 1995; Pierce and Kalivas 1997; Gnegy et al. 2004). The apparent interaction between amphetamine and DA/NE neurotransmission supports the current consensus hypothesis that ADHD symptomatology results from dysregulation of the release properties of catecholaminergic projections to the prefrontal cortex and/or striatum (Solanto et al. 2001; Biederman et al. 2002). The data support differences between the pharmacokinetic properties of D-amphetamine and L-amphetamine. L-amphetamine is noted to be slightly more potent than D-amphetamine in use as a sympathomimetic drug while the D-isomer is suggested to be 3 to 4 times more potent than the L-isomer in acting as a central nervous system stimulant (Goodman et al. 2001). Rats are known to self administer L-amphetamine and data support that it is effective in treating children with ADHD (Arnold et al. 1972, 1976; Yokel and Pickens 1973,1974).

In this study, we investigated clinical reports of differential response to Adderall® by comparing the dynamics of DA release evoked by D-amphetamine, D,L-amphetamine, and Adderall® in the striatum of anesthetized rats. Our prior studies support that there are differences in kinetics of evoked DA release that differ between D-amphetamine and D,L- amphetamine when locally-applied in rat striatum (Chapter 3). High speed chronoamperometry coupled with Nafion®-coated single carbon fiber microelectrodes was used to test the hypothesis that the enantiomers and/or components of Adderall® evoke greater DA release in rat striatum with a longer time course. This is the first *in vivo* demonstration of the effects of Adderall® versus D-amphetamine and D,L-amphetamine on DA neurotransmission using local applications of drugs to evaluate their effects in the rat brain. Drugs were applied in low levels to better simulate clinically relevant levels of these ADHD medications, and locally to eliminate drug pharmacokinetic issues from the study. The success of stimulant treatments for ADHD to decrease hyperactivity and increase attention is known to be highly variable from one patient to another (Rapoport et al. 1978; Elia et al. 1991). Since the primary purpose of these studies was to assess mechanistic properties using low levels of amphetamine, we chose normal, developmentally mature adult (3-6 month

old), F344 rats to study the effects of amphetamine isomers on striatal DA neurotransmission.

Methods

Drug Concentrations used for *In Vivo* Electrochemical Recordings

The volume of applied drug was kept constant at 500 nl and was measured using a dissection microscope fitted with a calibrated reticule (1 mm change=25 nl of fluid) (Cass et al. 1992, 1993a; Friedemann and Gerhardt 1992). Drugs were dissolved in 0.9% physiological saline and final drug solutions were brought to a pH of 7.4. Adderall®, D,L-amphetamine or D-amphtamine solutions were applied in the following amounts (0.68 nanomoles Adderall®, 1 nanomole D,L-amphetamine, and 0.5 nanomoles D-amhetamine). Therefore a constant 0.5 nanomoles of the D-amphetamine isomer were applied in all drug treatments in Chapter 4 in order to investigate the effects of differing amounts of L-amphetamine. **For methodological details, see Chapter 2.**

Results

Signal Confirmation

After local applications of the amphetamine solutions to the striatum, the resulting DA signals exhibited slow release and uptake properties as compared to the faster properties of DA signals produced by depolarization with potassium applications (Friedmann and Gerhardt 1992). We confirmed that our microelectrodes were measuring primarily DA by comparing the mean redox ratios obtained *in vivo* to those of *in vitro* calibrations, finding no significant differences similar to data shown in Figure 2.1 of Chapter 2. However, our redox ratios were significantly different from the much lower ratios obtained from *in vitro* measures of serotonin (5-HT) and ascorbic acid (Fig. 2.1). Taken together, our

measurements confirm that Nafion®-coated carbon fiber microelectrodes used for these studies were capable of measuring signals that match the chemical fingerprint for DA upon local applications of amphetamine solutions and that signals produced by locally-applied D-amphetamine were predominantly DA.

Comparisons of Adderall®, D-amphetamine, and D,L- amphetamine in the Rat Striatum

These studies investigated the effects of locally applied amphetamine solutions of D,L-amphetamine, Adderall®, and D-amphetamine. Significant differences were found among the resulting DA signal amplitudes. Figure 4.1 shows three representative DA release signals from applications of D-amphetamine, D,L-amphetamine, and Adderall® and the differences in amplitudes and time courses of the signals. The respective *in vivo* redox ratios (0.7-0.8) of the three drug-induced signals, indicative of predominantly DA release signals, are shown in the inset of Figure 4.1.

Applications of Adderall® resulted in significantly greater DA release amplitudes compared to D-amphetamine ($p<0.001$) and D,L- amphetamine ($p<0.001$) supportive of a greater effect on DA neurotransmission (Fig 4.2 a). Adderall® showed nearly a 40% greater effect on DA release amplitude in comparison to the other amphetamine isomers, providing the first *in vivo* data in parallel with the noted clinical efficacy of this drug. The amplitude of DA released per nanoliter of drug applied was significantly greater for Adderall® than for the other drugs tested indicating greater effects on evoked DA release at the same volume of drug applied into rat striatum (Fig. 4.2 b; $p<0.001$). This effect was achieved with the same number of equivalents of D-amphetamine in the Adderall® drug solution. This information supports the increased magnitude of DA evoked by D-amphetamine in the context of a smaller amount of L-amphetamine.

Temporal differences in the drug-evoked signals were also observed in the rise times (T_R) and 80% decay times (T_{80}) among Adderall®, D,L-

amphetamine, and D-amphetamine (Fig. 4.3 a, 4.3 b; $p<0.001$, $p<0.05$). Adderall®, as shown in Figure 4.3 a, evoked DA release over a significantly longer period of time as compared to the other drugs supporting the increased time course of activity associated with this drug in the clinic ($p<0.001$). D-amphetamine evoked DA release over a small but significantly longer time period in comparison to D,L-amphetamine, replicating our work described in Chapter 3 (Fig. 4.3 a; $p<0.05$). The 40% longer effect on T_R by Adderall® greatly contributes to the increased amplitude and total area of Adderall®-evoked DA signals. Figure 4.3 b shows the significant differences in decay times among Adderall®, D-amphetamine, and D,L-amphetamine ($p<0.001$) indicative of an increased effect on the time course of DA release. The 50% longer T_{80} associated with the clearance of Adderall®-evoked DA release is likely due to a greater amount of DA that remains to be cleared. This likely has implications on the clearance capacity/availability of the DAT that is available to remove DA from the extracellular space (Cass et al. 1993b; Giros et al. 1996; Saunders et al. 2000; Gulley et al. 2002; Madras et al. 2002; Kahlig and Galli 2003; Kahlig et al. 2004). The increased T_{80} associated with Adderall® theoretically supports that the components of Adderall® cause an initial DAT upregulation to the plasma membrane or changes DAT conformation to allow more DA to pass through. This is followed by a very potent blockade and/or a decrease in plasma membrane or functional DAT levels that contribute to the elongated Adderall® evoked DA release signals.

Discussion

In summary, these are the first *in vivo* data reporting differences between Adderall®, D-amphetamine, and D,L-amphetamine and their effects on DA release signal properties in rat striatum with potential implications to their clinical differences. Adderall® produced DA release signals with the longest rise time and decay time as compared to D-amphetamine and D,L-amphetamine. Differences were also observed between the effects of D- and D,L-amphetamine

on the kinetics of DA release. Taken together, these data support the hypothesis that Adderall® evokes greater DA release in rat striatum with a longer time course, which is produced by the combination of amphetamine enantiomers and/or salts present in the drug solution.

While these studies were designed to investigate the effects of amphetamine isomers on DA release and uptake properties, the clinical implications of these data should be interpreted with caution. Acute local applications of stimulants in anesthetized animals do not mimic repeated systemic administration of stimulants in ADHD patients. Route and time course of amphetamine administration have been shown to affect DA measures in microdialysis studies (Kametani et al. 1995; Purdom et al. 2003) supporting the use of drug-naïve animals for these studies. Although it would be most ideal to apply the stimulants in amounts that mimic the level striatal nerve endings would see physiologically with administration of oral medication for ADHD, data on what these concentrations may actually be, and the comparative dosing levels for a rat compared to the human are not exactly known. Therefore we chose to apply the stimulants at a concentration designed to both give us consistent and comparable DA responses, as well as be at an intermediate portion of the D-amphetamine concentration-response curve. Further data on the stimulant concentration-response curves are shown in Chapter 5.

These data are consistent with previous reports that this drug has been designed to work over a longer time scale, including its initial effects on DA release and its overall activity half-life (Cody et al. 2003). In addition, these data correlate with clinical reports of increased duration of action of Adderall® when compared to D-amphetamine in regards to measures of locomotor activity in humans (James et al. 2001). These findings replicate our previous *in vivo* voltammetric studies that investigated differences between D-amphetamine and D,L-amphetamine evoked DA release signals (Chapter 3). In Chapter 3, we concluded that local applications of D,L-amphetamine caused a faster release of DA than D-amphetamine alone in the striatum and nucleus accumbens core. Our present study showed similarities to our previous data in respect to DA

release signal amplitudes as well as significant differences in the rise time of the signals between D- and D,L-amphetamine.

The current study points to differential interactions between these stimulant drugs and the DAT. The Adderall® data support that it regulates the DAT in such a way that it remains capable of reverse transport of DA into the extracellular space and then blocks the uptake of DA both over a longer time period (Fig. 4.4). It is also important to note that nearly all of the DA signals recorded in the presence of Adderall® were prolonged and signal decay was typically longer than 5 minutes, almost double the signal decay times of the other drugs tested. The kinetic differences observed indicated an overall elongated Adderall® effect on DA release similar to previous DA uptake studies carried out in the rat striatum after exposure to a selective DA uptake inhibitor, GBR- 12909 (Cass and Gerhardt 1995). The amplitude of DA released per volume of drug applied was highest with Adderall®, indicating a greater evoked DA response elicited than if the same volumes of D-amphetamine and D,L-amphetamine were locally administered. While the current data provide evidence for amphetamine salt and/or isomer dependent differences in DA evoked release, additional studies are necessary to examine specific component interactions with the DAT.

Due to the elongated time course of the Adderall®-evoked DA release signals, we suggest that Adderall® may be causing reverse transport of DA through the DAT over a longer time period. However, a consideration of recent literature describing DAT-substrate effects poses other theoretical explanations. The DAT is a dynamic protein that shuttles to and from the plasma membrane or can undergo conformational changes to regulate its ability to transport DA in and out of the cell (Kahlig et al. 2004; Johnson et al. 2005). Johnson et al. (2005) performed *in vitro* synaptosome studies to assess substrate dependent changes in DAT function and found that plasma membrane availability of DATs increased in response to acute exposure of amphetamine (<1 min), however the available DATs differed in their capacity to transport DA.

While these studies provide acute exposure of drug over 30 seconds, similar to the 20 second application time for our studies, there was an increase in

DAT plasma membrane availability over this time frame. Previous work investigating hDAT transfected HEK 293 cells has indicated that both enantiomers of amphetamine are substrates with differing potencies and similar transporter currents (Sitte et al. 1998). Likewise, *in vitro* data support differential DAT states that determine the rate and amount of DA that can pass in response to amphetamine (Kahlig et al. 2005). Kahlig et al. (2005) described two independent mechanisms by which DAT-mediated DA efflux can occur: a highly regulated facilitated exchange mechanism or a more rapid process that allows for bursts of DA efflux through an open channel.

Considering the noted *in vitro* observations of the DAT, we propose L-amphetamine in Adderall® may be altering DAT function or availability to regulate the efficiency by which DA is allowed to pass through. Upon initial application of Adderall®, 1) externalization of the DAT may occur (Johnson et al. 2005) similar to initial cocaine induced upregulation of the DAT to the plasma membrane (Zahniser and Sorkin 2004) or 2) DATs may undergo conformation changes to an open channel mode (Kahlig et al. 2005) to allow large amounts of DA to exit the cell representing the large amplitudes seen in our DA release signals. Secondary modifications of plasma membrane DAT function or availability (trafficking) could decrease plasma membrane levels of DATs similar to DAT levels after chronic cocaine exposure (Gulley et al. 2002; Zahniser and Sorkin 2004). The decreased plasma membrane availability of the DAT would lead to accumulation of extracellular DA over a longer time period to explain the elongated Adderall®-evoked DA release signals. This could be accomplished via DA receptor and PKC dependent protein-protein interactions that regulate DAT conformation and functional states (Fig. 4.4) (Torres et al. 2003; Khoshbouei et al. 2004).

In summary, we have shown that Adderall® has a greater effect on DA release in the rat striatum compared to D- and D,L- amphetamine. This study documents important information concerning low levels of ADHD drugs and the differences seen in evoked DA release signals when varying the ratio of D-amphetamine to L-amphetamine and salt components. These findings are

similar to clinical data suggesting that Adderall® may be a more potent or a longer acting drug choice for certain behavioral symptoms of ADHD. Future studies are needed to elucidate the specific mechanisms governing D-amphetamine and Adderall®-induced release of DA *in vivo*.

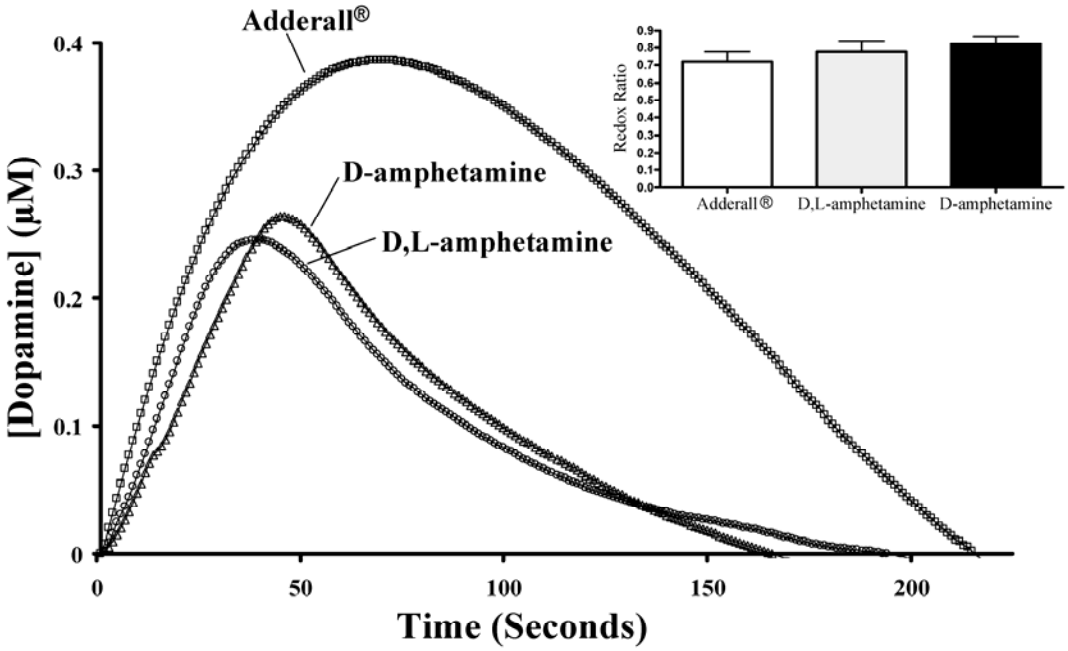

Figure 4.1 Representative DA Release Signals Caused by Local Applications of Adderall®, D-amphetamine, and D,L-amphetamine in the Striatum

Typical recordings indicating the time course of DA release in the striatum of rat brain induced by D,L-amphetamine (circles), D-amphetamine (triangles), and Adderall® (squares). All drug solutions contain an equivalent 0.5 nmol of D-amphetamine. **Inset:** Measured average *in vivo* redox ratios of all DA release signals, indicative of predominantly DA, included for statistical analysis (n=30, 42, 54 signals; error bars represent S.E.M).

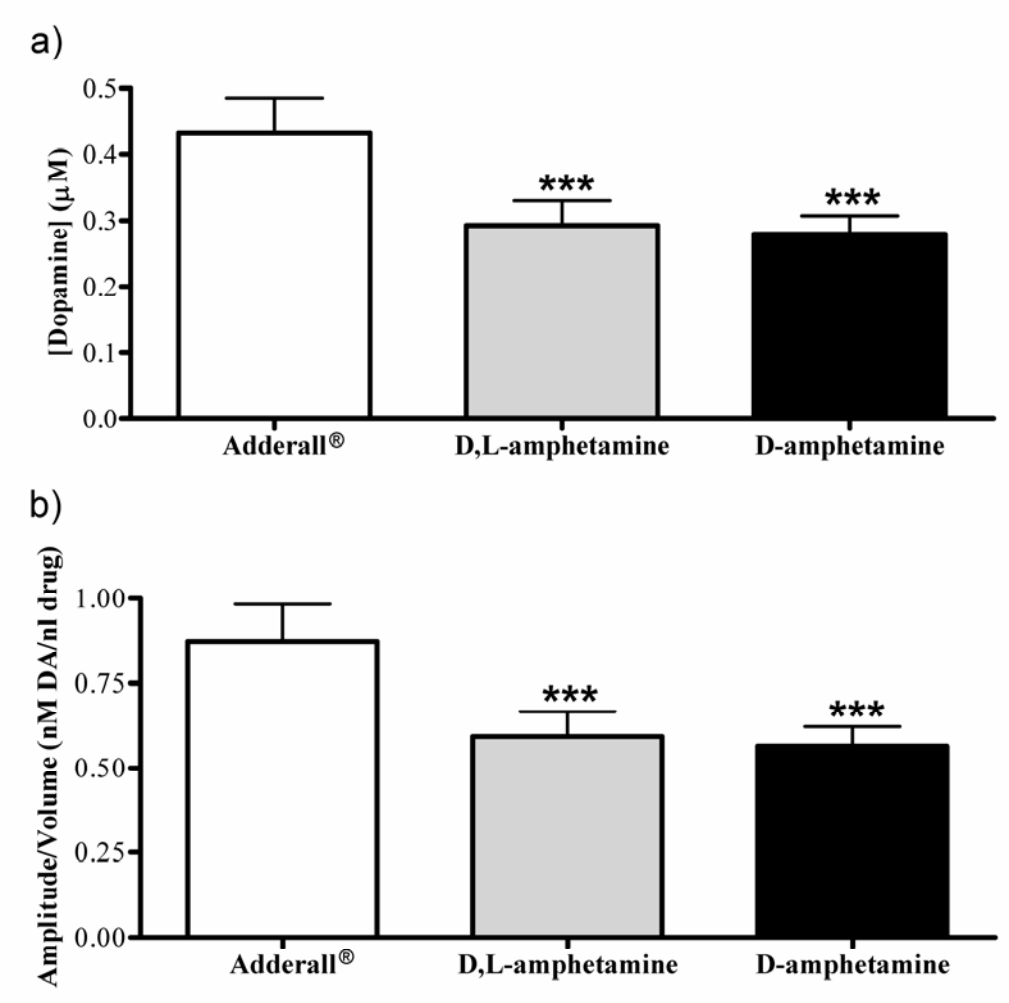

Figure 4.2 Amplitudes of DA Release Signals in the Striatum After Local Applications of Adderall®, D,L-amphetamine, and D-amphetamine

a. The amplitudes of DA release measured in the rat striatum after local application of Adderall® were significantly greater compared to D-amphetamine, or D,L-amphetamine (***$p<0.001$). **b.** Amplitude of DA (nM) recorded per volume of drug applied (nl) in the rat striatum was significantly greater for Adderall® in comparison to D-amphetamine and D,L-amphetamine (***$p<0.001$). All drug solutions contained an equivalent 0.5 nmol D-amphetamine. Data analyzed by MANOVA with Tukeys post-hoc comparisons; error bars represent S.E.M. (n=animals, signals; 10, 54; 10, 42; and 10, 30).

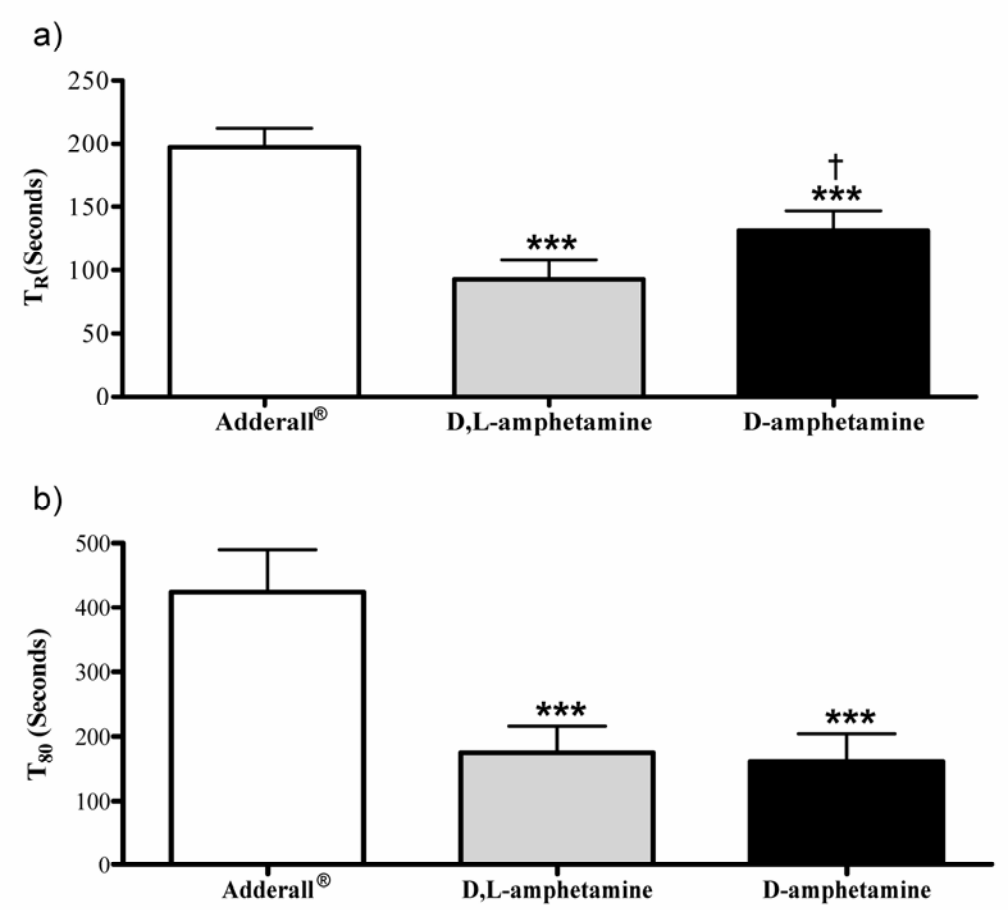

Figure 4.3 Kinetics of DA Release Signals After Local Applications of Adderall®, D,L-amphetamine, and D-amphetamine in the Striatum

a. Rise times for Adderall®-evoked DA release signals in the rat striatum were significantly longer than D-amphetamine or D,L-amphetamine evoked DA signals (***$p<0.001$). Rise times for D-amphetamine evoked DA release signals were significantly longer than D,L-amphetamine evoked DA signals similar to prior studies (†$p<0.05$). **b.** T_{80} decay times for Adderall®-evoked DA release signals in the rat striatum were significantly longer than those produced by D-amphetamine or D,L-amphetamine (***$p<0.001$). Data analyzed by MANOVA with Tukeys post-hoc comparisons; error bars represent S.E.M. (n=animals, signals; 10, 54; 10, 42; and 10, 30).

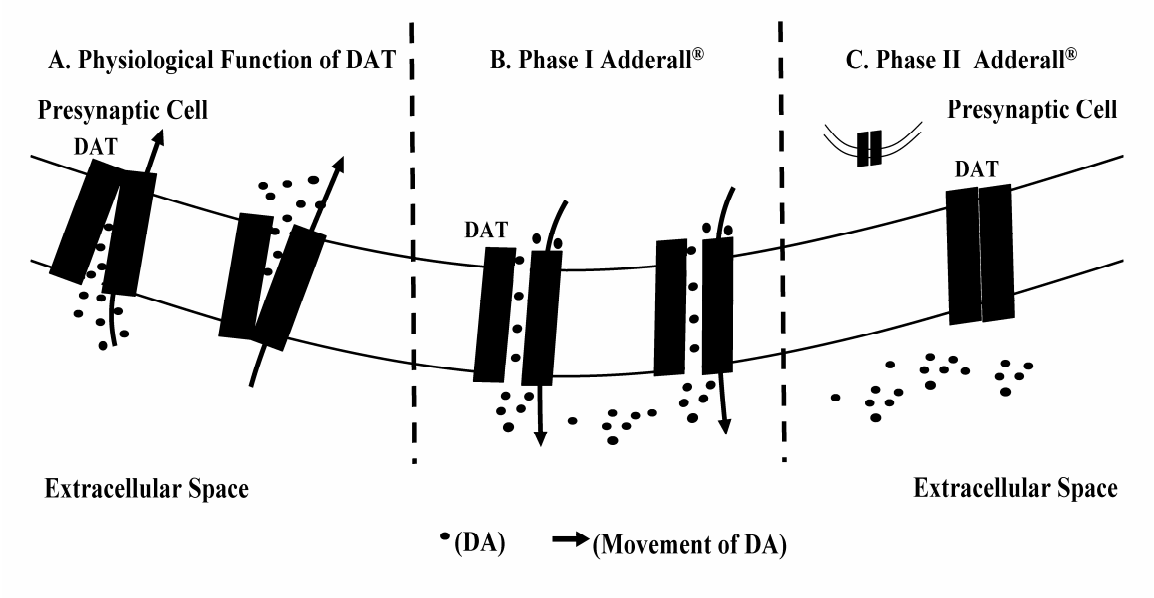

Figure 4.4 Theoretical Model of Activity Describing Adderall®
Evoked- DA Release Signals

A. Physiological Function of the DAT: Under normal conditions, the DAT functions to remove presynaptically released extracellular DA for recycling and intracellular degradation (Cooper et al. 1996). **B. Phase I Adderall®:** Amphetamine is theorized to cause a reversal of DA transport through the DAT while also blocking future DA reuptake (Seiden and Sabol 1993; Solanto et al. 2001). Down regulation of DAT activity results via amphetamine blockade, modulation of DAT function, or endocytosis of the DAT over a longer time period for Adderall® (Gulley and Zahniser 2003; Torres et al. 2003; Kahlig and Galli 2003; Khoshbouei et al. 2004; Kahlig et al. 2005). Early on, Adderall® may potently induce an open-pore mode of the DAT or cause an upregulation of plasma membrane DATs to allow increased levels of DA to pass through (Kahlig et al. 2005; Johnson et al. 2005). **C. Phase II Adderall®:** Down regulation of plasma membrane DATs or inhibition of DAT function supports the elongated decay times seen with Adderall® (Saunders et al. 2000; Gulley et al. 2002; Zahniser and Sorkin 2004; Kahlig et al. 2004).

Portions of this work have been published in Psychopharmacology

Joyce B.M., Glaser P.E.A., Gerhardt G.A. (2006) Adderall produces increased striatal dopamine release and a prolonged time course compared to amphetamine isomers. Psychopharmacology (Berl) In press.

Chapter 5: Reverse Microdialysis Studies of the Effects of Psychomotor Stimulants Used to Treat ADHD on Extracellular Dopamine

Introduction

Attention-deficit/hyperactivity disorder (ADHD) has an estimated prevalence of 3-17% in school age children with stimulant medications being used as the predominant mode of treatment (Lahey et al. 1999; Goldman et al. 1998; Solanto et al. 2001). Cardinal symptoms of ADHD, such as impulsivity, inattention, and motor restlessness, lead to impairment of function in social, school, and home settings that are improved by treatment with stimulant medications (DSM-IV-TR 2000). In 2004, prescription sales of stimulants for ADHD totaled $3.1 billion dollars, while 2.5 million children and 1.5 million adults were estimated to use these medications (Vedantam 2006). Until 2005, sales of methylphenidate exceeded the other treatment options. However, while collecting the data for these studies, sales of Adderall® ($1.16 billion) were similar or surpassed sales of methylphenidate ($929 million) medications during calendar year 2005 (Mathews 2006). At the current time, all ADHD medications are under scrutiny from the media in light of FDA hearings regarding potential risks and a need for further characterization.

Dexedrine® (D-amphetamine), Adderall® (mixed-salts amphetamine), and Ritalin® (methylphenidate) are thought to reduce the symptoms of ADHD via actions on dopamine (DA) and norepinephrine (NE) nerve endings in the CNS (Solanto et al. 2001). However, their mechanisms of action on neurotransmitter release and uptake remain speculative (Ohno 2003). Specifically, frontal cortex and subcortical neural networks (including structures of the basal ganglia) are implicated in MRI morphological studies of ADHD versus healthy controls (Durston 2003; Solanto et al. 2001; and Sowell et al. 2003). Within these brain structures, catecholaminergic membrane transporters are targets of most ADHD stimulants; however, previous data support differential mechanistic theories at the level of neurotransmission. Stimulants inhibit the dopamine transporter

(DAT) causing DA levels to increase (Bergman et al. 1989; Cadoni et al. 1995; Wall et al. 1993). While amphetamine is suspected to facilitate DA neurotransmission, in general, it is uncertain what differences exist between the optical isomers of amphetamine and how they compare to the effects of methylphenidate. In the 1990s the drug Adderall®, containing a mixture of 75-80% D- and 20-25% L-amphetamine across four component salts, was introduced and marketed as a robust treatment for the symptoms of ADHD compared to other medications (Popper 1994; Patrick et al. 1997). One clinical study compared Adderall® to D-amphetamine and found that Adderall® decreased specific symptoms of hyperactivity slightly faster and over a longer time period than D-amphetamine (James et al. 2001). Other clinical trials support that Adderall® is more effective than methylphenidate on outcomes measured 4 to 5 hours after dosing (Pelham et al. 1999). Adderall® given as a single morning dose was equivalent to methylphenidate received twice daily in regards to clinical improvements during the day (Pelham et al. 1999). In addition, methylphenidate has been characterized to have similar effects on DATs as cocaine. Sonders et al. (1997) categorized pharmacological agents that act on the human dopamine transporter (hDAT) into two groups: DA-like (including DA and amphetamine) and cocaine-like (including cocaine and methylphenidate).

Our previously published *in vivo* voltammetry data showed differences in kinetics between amphetamine optical isomers (Chapter 3). For these studies, we found that drugs with L-amphetamine produced faster rise times and signal decay times compared to D-amphetamine. Additionally, data collected by our group showed greater amplitudes and longer DA response signal kinetics following local applications of Adderall® in comparison with D-amphetamine and D,L-amphetamine (Chapter 4). Our previous studies support differential interactions with the mechanisms responsible for presynaptic DA release.

Due in part to the clinical reports of Adderall® efficacy in comparison to other ADHD treatments, we set out to test the hypothesis that D-amphetamine and Adderall® (75% D- 25% L- amphetamine) will differ in resulting extracellular DA response over a range of concentrationss. In addition, we speculated that

increased DA levels will be produced by amphetamine compared to little or no DA response after local applications of methylphenidate and cocaine in anesthetized rats. This concentration-response characterization was carried out using reverse microdialysis in drug-naïve animals to circumvent issues regarding DAT trafficking and/or change in function following substrate exposure (Kahlig and Galli 2003; Kahlig et al. 2004; Purdom et al. 2003). Finally, we compared these drugs with a novel combination of 25% D- 75% L- amphetamine and termed this “Reverse Adderall” to investigate the efficacy and potency of a drug with more L- than D-amphetamine. We sought to construct concentration-response curves for DA and 3,4 dihydroxyphenylacetic acid (DOPAC) of these drug solutions to further describe the properties of stimulants that make use of different combinations of amphetamine isomers. The concentration range included clinically relevant and high-dose effective tissue concentrations potentially relevant to stimulant abuse. Using the technique of microdialysis we were able to take measures of DA metabolites following degradation by monoamine oxidase. We suspected that differential effects on metabolite levels would result. Studies of individual concentration-response curves of stimulant drugs will demonstrate differential effects the drugs have on DA and metabolite levels. In addition, these are the first experiments, to our knowledge, that have investigated these drugs in the context of ADHD using a local administration method.

While we have described the use of voltammetric studies to investigate the properties of stimulants at low levels, it is difficult to accurately predict what the resulting effective concentrations were. Voltammetry affords the ability to study neurotransmission with high temporal and spatial resolution; however, we lose a magnitude of sensitivity that is available using High Performance Liquid Chromatography (HPLC) with electrochemical detection (EC). Using HPLC-EC to analyze samples collected during reverse microdialysis (local application) of stimulant drugs allows for studies to be carried out with more accurately projected drug concentrations. These studies were designed to complement our previous studies and mimic longer administration (over 20 minutes) in converse

to the rapid pressure ejection used earlier (20 seconds). As a final rationale of this work, we proposed to investigate complete concentration-response studies using reverse microdialysis coupled with HPLC-EC. Our data are the first characterizations of these drugs across low (clinical) and high (abuse) levels using local applications of stimulant drugs. Investigations of concentration-response patterns were intended to increase our understanding of ADHD drug mechanistic activity by looking at their effects on DA and metabolite levels.

For methodological details, see Chapter 2.

Results

Basal Levels of DA and DOPAC

Measures of the analytes were consistent with previous data of samples taken from the striatum. Average baseline levels of DA (<10 nM) were measured and found to be similar to previously collected data in the striatum of anesthetized and awake-behaving rats (Gerhardt and Maloney 1999; Ferguson et al. 2003; Garris et al. 1994; Kawagoe et al. 1992; Parsons and Justice 1992) (Fig. 5.1-5.4). Baseline DOPAC levels were determined to be (~800-1000 nM) in the rats used for the D-amphetamine and Adderall® studies and were similar to previously reported levels (Ferguson et al. 2003) and lower than other reports of DOPAC measures that have been above 1000 nM. The DOPAC data were represented in percent of baseline due to increased variance in baseline samples collected from the rats used for the Reverse Adderall and methylphenidate studies (Fig. 5.5-5.8). Homovanillic acid (HVA) levels were determined as percent of baseline, and no additional analysis was completed due to similarities between the data sets (Fig. 5.11).

Comparisons of DA and Metabolite Response After Reverse Microdialysis of Stimulants in the Striatum

The twenty minute local tissue perfusions of drugs (including methylphenidate) induced a dose-dependent increase in DA overflow followed by a 60 minute time period to return to baseline supporting the DAT and DA uptake blocking effects of the tested stimulants (Fig. 5.1-5.4) (Wise and Hoffman 1992; Sulzer et al. 1993; Castellanos et al. 1996; Schweri et al. 1985). The resulting DA levels, at the highest concentration of drug, were similar to previous microdialysis measures of ~150 nM (Seeman and Madras 2002). The measures of DA were elongated over 40 minutes (2 samples) after stimulus compared to local perfusions of potassium, which depolarizes the cell and causes subsequent return of DA levels to baseline within 20 minutes (1 sample) after removal of potassium solution (Hebert et al. 1996; Purdom et al. 2003; Stanford et al. 2001). Furthermore, applications of low concentrations of stimulants did not result in DA levels being higher than levels after artificial cerebral spinal fluid (aCSF) control (Fig. 5.1-5.4).

The resulting D-amphetamine concentration-response curve of DA in rat striatum displayed a double-sigmoidal pattern that supports biphasic effects on DA stores and/or DAT trafficking (Fig 5.1) (Kahlig et al. 2005). Plateaus in the DA response occurred at the lower concentration (1 μM D-amphetamine) and at a higher concentration (100 μM D-amphetamine). At 0.1 μM D-amphetamine, little or no increase in DA overflow resulted in comparison to aCSF control; and no significant differences were found between 100 μM and 400 μM D-amphetamine supporting an upper plateau in DA measures (Fig 5.1). While two half-maximal effective concentration (EC_{50}) values are indicated for the lower (D-amphetamine I) and upper (D-amphetamine II) portions of this concentration-response curve (Table 5.1), the EC_{50} of D-amphetamine II was used to make conclusions regarding potency and efficacy.

The resulting methylphenidate concentration-response curve of DA in the rat striatum supports a dose-dependent increase in DA levels (Fig 5.2). Since

methylphenidate had previously been characterized as a DAT blocker and not a substrate that undergoes transport through the DAT, we hypothesized that we would see little or no change in DA levels in an anesthetized rat. Applications of 0.5-400 μM methylphenidate increased DA concentrations significantly greater than aCSF control. 0.1 μM methylphenidate did not cause increased DA levels significantly different from control indicating the lower plateau of the concentration-response curve. The two highest concentrations tested (100 and 400 μM) were not significantly different supporting an upper level plateau (Fig. 5.2).

Figure 5.3 displays the resulting Adderall® concentration-response curve of DA measured in the rat striatum. An upper plateau in DA levels occurred at 100 μM Adderall, as 100 μM and 400 μM Adderall were not significantly different in response. At 0.1 μM Adderall, DA levels were not significantly different from local application of aCSF control. Finally, the Reverse Adderall (75% L-amphetamine, 25% D-amphetamine) concentration-response curve of DA showed a dose-dependent increase in evoked DA at all concentrations tested except for 0.1 μM; which was not significantly different from aCSF control (Fig. 5.4). While Reverse Adderall was predominantly made of L-amphetamine, it did not increase DA levels to the extent of Adderall® at some concentrations (Table 5.1). The highest two concentrations of Reverse Adderall tested were significantly different supporting that a plateau of DA measures will likely occur at a higher concentration.

Comparisons of Potencies of Stimulants after Reverse Microdialysis in the Striatum

Potencies were extrapolated from the median concentration along the concentration-response curves for D-amphetamine II, methylphenidate, Adderall®, and Reverse Adderall and are represented as EC_{50} in Table 5.1. The stimulants in order of their potency on DA overflow were: methylphenidate (10 μM) > Adderall® (25 μM) > D-amphetamine II (50 μM) = Reverse Adderall (50

μM). The two potency concentrations for D-amphetamine represent EC_{50} values for both plateaus of the concentration-response curve of DA, however the most similar response in effects on DA levels to the other stimulants tested is represented in the above order of potency. The effects of methylphenidate on increasing DA levels could be due to an action of methylphenidate only seen with local application, but it is more likely that our anesthesia does not completely remove spontaneous neuronal activity supporting that methylphenidate blocked uptake of spike-dependent DA release (Sabeti et al. 2003; Kish et al. 1999). These data support the increased potency of Adderall® and replicate data from Chapter 4 that indicate that Adderall® had the greatest effect on DA release in comparison to D- and D,L-amphetamine. Finally, Reverse Adderall (made of predominantly L-amphetamine) and D-amphetamine were similar in potency supporting the DA releasing properties of L-amphetamine and replicating the data presented in Chapter 3 that DA signal amplitudes were similar between D- and L- amphetamine.

Comparisons of Efficacies of Stimulants after Reverse Microdialysis in the Striatum

At the highest effective concentrations, Adderall® and Reverse Adderall were similar in their effects on DA levels and caused greater increases in DA levels than D-amphetamine and methylphenidate. Measures of efficacy in regards to increased DA levels resulting from D-amphetamine and methylphenidate were similar. In a comparison of these stimulants versus cocaine, Adderall® ($p<0.001$) and D-amphetamine ($p<0.05$) caused significantly greater increases in DA levels than cocaine (Fig. 5.9 a). While these data did not support that methylphenidate caused significantly greater DA levels compared to cocaine, average DA increases resembled that caused by amphetamine isomers more so than cocaine (Fig. 5.9a). These data support an unpredicted dissociation of methylphenidate and cocaine, likely visible due to the local application of drugs used in these studies. Reverse Adderall ($p<0.01$) and L-

amphetamine ($p<0.05$) caused significantly greater DA levels when compared to the effects of cocaine. The lower DA levels produced by D,L-amphetamine are supported by previous voltammetric data that indicated faster kinetics of DA release and uptake in comparison to D-amphetamine and Adderall® (Chapters 3 and 4) (Fig. 5.9b) and also the approximate D-amphetamine in this drug solution was only half of the other amphetamine isomers in attempt to make comparisons with the data in Chapter 3. L-amphetamine caused similar DA levels to the other stimulants and replicated previous voltammetric data that demonstrated no differences in evoked DA amplitudes when compared to D-amphetamine (Chapter 3) (Fig. 5.9b).

Changes in Metabolite Levels Following Reverse Microdialysis of Adderall®, D-amphetamine, Methylphenidate, and Reverse Adderall

Figure 5.5 shows the individual tracings of detected DOPAC levels (represented as % of baseline) following reverse microdialysis of D-amphetamine at multiple concentrations. While some variability was found, D-amphetamine generally caused a dose-dependent decrease in DOPAC levels similar to Adderall and Reverse Adderall. The resulting response curve for changes in DOPAC levels did not mimic the double-sigmoidal pattern seen in the response curve of DA. D-amphetamine, Adderall®, and Reverse Adderall decreased DOPAC levels in a similar manner following local perfusion of drug at 120 minutes and continued to decrease DOPAC production up to one hour when DOPAC levels returned to baseline (Fig. 5.5-5.8). While methylphenidate caused increased DA levels similar to the other stimulants, it did not affect DOPAC levels in a consistent manner and was similar in this aspect to the effects of cocaine. Table 5.1 shows the concentrations that caused a half-maximal response extrapolated from the response curves for DOPAC. Differences in the maximal stimulant concentration effects on DOPAC levels are shown in Figures 5.10 a, b. DOPAC production was less significantly affected by methylphenidate and cocaine in comparison to Adderall® ($p<0.001$; Fig. 5.10a), and D-amphetamine

($p<0.01$, $p<0.05$; Fig. 5.10a). Reverse Adderall, L-amphetamine, and D,L-amphetamine all caused significantly greater effects on DOPAC levels in comparison to cocaine ($p<0.001$; Fig. 5.10b). An initial increase in DOPAC was seen following application of 100 µM and 400 µM methylphenidate followed by a decrease similar to that of other concentrations without a dose-dependent pattern (Fig. 5.6). Figure 5.11 shows measures of HVA following reverse microdialysis of D-amphetamine, methylphenidate, Adderall®, and Reverse Adderall. No clear dose-response pattern was detected across measures of HVA supported by the decreased levels of HVA in the rat CNS compared to nonhuman primates (Cooper et al. 1996).

Discussion

These data represent novel findings regarding the effects of D-amphetamine across a range of concentrations and the activity of local applications of methylphenidate compared to its analog, cocaine. The concentration-response curve for D-amphetamine displayed a double-sigmoidal pattern that supported dual-functionality properties of the DAT and/or differential mechanisms by which high and low concentrations of D-amphetamine affect DA efflux and DAT trafficking. These data support potent increased DA levels caused by local application of methylphenidate in a dose-dependent pattern. Replicating our data in Chapter 4, Adderall® resulted in the most efficacious effects on increased DA levels; while the EC_{50} (DA) of methylphenidate supported the greatest potency when compared to the other stimulant concentration-response data. Decreased DA levels caused by cocaine compared to higher DA levels after local application of methylphenidate indicate a dissociation between the local effects of methylphenidate and cocaine. Measures of DOPAC support differential interactions with the process of MAO degradation of DA between amphetamine stimulants and methylphenidate. Cocaine and methylphenidate were similar in effects on DOPAC production at the highest concentrations.

The data shown here were consistent with the known DA releasing properties of amphetamine, predominantly due to the DAT reversal of normal reuptake into the presynaptic terminal (Giros et al. 1996). Likewise, amphetamine has been shown to impair DA reuptake, inhibit MAO activity, and affect vesicular conditions that lead to emptying of vesicular stores via the vesicular monoamine transporter 2 (VMAT2) (Horn et al. 1971; Sulzer et al. 1995; Dubocovich et al. 1985; Heikkila et al. 1975; Uretsky and Snodgrass 1977; Green and El Hait 1978; Cadoni et al 1995). To our knowledge, this is the first *in vivo* study making use of local applications of a range of concentrations in drug naïve animals. We chose to carry out these studies in this manner based on information supporting the dynamic changes that occur in DA neuronal systems in response to DAT substrates and inhibitors. Purdom et al. (2003) showed data supporting that the order of administration of different concentrations of D-amphetamine significantly affected DA and DOPAC levels. These results were attributed to persistent changes in DAT membrane availability and/or function. Other *in vitro* studies have shown substrate dependent trafficking of the DAT to and from the plasma membrane and subsequent ability to transport DA (Kahlig et al. 2005; Johnson et al. 2005; Saunders et al. 2000; Kahligh et al. 2004; Kahlig and Galli 2003).

One possible mechanism for the D-amphetamine double-sigmoidal concentration-response curve involves targeting of specific DA pools and amphetamine concentration-dependent effects. Some data support contribution of both cytosolic and vesicular stores to the released DA following exposure to amphetamine (Pifl et al. 1995); while other data indicate a predominant vesicular DA contribution (Jones et al. 1998). Jones et al. (1998) measured DA released following electrical stimulation and amphetamine perfusion of striatal brain slices and noticed a delay in DA release with amphetamine, supporting that the DA had to be redistributed to the cytosol prior to being released from the cell. Based on these different contributions to amphetamine-evoked DA increases, our data are in agreement with previous investigations that support lower concentrations of D-amphetamine release “newly synthesized” DA pools in the cytosol, and higher

concentrations contributed to the emptying of vesicular stores. As a result a biphasic pattern and a marked increase in the amount of DA released at the higher concentrations (Seiden et al. 1993; Langeloh and Trendelenburg 1987; Sulzer et al. 1993, 2005).

An alternative mechanism for the D-amphetamine concentration-response curve might be explained by an upregulation of DAT levels caused by stimulation of D_2R autoreceptors leading to second messenger regulation. Previous data support a link between stimulation of D_2R autoreceptors and levels of membrane DATs (Parsons et al. 1993; Cass and Gerhardt 1994; Rothblat and Schneider 1997; Dickinson et al. 1999; Hoffman et al. 1999; Mayfield and Zahniser 2001). For example, *in vivo* measures of DA have shown decreased DA clearance in the striatum, prefrontal cortex, and nucleus accumbens after administration of the D_2R antagonist raclopride (Cass and Gerhardt 1994). In addition, acute amphetamine stimulation has been shown to cause increased synaptosomal DAT surface expression that occurs within 30 seconds of treatment (Johnson et al. 2005), while other reports have indicated rapid trafficking of the DAT. These studies support that the effects we observed over 20 minutes could be caused by DAT trafficking (Fleckenstein et al. 1999; Saunders et al. 2000). Due to the comparatively increased sensitivity of D_2R autoreceptors, low levels of extracellular DA are sufficient to stimulate these autoreceptors that would result in increased DA clearance (Cooper et al. 2003) (Fig. 5.12). The small amounts of released DA required to stimulate these autoreceptors would be taken up quickly through increased levels of membrane DATs, supporting the effects we see with the first plateau of the D-amphetamine concentration-response curve. At higher concentrations of D-amphetamine, increased DAT trafficking to the membrane will likely occur; however, the higher concentrations of D-amphetamine will more potently cause reverse transport of DATs to move DA into the extracellular space (Khoshbouei et al. 2004; Gorentla and Vaughan 2005) (Fig. 5.12). The unaffected DATs will not be able to counteract this response. Even though DAT levels are increasing, the increased levels of amphetamine will also potently block eventual clearance of DA from the

extracellular space. It is possible that D_2R autoreceptor desensitization is likely to occur which would decrease upregulation of DATs to the plasma membrane (Kim et al. 2001; Namkung and Sibley 2004; Ferguson et al. 1996; Tang et al. 1994). Finally, data support that interactions of amphetamine and the DAT lead to DAT internalization via phosphorylation of target residues in the C- and N- termini (Khoshbouei et al. 2004; Kahlig et al. 2006; Fog et al. 2006), providing theoretical support for the effects we see in the second plateau of the D-amphetamine concentration-response curve.

While the double plateaus we note here are in regards to increasing concentrations of D-amphetamine, other reports suggest biphasic effects of catecholamine transporters over different parameters. Johnson et al. (2005) described the effects of amphetamine on DAT surface expression in rat synaptosomes. They described initial amphetamine upregulation of DATs to the plasma membrane leading to DA efflux followed by amphetamine induced internalization of DATs after repeated doses of amphetamine. Jayanthi et al. (2005) described mechanisms that contribute to a biphasic regulation of endogenous serotonin transporters (SERTs) expressed in platelets. Protein kinase c (PKC) activation in platelets resulted in the initial reduction of functional SERTs followed by enhanced endocytosis of SERTs. Finally, due to the high percentage of D-amphetamine in Adderall®, it is surprising that two plateaus of the D-ampheatmine concentration-response curve are not distinguishable in the Adderall® concentration-response curve. Taking into consideration data from Chapter 3, the faster kinetics of the effects of L-amphetamine in combination with the slower kinetics of D-amphetamine could either mask 1) the noticeable differences of source (cytosolic versus vesicular) contribution of DA or 2) the effects on DAT trafficking.

The increased DA levels we report after reverse microdialysis of methylphenidate were in contrast to our hypothesis that local applications of methylphenidate would not cause increased DA levels in an anesthetized rat. Due to reports that methylphenidate mainly works to block reuptake of impulse-released DA from predominantly vesicular stores (Sonders et al. 1997; Clemens

et al. 1979; Volkow et al. 1998) and is thought to mainly be a DAT blocker (Riddle et al. 2005; Bergman et al. 1989), it was surprising that enough spontaneous release occurred in anesthetized rats to cause accumulation of DA. These studies were carried out in anesthetized F344 rats and the urethane anesthesia that we use here has been shown to markedly decrease intrinsic neuronal firing rates but not affect DAT activity mediated by local applications of stimulants (Warenycia and McKenzie 1988; Sabeti et al. 2003). While spontaneous firing rates measured by multiple single-unit electrophysiology were significantly decreased after anesthesia in a freely-moving animal, they were not completely ablated in the striatum (Kish et al. 1999). Sonders et al. (1997) classified methylphenidate in a group of pharmacological agents that are cocaine-like in terms of voltage dependence of their subtractive currents from control current and placed amphetamine in a category similar to measures in the presence DA, the endogenous substrate for the DAT. Our results are consistent with the effects of intraperitoneal administration of methylphenidate in freely-moving rat microdialysis studies (Berridge et al. 2006). Additionally, our data parallel a study that investigated the neurochemical effects of subcutaneously administered DA uptake inhibitors and releasers in anesthetized rats. Hurd and Ungerstedt (1989) found that amphetamine and methylphenidate caused similar increases in DA levels however methylphenidate caused these levels over a longer time period. This study also reported that methylphenidate had less of an effect on decreasing DOPAC levels compared to the more pronounced decrease caused by amphetamine (Hurd and Ungerstedt 1989). Our data were similar to others in the effects of methylphenidate in comparison to amphetamine on behavior. Similar to D-amphetamine, methylphenidate has been shown to induce locomotor activity at low doses and cause stereotypies at higher doses (Fessler et al. 1980; Hughes and Greig 1976; Scheel-Kruger 1971). Additionally, methylphenidate has also been found to be reinforcing in regards to drug abuse potential in humans, and it has been self-administered by animal models (Stoops et al. 2005; Rush et al. 2001; Risner and Jones 1976). In general, cocaine and methylphenidate are thought to work in a similar manner by predominantly acting

as competitive inhibitors of the DAT and increases in extracellular DA result predominantly from this blockade after impulse-dependent release of DA.

Other studies have brought forward some inconsistencies and support that cocaine-like, DAT blockers may have DA releasing properties (Stamford et al. 1989; Shore 1976; Ewing et al. 1983; Venton et al. 2006). Additional evidence support that these DAT inhibitors must act in a local manner due to studies that have shown cocaine-evoked DA release in striatal terminals isolated from cell bodies (Lee et al. 1996). Finally, Russell et al. (1998) demonstrated that methylphenidate caused DA release in brain slices mainly from vesicular stores and suggested that vesicular function may be impaired in ADHD neuropathology, and another study also showed methylphenidate induced DA increases with microdialysis (Butcher et al. 1991).

These data support the differential local effects of methylphenidate and a potential dissociation of the effects of cocaine and methylphenidate. Increased DA levels caused by local applications of methylphenidate were not significantly different from DA levels caused by cocaine. However, on average, DA levels after cocaine were lower than DA levels after applications of methylphenidate, and methylphenidate caused DA levels similar to the amphetamine isomers. It is possible that methylphenidate has DA releasing properties other than just blockade of the DAT.

While the argument can be made that these local applications failed to account for pharmacokinetic differences between these stimulants, we propose that this is a particular strength of our study. For these experiments, drugs were applied over a range of concentrations, including clinically relevant concentrations (10-50 μM) and potentially drug abuse levels (>400 μM) (West et al. 1999; Shader et al. 1999; Kuczenski and Segal 2001; Solanto et al. 2001; Grilly and Loveland 2001). The low concentrations were projected to simulate potential levels of drug that would be present in brain tissue following systemic or oral administration. Finally, administering the drugs via reverse microdialysis eliminated pharmacokinetic issues from the study.

In summary, we have shown that the D-amphetamine concentration-response curve of DA displayed a double plateau pattern indicating effects on DA stores and/or rapid regulation of DAT trafficking and/or function. This study provides important information describing the effects of stimulants over clinically relevant and possibly abuse levels on DA neurotransmission and metabolite production. These data support that methylphenidate may cause DA release in addition to acting as a DA uptake inhibitor. Taken together, these data explain the effects of clinically available stimulants on DA levels over a range of concentrations and how alternative combinations of amphetamine isomers show promising effects.

ACKNOWLEDGEMENTS

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Drug	EC_{50} [Drug] (μM) For DA	EC_{50} [Drug] (μM) For DOPAC
D-amphetamine I	0.5	Not Determined
D-amphetamine II	50	5
Methylphenidate	10	1
Adderall®	25	10
Reverse Adderall	50	25

Table 5.1 Stimulant Potency on DA and DOPAC Measures

Values determined for EC_{50} represent extrapolation of potency measures from the stimulant concentration response curves of DA and DOPAC.

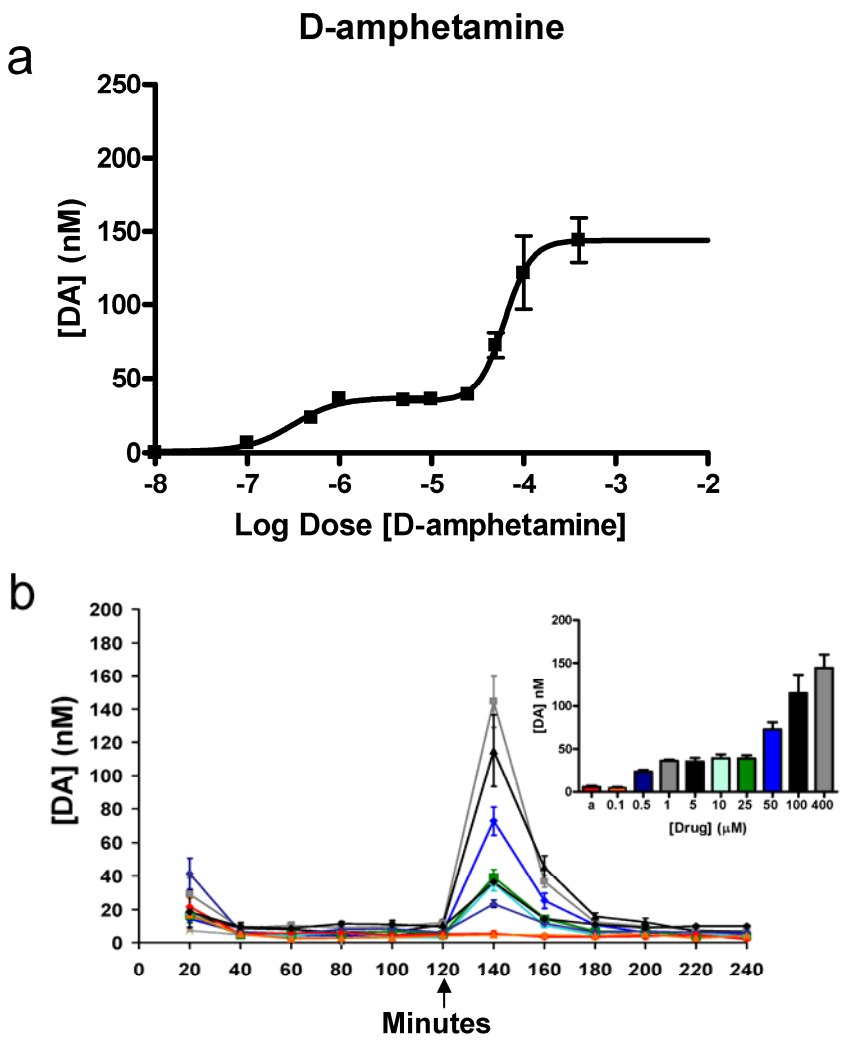


Figure 5.1 D-amphetamine Concentration-Response Curve for DA with a Double Plateau

a. Complete D-amphetamine concentration-response curve of DA levels after reverse microdialysis in the striatum of rat brain with two plateaus. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DA measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** Representation of each average peak response including aCSF (a) control (n=5 rats per concentration; error bars represent S.E.M.).

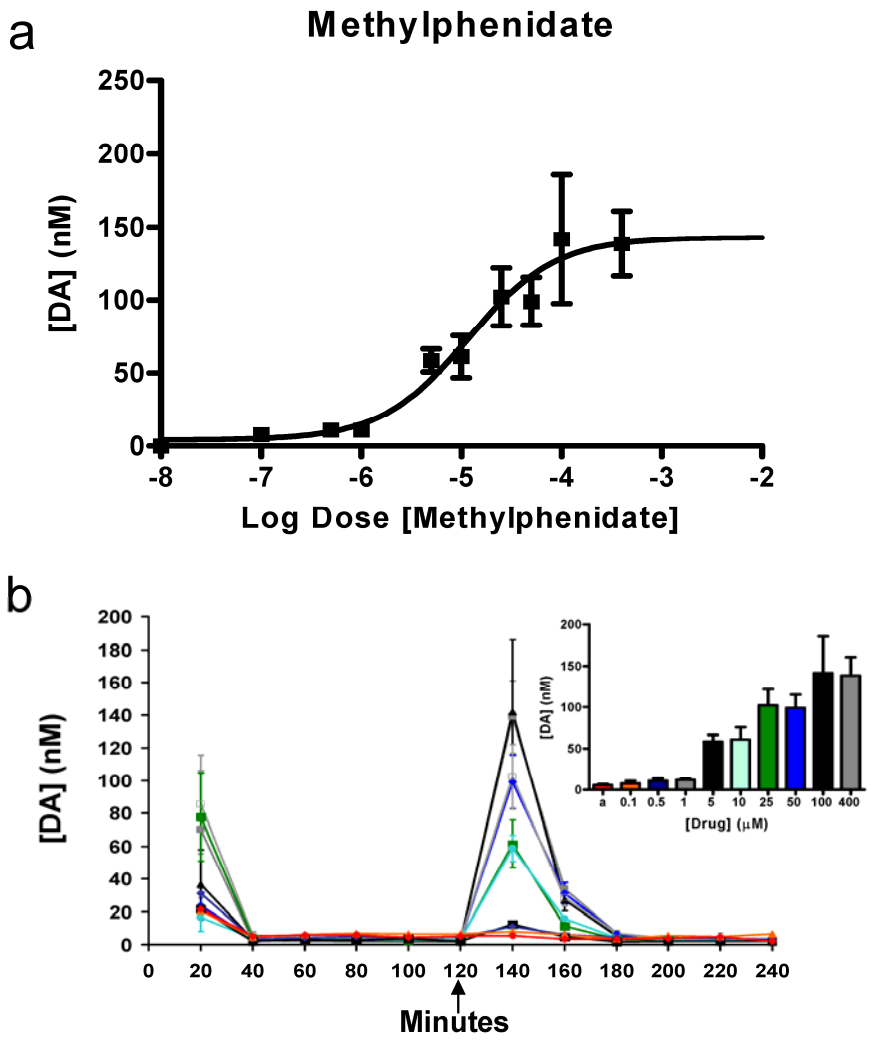


Figure 5.2 Methylphenidate Concentration-Response Curve of DA

a. Complete methylphenidate concentration-response curve of DA levels after reverse microdialysis in the striatum of rat brain. **b.** Mean microdialysis sample runs are shown including collection over two hours for basal DA measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control (n=5 rats per concentration; error bars represent S.E.M.).

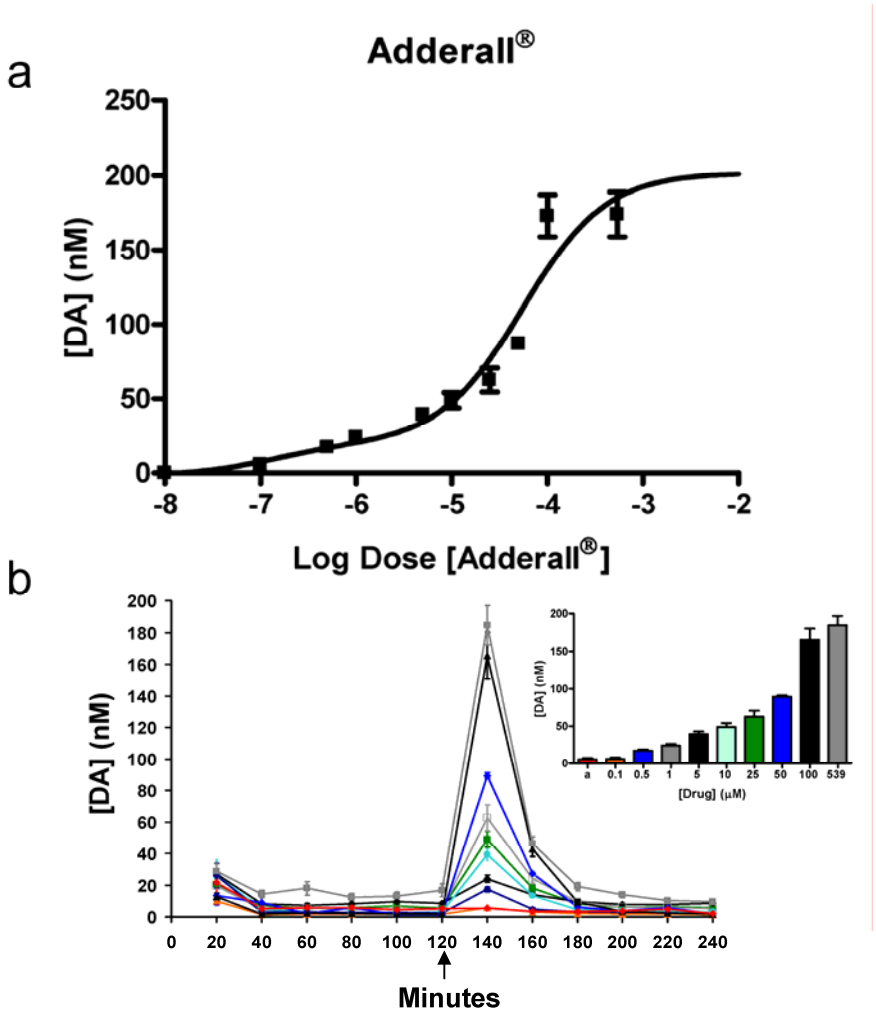


Figure 5.3 Adderall® Concentration-Response Curve of DA

a. Complete Adderall® concentration-response curve of DA levels after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DA measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control (n=5 rats per concentration; error bars represent S.E.M.).

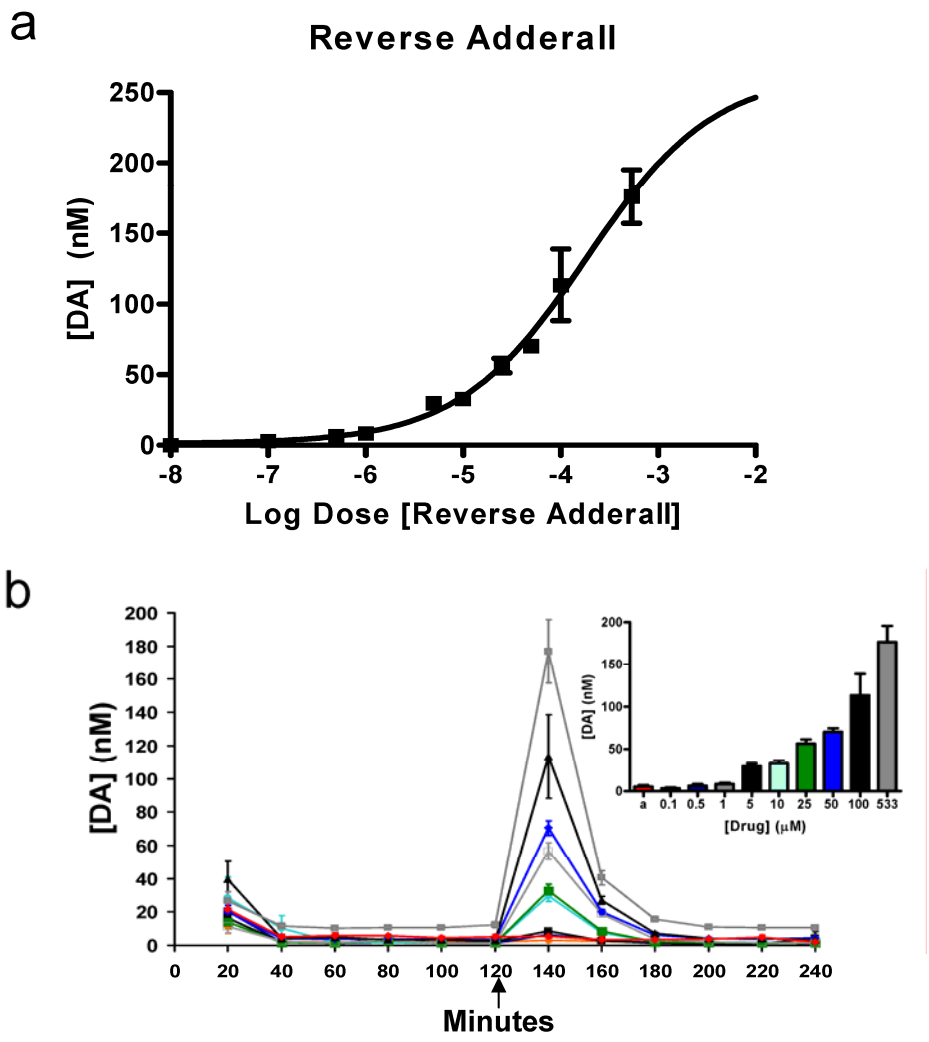


Figure 5.4 Reverse Adderall Concentration-Response Curve of DA

a. Complete Reverse Adderall concentration-response curve of DA levels after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DA measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control (n=5 rats per concentration; error bars represent S.E.M.).

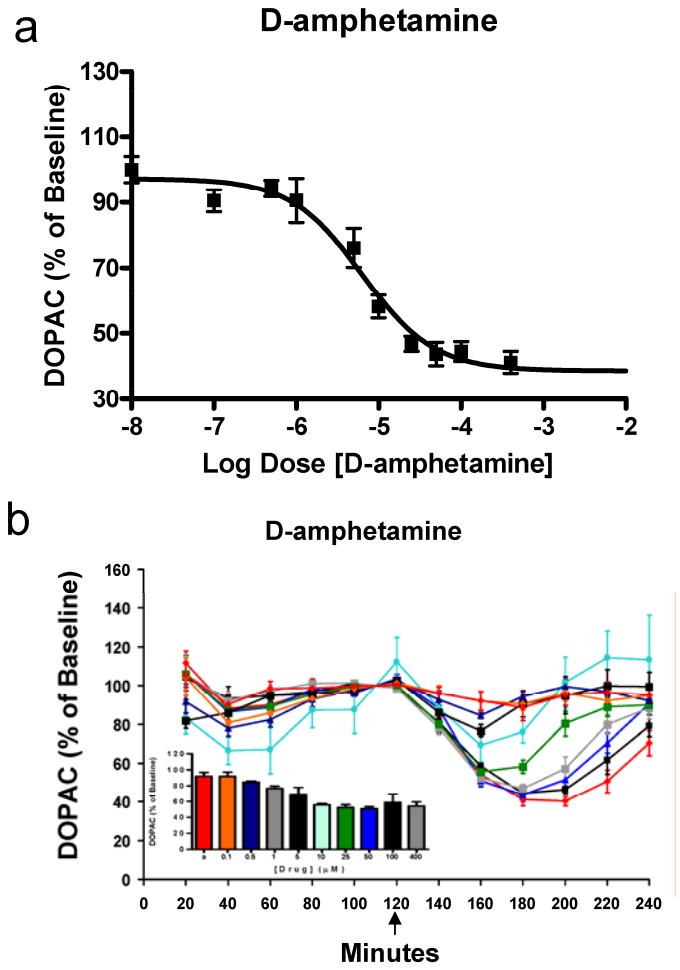


Figure 5.5 D-amphetamine Response Curve for DOPAC (% of Baseline)

a. Complete D-amphetamine response curve of DOPAC production after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DOPAC measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** Average representation of each peak response including aCSF (a) control (n=5 rats per concentration; data are represented in percent of baseline calculated from the change occurring 20 minutes after stimulation at time point 140 minutes; error bars represent S.E.M. for percent of baseline).

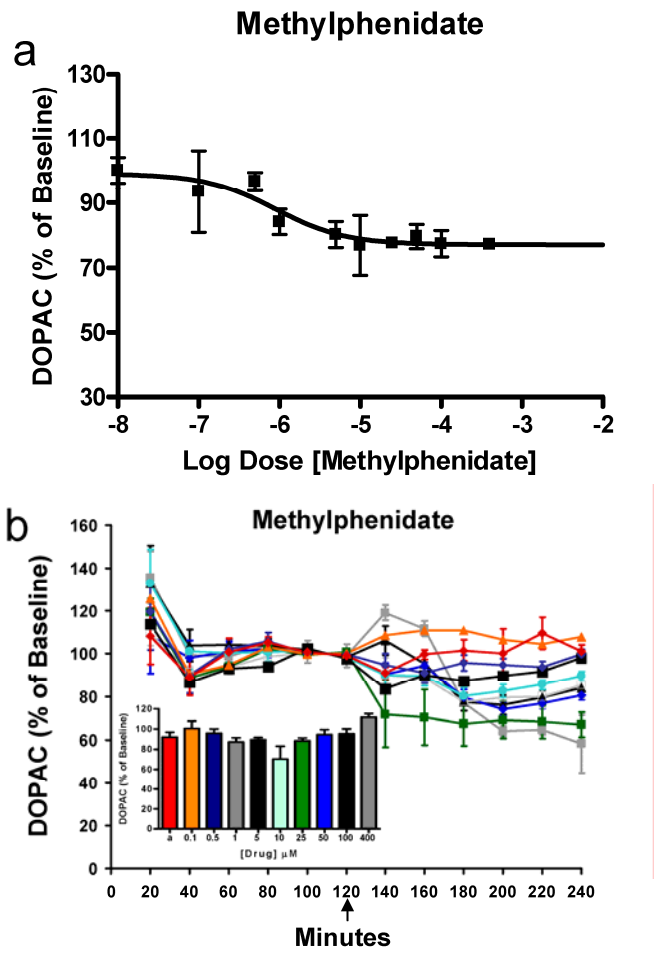


Figure 5.6 Methylphenidate Response Curve of DOPAC (% of Baseline)

a. Complete methylphenidate response curve of DOPAC production after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DOPAC measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control (n=5 rats per concentration; data are represented in percent of baseline calculated from the change occurring 20 minutes after stimulation at time point 140 minutes; error bars represent S.E.M. for percent of baseline).

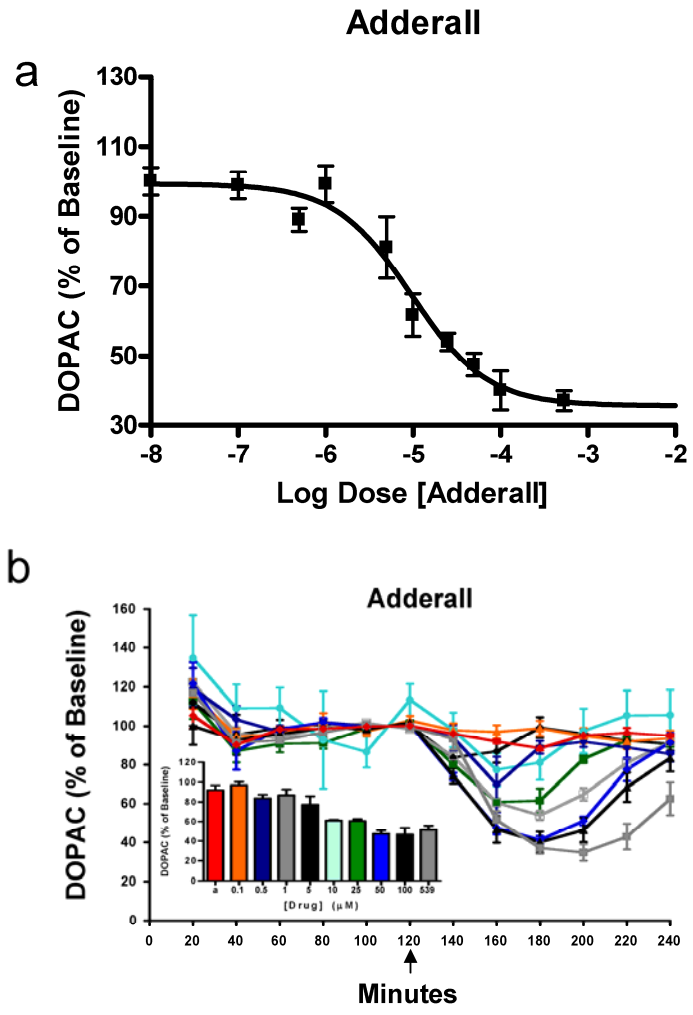


Figure 5.7 Adderall® Response Curve of (DOPAC % Baseline)

a. Complete Adderall® response curve of DOPAC production after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DOPAC measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control (n=5 rats per concentration; data are represented in percent of baseline calculated from the change occurring 20 minutes after stimulation at time point 140 minutes; error bars represent S.E.M. for percent of baseline).

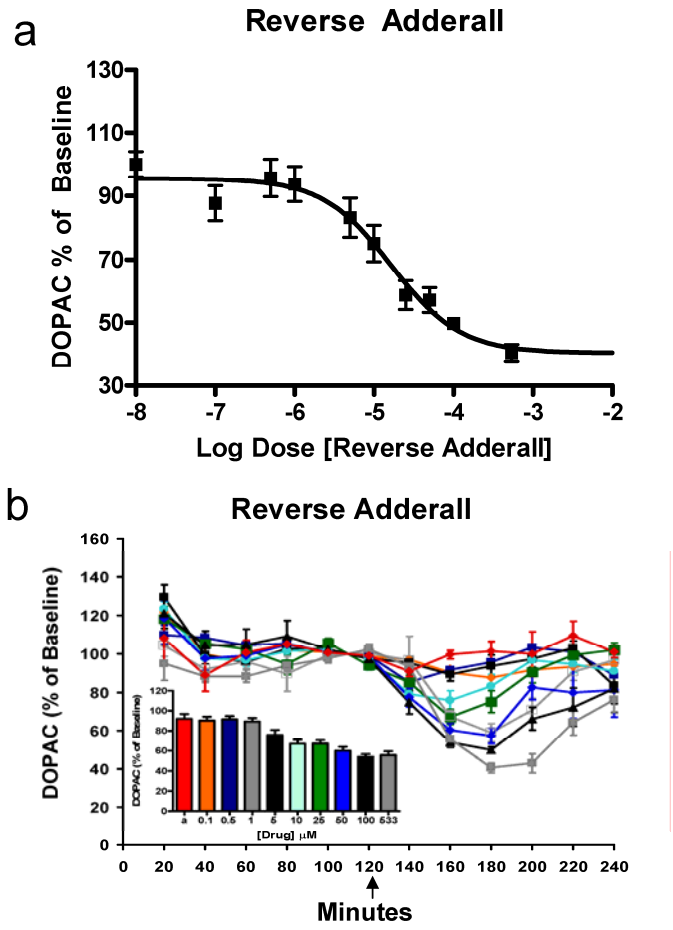


Figure 5.8 Reverse Adderall Response Curve of (DOPAC % of Baseline)

a. Complete Reverse Adderall response curve of DOPAC production after reverse microdialysis in the striatum of rat brain. **b.** Mean individual microdialysis sample runs are shown including collection over two hours for basal DOPAC measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer. **Inset:** representation of each average peak response including aCSF (a) control. (n=5 rats per concentration; data are represented in percent of baseline calculated from the change occurring 20 minutes after stimulation at time point 140 minutes; error bars represent S.E.M. for percent of baseline).

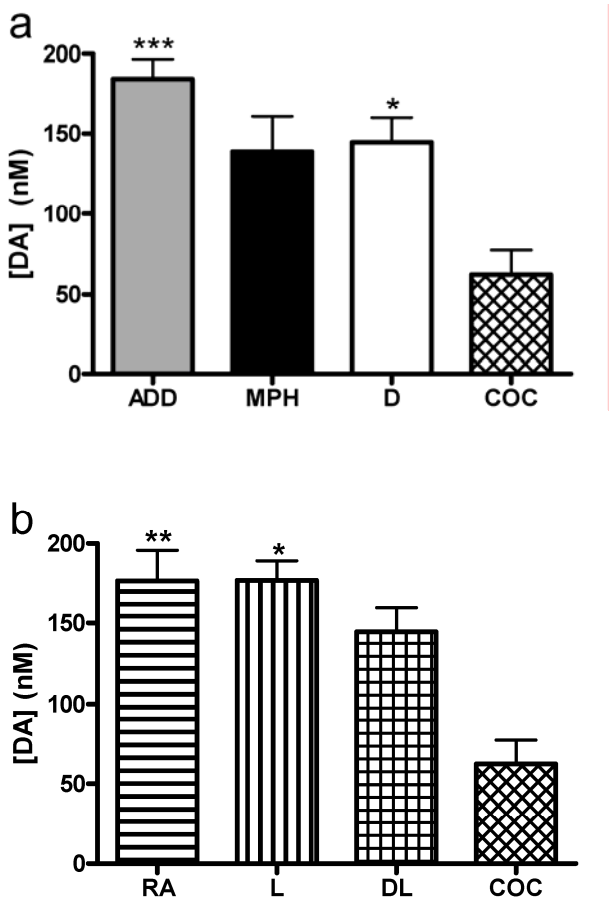


Figure 5.9 Comparisons of Stimulant Efficacies on DA Levels

a. ADHD Drugs Versus Cocaine: Comparisons of DA levels after reverse microdialysis of the highest concentrations of ADHD medications tested in the F344 rat striatum versus cocaine (all solutions are equimolar to D-amphetamine). Adderall® (***p<0.001) and D-amphetamine (*p<0.05) caused significantly greater DA levels than cocaine. There was a trend for methylphenidate to cause greater DA levels than cocaine supporting a dissociation between the effects of these two stimulants. **b. Non-ADHD Drugs Versus Cocaine:** Comparisons of DA levels after reverse microdialysis of the highest concentrations of non-ADHD amphetamine isomers tested in the F344 rat striatum versus cocaine. Reverse Adderall (**p<0.01) and L-amphetamine (*p<0.05) caused significantly greater DA levels than cocaine. (n=5 rats per concentration; data represent mean ± S.E.M.).

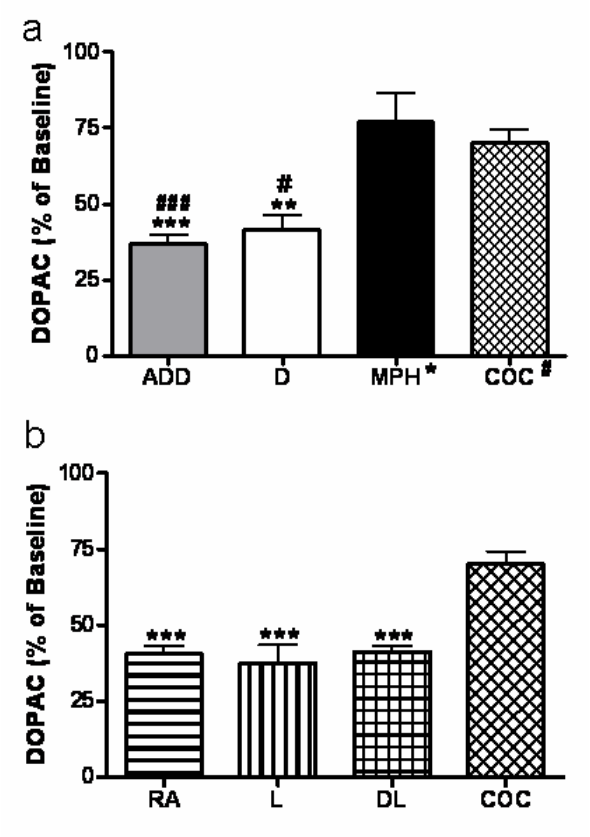
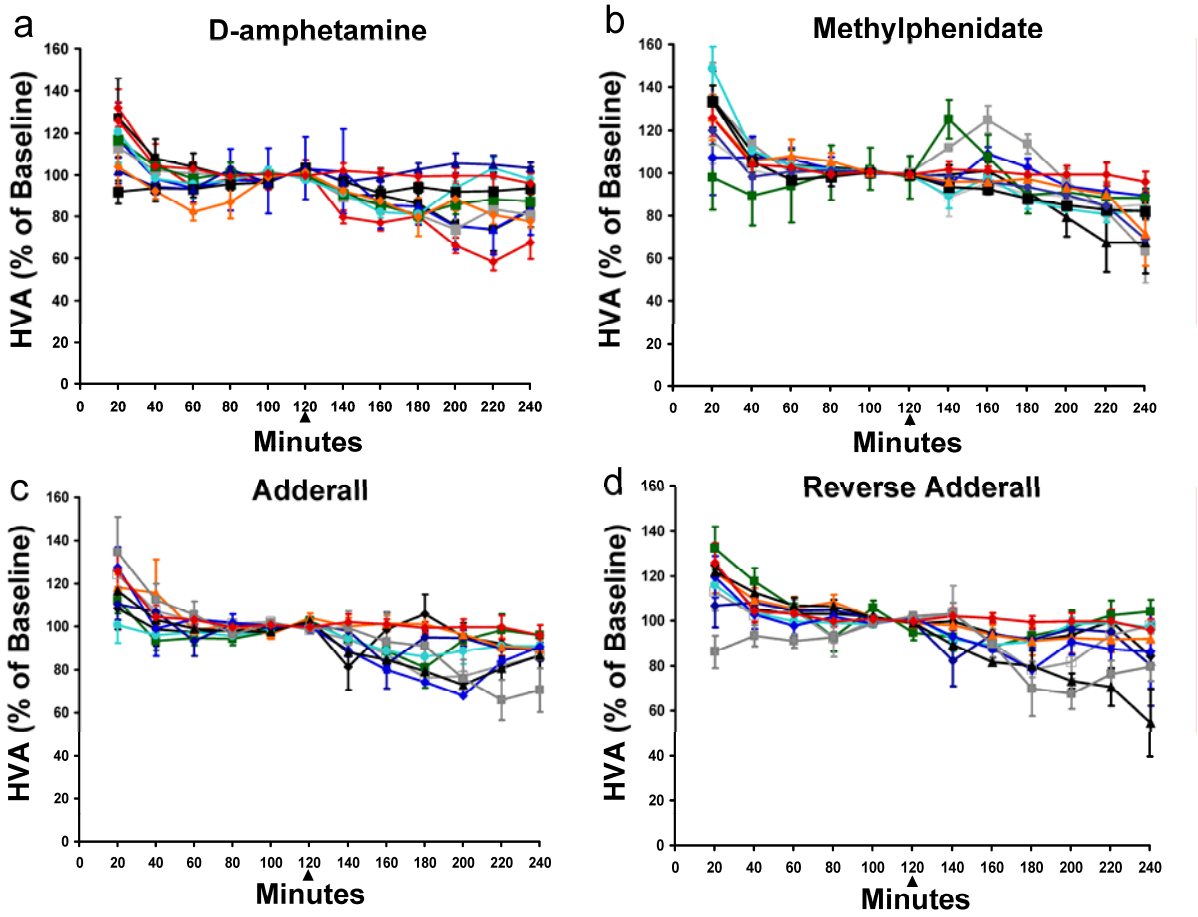


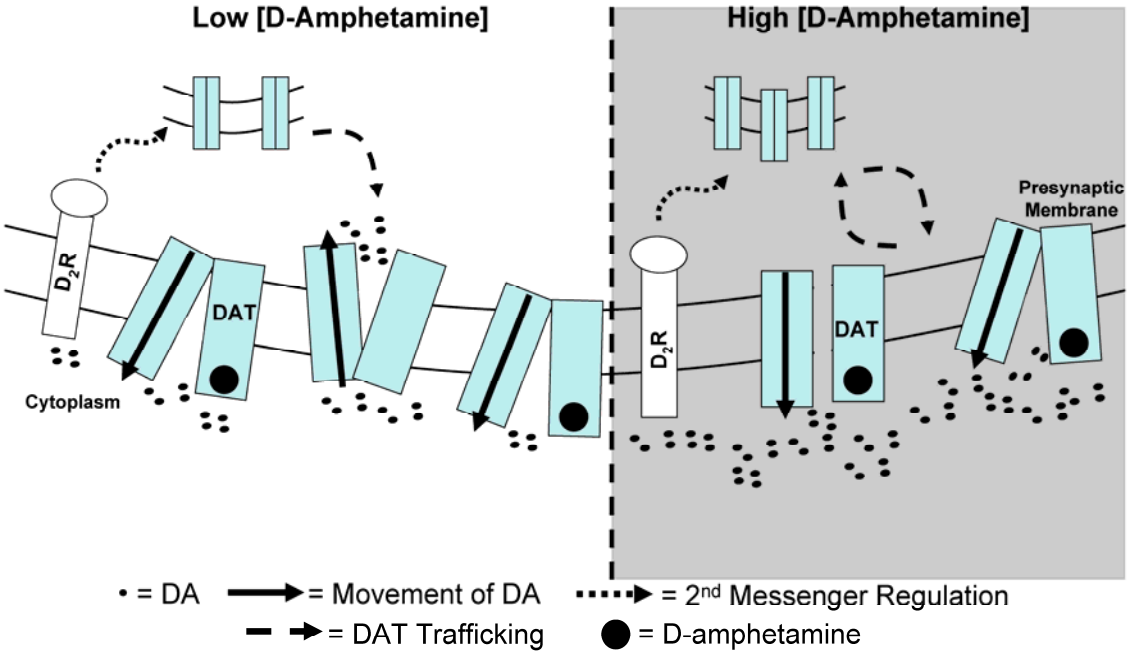
Figure 5.10 Comparison of Stimulant Efficacies on DOPAC Levels (% of Baseline)

a. ADHD Drugs Versus Cocaine: DOPAC levels following reverse microdialysis of the highest concentrations of ADHD stimulants tested in the striatum of rat brain represented as percent of baseline (based on DOPAC measures 20 minutes following stimulus). Adderall® (***p<0.001; ###p<0.001) and D-amphetamine (**p<0.01; #p<0.05) had significantly greater effects on DOPAC production in comparison to methylphenidate and cocaine respectively.
b. Non-ADHD Drugs Versus Cocaine: DOPAC levels following reverse microdialysis of the highest concentrations of non-ADHD stimulants tested in the striatum of rat brain represented as percent of baseline (based on DOPAC measures 20 minutes following stimulus). Reverse Adderall, L-amphetamine, and D,L-amphetamine (***p<0.001) had significantly greater effects on DOPAC production in comparison to cocaine (n=5 rats per concentration; data represent mean ± S.E.M.).



5.11 HVA Levels After Reverse Microdialysis of D-amphetamine, Methylphenidate, Adderall®, and Reverse Adderall in the Striatum

HVA levels (% of Baseline) after local application of **a.** D-amphetamine, **b.** methylphenidate, **c.** Adderall, and **d.** Reverse Adderall did not reveal a dose-response pattern similar to DOPAC. Individual microdialysis sample runs are shown including collection over two hours for basal DA measures followed by a 20 minute local application of drug (respective concentration) in the aCSF (arrow indicates time point of stimulation) and completed with perfusion of aCSF for 100 minutes longer (n=5 rats for each concentration; data represent mean ± S.E.M.). Refer to Fig. 5.1-5.8 for representation of concentrations and corresponding colors.



5.12 Theoretical Model of Activity Describing the Double Plateaus of the D-amphetamine Concentration Response Curve for DA

Plateau I Low [D-amphetamine]: Lower concentrations of D-amphetamine cause reverse transport of low levels of DA through the DAT leading to stimulation of DA sensitive D_2R autoreceptors. Autoreceptor stimulation has been shown to signal the upregulation of functional DATs to the plasma membrane to increase DA clearance (Parsons et al. 1993; Cass and Gerhardt 1994; Rothblat and Schneider 1997; Mayfield and Zahniser 2001). Due to the increased clearance of DA, the first plateau of the concentration-response curve potentially results. **Plateau II High [D-amphetamine]:** Amphetamine has been shown to interact with DATs and facilitate DA release followed by DAT internalization (Johnson et al. 2005; Fleckenstein et al. 1999; Saunders et al. 2000; Khoshbouei et al. 2004; Gorentla and Vaughan 2005). Higher concentrations of D-amphetamine will likely cause increased DA release and DAT internalization. D_2R autoreceptor desensitization is likely to occur and interrupt DAT expression (Kim et al. 2001; Namkung and Sibley 2004; Ferguson et al. 1996; Tang et al. 1994). Higher levels of extracellular DA and decreased DA clearance could cause the second plateau.

Chapter 6: Discussion and Conclusions

Numerous clinical studies have provided data to support that amphetamine isomer and stimulant dependent differences exist (James et al. 2005; Gross 1976; Arnold et al. 1972; 1976; Ahmann et al. 2001; Greenhill et al. 2001; Swanson et al. 1998; Biederman et al. 2006; Pelham et al. 1999; Bradley 1950; Smith and Davis 1977). Additionally, animal studies have often been inconclusive in describing amphetamine enantiomer differences (Stromberg and Svensson 1975; Jones et al. 1974; Yokel and Pickens 1973; Kanbayashi et al. 2000; Hutchaleelaha et al. 1994; Risner and Jones 1975). Previous findings from basic science studies have been limited by decreased sensitivity of the techniques employed. The differences described in this dissertation are novel and important findings generated via techniques with capabilities to measure low levels of analyte *in vivo*.

Due to the known safety and efficacy of these medications, their clinical use to treat ADHD has increased over the last few decades. While the stimulants we tested are known to be safe, there are unanswered questions that merit further investigation. If we can determine the specific interactions of these medications and their cellular targets then it will be possible to design analogue drugs that have less abuse potential, increased efficacy, increased consistency across patients, more accurate targeting of specific symptoms, and decreased side effects. Finally, if different behavioral outcomes result following prescribed use of stimulant medications, then correlations to specific patterns of neurotransmission can allow for accurate determination of the necessary components of stimulant treatments that may be forthcoming. Our studies have sought to address some of these questions by completing the first studies, to our knowledge, that look at these drugs and their components' interactions with DA neurotransmission.

In Chapter 3, we used microelectrodes and local applications of amphetamine enantiomers to investigate differences in the effects on DA release and regulation. The majority of the *in vivo* investigations of amphetamine

isomers have made use of only systemic amphetamine administration while observing changes in measures of DA uptake or analyte level changes across microdialysis samples. Therefore, our first objective of these studies was to determine if we could accurately and consistently evoke DA release signals at a maximally effective concentration. After determining an effective concentration in which we could consistently record DA release signals, we were able to test multiple amphetamine isomers. These recordings were completed in the striatum, a region rich in DA nerve endings and an area implicated in human anatomical studies of ADHD (Durston 2003; Solanto 2001; and Sowell et al. 2003). An important finding from these studies addressed previously reported differences between D- and L- amphetamine; our data support that the two enantiomers were similar in efficacy when applied locally to the striatum. However, kinetic differences were found between D- and L-amphetamine that support differential interactions and/or regulation of the DAT, the primary target of amphetamine. When the two enantiomers were applied in combination, these data indicate that resulting DA signal amplitudes were dependent on the amount of D-amphetamine. Finally, kinetic differences were observed; D,L-amphetamine caused transient DA release signals compared to the longer signals recorded after local application of D-amphetamine. While these initial findings were some of the first to show *in vivo* differences in amphetamine isomer effects on DA release and uptake, we concluded that these concentrations were either in the upper range or higher than clinically effective concentrations in the brain after clinical use of amphetamine in humans (Shader et al. 1999; Solanto et al. 2001; West et al. 1999; Grady et al. 1996; Seeman and Madras 2002).

In converse to studies completed for Chapter 3 in which we did not make use of Adderall® to evoke DA release, we completed studies to investigate the effects of clinically available Adderall® and D-amphetamine in Chapter 4. Based on the data from Chapter 3, we maintained equimolar amounts of D-amphetamine across all drug solutions and tested these at similar concentrations to those in Chapter 3. To address concerns that the concentrations tested were too high for clinical relevance, we also used lower drug concentrations. After

determining that we could consistently measure the effects of decreased levels of amphetamine isomers on DA release signal recordings; a complete analysis was conducted of amphetamine isomers at these levels. An important finding from these studies was that low-level Adderall® produced increased striatal DA release and a prolonged time course compared to amphetamine isomers. While the amplitude of DA release was significantly increased compared to D- and D,L-amphetamine, the kinetics of DA release signals were also significantly elongated for Adderall®. These data support that the combination of salts and/or enantiomers in Adderall contribute to a greater effect on the DAT in causing reverse transport of DA over a longer period and a DAT blocking effect lasting longer than the effects caused by amphetamine isomers. Since it is hard to speculate what this increased effect on DA release and uptake caused by Adderall® indicates without specific mechanistic investigations, the effect does correlate with findings that Adderall® had longer lasting effects on symptoms of locomotor activity compared to D-amphetamine in a recent clinical study (James et al. 2001). We also replicated the significant difference in rise time between D-amphetamine (slower) and D,L-amphetamine (faster) from Chapter 3 at these low levels. While these concentrations of amphetamine isomers were lower in magnitude, it is still difficult to verify the resulting effective concentration in the brain and if these concentrations were maximally effective. Therefore reverse microdialysis studies were necessary to identify and locally apply specific effective concentrations that could be tested at much lower levels than were studied in Chapters 3 and 4.

In Chapter 5 we used a different *in vivo* technique, intracerebral reverse microdialysis, having decreased temporal and spatial resolution compared to the electrochemical methods used in Chapters 3 and 4. This technique was chosen to complete the concentration-response studies carried out in Chapter 5 because of the ability to more accurately determine the effective concentrations in which we wanted to locally apply in the striatum and the ability to sample the effects on analytes of lower levels of amphetamine isomers due to the coupling of High Performance Liquid Chromotography with electrochemical detection (HPLC-EC).

Finally, using microdialysis, we had the added ability to monitor the effects of methylphenidate not possible with our current *in vivo* voltammetric techniques. We used drug-naïve animals to make the concentration-response characterizations due to data that support changes in measures of DA levels before and after exposure to amphetamine (Purdom et al. 2003).

One major finding in Chapter 5 was that the concentration-response curve of D-amphetamine had two plateaus. These data are among the first to directly support a dissociation in the effects of high and low doses of amphetamine on the DAT. These data correlate with previous findings that 1) different sources of cellular DA are affected by different stimulants or 2) biphasic regulation of the DAT (Langeloh and Trendelenburg 1987; Sulzer et al. 2005,1993; Jones et al. 1998; Johnson et al. 2005; Jayanthi et al. 2005; Kahlig et al. 2005). The concentration-response curve of D-amphetamine supports differences in the effects of low versus high-level stimulants that could correlate with clinical versus drug abuse levels. The biphasic properties of the D-amphetamine concentration-response curve are supported by other studies that have provided evidence in biphasic regulation of catecholamine transporters and data that support the release of different DA stores. Finally, this concentration-response curve could be the first *in vivo* observation of the paradoxical effects of stimulants in humans. Low doses are known to reduce locomotor activity and distractibility in humans, while higher doses have caused sleeplessness and restlessness (Seeman and Madras 2002).

A second major finding of the data in Chapter 5 is the robust increase in DA levels caused by methylphenidate in the anesthetized rat. Methylphenidate is generally regarded to contribute to impulse-dependent accumulation of DA (Bergman et al. 1989). Based on the similar increases in DA levels caused by methylphenidate and the other amphetamine analogs, methylphenidate either blocked reuptake of impulse-released DA or displaced DA from DA neurons when applied locally. Due to the decreased sensitivity of temporal measures with this technique, we no longer saw distinct differences in the effects of these stimulants, in particular at the suspected clinical levels. Finally, we compared

these stimulants with cocaine, a drug that has been grouped with methylphenidate and impulse-dependent accumulation of DA (Sonders et al. 1997). Our data support that methylphenidate increased DA levels more similar to the effects of amphetamine than cocaine. These data have important implications regarding the potential dissociation of the properties of cocaine and methylphenidate and promote a unified theory that initially increasing DA neurotransmission is important for decreasing the symptoms of ADHD.

Combined, these *in vivo* data have replicated clinical and animal behavioral observations that stimulants have different effects on behavioral outcomes. Chapter 3 brought about the relationship of L-amphetamine's effects on D-amphetamine-evoked DA release, since the two were not additive in their effects on DA signal amplitudes when applied together but demonstrated a regulatory mechanism. In regards to these data, we proposed that differential amphetamine enantiomer interactions with the DAT and resulting effects on DAT function/expression occur. In Chapters 4 and 5 we propose more theoretical explanations of the effects on the DAT and what these data represent. Not only do these data reveal differences between enantiomers but also differences that are present between varying ratios of D and L-amphetamine. While the effects of D- and L- amphetamine were not additive in Chapter 3, the sum of their effects may indicate why a 75% D- and 25% L-amphetamine mixture had the greatest effects on DA in Chapter 4. According to the data in Chapter 3, L-amphetamine caused faster DA release and therefore a relatively small amount of L-amphetamine in Adderall® could prime the DAT for a relatively greater proportion of D-amphetamine to cause a more robust and elongated effect on DA neurotransmission. In regards to our robust effects with Adderall in Chapter 4, most of the literature support that stimulants that cause fast abrupt increases in DA are more reinforcing or likely to be abused (Volkow 2006; Volkow and Swanson 2003). Conversely, drugs and delivery systems that cause slower effects on DAT blockade and DA increases are thought to have less abuse liability (Spencer et al. 2006). It is possible that Adderall displays a trend to fall

into the latter category of medications due to our data that support the elongated effects of Adderall.

Future Investigations of Stimulant Medications

In conclusion, we have made progress in conducting some of the first *in vivo* experiments to date looking at stimulant drugs in the context of their clinical and therapeutic use. The differences that we report are novel because techniques with the sensitivity to measure the effects of low-levels of drugs have not been available previously, the studies were carried out in drug-naïve animals to study the effects in brain tissue with decreased variability, and the drugs were applied locally to eliminate drug pharmacokinetic issues from these studies. Future studies are necessary to characterize the effects of amphetamine isomers and methylphenidate on other neurotransmitters and brain regions such as the prefrontal cortex. In addition, animal models of ADHD are forthcoming and locally applied stimulant effects should be studied in these models (Solanto et al. 2001; Giros et al. 1996; Wultz et al. 1990; Van Den Buuse and De Jong 1989; Hess et al. 1992, 1996). The development of microelectrode arrays that have capabilities to assess multiple neurotransmitters and target recordings in more discrete brain loci by our lab will allow for investigations of the effects of stimulants on glutamate, norepinephrine, and 5-HT neurotransmission in the striatum, prefrontal cortex, and cerebellum (Nickell et al. 2005, 2006; Day et al. 2006; Pomerleau et al. 2003; Burmeister et al. 2002). According to current estimates, 120,000,000 prescriptions have been given since we began collecting the data for this dissertation (Vendentam 2006). Knowing that these drugs are safe and effective is no longer sufficient to neglect necessary studies to characterize their properties that could yield more efficient, selective ADHD medications with less side effects and abuse potential.

Appendix 1: Comparisons of High Concentrations of D-amphetamine and Adderall®- Evoked DA Release in the Rat Striatum

For these *in vivo* voltammetric studies, two different amphetamine solutions were locally applied to evoke DA release signals in the striatum at higher amounts than used in Chapter 4: D-amphetamine and Adderall® (mixed from only amphetamine sulfate components of Adderall® to allow for the 75% D-amphetamine: 25% L-amphetamine ratio) and D-amphetamine. The volume of applied drug was kept constant at 500 nl and was measured using a dissection microscope fitted with a calibrated reticule (1 mm change=25 nl of fluid) (Cass et al. 1992, 1993a; Friedemann and Gerhardt 1992). Drugs were dissolved in 0.9% physiological saline and final drug solutions were brought to a pH of 7.4. High concentrations of D-amphetamine and Adderall were selected for use and were applied in the following amounts 2 nanomoles D-amphetamine and 2.72 nanomoles Adderall®. Figure A1.1 shows a complete representative signal for both drugs indicating no difference in amplitudes or signal decay, but a difference in the time course of DA release. When considering the amplitudes of DA recordings in the striatum after application of Adderall® and D-amphetamine, the mean amplitudes after local application of these drugs were not significantly different (Fig. A1.2a). When comparing amplitude of DA release per nanoliter of drug applied, there was no difference, indicating that either drug solution applied at a similar volume is capable of producing a similar DA response signal (Fig A1.2b). A comparison of rise times (T_R) of the amphetamine-induced DA signals in the striatum indicate significantly faster rise times in the presence of D-amphetamine when compared to DA signals produced by Adderall® ($p<0.01$; Fig A1.3a). When comparing the T_{80} signal decay times, indicative of DA uptake after release, there was no significant difference between the average T_{80} decay time of Adderall® and D-amphetamine (Fig A1.4b).

At the higher concentrations of Adderall® and D-amphetamine tested, there were no differences in the amplitude of DA release signals which may be

interpreted in two ways. The first description may reveal a caveat that these concentrations result in a local tissue concentration that may be too high to notice the differences, however this is unlikely when noticing the higher amplitude generated in the presence of lower drug concentrations. The similar amplitudes of the DA release signals caused by the higher concentrations of D-amphetamine and Adderall® are smaller than the amplitudes of the DA release signals evoked by the lower drug concentrations tested. This supports a potential inhibition of DAT reverse transport of DA at these levels of D-amphetamine that likely has implications in situations of abuse or the paradoxical effects that have classically been associated with stimulants (Stromberg and Svensson 1975; Grilly and Loveland 2001). It is interesting that across the high and low concentrations (from Chapter 4), the kinetics are similar. These data strengthen the argument that studies with decreased stimulant concentrations are necessary to determine potential differences in effects on DA neurotransmission as they relate to clinical use. While these data replicated the major finding in Chapter 3, that the amount of DA release is dependent on the amount of D-amphetamine applied; further investigations of these effects are warranted in future studies.

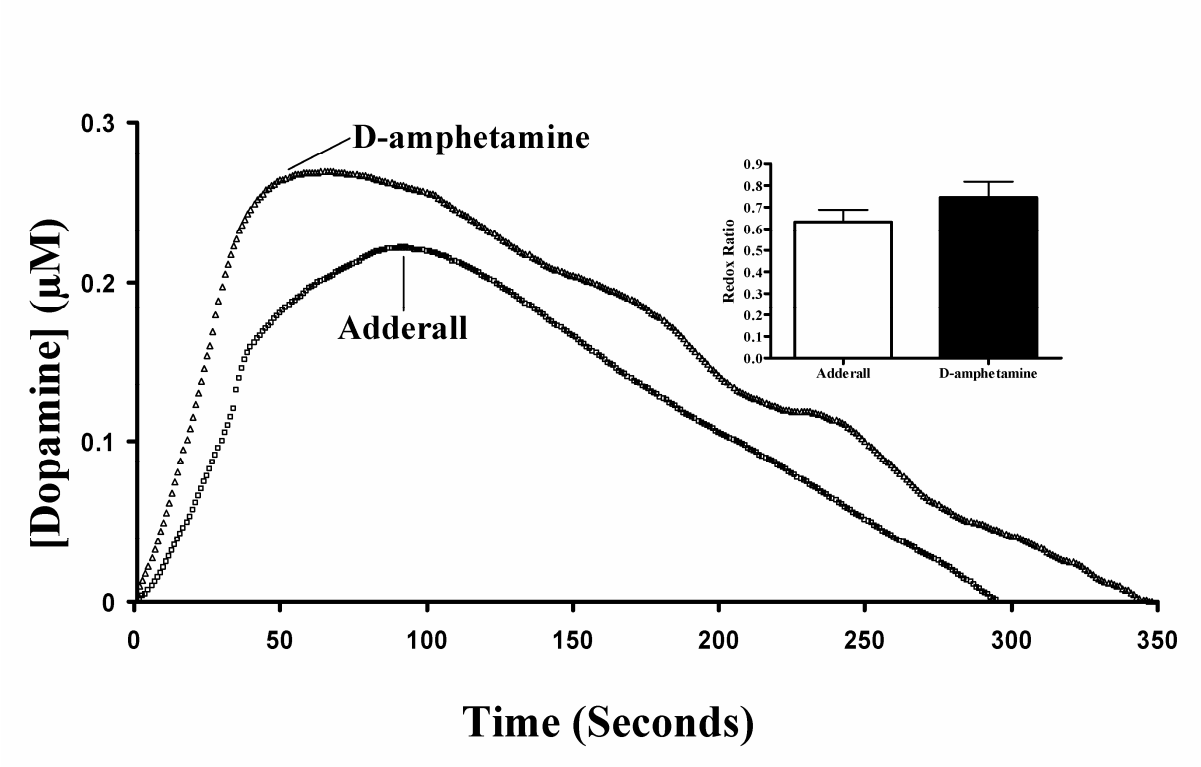


Figure A1.1 Representative DA Release Signals in the Striatum Caused by Local Applications of Higher Levels of D-amphetamine and Adderall®

Typical recordings indicating the time course of DA release in the striatum of rat brain induced by D-amphetamine (Triangles), and Adderall® (squares). All drug solutions contain an equivalent 2 nmol of D-amphetamine. **Inset:** Measured average *in vivo* redox ratios of all DA release signals, indicative of predominantly DA, included for statistical analysis (n= 34, 49 signals; error bars represent S.E.M.)

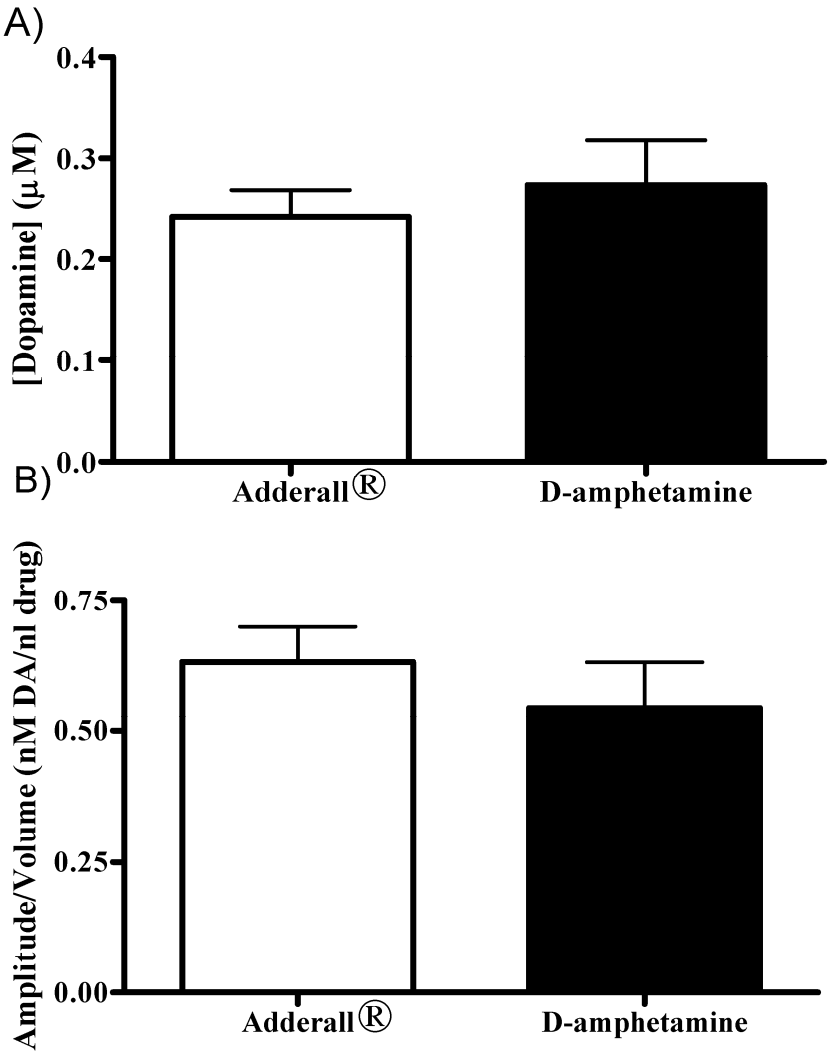


Figure A1.2 Amplitudes of DA Release Signals in the Striatum After Local Applications of Higher Levels of Adderall®, and D-amphetamine

a. The amplitudes of DA release measured in the rat striatum after local application of Adderall® were not significantly different compared to D-amphetamine. **b.** Amplitudes of DA (nM) recorded per volumes of drug applied (nl) in the rat striatum were not different between Adderall® and D-amphetamine. All drug solutions contained an equivalent 2 nmol of D-amphetamine. Data analyzed by MANOVA with Tukeys post-hoc comparisons; error bars represent S.E.M. (n=animals, signals; 11, 49; 15, 34).

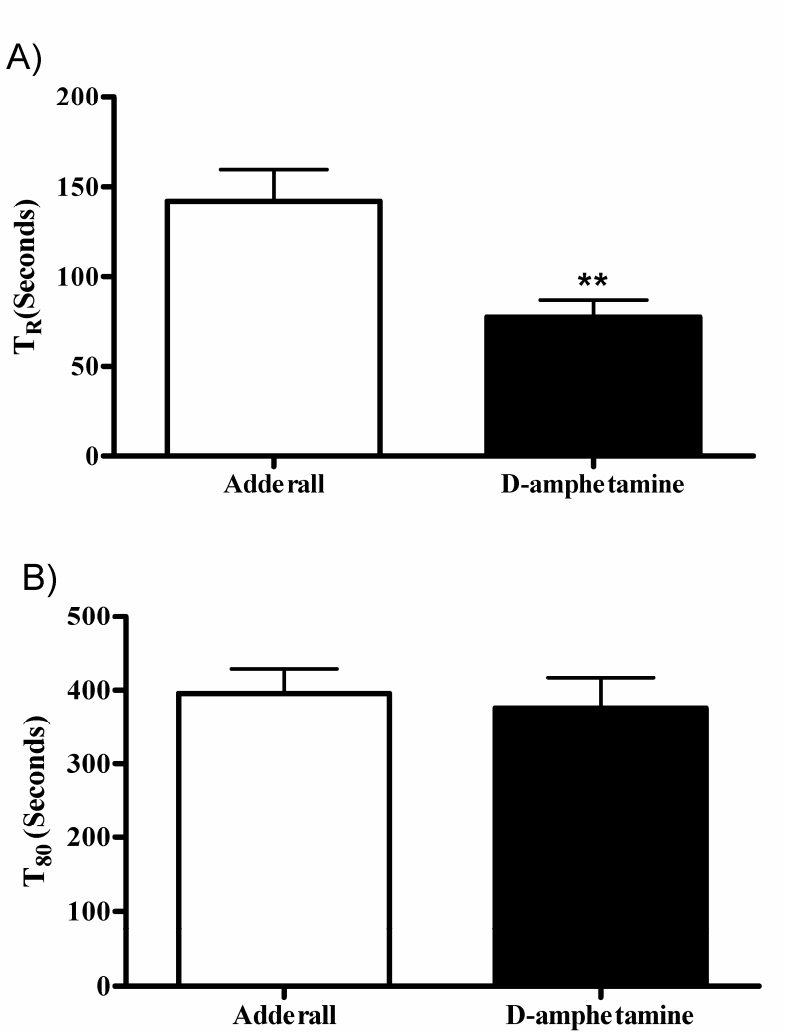


Figure A1.3 Kinetics of DA Release Signals After Local Applications of Higher Levels of Adderall® and D-amphetamine in the Striatum

a. Rise times for Adderall® evoked DA release signals in the rat striatum were significantly longer than D-amphetamine evoked DA signals (**p<0.01). **b.** T_{80} decay times for Adderall® evoked DA release signals in the rat striatum were not significantly longer than those produced by D-amphetamine. All drug solutions contained an equivalent 2 nmol D-amphetamine. Data analyzed by MANOVA with Tukeys post-hoc comparisons; error bars represent S.E.M. (n= animals, signals; 11, 49; 15, 34).

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VITA

Name: Barry Matthew Joyce

Date of Birth: 08/23/1978

Birthplace: Louisville, Kentucky

Education

2001-2006	Doctoral degree candidate Department of Anatomy and Neurobiology The Graduate School at the University of Kentucky Lexington, KY 40536
2000	B.S. Biology and Psychology, University of the Cumberlands, Williamsburg, Kentucky 40769
1996	High School Diploma, Trimble County High School Bedford, Kentucky 40006

Professional Experience

2001-2006	Doctoral candidate, University of Kentucky, Department of Anatomy and Neurobiology, Greg Gerhardt, Ph.D., Supervisor
2001	Student researcher, University of Kentucky, Department of Anatomy and Neurobiology, Jane Joseph, Ph.D., Supervisor
2001	Student researcher, University of Kentucky, Department of Anatomy and Neurobiology, Stephen Scheff, Ph.D., Supervisor

Scholastic and Professional Honors

July 2006	Invited Speaker Wake Forest University, Winston- Salem, North Carolina Department of Physiology and Pharmacology Contact: Sara Jones, Ph.D.
April 2006	Graduate Student Poster Award

	Lexington Conference on RNA Therapy for Neurodegenerative Diseaseas University of Kentucky Don Gash, Ph.D., Chairman
March 2006	Invited Speaker University of Kansas Medical Center, Kansas City, Kansas Contact: John Stanford, Ph.D.
March 2006	Graduate Student Poster Award Bluegrass Chapter, Society for Neuroscience University of Kentucky Joe Springer, Ph.D., Chairman
February 2006	Invited Speaker University of Tennessee at Martin, Martin, Tennessee Contact: Ann Gathers, Ph.D.
January 2006	Invited Speaker Wake Forest University, Winston- Salem, NC Deparment of Neurology Contact: James Eisenach, M.D.
November 2005	Graduate Student Delegate Graduate Student Leadership Conference convened by the Woodrow Wilson National Fellowship Foundation George Washington University, St. Louis, Missouri
March 2004	Graduate Student Poster Award Bluegrass Chapter, Society for Neuroscience University of Kentucky Linda Dwoskin, Ph.D., Chairwoman
2004-2005	Bluegrass Chapter-Society for Neuroscience Graduate Student Representative on the Executive Committee Greg Gerhardt, Ph.D., Chairman
2003-2005	Research Challenge Trust Fund Fellowship University of Kentucky Greg Gerhardt, Ph.D., Chairman
2002	Morris K. Udall Fellowship University of Kentucky

	Greg Gerhardt, Ph.D., Chairman
2002	Cellular and Molecular Basis of Aging Training Grant Fellowship University of Kentucky Don Gash, Ph.D., Chairman
2000	Hutton Leadership Scholar University of the Cumberlands, Williamsburg, Kentucky Michael Colegrove, Ph.D., Chairman
1999-2001	Psi Chi Psychological National Honor Society University of the Cumberlands, Williamsburg, Kentucky Charles Huffman, Ph.D., Chairman
1998-2001	Tri-Beta Biological National Honor Society University of the Cumberlands, Williamsburg, Kentucky Brett Kuss, Ph.D., Chairman
1996-2000	University of the Cumberlands Tuition Scholarship

Publications

Manuscripts:

Joyce, B.M., Glaser, P.E.A., Gerhardt, G.A. Adderall® produces increased striatal dopamine release and a prolonged time course compared to amphetamine isomers. In press- Psychopharmacology.

Stanford, J.A., Salvatore, M.F., Joyce, B.M., Zhang, H., Gash, D.M., Gerhardt, G.A. Bilateral effects of unilateral intrastriatal GDNF on locomotor-excited and nonlocomotor-related striatal neurons in aged F344 Rats. Neurobiology of Aging (Epub ahead of print). 2005 Nov.

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Submitted Manuscript:

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Selected Published Abstracts:

Joyce, B.M., Epperly, G.D., Pomerleau, F., Huettl, P., Glaser, P.E.A., Gerhardt, G.A. Reverse Microdialysis Studies of Stimulant Treatments for ADHD. Monitoring Molecules in Neuroscience: 11th International Conference on *In Vivo* Methods, Sardegna, Italy, May 2006.

Joyce, B.M., Epperly, G.D., Gerhardt, G.A., Glaser, P.E.A. Reverse Microdialysis Studies of Stimulant Treatments For ADHD. Bluegrass Chapter of SFN- Spring Neuroscience Day, University of Kentucky, March 2006. Lexington Conference on RNA Therapy For Neurodegenerative Diseases, University of Kentucky, April 2006.

Glaser, P.E., Joyce, B.M., Epperly, G.D. Gerhardt, G.A. Dose-response of local applications of D-amphetamine and adderall® on dopamine overflow in the striatum of F344 rats, The American College of Neuropsychopharmacology Annual Meeting, Waikoloa, Hawaii, December 2005.

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Glaser, P.E.A., Joyce, B.M., Thomas, T.C., Castellanos, F.X., Gerhardt, G.A. Differential effects of D-amphetamine, adderall®, and other amphetamine isomers on dopamine neurotransmission in the striatum and nucleus accumbens core. The American College of Neuropsychopharmacology, December 2004.

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Glaser, P.E.A., Joyce, B.M., Currier, T.D., Gerhardt, G.A.The effects of amphetamines on dopamine neurotransmission in the rat, Fourth Annual Future Leaders in Psychiatry Meeting- April 2002.

Signed: Barry Matthew Joyce