

# The Neuropsychopharmacology of Stimulants: Dopamine and ADHD

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## 1. Introduction

In this chapter we consider the neuropsychopharmacology of ADHD in general and dopamine and the stimulants more specifically. Attention will be given to the various neurotransmitter theories for ADHD. We will consider the theoretical mechanisms of actions for the various medicines used to treat ADHD. We will look at how the stimulants, although often assumed to be similar, actually show evidence of differential mechanisms of action. We will look at new data that utilizes the technique of reverse microdialysis to demonstrate how different the dose-response curves are for dopamine release in the striatum following local application of the different stimulants.

Throughout the text we will use ADHD (Attention-Deficit/Hyperactivity Disorder) without reference to the DSM-IV type, unless a specific reference pertains to combined, inattentive or hyperactive subtypes.

## 2. Neuropsychopharmacology of stimulants

The stimulant medications were discovered serendipitously with the indirect observation that amphetamines calmed and focused children who were given the medicine to try to treat headache that was caused by the technique of pneumoencephalography, a largely outdated procedure where the spinal fluid was drained and replaced with air in order to see the brain more clearly on X-ray (Bradley, 1937, Strohl, 2011). The form of amphetamine used by Bradley was Benzedrine, the racemic mixture, or 50/50 mixture of d- and l-amphetamine. Because of research pointing to the dopamine releasing qualities of the stimulants, the earliest theory for ADHD was that it represented a hypodopaminergic state. This hypodopaminergic state theoretically led to alterations in reward sensitivity if it was in the nucleus accumbens, hyperactivity if lowered dopamine was in the striatum, and decreased inhibitory control if the lowered dopamine was in the frontal cortex. Although the collective data never supported such clean demarcations in brain structure and dependence solely on dopamine, the “hypodopaminergic” theory of ADHD is still a popular teaching in the clinical setting.

### 2.1 Dopamine and ADHD

Dopamine was not always considered a neurotransmitter. As details about the neurotransmitters were emerging dopamine was noted as the penultimate molecule in the

synthesis of norepinephrine. The concept emerged of the monoamines being packaged into discrete vesicles that could be released when an action potential brought on an influx of calcium. Dopamine was transported into these synaptic vesicles by VMAT (Vesicular Monoamine Transporter). Then the enzyme Dopamine  $\beta$ -Hydroxylase inside the vesicle converted the dopamine to norepinephrine. Work by Carlsson and others in the 1950s showed that some regions of the brain, particularly the basal ganglia that includes the striatum and nucleus accumbens, were enriched in dopamine and had very little norepinephrine (Cooper et al., 2003). Following these discoveries, dopamine's importance in coordinating motor control, Parkinson's Disease, and reward were established. It was found that following release of dopamine from presynaptic vesicles that dopamine had specific receptors postsynaptically that could modulate the neurons function (both stimulatory and inhibitory modulation depending on the dopamine receptors and second messenger systems). Dopamine receptors were also found presynaptically and thought to allow for feedback mechanisms for precise regulation of dopamine release. Finally, dopamine's effects were terminated both through reuptake into the presynaptic cytoplasm by the dopamine transporter (DAT), and by metabolism either inside the neuron by MAO (monoamine oxidase) or extracellularly by COMT (catechol O-methyl transferase) (see Figure 1).

As discoveries about dopamine were evolving, stimulants were being used for many purposes in the mid to late 20<sup>th</sup> century. Bradley's observations on amphetamine's benefit for children with features of ADHD went largely ignored for several decades. The stimulants found use for their ability to keep people awake despite fatigue. Several militaries in World War Two used both amphetamine and methamphetamine for this purpose, although it was soon found that soldiers would "crash" following this use and need time to recover. Tolerance was also noted with increasing doses needed for effects such as euphoria. Abuse was reported for several decades before the FDA banned Benzedrine inhalers and limited amphetamines to prescription use only in 1959. Researchers in the 1970s and 1980s connected and clarified the stimulants function in increasing dopamine in the synaptic cleft, as well as its connection to treating ADHD and the role of both tonic and phasic levels of dopamine (Robbins & Sahakian, 1979). Perhaps due to the ease of measurement and abundance of dopamine in the striatum and nucleus accumbens, dopamine research predominated over norepinephrine. In truth, amphetamines exert most of its CNS effects through dopamine and norepinephrine, with very little effects on serotonin. Methylphenidate is strongest at blocking dopamine and much less so norepinephrine, and even less so for serotonin (Gatley et al. 1996). Finally cocaine and methamphetamine seem to affect all three neurotransmitters, with their effect on serotonin theoretically leading to the greater euphoria. When this serotonin function is coupled to the reward function of dopamine release in the nucleus accumbens, it theoretically makes methamphetamine and cocaine have greater overall abuse potential compared to amphetamine and methylphenidate. To this day, stimulants are approved for use in ADHD, narcolepsy, and severe obesity; but with strict control by the FDA and other governmental agencies around the world.

## 2.2 Other Neurotransmitters and ADHD

As more intricacies have been revealed through animal models of ADHD and human research, other neurotransmitters have been implicated in ADHD. Perhaps the strongest case can be made for norepinephrine. Arnsten and colleagues have suggested that

norepinephrine is as important as dopamine in attention and ADHD. Recent elegant work in non-human primates suggest that alpha-2 adrenergic input in the frontal cortex is critical in maintaining working memory in a visual attention task constructed by Arnsten's group (Wang et al., 2007). Interestingly, dopamine-1 receptor input is needed in the areas surrounding the circuitry of working memory to suppress areas of the frontal cortex that were not needed for that specific memory. One might say that norepinephrine was allowing for saliency and attention, and dopamine for signal-to-noise adjustment or inhibition of inappropriate information (Gamo et al., 2010).

Initially one might think that atomoxetine lends credence to just the norepinephrine theories of ADHD in that it is a NET (norepinephrine transporter) inhibitor. But research has shown that the NET transports dopamine as well as NE. Thus atomoxetine raises NE and DA in the prefrontal cortex. Since NET is primarily present in the frontal cortex and not the nucleus accumbens or striatum, the neurotransmitter modulating effects of atomoxetine are only in the frontal cortex. This accounts for its lack of abusability, and perhaps the fact that atomoxetine overall is a less efficacious medicine for ADHD compared to amphetamine and methylphenidate (Lile et al., 2006). The stimulants in blocking DAT (dopamine transporter) also create increases in both DA and NE, since like NET, DAT transports both DA and NE.

Other neurotransmitters implicated in ADHD include acetylcholine, histamine, adenosine receptors, and glutamate. Nicotinic receptors are involved in various tasks requiring attention and this has led to the speculation that the high rate of smoking seen in people with ADHD may be due in part to "self-medication". Although most nicotinic medications have targeted Alzheimer's, their use in memory may prove beneficial to ADHD. Several histamine-3-receptor antagonists are in the stages of being tested for ADHD and other cognitive disorder (Sander et al., 2008). Only a few studies have been reported thus far and their results using these histamine modulating drugs for ADHD have been mixed (Brioni et al., 2011). Caffeine, an adenosine receptor antagonist, can improve symptoms of ADHD in some animal models perhaps through interactions of adenosine receptors and dopamine systems. Caffeine is poorly studied in ADHD but appears to help alertness more than actual symptoms of ADHD (Smith 2002). Glutamate has recently been implicated from both neuroimaging and neuroscience. One open labeled trial has shown that glutamate modulating drugs, such as NMDA antagonist memantine shows some efficacy in treating ADHD (Findling et al., 2007). Interestingly a recent patch clamp study suggests that atomoxetine is also an NMDA antagonist at clinical levels (Ludolph et al., 2010).

### 2.3 Heterogeneity amongst the stimulants

Returning to the dopamine mechanisms of action involved in ADHD let us now focus on how the separate stimulants used in treating ADHD are different from each other. Methylphenidate has been shown to have a mechanism of action similar to cocaine in that it specifically blocks DAT (see Figure 1). D-amphetamine (the dextro-isomer of amphetamine) has been shown to have three potential mechanisms. The first is direct effect on the DAT by allowing reverse transport of DA from the cytoplasm presynaptically into the synapse, this is a calcium-independent DA release that is perhaps coupled to overall decrease in DA uptake. Secondly d-amphetamine inhibits MAO-B (Monoamine oxidase-B isoform) which catabolizes DA. Thirdly, d-amphetamine inhibits VMAT (vesicular monoamine transporter) leading to an increase in cytoplasmic DA that can be reverse transported out by DAT (see

Figure 1) (Bergman et al. 1989; Cadoni et al. 1995). Although it is not known which of these three mechanisms is the most important of note is that all three are different than methylphenidate. This agrees with the clinically observed phenomena that responses to methylphenidate and d-amphetamine are not always equal in patients. Thus, if a patient is not doing well on one stimulant, say methylphenidate, then it is the recommended standard of care to then try an amphetamine preparation. Sonders et al. (1997) categorized pharmacological agents that act on the human dopamine transporter (hDAT) into two groups: substrates for DAT (including dopamine and amphetamine) and cocaine-like (including cocaine and methylphenidate). Thus, amphetamine can actually serve as a substrate for DAT, like dopamine itself; whereas methylphenidate is not a substrate for DAT.

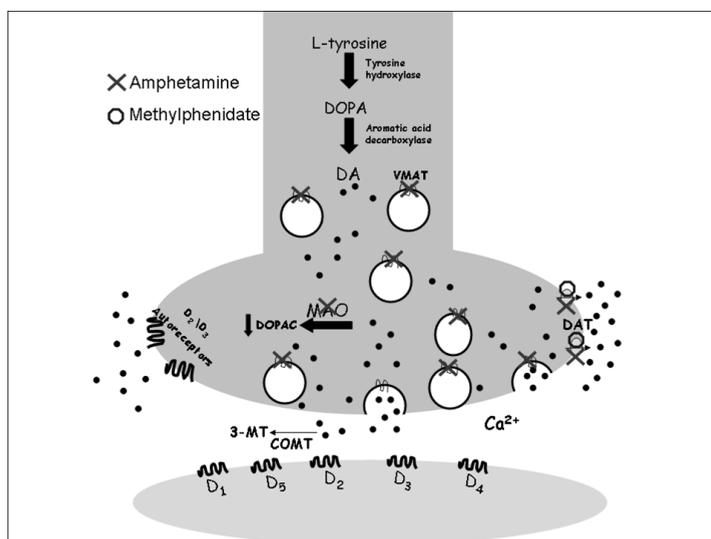


Fig. 1. Simplified Model of Dopamine synapse with putative mechanisms of action for amphetamine and methylphenidate.

But are all amphetamine preparations equivalent? What about the preparations such as Adderall that have some l-amphetamine (the opposite stereo isomer of d-amphetamine). In the 1990s the drug Adderall 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), but this was a minor difference. Other clinical trials support that Adderall is more effective than immediate-release methylphenidate on outcomes measured 4 to 5 hours after dosing (Pelham et al. 1999). A majority of data supports that population comparison of efficacy for stimulants in treating ADHD show little difference. It is only when you get to the individual patient that you find differences in the stimulants. For example, l-amphetamine alone has been tested and shown in a smaller study to be useful for some patients with ADHD, even a few which did not respond as well to d-amphetamine (Segal 1974). More recent comparison of controlled-release preparations of

amphetamines and methylphenidate show little differences in overall efficacy. Previous *in vivo* voltammetry data in our laboratory showed differences in kinetics between amphetamine optical isomers (Glaser et al. 2005). In these studies, preparations with L-amphetamine evoked faster DA 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 (Joyce et al. 2007) supporting different mechanistic effects of these drugs on DA release.

#### **2.4 Reverse microdialysis of stimulants in the rat striatum: hypothesis**

When comparing different stimulant medications and their effects on dopamine levels, several caveats have limited direct comparison. First of all, stimulants are often given by intraperitoneal injection, due in part to its ease and the fact that the rapid rise in blood levels makes dopamine easier to measure in brain regions. However variability in absorption and first pass effects of the liver make it difficult to compare concentrations between medications. Gavage or oral delivery of food, while simulating the clinical experience for ADHD, has even more pharmacokinetic factors involved due to gut absorption factors as well. Finally, many injection and oral stimulant studies have to use larger, more abuse related dosing, because there are often little appreciable changes in dopamine at drug dosing similar to that used in ADHD, although a few studies have been able to accomplish this (Berridge et al, 2006). In order to circumvent some of these caveats, and yet still look at the *in vivo* effects of these drugs and their differences on striatum, we chose the technique of reverse microdialysis. This technology places the medication in the dialysate that goes directly to the striatum and allows for direct and sensitive dose-response curves for stimulant-evoked dopamine.

The technique of reverse microdialysis coupled with high performance liquid chromatography with electrochemical detection (HPLC-EC) was used to study local drug-evoked increases in extracellular dopamine (DA) levels and changes in DA metabolites in the striatum of anesthetized rats. 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 likely attributable to changes in the surface expression of DAT on DA nerve endings and/or DAT function. Other *in vitro* studies have shown substrate dependent trafficking of the DAT to and from the plasma membrane and subsequent changes in the ability to transport DA (Kahlig et al. 2005; Johnson et al. 2005; Saunders et al. 2000; Kahlig et al. 2004; Kahlig and Galli 2003). Therefore to have the most accurate dose-response curves the same animal should not be used to test several doses. For these experiments drug-naïve animals were used 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). We tested the hypothesis that stimulant concentration-response curves of DA and its metabolites will display differential patterns of DA overflow that correlate with their mechanistic properties at the level of DAT function. In addition, we tested a unique formulation of 25% D- and 75%L-amphetamine and termed this mixture "Reverse Adderall", to contrast it with Adderall that is ~75% D- and 25% L-amphetamine. We hypothesized that the Reverse Adderall would also have a differential dose-response curve than the other amphetamine preparations.

## 2.5 Reverse microdialysis of stimulants in the rat striatum: methods

Male Fischer 344 (F344) rats (3-6 months old) were anesthetized with urethane (1.25 g/kg i.p. in 0.9% saline). After placement into a stereotaxic frame (Kopf, Tujunga, CA, USA) with the incisor bar set at -2.3 mm, the rat striatum was prepared for study. Body temperature was maintained by use of an isothermal heating pad (Braintree Scientific, Braintree, MA, USA) at 37° C and periodically monitored by a rectal thermometer. After the retraction of the skin and tissue and exposure of the skull overlying the striatum, a small craniotomy (2 x 2 mm) was carried out in the right hemisphere. The microdialysis probes were stereotactically placed with respect to bregma: +1.0 mm AP, ±2.2 mm ML, DV -6.0 mm) (Paxinos and Watson, 1986). The 2-mm length membrane probes (CMA/11, CMA Microdialysis, Stockholm, Sweden) remained at this location for the duration of the experiment. 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.

Fluid flow through the microdialysis probes was achieved using a syringe pump (KDS230, KD Scientific, Holliston, MA) fitted with 1ml gastight syringes (1001 LTN, Hamilton USA, Reno, NV) containing dialyzing fluid. Dialysis probes were perfused at a flow rate of 1 µl/min. Syringes were connected to a liquid switch (CMA/110, CMA Microdialysis, Stockholm, Sweden) that allowed for alternation between treatments: artificial cerebral spinal fluid (aCSF) (in mM: NaCl 123, KCl 3, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 1, and glucose 5.9) and aCSF + [drug]. Teflon tubing (FEP tubing, 0.12 mm i.d.) and tubing adapters (CMA Microdialysis, Stockholm, Sweden) were used to establish all connections. Samples were collected at twenty minute intervals into a 0.2 ml microcentrifuge tube and manually injected into an HPLC-EC system. The order of administration for each of the drug solutions tested was as follows: samples 1-6 (0-120 minutes, aCSF), sample 7 (120-140 minutes, aCSF + stimulant drug solution), samples 8-12 (160-240 minutes, aCSF).

Probe recoveries were collected using a standard solution with known concentrations of DA, norepinephrine (NE), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic Acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA). In order for a probe to be used in these studies, *in vitro* recoveries of 10% ± 1 were required. Based on this exchange rate, seen for molecules similar in size to amphetamine such as DA, NE, and 5-HT, we were able to more accurately adjust the effective concentrations of stimulant drugs being studied.

Stimulant concentrations used for reverse microdialysis studies were chosen to represent a range that included clinically relevant levels and abuse levels that were normalized for the amount of D-amphetamine. Our prior studies support that D-amphetamine determines the amount of DA released in the presence of both enantiomers (Glaser et al. 2005). The following concentrations were used based on ~10% exchange rate for the microdialysis probes: for D-amphetamine and methylphenidate, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 400 µM were studied; for Reverse Adderall, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 400 µM and 533 µM; ; and for Adderall , 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 400 µM and 539 µM solutions were studied . For D,L-amphetamine (normalized to D-amphetamine/2), L-amphetamine, and cocaine, only a concentration of 400 µM was tested for maximum effect comparisons. Prior to each

experiment, 20 mM ascorbic acid was added to each solution and solutions were aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Solutions were immediately added to individual 1 ml gastight syringes. 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 sectioned stained with cresyl violet to verify probe placement in the striatum.

High-Performance Liquid Chromatography Coupled with Electrochemical Detection (HPLC-EC) analysis followed the methods previously 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), E1 = +0.35 mV and E2 = -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-octanesulfonic acid delivered at a flow rate of 2.0 ml/min. Peaks for the analytes were identified by retention times from known standards.

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  $1 \times 10^{-7}$  M mixed standard of known analytes and probe recoveries of ~ 10%. 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<sub>50</sub>) 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 release produced following reverse microdialysis of clinically relevant drug concentrations and maximum concentrations. Potency measures were defined by the stimulant that reached its half-maximal response on the concentration-response curve with the lowest effective concentration of stimulant. Efficacy measures were defined by the highest amount of DA overflow evoked when all stimulant concentrations were at maximal levels. Statistical significance was defined as  $p < 0.05$ .

## 2.6 Reverse microdialysis of stimulants in the rat striatum: results

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). Baseline DOPAC levels were determined to be (~800-1000 nM) in the rats used for the D-amphetamine and Adderall studies and are similar to previously reported levels (Ferguson et al. 2003). The DOPAC data are reported as percent of baseline due to increased variance in baseline samples collected from the rats used for the Reverse Adderall and

methylphenidate studies (Fig. 3). Levels of the DA metabolite homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and serotonin (5-HT) levels were measured and concentration-dependent effects were not detected (data not shown).

The twenty minute local tissue perfusions of drugs induced a concentration-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 (Wise and Hoffman 1992; Sulzer et al. 1993; Schweni 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 seen to decline over 2 more fractions post drug administration. Furthermore, applications of the lowest stimulant concentration resulted in DA levels that were not statistically different from those seen after reverse microdialysis of the artificial cerebral spinal fluid (aCSF) control.

The resulting D-amphetamine concentration-response curve for extracellular DA in rat striatum displayed an unexpected double-sigmoidal pattern with two plateaus. Plateaus in the amount of DA overflow occurred at the lower concentration (1  $\mu\text{M}$  D-amphetamine) and at a higher concentration (100  $\mu\text{M}$  D-amphetamine). At 0.1  $\mu\text{M}$  D-amphetamine, little or no

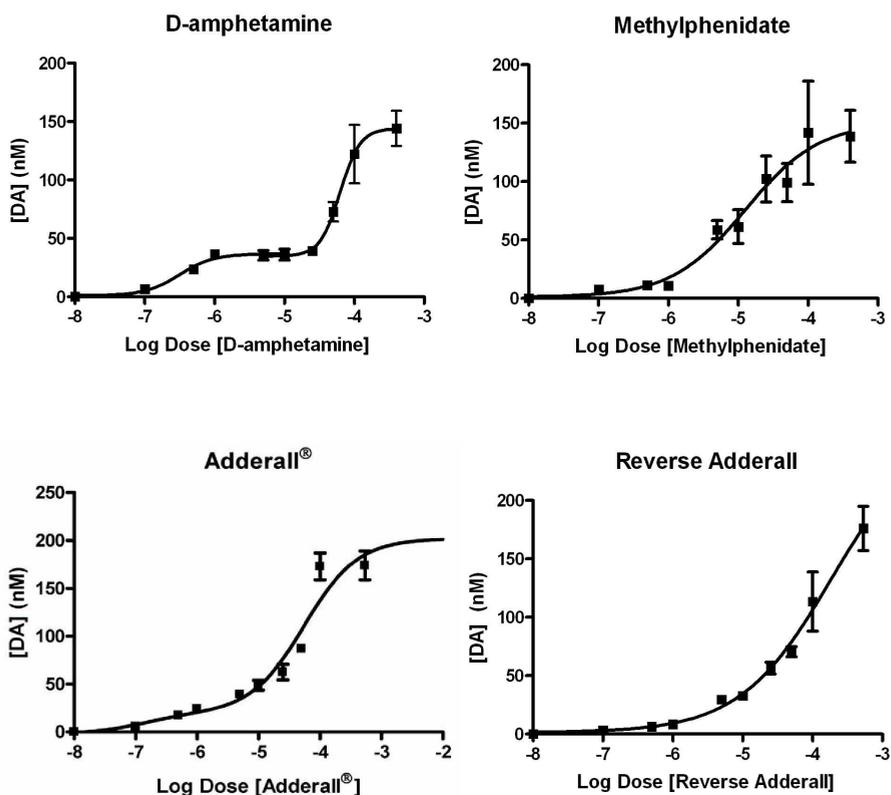


Fig. 2. Dose-Response curves for evoked overflow of dopamine in the rat striatum by various stimulants.

increase in DA overflow resulted in comparison to aCSF control; and no significant differences were found between 100  $\mu\text{M}$  and 400  $\mu\text{M}$  D-amphetamine supporting an upper plateau in DA measures (Figure 2). Two half-maximal effective concentration ( $\text{EC}_{50}$ ) values are indicated for the higher potency (lower plateau) and lower potency (upper plateau) portions of this concentration-response curve (Table 1).

| Drug             | $\text{EC}_{50}$ [Drug] ( $\mu\text{M}$ ) For DA | Maximum Response ( $\mu\text{M}$ ) For DA |
|------------------|--|---|
| Methylphenidate  | 10   | 138.7 $\pm$ 22.2                          |
| Adderall         | 25   | 184.6 $\pm$ 12.3                          |
| D-amphetamine II | 50   | 144.5 $\pm$ 15.6                          |
| Reverse Adderall | 50   | 176.7 $\pm$ 19.1                          |
| D-amphetamine I  | 0.5  | N/A                                       |

Table 1. Stimulant Potency and Efficacy on DA Measures

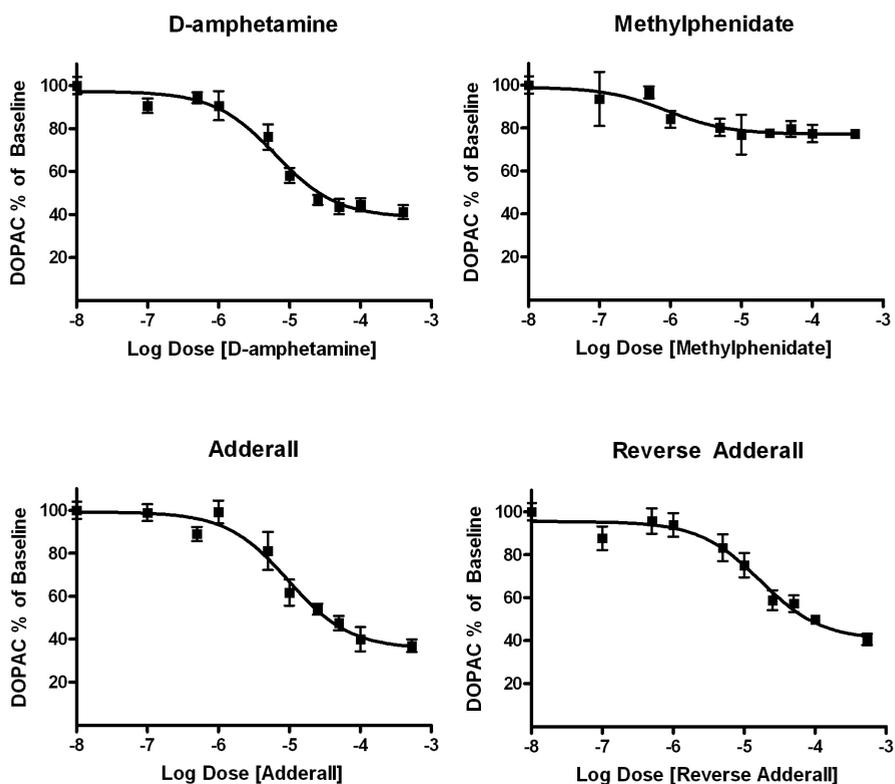


Fig. 3. Dose-Response curves for overflow of dopamine metabolite DOPAC in the rat striatum by various stimulants.

The methylphenidate concentration-response curve for extracellular DA in the rat striatum also supports a concentration-dependent increase in DA levels (Figure 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 much lower levels of DA release in an anesthetized rat. It was therefore surprising to see that applications of 0.5-400  $\mu\text{M}$  methylphenidate increased DA concentrations significantly greater than aCSF control. However, in contrast to d-amphetamine, 0.1  $\mu\text{M}$  methylphenidate did not cause increased DA release that was significantly different from control. The two highest concentrations tested (100 and 400  $\mu\text{M}$ ) were not significantly different in the amount of DA release (Figure 2).

The Adderall concentration-response curve for extracellular DA measured in the rat striatum demonstrated a similar range of evoked DA overflow, although the dose-response curve was closer to a single sigmoidal curve (Figure 2). An upper plateau in DA levels occurred at 100  $\mu\text{M}$  Adderall, as 100  $\mu\text{M}$  and 400  $\mu\text{M}$  Adderall were not significantly different in response. At 0.1  $\mu\text{M}$ , Adderall did not produce DA levels that were significantly different from local application of aCSF control.

Finally, the Reverse Adderall (75% L-amphetamine, 25% D-amphetamine) concentration-response curve for extracellular DA showed a concentration-dependent increase in evoked DA at all concentrations tested except for 0.1  $\mu\text{M}$ ; which was not significantly different from aCSF control (Figure 2). While Reverse Adderall was predominantly made of L-amphetamine, it did not increase DA levels to the extent of Adderall at some concentrations (Table 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.

Figure 3 shows the individual tracings of detected DOPAC levels (represented as % of baseline) following reverse microdialysis of D-amphetamine at multiple concentrations. D-amphetamine, Adderall, and Reverse Adderall inhibited 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. 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. DOPAC production was less affected by methylphenidate and cocaine in comparison to Adderall ( $p < 0.001$ ), and D-amphetamine ( $p < 0.01$ ,  $p < 0.05$ ). Reverse Adderall, L-amphetamine, and D,L-amphetamine all caused significantly greater effects on DOPAC levels in comparison to cocaine ( $p < 0.001$ ). An initial increase in DOPAC was seen following application of 100  $\mu\text{M}$  and 400  $\mu\text{M}$  methylphenidate followed by a decrease similar to that of other concentrations without a pronounced concentration-dependent pattern (data not shown).

## 2.7 Reverse microdialysis of stimulants in the rat striatum: implications

These data represent novel findings regarding the effects of various stimulants across a range of concentrations on dopamine release in the striatum. 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 levels of D-amphetamine affect DA efflux. In addition, these data show for the first time that local applications of methylphenidate increased DA levels in a concentration-dependent

pattern and even demonstrated a greater EC<sub>50</sub> (DA) when compared to other stimulants. These data are in agreement with our previous *in vivo* high speed chronoamperometric data that support the robust local activity of Adderall compared to other ADHD medications (Joyce et al. 2007). Decreased DA levels caused by cocaine compared to higher DA levels after local application of methylphenidate suggest dissociation between the local effects of methylphenidate and cocaine. DOPAC levels in these studies showed significant decreases following additions of any of the amphetamine preparations in a dose- dependent fashion, whereas cocaine and methylphenidate were less effective in inhibiting DOPAC production.

The data shown here are consistent with the known DA releasing properties of amphetamine predominantly due to 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). Our previous data support amphetamine enantiomeric differences that could not be accounted for across multiple concentrations due to technical limitations; particularly with the current difficulties of studying the effects of low levels of these drugs on DA release using *in vivo* electrochemical methods (Glaser et al., 2005). 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 likely attributable to changes in the surface expression of DAT on DA nerve endings and/or DAT function. Other *in vitro* studies have shown substrate dependent trafficking of the DAT to and from the plasma membrane and subsequent changes in the ability to transport DA (Kahlig et al. 2005; Johnson et al. 2005; Saunders et al. 2000; Kahlig et al. 2004; Kahlig and Galli 2003). Therefore we used stimulant-naïve animals for these studies, an important but often neglected consideration in many mechanistic studies of stimulant medications.

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 in these studies. Voltammetry affords the ability to study neurotransmission with high temporal and spatial resolution; however, we lose a magnitude of sensitivity that is available using microdialysis coupled with HPLC-EC. Using HPLC-EC to analyze samples collected during reverse microdialysis (local application) of stimulant drugs allows for studies to be carried out with lower 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. 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.

Although we did measure norepinephrine (NE) with our HPLC methods, the peak was not consistently measurable due to its proximity to the solvent edge. In addition, NE is not a

common neurotransmitter in the striatum. Therefore, one caveat of our study is that it does not investigate the possibility that NE elevation, and not DA, in the PFC (and not the striatum) is most important for clinical efficacy in ADHD. This theory goes on further to state that DA is elevated in the synapse only at higher doses of stimulants and that this leads more to the rewarding symptoms and drug-abuse potential (the downward slope of the theoretical inverted-U of stimulant action). Our data would suggest that this may be true for methylphenidate, but that d-amphetamine does involve appreciable DA at lower-doses that may work in concert with NE in the PFC. This would also give credence to the fact that the two main stimulants are both tried on patients with ADHD because some will respond well to one and not the other, where as other patients respond to both. Obviously, a repeat of this study using microdialysis in the PFC and an HPLC method to pick up the lower levels of DA and NE in the PFC would be needed to answer this mechanism of action question.

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 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, we suggest that lower concentrations of D-amphetamine release "newly synthesized" DA pools in the cytosol, and higher concentrations contribute to the emptying of vesicular stores. Together this produces 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 D2R autoreceptors leading to second messenger regulation. Others have reported a link between stimulation of D2R 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, decreased DA clearance in the striatum, prefrontal cortex, and nucleus accumbens after administration of the D2R agonist raclopride has been demonstrated (Cass and Gerhardt 1994). In addition, acute amphetamine stimulation caused increased synaptosomal DAT surface expression that occurred within 30 seconds (Johnson et al. 2005) indicating the rapid trafficking of the DAT and supporting that these changes would have occurred during the time frame we were sampling (Saunders et al. 2000). Due to the comparatively increased sensitivity of D2R autoreceptors, low levels of extracellular DA are sufficient to stimulate these autoreceptors that would result in increased DA clearance (Cooper et al. 2003) (Fig. 4). 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 DA clearance will likely be followed by autoreceptor desensitization caused by the high levels of DA released after such a robust concentration of drug (Khoshbouei et al. 2004; Gorentla and Vaughan

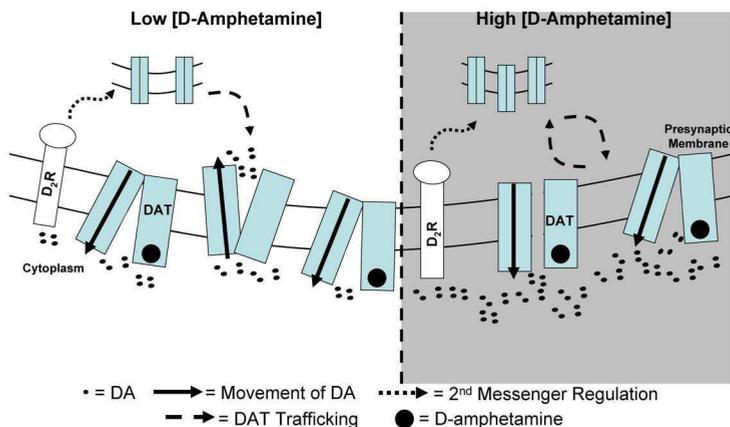


Fig. 4. Theoretical model of activity describing the double Plateaus of the D-amphetamine concentration-response curve for DA: Plateau I Low [D-amphetamine]: Lower concentrations of D-amphetamine cause reverse transport of low levels of DA through the DAT. In addition, DA sensitive D2R autoreceptors are stimulated. Due to the increased clearance of DA, the result is the first plateau of the concentration-response curve. Plateau II High [D-amphetamine]: Amphetamine has been shown to interact with DATs and facilitate DA release followed by DAT internalization. Higher concentrations of D-amphetamine will likely cause increased DA release and DAT internalization. D2R autoreceptor desensitization is likely to occur and interrupt DAT expression. Higher levels of extracellular DA and decreased DA clearance likely cause the second plateau.

2005; Kim et al. 2001; Namkung and Sibley 2004; Ferguson et al. 1996; Tang et al. 1994) (Fig. 4). 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), that may contribute to the effects we see in the second plateau of the D-amphetamine concentration-response curve. We propose that the amount of D-amphetamine in Adderall leads to a similar but slightly different concentration-response curve. Previous in vivo electrochemical data support the faster kinetics of the effects of L-amphetamine in combination with the slower kinetics of D-amphetamine could allosterically modulate DAT trafficking rendering a concentration-response with less apparent plateaus (Glaser et al. 2005; Joyce et al. 2007). Another possible correlation is the similar EC50 for both the second D-amphetamine sigmoidal curve and the DOPAC decrease consistent with MAO inhibition.

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.

Intraperitoneal administration of methylphenidate in freely-moving rat has been shown to cause increases in DA levels measured in dialysates and some argue that the greatest effects were seen in the prefrontal cortex (Hurd and Ungerstedt 1989; Berridge et al. 2006). In particular, Hurd and Ungerstedt found that amphetamine and methylphenidate caused similar increases in DA levels; however, methylphenidate caused these levels over a longer time period correlating with the robust effects of methylphenidate we present here. 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). In terms of behavioral effects, D-amphetamine and methylphenidate have 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 in animal models (Stoops et al. 2005; Rush et al. 2001; Risner and Jones 1975). In general, cocaine and methylphenidate are thought to work in a similar manner by predominantly acting as competitive inhibitors of the DAT (Wu et al. 2001) and increases in extracellular DA result predominantly from this blockade after impulse-dependent release of DA. However, the robust extracellular effects of methylphenidate in this study argue against this concept as the effects observed mirror d-amphetamine and not cocaine.

While the argument can be made that the studies herein that involve local applications of drugs fail 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 levels, including clinically relevant concentrations (10-50  $\mu\text{M}$ ) and potentially drug abuse levels (>400  $\mu\text{M}$ ) (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 allowing for more of the pure effects of the drugs on DA nerve terminals.

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. 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 confirm that methylphenidate, d-amphetamine, and combinations of amphetamine isomers have potent, yet different, effects on dopamine in the striatum.

## 2.8 Future directions in the neuropsychopharmacology of ADHD

The data presented herein demonstrates the translational aspect of how the currently available stimulants are different from each other, and therefore backs up the current practice of trying different stimulants on patients to maximize efficacy and minimize side effects. It also suggests that other percentages of l-amphetamine may be useful to test in the future for some patients with ADHD may respond better to them. Yet, this data does little to address some of the larger problems that face us in understanding ADHD and in finding superior treatments for ADHD. Some might argue that the stimulants are largely effective and safe already. But with the risk of abuse and diversion, the fact that many

adolescents and adults do not like how they feel on them and many other factors such as whether or not they truly decrease a person's subsequent risk for substance abuse, there is still room for improved medications. Perhaps one goal may be to find a medication that truly helps only those with ADHD, since stimulants actually can be "performance enhancing" drugs that can give people such as college students without ADHD benefits in studying or taking tests with little knowledge of their possible dangers or ethical implications.

In the future it will be useful to more fully understand not only the neurotransmitters involved in the various aspects of ADHD, but the way that circuitry and brain region interact to lead to dysfunction. Neuroimaging may contribute greatly to this as it obtains greater resolution and ways to measure separate neurotransmitter systems. Our lab and others have begun to use neurotransmitter specific probes to measure more accurately second by second changes in neurotransmitters. We are finding the milieu is much more heterogeneous than previously understandable by microdialysis. As microelectrode technology improves and gets more compact, real-time recordings of multiple brain areas while the animal is awake will answer more questions and allow for more precise drug development. Finally, pharmacogenomics is starting to yield some benefit and may help in more targeted use of the right stimulants for the right patient instead of the trial and error method now employed.

### 3. Conclusion

In this chapter we have reviewed the case for dopamine's role in ADHD especially as it pertains to the mechanisms of action of the stimulants methylphenidate and the various amphetamines. We have also shown how many other neurotransmitters are involved in ADHD and alternative medications for ADHD. No doubt as the neuropsychopharmacology of ADHD evolves, we will discover more intricate details about the relative contributions of the neurotransmitters and how they relate to the genetic and neurocircuitry levels of our understanding. The ultimate goal of this knowledge is to improve treatment and maximize safety for people of all ages with ADHD.

### 4. Acknowledgment

Special thanks to B. Matthew Joyce, Ph.D., Garretson D. Epperly, M.S., Theresa Currier Thomas, Ph.D for their contributions in conducting the experiments in this chapter. These studies were supported by USPHS grants MH066393, MH01245, DA14944, and NS39787. Pure substance Adderall® was provided by Shire Pharmaceuticals, Hampshire, Chineham, England.

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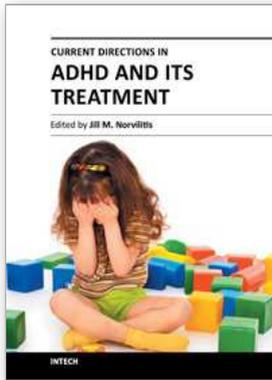
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## **Current Directions in ADHD and Its Treatment**

Edited by Dr. Jill M. Norvilitis

ISBN 978-953-307-868-7

Hard cover, 302 pages

**Publisher** InTech

**Published online** 15, February, 2012

**Published in print edition** February, 2012

The treatment of Attention Deficit Hyperactivity Disorder is a matter of ongoing research and debate, with considerable data supporting both psychopharmacological and behavioral approaches. Researchers continue to search for new interventions to be used in conjunction with or in place of the more traditional approaches. These interventions run the gamut from social skills training to cognitive behavioral interventions to meditation to neuropsychologically-based techniques. The goal of this volume is to explore the state-of-the-art in considerations in the treatment of ADHD around the world. This broad survey covers issues related to comorbidity that affect the treatment choices that are made, the effects of psychopharmacology, and non-medication treatments, with a special section devoted to the controversial new treatment, neurofeedback. There is something in this volume for everyone interested in the treatment of ADHD, from students examining the topic for the first time to researchers and practitioners looking for inspiration for new research questions or potential interventions.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Paul E.A. Glaser and Greg A. Gerhardt (2012). The Neuropsychopharmacology of Stimulants: Dopamine and ADHD, *Current Directions in ADHD and Its Treatment*, Dr. Jill M. Norvilitis (Ed.), ISBN: 978-953-307-868-7, InTech, Available from: <http://www.intechopen.com/books/current-directions-in-adhd-and-its-treatment/the-neuropsychopharmacology-of-stimulants-dopamine-and-adhd>

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