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Effects of Early Adolescent Exposure to $d$-Amphetamine in a Rodent Model of Nicotine Reward

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EFFECTS OF EARLY ADOLESCENT EXPOSURE TO d-AMPHETAMINE
IN A RODENT MODEL OF NICOTINE REWARD

by

Eric L. Harvey

A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
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EFFECTS OF EARLY ADOLESCENT EXPOSURE TO \textit{d}-AMPHE TAMINE IN A RODENT MODEL OF NICOTINE REWARD

Eric L. Harvey, Ph.D.
Western Michigan University, 2017

Previous studies with rodents have found that adolescent exposure to psychoactive drugs can alter neurophysiology and produce behavioral effects that persist into adulthood. While several studies have examined the effects of adolescent exposure to nicotine on the subsequent rewarding value of various drugs of abuse in adulthood, to date, no known studies have examined the converse of this relationship. \textit{d}-Amphetamine (i.e., dextroamphetamine, ProCentra\textsuperscript{®}) is a potent psychostimulant that is commonly used in the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD) in adolescents. The present study assessed the effects of adolescent exposure to \textit{d}-amphetamine on the rewarding value of nicotine in adulthood.

Seventy-two male Sprague-Dawley rats received subcutaneous (sc) injections of \textit{d}-amphetamine (\textit{d}-AMPH) (0.5 mg/kg; \textit{n} = 36) or saline (1.0 ml/kg; \textit{n} = 36) once-daily for 10 days during adolescence, beginning on postnatal day (PND) 31. Subjects were then allowed to mature to early adulthood (PND 70), and the reward potential of nicotine was evaluated using a 10-day biased conditioned place preference (CPP) procedure. Side preference for a two-compartment CPP apparatus was assessed for each subject during a pre-conditioning test session, followed by eight once-daily 30-minute conditioning trials. During conditioning trials, subjects were confined to one side of the CPP apparatus and received injections of nicotine (0.04 or 0.10 mg/kg sc; \textit{n} = 24 each) or saline (1.0 ml/kg sc; \textit{n} = 24) in the non-preferred compartment (conditioning trials 1,
3, 5 and 7). All subjects received injections of saline in the preferred compartment (conditioning trials 2, 4, 6 and 8). Following conditioning trials, a post-conditioning test session assessed for the emergence of place preference for the drug-paired compartment. As a secondary measure of drug effects, locomotor activity was recorded during all sessions.

Subjects in the d-AMPH pre-treatment group exhibited a significant reduction in locomotor activity across nicotine drug trials not seen in saline pre-treated subjects. Both doses of nicotine tested produced significantly less activity compared to saline in the d-AMPH pre-treated group, but not in the saline pre-treatment group. Subjects pre-treated with d-AMPH did show a greater increase in preference for the drug-paired compartment with both nicotine doses versus saline pre-treated subjects, though this difference was not statistically significant. These results indicate that adolescent exposure to d-AMPH produces moderate behavioral effects that may persist into adulthood. Additional studies investigating the neurobehavioral effects of adolescent d-AMPH exposure on the rewarding properties of drugs of abuse are warranted to further elucidate this relationship.
I would like to begin by thanking all of the members of the Baker and Poling labs who gave their time and effort in helping me on this project and others. A special thank you to Robert Kohler and Harmony Risca for their contributions to this research. I would also like to thank my friend and colleague, Dr. Michael Berquist II, for his invaluable advice and feedback throughout the dissertation process.

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Ad astra per aspera

Eric L. Harvey
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INTRODUCTION

Adolescent Drug Exposure

Adolescence signifies a period of significant neural growth and development (Giedd et al., 1999; Gogtay et al., 2004). During this time the volume of gray matter (primarily neuronal cell bodies) in the CNS reaches its peak, in conjunction with a continual increase in CNS white matter (neuronal axons) (Ashtari & Cyckowski, 2012). It is thought that disruptions to the neural development process during this critical period represent a significant risk factor in the development of several adolescent psychiatric disorders (Paus, Keshavan, & Giedd, 2008).

Concordantly, adolescent exposure to psychoactive drugs has been shown to produce neurobehavioral effects that persist into adulthood (Achat-Mendes, Anderson, & Itzhak, 2003; Bambico, Nguyen, Katz, & Gobbi, 2010; Pistis et al., 2004).

A growing body of evidence has suggested that drugs of abuse can have differential effects in adolescents versus adults (Smith, 2003), and that early adolescent drug use is most commonly associated with addiction and substance use disorders in adulthood (Clark, Kirisci, & Tarter, 1998; Jordan & Andersen, 2016). While several studies have now examined the between-group differences that exist between the effects of adolescent and adult drug exposure, fewer have attempted to assess the within-subject relationship between adolescent drug exposure and the subsequent effects of drug exposure in adulthood. Of the limited number of studies that have investigated this relationship, a substantial number have concerned the potential for adolescent exposure to tobacco (i.e., nicotine), to alter the behavioral effects of other drugs of abuse during adulthood (e.g., Alajaji et al., 2016; McQuown, Belluzzi, & Leslie, 2007; Nolley & Kelley, 2007; Pipkin et al., 2014; Pomfrey, Bostwick, Wetzell, & Riley, 2015; Weaver et al., 2012). This focus on adolescent nicotine exposure has presumably been due to the prevalent use of tobacco
and other nicotine containing products by adolescents (Alajaji et al., 2016). Curiously, despite the serious health risks posed by tobacco/nicotine use itself, there exists a paucity of research evaluating the converse of the ‘adolescent nicotine—adult drug use’ relationship. Amphetamine is a potent psychostimulant that is increasingly prescribed in the treatment of adolescent Attention-Deficit/Hyperactivity Disorder (ADHD), a disorder that currently affects up to 11% of school age children, and is rising (Visser et al., 2014). To date, no known studies have examined the relationship between adolescent exposure to amphetamine and the reward properties of nicotine in adulthood.

**Amphetamine**

**Use in Attention-Deficit/Hyperactivity Disorder Treatment.** Amphetamine was first synthesized in 1887, but was not examined for medical use until several years later (Anglin, Burke, Perrochet, Stamper, & Dawud-Noursi, 2000). In 1927, G. A. Alles re-discovered amphetamine and began testing it as a potentially cheaper replacement for ephedrine, finding that it produced similar stimulant effects (Heal et al., 2013). The pharmaceutical company Smith, Kline and French first marketed a form of racemic amphetamine as Benzedrine® beginning in 1933, for use as a decongestant and later as a treatment for narcolepsy, asthma, and various other ailments. A report by Bradley (1937) first described the use of amphetamine to treat children with “behavioral problems”, many of which would later be considered symptoms of ADHD. Subsequent experiments by Bradley and others led to the use of amphetamine and its isomers in the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD), which first appeared as a psychiatric diagnosis under the name “Hyperkinetic Reaction of Childhood (or adolescence)” in the second edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-II) in 1968 (American Psychiatric Association, 1968).
Attention-Deficit/Hyperactivity Disorder (ADHD) is a psychiatric disorder characterized by symptoms of inattention, hyperactivity, and impulsivity (American Psychiatric Association, 2013). It is the most common neurodevelopmental disorder diagnosed in children and adolescents (Booth, 2016; Childress et al., 2017), with an average estimated prevalence of 7.2% in that population (Thomas, Sanders, Doust, Beller, & Glasziou, 2015). ADHD in adolescents is associated with poor academic achievement, deficits in social relationships, and emotional difficulties (Booth, 2016; Ratzliff et al., 2016).

Central nervous system (CNS) stimulants are considered the first line of pharmacotherapy treatment for ADHD in children and adolescents (Antshel et al., 2011; Brahmbhatt et al., 2016), with clinical studies showing them to be highly efficacious in core symptom reduction (Jensen, 1999; Pliszka, 2007; Spencer et al., 1996). Zuvekas and Vitiello (2012) found that between 1996 and 2008, the number of stimulant prescriptions for adolescent ADHD grew by 6.5% annually. More recent reports have indicated that this trend has continued (Bachmann et al., 2017; Burcu, Zito, Metcalfe, Underwood, & Safer, 2016). One of the most commonly prescribed treatments include drugs in the amphetamine class, such as ProCentra® (d-amphetamine) and Adderall® (3:1 mixture of d- to l-amphetamine enantiomers) (Bachmann et al., 2017; Heal, Smith, Gosden, & Nutt, 2013; Rubia et al., 2014).

**Pharmacology of Amphetamine.** Amphetamine (a contraction of alpha-methylphenethylamine) belongs to the phenethylamine class of compounds, which includes other drugs such as cathinone, ephedrine, methamphetamine, and MDMA. It is a molecule with a stereocenter and exists in the form of two stereoisomers (enantiomers), dextroamphetamine and levoamphetamine, or d-amphetamine and l-amphetamine, respectively. Racemic amphetamine (generally referred to as simply ‘amphetamine’) contains a 1:1 ratio of the d- and l-enantiomers.
Of the two enantiomers, $d$-amphetamine has been found to be the more potent (Easton, Steward, Marshall, Fone, & Marsden, 2007; Segal, 1975).

Amphetamine is a potent psychostimulant with a mechanism of action that involves numerous receptors and signaling pathways in the CNS (Hutson, Tarazi, Madhoo, Slawecki & Patkar, 2014; Miller, 2011). However, its effects are chiefly exerted through the disruption of presynaptic vesicles and the reversal of dopamine and norepinephrine transporters (DAT & NET), causing an efflux of monoamines into the synapse (Jones, Gainetdinov, Wightman, & Caron, 1998; Robertson, Matthies, & Galli, 2009). Amphetamine can also produce sympathomimetic effects in the peripheral nervous system (PNS) including tachycardia, hypertension, and diaphoresis (Greene, Kerr, & Braitberg, 2008).

**Nicotine**

*Nicotine and Tobacco.* Nicotine is a naturally occurring alkaloid found primarily in the Solanaceae (nightshade) family of plants. The tobacco plant (*Nicotiana tabacum*) is a member of this family and it was from that plant that the compound was first isolated by German chemists Wilhelm Heinrich Posselt and Karl Ludwig Reimann in 1828 (Henningfield & Zeller, 2006). In his 1924 book *Phantastica*, the renowned pharmacologist Louis Lewin first identified nicotine as being “[t]he decisive factor in the effects of tobacco, desired or undesired…” (1924/1998, p. 256). Although various reports of the addictive properties and health harming effects caused by tobacco had existed for nearly half a century or more, public health policy in the U.S. was slow to react to the problem. The issue was first addressed starting with the publication of 1964’s *Smoking and Health: Report of the Advisory Committee to the Surgeon General*, which referred to tobacco use as “habituating” but stopped short of identifying it as an addiction (USPHS, 1964). It wasn’t until 1979’s *Smoking and Health: A Report of the Surgeon General* that
smoking was first identified as a “substance abuse dependency” (USPHS, 1979). Finally, in 1988 nicotine was officially identified by the Surgeon General as the powerfully addictive component of tobacco (USDHHS, 1988).

Today, despite public awareness campaigns and stricter laws, tobacco use continues to be one of the greatest public health concerns in the U.S. and other parts of the world. As recent as 2015, it was estimated that 15% of U.S. adults (approximately 36.5 million people) were using tobacco via cigarette smoking alone (Jamal et al., 2016). Approximately $170 billion in U.S. annual healthcare costs are attributable to tobacco use and it is currently responsible for an estimated 480,000 deaths per year in the U.S. alone (USDHHS, 2014; Xu et al., 2015). More recently, the last decade has also seen the introduction and rise in popularity of “electronic cigarettes” or “e-cigarettes” (Schraufnagel, 2015). Despite often being touted as a less harmful alternative to conventional cigarettes, e-cigarettes have been found to frequently contain many of the same toxic substances, such as formaldehyde, acetone, and lead (Cheng, 2014). Due to their novelty, the long-term effects of e-cigarettes are unclear, and it is possible that they may ultimately pose a similar health risk to tobacco products.

**Reinforcing / Rewarding Effects.** Following the 1964 Surgeon General report in which the deleterious health effects were first officially recognized, an emphasis was placed on tobacco and nicotine research. Several early studies on nicotine were focused on determining its role in maintaining tobacco use – primarily smoking. Lucchesi, Schuster, and Emley (1967) examined the effects of intravenous injections of nicotine on the frequency of cigarette smoking. The experimenters administered nicotine to adult male and female smokers at doses that previously were found to not produce salient subjective effects. When the participants were given nicotine doses in the range of 2 – 4 mg/kg per hour, the average number of cigarettes smoked and the
The amount of each cigarette smoked was significantly reduced during the drug sessions compared to saline sessions. A similar study by Jarvik, Glick, and Nakumura (1970) examined the effects of orally administered nicotine on cigarette smoking frequency. In that study, subjects were given capsules containing either lactose (placebo) or nicotine for four days and allowed to smoke *ad libitum*. The results of the study showed that subjects smoked significantly fewer cigarettes on nicotine days versus placebo days. Ashton and Watson (1970) investigated the differences in cigarette “puffing” between subjects given cigarettes with high-retention (nicotine limiting) filters and those given cigarettes with low-retention filters. They found that subjects given the high-retention cigarettes took significantly more puffs than subjects with low-retention cigarettes and both groups received approximately equal amounts of nicotine throughout the trials.

Stolerman et al. (1973) found that mecamylamine, an antagonist of nicotinic acetylcholine receptors (nAChR) in the central nervous system (CNS), increased the rate of smoking in human subjects, while the peripherally acting nAChR antagonist pentolinium, did not. Deneau and Inoki (1967) used intravenous self-administration in rhesus monkeys to directly study the reinforcing properties of nicotine itself. They found that the animals would reliably self-administer a 0.025 mg/kg dose of nicotine, with some continuing to doses as high as 2.0 mg/kg. The subjects continued to self-administer high doses of nicotine despite the fact that at doses higher than 0.2 mg/kg, clear signs of distress including dyspnea, vomiting, and muscular weakness were observed. These studies served to validate the theory of nicotine as the primary psychoactive component maintaining tobacco use, and provided the impetus for more than five decades of subsequent nicotine research.

**Pharmacology of Nicotine.** Nicotine is psychostimulant that belongs to a chemical class of compounds known as tertiary amines. It is an optically active molecule that exists in the form
of two stereoisomers (enantiomers), (+)-nicotine (i.e., (R)-nicotine) and (-)-nicotine (i.e., (S)-nicotine). The (-)-nicotine isomer accounts for nearly all nicotine’s psychoactive effects and represents more than 99% of the nicotine found in tobacco products (Benowitz, 2009; Benowitz, Hukkanen, & Jacob III, 2009). Nicotine is a nicotinic acetylcholine receptor (nAChR) agonist, and exerts its psychoactive effects primarily through the mediation of nAChRs in the CNS (Balfour & Fagerström, 1996). Stimulation of CNS nAChRs by nicotine imitates the release of multiple neurotransmitters, most notably dopamine, which is thought to be the primary mechanism underlying nicotine reward and addiction (Dani & De Biasi, 2001).

**Animal Models of Drug Abuse Liability**

In psychopharmacology research, animal models are commonly employed to assess the rewarding/reinforcing value and abuse liability of psychoactive compounds. Nonhuman models are often used due to the practical limitations and ethical restraints involved with human drug research. While no animal model is capable of fully emulating human drug use, these models do allow for specific aspects of drug use to be investigated, and as such can have moderate to high construct and predictive validity (Heidbreder, 2011).

The conditioned place preference (CPP) assay is a well-established animal model used to assess the rewarding and/or aversive properties of a drug (Bardo & Bevins, 2000; Huston, de Souza Silva, Topic, & Müller, 2013). It is often used to evaluate abuse liability as well as measures of drug “craving” and pharmacological mechanisms of action (Tzschentke, 1998; Tzschentke, 2007).

**Conditioned Place Preference (CPP)**

**History of CPP.** The likely earliest progenitor to what would become the conditioned place preference paradigm was a study by Spragg (1940) examining morphine addiction in
chimpanzees (Bardo & Bevins, 2000). In that study, chimpanzees developed a dependence on morphine through daily injections given by the researchers. The chimpanzees were then taught to discriminate between two boxes containing different reinforcers. One box contained a syringe of morphine (to be injected by the experimenter) and the other box contained a banana. When the subjects were deprived of their daily morphine injection, they would immediately open the morphine containing box when given the choice. However, if the subjects received their daily morphine injection prior to the opportunity to choose a box, they would first open the box containing the banana. In a closer approximation to modern CPP procedures, Beach (1957) used a Y-maze apparatus to study morphine ‘‘addiction’’ and reward in rats. In that study, animals were first assessed for an initial preference for either a white goal box or a black goal box, each located at the end of opposite maze arms. The subjects then received twelve days of ‘‘addiction training’’ (i.e., conditioning) in which each was given a saline injection and directed into the initially preferred goal box, followed by an injection of morphine (1–20 mg/kg) and then directed into the initially non-preferred goal box. Interestingly, this assignment of the drug condition to the non-preferred context resembles what would eventually be referred to as a biased CPP procedure (see below). Beach (1957), found that during post-conditioning trials (testing), rats would select the drug-paired arm/goal box significantly more often than the saline-paired box. This result held true even when rats were in a drug satiated state, indicating that the preference for the drug-paired box was not due to relief from withdrawal stress alone but drug-induced euphoric effects as well. Finally, Rossi and Reid (1976) first described the use of procedures that still closely resemble those commonly used in modern CPP studies. In that study, an ‘‘alley’’ (i.e., a rectangular chamber) was divided into three compartments, each with distinct contextual cues and separated by guillotine style doors that could be used to restrict or allow
access between compartments. Beginning with a two-day habituation period, subjects were placed in the center compartment and allowed to move freely between all compartments for 30 minutes. Following habituation were three days of 30-minute conditioning trials in which half of the subjects received injections of either saline or morphine and were confined to one side of the apparatus. During drug trials, the post injection time that the animals were placed in the apparatus varied from 1.75 to 4.75 hours, in order to study the time course of morphine’s rewarding effects. On the final day, subjects did not receive an injection and were again placed in the center compartment and allowed to move freely between compartments. The researchers found that animals who received morphine spent more time in the drug-paired compartment than those who received saline. Additionally, because the rats were placed in the apparatus without being in a state of withdrawal, they concluded that morphine must be producing rewarding effects that are independent of any negative reinforcement (e.g., termination of withdrawal symptoms) which was previously thought by many to account for its reward properties (Rossi and Reid, 1976).

The methods described by Rossi and Reid (1976) were soon adopted by other researchers, with most CPP studies still using the same fundamental procedures today. Advancements in technology and purpose-built hardware have allowed researchers to conduct place preference studies with ever increasing efficiency and accuracy. As such, beginning in the early 1980s, the number of published place preference studies has continued to grow (see Figure 1).
Figure 1. The number of published studies per year that have utilized place conditioning procedures to investigate the rewarding and/or aversive properties of drugs or other stimuli. The counts are based on a PubMed database search using the search terms ‘place preference’, ‘place aversion’, or ‘place conditioning’ (see Tzschentke, 2007).

The CPP Paradigm. While specific CPP procedures can vary among laboratories and experimenters, they largely share a common set of properties. The most regularly used CPP apparatus consists of an appropriately sized animal chamber divided into two separate compartments or “sides” (with a middle compartment sometimes used as “neutral” area) (Tzschentke, 1998). Each compartment features a door that can be opened or closed in order to allow or restrict movement to other neighboring compartments. Within each compartment are contextual cues that are unique to that compartment and often vary along multiple stimulus
dimensions. For example, it is common for each compartment to have a unique wall color (e.g., black vs. white) or have walls with different patterns (e.g., vertical stripes vs. horizontal stripes). It is also common for each compartment to have dissimilar flooring types (e.g., mesh flooring vs. rod flooring). Occasionally scents are used to further differentiate compartments (e.g., banana scent vs. lemon scent). It is key to the procedure that the cues in each compartment be both sufficiently salient and distinct to allow for adequate stimulus pairing to occur during conditioning trials.

Often a CPP experiment to evaluate a drug will begin with a pre-test or habituation phase. During this time, each animal is placed into an individual chamber (with no injection) and allowed to roam freely between compartments for the pre-determined session time. This phase of the experiment can consist of one or more of these sessions. Generally during pre-test sessions, the time spent by each animal in each individual compartment is recorded for use in later analyses. Following the pre-test phase, subjects begin conditioning sessions or “trials” (i.e., stimulus pairings). During conditioning trials, animals receive an injection and then are confined to a single compartment for the duration of the session. Each subject is given only saline injections in one compartment and only drug injections (or sometimes saline again to serve as a control) in the opposite compartment. When animals are assigned to receive drug injections in the initially non-preferred compartment of the apparatus (as determined from the pre-test data), the CPP procedure is termed a “biased” procedure (Cunningham, Ferree, & Howard, 2003). Conversely, if the assignment of the drug condition is random (usually counterbalanced) between both compartments, regardless of initial preference, the CPP procedure is termed an “unbiased” procedure. The number and frequency of conditioning sessions can vary. Most CPP studies commonly employ between two and four drug/saline conditioning trials, though some have used
as little as one drug/saline pairing (e.g., Brielmaier, McDonald, & Smith, 2012; Edwards, Konz, Hirsch, Weedon, & Dow-Edwards, 2014), and others as many as 16 drug/saline pairings (e.g., Contarino et al., 1997). Conditioning trials may occur once per day (drug session or saline session only) or twice per day (generally a saline session followed by a drug session), depending on the study design.

Following the conditioning phase, the post-conditioning test session assesses for the development of place preference and/or place aversion. Post-test conditions are often identical to those of the pre-test. Subjects do not receive an injection and are allowed to move freely between compartments, with the time spent in each recorded. Evaluating for place preference/aversion can be done through a variety of different methods (Bardo, Rowlett, & Harris, 1995). One method involves comparing subjects’ time spent in the drug-paired compartment during the pre-test (before pairing) to the time spent in the drug-paired compartment during the post-test. A significant increase in time spent in the drug-paired compartment during post-test indicates a place preference. Another method involves simply comparing time spent in the drug-paired compartment versus the saline-paired compartment during the post-test. CPP is then defined by subjects spending significantly more time in the drug-paired compartment. A final method for assessing place preference involves comparing time spent by drug conditioned subjects in the drug-paired compartment to saline control animals that received saline pairings in both compartments.

**CPP vs. Self-administration.** Although conditioned place preference and self-administration are the most frequently used paradigms to investigate the abuse liability of drugs, they do so through very different methods (Le Foll & Goldberg, 2005). From a behavioral standpoint, the assays differ fundamentally in type of conditioning that occurs. CPP makes use of
Pavlovian (i.e., respondent) conditioning. The drug serves as an unconditioned stimulus (US) that is paired with the contextual cues of a specific “place” (e.g., compartment), which serves as a conditioned stimulus (i.e., CS+) (Bardo and Bevins, 2000). The absence of drug is in turn paired with the opposite context (i.e., CS-). The important aspect of this pairing procedure is that the drug is administered without being contingent upon any response. If during subsequent testing, a subject shows a preference for the drug-paired context, then the drug is assumed to have rewarding properties.

The self-administration assay, on the other hand, makes use of operant conditioning principles. The subject receives the drug only after emitting a specific response (or specific responses). Generally, this involves the subject pressing a lever or a similar such task. Once the subject emits the required response, a pre-determined dose of drug is administered (most commonly by automatic intravenous infusion). Here the key aspect is that the drug is only received contingent upon responding. If a subject will reliably respond (i.e., self-administer), then the drug is assumed to have reinforcing properties.

While the rewarding properties and the reinforcing properties produced by a drug are generally presumed to be similar phenomena, the distinction in terminology between assays comes from the type of conditioning that occurs in each. Specifically, for a stimulus to be deemed reinforcing, it must increase a behavior which precedes it (e.g., a lever press). This distinction between rewarding versus reinforcing (i.e., non-contingent versus contingent drug administration) has sometimes led to differences in the interpretation of results. This has primarily stemmed from the perceived translational value of the assays. It has been argued that CPP does not have high face validity compared to self-administration due to the fact that the drugs are delivered non-contingently, and therefore does not model the human condition of drug
use (Carter and Griffiths, 2009). It should be noted, however, that research has found that a vast number of drugs that support self-administration do also produce CPP (Bardo and Bevins, 2000). This gives evidence that the CPP paradigm does have high predictive validity in terms of assessing the abuse liability of drugs. Further, the CPP paradigm has the benefit of using less costly equipment and does not require invasive surgical procedures.

**Nicotine CPP.** The first known study to examine nicotine using conditioned place preference was published by Fudala, Teoh, and Iwamoto (1985). In that study, the researchers used a biased CPP procedure to test nicotine at 0.1 and 1.2 mg/kg in rats. Both doses of nicotine were found to produce place preference. Follow up studies examined the temporal relationship between nicotine administration and the production of place preference. Those studies found that nicotine only produced place preference when administered immediately prior to the animals being placed in the CPP apparatus versus following a delay of 20 – 120 minutes (Fudala & Iwamoto, 1986), and indeed could produce place aversion if the drug was administered immediately after the conditioning session (Fudala & Iwamoto, 1987). Several studies have since used CPP to examine the reward properties of nicotine under different conditions and have occasionally produced somewhat variable results.

The dose used in conditioning is one area of variability influencing CPP results. Doses of nicotine that have produced CPP in some studies have failed to do so in others. The procedural differences that exist within and between biased and unbiased designs are key factors identified as a likely cause of this discrepancy (Brielmaier, McDonald, & Smith, 2008; Tzschentke, 2007). In an example of differential results found using a biased design, Fudala and Iwamoto (1986) found that 0.8 mg/kg nicotine produced CPP when administered on the non-preferred chamber side, while Calcagnetti and Schechter (1994) found that the same dose did not produce CPP
when administered on the preferred side. This is further contrasted with the results of Jorenby, Steinpreis, Sherman, and Baker (1990), who found that 0.8 mg/kg nicotine produced conditioned place aversion (CPA) when using an unbiased (counterbalanced) design.

Physiological factors have also been identified in the differential outcomes of nicotine CPP studies. Age appears to have a significant impact. Vastola, Douglas, Varlinska, and Spear (2002) found that 0.6 mg/kg nicotine produced CPP in adolescent rats (PND 40 at test), but failed to produce CPP under the same conditions in adult rats (PND 70 at test). Similarly, Belluzzi, Lee, Oliff, and Leslie (2004) found that a single drug conditioning trial with 0.5 mg/kg nicotine was enough to induce place preference in early adolescent rats (PND 30 at test), but not late adolescent (PND 41 at test) or adult (PND 94 at test) rats. This result persisted even when separate groups of late adolescent and adult rats were given a total of four drug conditioning trials. Sex differences also appear to play a role in the rewarding effects of nicotine. Yararbas, Keser, Kanit, and Pogun (2010) and Lenoir et al. (2015) both found that adult male rats were more sensitive to the rewarding properties (i.e., produced CPP at lower doses) of nicotine than adult females. Interestingly, Edwards et al. (2014) found the opposite of this was true when investigating nicotine CPP in young (PND 25-27) male and female rats.

CPP has been used to examine the extent to which certain environmental/behavioral factors influence nicotine reward. Falco, McDonald, and Smith (2014) found that rats deemed to have high anxiety levels (as determined from elevated plus maze experiments) developed a place preference to 0.5 mg/kg nicotine, while those displaying low anxiety did not. Biala et al. (2017) found comparable results in that rats exposed to three weeks of chronic unpredictable mild stress (CUMS) developed a place preference with 0.175 mg/kg nicotine, while unstressed controls did not. Thiel, Sanabria, and Neisewander (2009) investigated the interaction of social reward and
nicotine in CPP. Nicotine (0.1 – 0.6 mg/kg) and social reward (social play with another rat) each failed to produce CPP on their own in adolescent rats (PND 28-42). However, the combination of nicotine and social reward in the same context, did produce place preference.

Finally, the pharmacological properties of nicotine have also been assessed using CPP. Carboni, Acquas, Leone, and Chiara (1989) found that the 5-HT₃ receptor antagonists ICS 205-930 and MDL 72222, blocked CPP induced by 0.6 mg/kg of nicotine in rats. Similarly, the D₁ receptor antagonists SCH 23390 (Acquas, Carboni, Leone, & Chiara, 1989) and SCH 39166 (Spina, Fenu, Longoni, Rivas, & Di Chiara, 2006) were shown to block CPP induced by 0.6 mg/kg and 0.4 mg/kg nicotine, respectively.

Despite some of the noted complexities, conditioned place preference continues to be a powerful tool in pharmacology research. It remains one the preeminent preclinical animal models for investigating drug reward as well as the pharmacological, behavioral, and environmental influences on drug reward.

**Current Research Objective**

The number of newly diagnosed cases of adolescent ADHD has been increasing every year over the past two decades and continues to accelerate. As the prevalence of ADHD has rapidly increased, so too has the controversy surrounding it. Perhaps no other aspect of ADHD is more controversial than the use of psychostimulant pharmacotherapies, especially in children and adolescents. Stimulants are the most commonly prescribed medical treatment for ADHD, with amphetamine preparations accounting for a large portion. The long-term effects of adolescent amphetamine use are still relatively unknown. Exposure to stimulants like amphetamine during the critical developmental period of adolescence could have profound neurobehavioral effects that alter the reward properties of other stimulants such as nicotine in adulthood.
To assess the effects of adolescent exposure to *d*-Amphetamine on the subsequent rewarding properties of nicotine in adulthood, groups of adolescent male Sprague-Dawley rats were pre-treated with either *d*-Amphetamine or saline, then tested using nicotine conditioning in a conditioned place preference procedure as young adults (PND 70). The main objective of this experiment was to determine if adolescent exposure to a commonly used ADHD treatment would potentiate nicotine reward in adulthood.

**METHODS**

**Subjects**

Seventy-two male Sprague-Dawley rats (Charles River Laboratories Inc., Kingston, NY, USA) were used in the study. Animals were delivered to the animal facility on postnatal day (PND) 24, and upon arrival, were randomly pair housed in polycarbonate cages with Teklad corncob bedding (#7097, Envigo, Madison, Wisconsin, USA). Subjects were given *ad libitum* access to water and commercial rodent diet (LabDiet® 5001, PMI Nutrition Int. LLC, Brentwood, Missouri, USA) in the home cages. The animal facilities were maintained at a (20±2°C) and humidity (50±5%) under a 12:12 light/dark cycle, (lights on from 0700 to 1900). All procedures were reviewed and approved by the Western Michigan University Institutional Animal Care and Use Committee and were in accordance with the guidelines of the *Guide for the Care and Use of Laboratory Animals* (National Research Council of the National Academies, 2011) and EU Directive 2010/63/EU.

**Apparatus**

Six two-compartment conditioned place preference (CPP) chambers (ENV-013C, Med Associates Inc., St. Albans, Vermont, USA), housed inside individual sound-attenuating cabinets
equipped with electric fans for ventilation and masking noise (ENV-020M, Med Associates Inc.), were used for CPP conditioning and testing procedures. Time spent in each compartment and locomotor activity for each subject was recorded by a series of 12 infrared photobeams evenly spaced longitudinally along the walls of each chamber (see Figure 2). Experiments were controlled by an IBM compatible PC running Med-PC software (version IV, Med Associates Inc., St. Albans, Vermont, USA).

A photograph of the CPP apparatus is shown in Figure 3. The apparatus was composed of two compartments, each 30 x 21 x 21 cm in size with opaque acrylic walls, a clear acrylic top with vent holes, and a metal floor. A center guillotine style door approximately 8 x 10 cm in size separated the two compartments and could be manually raised or lowered to allow or restrict movement between compartments. Each compartment was visually and texturally distinct. One compartment had black walls with a floor of 0.48 cm diameter rods spaced every 1.6 cm, running in a single direction. The other compartment had white walls with a wire grid floor with 1.3 cm² openings. Both compartments had a shielded overhead 3-watt light bulb that was adjusted to provide approximately equal illumination on each side.
Figure 2. The two-compartment conditioned place preference apparatus (front view). The left compartment consisted of white walls with wire grid flooring. The right compartment consisted of black walls with metal rod flooring. A center guillotine style door was raised or lowered to allow or restrict access between the two compartments. Infrared photobeams (shown 1–15) recorded time spent in each compartment and locomotor activity counts (beam breaks). (MED Associates Inc, St. Albans, VT)
Figure 3. A top-down photograph of the CPP apparatus with a subject in the right (black) compartment. The differences between the grid flooring (left/white compartment) and rod flooring (right/black compartment) are visible. A center guillotine style door (not visible) was raised or lowered to allow or restrict movement between compartments.

Drugs

d-amphetamine-hemisulfate and (-)-nicotine hydrogen tartrate were purchased from Sigma-Aldrich Corp. (St. Louis, Missouri, USA). Drug solutions were prepared by dissolving the salt in 0.9% (wt/vol) bacteriostatic saline. All doses are expressed as the weight of the salt and were given at a constant injection volume of 1 ml/kg administered subcutaneously (sc).
Procedures

Figure 4 shows the experimental group assignments. Figure 5 shows the timeline of experimental procedures.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
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<tbody>
<tr>
<td>$d$-Amphetamine Pre-Treatment</td>
</tr>
<tr>
<td>CPP Conditioning</td>
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<tr>
<td>mg/kg</td>
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<tr>
<td>Subjects ($n$)</td>
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*Figure 4. A graph of showing the experimental group assignments and number of subjects per group.*
Figure 5. Timeline of experimental procedures. During early adolescence, animals receive a 10-day pre-treatment (days 1 – 10) of either d-amphetamine (0.5 mg/kg) or saline (1.0 ml/kg), followed by a maturation period (days 11 – 39) to early adulthood. Conditioned place preference procedures begin on day 40. During the pre-test, animals are allowed to move freely between both compartments of the CPP chamber for 15 minutes. Initial side preference is then assessed. During drug conditioning trials (days 41, 43, 45, and 47), animals receive an injection of 1.0 ml/kg saline, 0.04 mg/kg nicotine, or 0.10 mg/kg nicotine and are confined to the non-preferred side of the chamber for 30 minutes. During saline conditioning trials (days 42, 44, 46, and 48), all animals receive 1.0 ml/kg of saline and are confined to the preferred side for 30 minutes. Post-test procedures are identical to pre-test. Time spent in each compartment (pre-test and post-test days) and locomotor activity (all days) is recorded for each animal.
Pre-Treatment

In order to facilitate behavioral testing, subjects were received as two separate cohorts of 36 animals each, two weeks apart. All experimental variables and procedures were identical for both cohorts.

Upon arrival, subjects were first allowed to habituate to the animal facility environment for a period of seven days prior to the start of experiments. During this time, they received individual handling for approximately two minutes each day for 3 – 4 days in an effort to reduce handling stress during subsequent procedures. The pre-treatment phase of the study began on PND 31 and was considered early adolescence (Belluzi et al., 2004; Spear, 2000). In this phase, subjects received injections of 0.5 mg/kg d-amphetamine (n=36) or 1.0 ml/kg saline (n=36) in their home cages, once per day for 10 consecutive days. Pre-treatment assignments were counterbalanced across position on the cage rack and cagemates all received the same pre-treatment condition. All injections were given at approximately the same time of day during the animals’ light cycle. Following the 10-day pre-treatment period, subjects were allowed to mature to adulthood. During this time, no experimental procedures were conducted and animals were handled twice per week for approximately 2 – 3 minutes each.

Conditioned Place Preference

Behavioral testing began on PND 70, defined as early adulthood (Varlinskaya & Spear, 2008), and consisted of a 10 day, biased CPP procedure with three phases. For all phases, subjects were first weighed and then brought from the animal facility to the procedure room in squads of six animals (three pairs) at a time. On the pre-test day (day 1), subjects were placed into the CPP apparatus with half starting in the black compartment and half starting in the white compartment for counterbalance. The center door was open and the animals were allowed to
move freely between the two compartments of the chamber. Data collection began immediately after the subject was placed in the chamber (after the first photobeam break). Pre-test sessions lasted for 15 minutes, during which the total time spent in each compartment and total locomotor activity (beam breaks) was recorded for each subject. Subjects were then removed from the chambers and returned to their home cages in the animal facility. Following the pre-test session, initial side (i.e., compartment) preference for each animal was determined to be the side on which the greater amount of time was spent. Subjects were then assigned to one of the following treatment groups: 1.0 ml/kg saline ($n=24$), 0.04 mg/kg nicotine ($n=24$), or 0.10 mg/kg nicotine ($n=24$). Treatments were equally assigned between both pre-treatment groups, so that 12 rats were assigned to each pre-treatment + treatment combination. Cagemates received the same treatment and treatments were counterbalanced with respect to position on the cage rack. Counterbalancing of each pre-treatment + treatment combination across chamber assignments was also done to the extent possible.

During the conditioning phase (days 2 – 9), subjects received four drug trials and four saline trials, alternating one per day for eight consecutive days. For these sessions, the center door was closed and subjects were confined to one compartment. During drug conditioning trials, each subject received their assigned treatment injection and was immediately placed in the initially non-preferred side of the chamber (a biased stimulus assignment procedure). For saline conditioning trials, all subjects received a 1.0 ml/kg injection of saline and were immediately placed in the initially preferred side of the chamber. All conditioning trials were 30 minutes in duration and locomotor activity was recorded throughout. At the end of each trial, subjects were immediately returned to their home cages.
The final phase of the CPP procedure consisted of the post-test (day 10). Post-test procedures were identical to those used for the pre-test (i.e., no injection, unrestricted movement, and 15 minute sessions). Post-test time spent in each compartment was then analyzed for each subject to assess for the expression of conditioned place preference.

**Data Analysis**

**Locomotor Activity.** A two-factor ANOVA [2 (Pre-Treatment) × 3 (Nicotine Dose)] was used to analyze overall locomotor activity data between all pre-treatment + treatment combinations, with differences between combinations analyzed using Sidak’s multiple comparison tests. Individual one-way ANOVAs were used to analyze simple main effects of nicotine dose within each pre-treatment group averaged locomotor activity. Lastly, a two-factor mixed-model ANOVA (with nicotine dose as the between-subjects factor and drug trial as the within-subjects factor) was used to analyze the change in activity levels across drug days within each pre-treatment group, with Dunnett’s multiple comparisons testing for significant changes from Drug Trial 1 to all other drug trials.

**Conditioned Place Preference.** Time spent in each compartment during the pre-test was analyzed using a paired *t*-test. Difference scores were calculated by subtracting the pre-test time on the drug-paired side (in sec.) from the post-test time (in sec.) spent on the drug-paired side for each animal. Analysis of difference scores was done by way of a two-factor analysis of variance (ANOVA) [2 (Pre-Treatment) × 3 (Nicotine Dose)].

**Software.** Graphical and statistical analyses were conducted using GraphPad Prism (version 6, GraphPad Software, Inc., La Jolla, California, USA).
RESULTS

Locomotor Activity

Overall Analysis. Average activity counts across all drug conditioning trials for each pre-treatment plus nicotine treatment combination are shown in Figure 6. In the saline pre-treated animals, nicotine produced an increase in average (±SEM) activity of 242±230 at the 0.04 mg/kg nicotine dose, and a decrease of 221±127 at the 0.10 mg/kg dose, compared to saline controls (1606±100). In d-amphetamine pre-treated subjects, nicotine 0.04 mg/kg and 0.10 mg/kg produced decreases in average activity of 330±62 and 343±89, respectively, compared to saline controls (1652±118). A two-factor ANOVA did not reveal a significant effect of pre-treatment ($F_{1, 66} = 2.933$, $p = .092$), nicotine dose ($F_{2, 66} = 2.638$, $p = .079$), or a significant pre-treatment × nicotine dose interaction ($F_{2, 66} = 2.606$, $p = .081$). Sidak’s multiple comparison tests did indicate a significant difference between overall activity levels of the two pre-treatment groups at the 0.04 mg/kg nicotine dose ($p < .05$).
Figure 6. Average activity counts across all drug conditioning trials for each pre-treatment plus nicotine treatment combination. White = saline pre-treatment; Black = d-Amphetamine pre-treatment. *p < .05

Within Pre-Treatment Groups Analysis. Average drug trial activity counts for each pre-treatment group are shown in Figure 7. Statistical analysis of average drug trial activity counts for saline pre-treated subjects, showed no significant effect of nicotine dose on activity ($F_{2, 33} = 2.045, p = .145$). Analysis of average drug trial activity in d-amphetamine pre-treated
subjects revealed a significant effect of drug dose on activity \( (F_{2, 33} = 4.383, p < .05) \). Dunnett’s multiple comparisons showed both the 0.04 mg/kg and 0.10 mg/kg nicotine doses significantly lowered activity compared to saline controls \( (p < .05, \text{each}) \).

![Average Activity Across Drug Trials](image)

**Figure 7.** Average activity across all drug conditioning trials for saline pre-treated animals (left graph) and d-amphetamine pre-treated animals (right graph). *\( p < .05 \) compared to saline controls.

Activity within each pre-treatment group during individual drug conditioning trials (days) is shown in Figure 8. In saline pre-treated animals, there was no significant effect of nicotine dose \( (F_{2, 33} = 2.045, p = .145) \), drug trial \( (F_{3, 99} = 1.360, p = .260) \), or a nicotine dose \( \times \) drug trial interaction \( (F_{6, 99} = 1.049, p = .399) \). The analysis of the d-amphetamine pre-treated subjects found a significant effect of nicotine dose \( (F_{2, 33} = 4.383, p < .05) \), a significant effect of drug trial \( (F_{3, 99} = 6.474, p < .001) \), and no significant nicotine dose \( \times \) drug trial interaction \( (F_{6, 99} = 28) \).
Dunnett’s multiple comparison tests found that in the 0 mg/kg nicotine group, activity during drug trial 4 was significantly lower compared to the first drug trial ($p < .001$). Activity levels in the 0.04 mg/kg nicotine group were significantly lower during both drug trial 3 and 4, compared to the first drug trial ($p < .05$).

**Figure 8.** Activity during drug conditioning trials (days) for the saline pre-treated animals (left) and d-amphetamine pre-treated animals (right). *$p < .05$, ***$p < .001$.

**Conditioned Place Preference**

**Apparatus Bias.** During the pre-test, subjects showed a slight overall preference for the white side of the apparatus compared to the black side. Throughout the 15-minutes (900 sec.) of the session, the animals spent an average ($\pm$SEM) of 465±5 sec. (51.7% of total time) in the white compartment (see Figure 9). A paired $t$ test indicated a significant difference in the time spent on each side ($t(71) = 3.01, p < .01$). Correspondingly, a greater number of subjects showed
an initial side preference (greater time spent) for the white side versus the black side during the pre-test ($n=48$ and $n=24$, respectively) (see Figure 10).

*Figure 9.* Percentage of total time spent on the black and white sides of the CPP apparatus during the 15-minute pre-test session ($n=72$). **$p < .01$
Figure 10. Initial side preference as determined by the side of the CPP apparatus in which each subject spent the majority of time during the 15-minute pre-test session. During subsequent conditioning trials, saline was paired with the initially preferred side and nicotine was paired with the initially non-preferred side of the chamber.

**Overall Analysis.** The time spent on the drug-paired side of the chamber during the pre-test and post-test for both pre-treatment groups is shown in Figure 11. Difference scores (post-test - pre-test) calculated for both groups are depicted in Figure 12. All but the saline pre-treatment + 0.10 mg/kg nicotine group exhibited increases in the mean time spent on the drug-
paired side. The saline pre-treated animals showed an average (±SEM) increase in time spent in the nicotine-paired side of 9.51±27.93 sec. at the 0 mg/kg dose, 2.91±29.83 sec. at the 0.04 mg/kg dose, and an average decrease of 18.03±24.49 sec. at the 0.10 mg/kg dose. The d-amphetamine pre-treated animals showed average increases in time on the nicotine-paired side of 20.95±36.41 sec. at the 0.00 mg/kg dose, 47.80±22.61 sec. at the 0.04 mg/kg dose, and 36.20±31.31 sec. at the 0.10 mg/kg dose. Statistical analysis of difference scores did not reveal a significant effect of pre-treatment ($F_{1, 66} = 2.404, p = 0.126$), nicotine dose ($F_{2, 66} = 0.1590, p = 0.853$), or a significant pre-treatment × nicotine dose interaction ($F_{2, 66} = 0.2985, p = 0.743$).
Figure 11. Time spent in seconds (±SEM) on the nicotine-paired side of the chamber during the 15-minute pre-test (solid bars) and 15-minute post-test (checkered bars) sessions for the saline pre-treatment group (white) and the d-amphetamine pre-treatment group (gray).
Figure 12. Difference scores (post-test - pre-test in seconds) for the saline pre-treatment (white) and d-amphetamine pre-treatment (gray) groups receiving 0.0 mg/kg nicotine (vehicle), 0.04 mg/kg nicotine, or 0.10 mg/kg nicotine during drug conditioning trials.
The difference scores for each individual subject in the saline pre-treatment group are shown in Figure 13, and individual difference scores for the d-AMPH pre-treatment group subjects are shown in Figure 14. In the saline pre-treated animals, the data show an approximately equal number of subjects had positive and negative difference scores in each nicotine treatment condition combination (i.e., approximately the same number of subjects spent increased time on the drug-paired side as spent decreased time on the drug-paired side). This is also true for subjects in the d-AMPH pre-treatment plus 0 mg/kg nicotine (saline) treatment combination. In the d-AMPH pre-treatment plus 0.04 mg/kg nicotine or 0.10 mg/kg nicotine treatment combination, 9 out 12 animals had positive difference scores.
Figure 13. Individual subject difference scores (saline pre-treatment group).
Figure 14. Individual subject difference scores (d-amphetamine pre-treatment group).
DISCUSSION

d-Amphetamine (d-AMPH) is used as a common treatment for adolescent ADHD, either alone (e.g., ProCentra®) or in combination with its optical isomer l-amphetamine (e.g., Adderall®). As such, it is of importance to understand the long-term neurobehavioral effects of these drugs when used in adolescence. Conditioned place preference provides a model for assessing nicotine reward in adult rats with a history of adolescent d-amphetamine exposure.

The present study found notable differences between adolescent animals treated with d-amphetamine and those treated with saline. Overall, the 0.04 mg/kg nicotine dose showed the greatest differential effects on activity. This dose produced overall locomotor activity across drug trials that was significantly higher in the saline pre-treated subjects versus those pre-treated with d-AMPH. In saline pre-treated animals, 0.04 mg/kg nicotine also produced greater overall drug trial activity than saline or 0.10 mg/kg, an effect that was not seen in the d-AMPH group.

On measures of conditioned place preference, 0.04 mg/kg nicotine produced the greatest shift in time spent on the drug-paired side for the d-AMPH pre-treated animals but not in those pre-treated with saline. Examining the individual subject difference score data, 9 out of 12 subjects in the d-AMPH pre-treatment group had positive difference scores at the 0.04 mg/kg nicotine dose. In comparison, the same nicotine dose resulted in only 5 out 12 subjects with positive difference scores in the saline pre-treatment group.

Differences between pre-treatment groups were also seen in the changes in locomotor activity levels observed progressively across drug conditioning trials. In d-AMPH pre-treated animals, 0.04 mg/kg nicotine produced significant declines in activity during the third and fourth drug trials, compared to drug trial one. Curiously, a significant decline in activity was also seen in the 0 mg/kg nicotine (vehicle) treatment group during drug trial four compared to trial one.
The locomotor activity data indicate that the nicotine treatment doses were behaviorally active, and appear to have differential effects not only between doses, but between pre-treatments as well. Overall, $d$-AMPH pre-treatment attenuated the locomotor effects of nicotine. The fact that the attenuation occurred as a progressive reduction across drug trials, rather than an immediate effect on day one, may suggest that the $d$-AMPH pre-treated animals developed a rapid tolerance to the locomotor stimulant effects of nicotine, whereas the saline pre-treated animals did not. Interestingly, in one of the only known studies to investigate a long-term behavioral consequence of adolescent exposure to amphetamine, Santos, Marin, Cruz, DeLucia, and Planeta (2009) found that adolescent rats that were pre-treated with amphetamine (PND 28–34) showed significantly higher locomotor activity when tested with nicotine in adulthood (PND 70), compared to those that were pre-treated with saline. Though the drug doses used in that study (5.0 mg/kg amphetamine and 0.4 mg/kg nicotine) were tenfold higher.

On measures of place preference, although greater difference scores were observed in the $d$-AMPH pre-treated subjects, high variability between subjects within some treatment groups precluded a statistically significant result. Within treatment group variability is not entirely uncommon in the CPP paradigm. Adams, Careri, Efferen, and Rotrosen (2001) used a conditioned place preference procedure to assess the ability for selected dopamine antagonists to block cocaine reward. In at least one of their tests, they noted that the subjects were almost equally divided into groups showing preference, no preference, or aversion. A study by Daza-Losada et al. (2007) found that a dose of 3,4-methylenedioxyamphetamine (MDMA) that produced robust place preference in some animals, produced a considerable place aversion in others. Based in part on these studies, dela Cruz, Herin, Grady, and Cunningham (2009) proposed a method for identifying separate subgroups within a CPP conditioning group, that are
often hidden by the averaging of group data. These differences between subjects expressing CPP and those not, may have clinical relevance in that some human drug users tend to develop strong drug dependency, while others do not.

One potential limitation to the present study, was the use of relatively low drug doses in both the pre-treatment and CPP phases. The 0.5 mg/kg pre-exposure dose of d-amphetamine is generally considered a low to moderately-low dose in laboratory animal studies (Grilly & Loveland, 2001). However, it is important to note that this dose falls within the clinically relevant range for ADHD treatment in humans (Heijtz, Kolb, & Forssberg, 2003). The nicotine doses selected were also at the low end of the spectrum of doses typically used in nicotine CPP studies (Le Foll, & Goldberg, 2005). The decision to use low doses of nicotine was made in an attempt to identify a potential threshold dosage at which the d-AMPH pre-treated subjects would show place preference while the saline pre-treated subjects would not.

Other possible influences on the results of this study include age, pre-treatment conditions, and stress factors. As mentioned previously, CPP studies using relatively low doses of nicotine have often found that the drug’s effects are more pronounced in adolescent animals than in adult animals. It is possible that testing for nicotine CPP in late adolescence versus early adulthood would have produced more robust results. The pre-treatment phase of the present study began in early adolescence, was discontinued near mid-adolescence, and was followed by a four-week gap to early adulthood. Though this was done in an attempt to assess the effects of early adolescent d-AMPH exposure on adult nicotine reward in the absence of d-AMPH itself, it may not accurately model typical clinical conditions. It is possible that continuing d-AMPH pre-treatment throughout adolescence (as is common in adolescent ADHD treatment), would have
had a greater impact on nicotine place preference. Finally, Le Foll and Goldberg (2005) noted that the animals’ stress levels, could have a substantial influence in the expression of nicotine CPP. Animals with high stress levels are less likely to show nicotine induced place preference. In the present study, animals were handled frequently in an attempt to reduce stress during the pre-treatment and CPP phases. However, it is possible that further measures such as increased acclimation time to housing conditions and habituation to the test environment, may have reduced stress further and influenced CPP results.

**Conclusion**

Attention-Deficit/Hyperactivity Disorder is a problem affecting an increasing number of adolescents worldwide. Psychostimulants are currently the most commonly prescribed pharmacotherapies for adolescent ADHD. Given the evidence that adolescent exposure to these drugs may produce behavioral effects that endure into adulthood, it is of importance to investigate the long-term consequences of these treatments. This study serves as an attempt to further elucidate this relationship.
REFERENCES


APPENDIX

IACUC Approval Letters
Date: May 2, 2016

To: Lisa Baker, Principal Investigator

From: Kathryn Eckler, Vice Chair

Re: IACUC Protocol Number 16-03-01

Your protocol titled “Conditioned Place Preference Procedures in Rats” has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: May 1, 2017
Date: February 7, 2017

To: Lisa Baker, Principal Investigator

From: Kathryn Eckler, Vice Chair

Re: IACUC Protocol Number 16-03-01

This letter will serve as confirmation that the changes to your research project “Conditioned Place Preference Procedures in Rats” requested in your memo received February 6, 2017 (to increase the total number of rats to be used in this protocol by adding 120 adolescent rats; to include a pre-treatment phase as part of the added experiments involving adolescent rats; to add the following three drugs: amphetamine, methylphenidate, and nicotine

With these additions, the amended protocol will include the following procedures:

Rats will be received on post-natal day (PND 21) in cohorts of 36 animals each, with no more than two cohorts (72 total animals) in the animal facility at one time. Upon arrival, rats will be assigned to pair housing and allowed to habituate to the animal facility for seven days. During this time, animals will be handled regularly to decrease handling stress during subsequent procedures. Beginning on PND 28, animals will be randomly assigned to one of three groups. Rats will receive once-daily injections of saline (1 ml/kg SC, N=40) d-amphetamine (0.5-1.0 mg/kg SC, N=40), or methylphenidate (0.5-1.0 mg/kg SC, N=40) for a total of 10 days (the pre-treatment period). Rats assigned to the drug treatment in these added experiments will receive only one of the aforementioned drugs once daily for 10 days.

Following the pre-treatment period, rats will be allowed to mature to early adulthood in their home cages and with routine cage maintenance and handling. On PND 70, conditioned place preference (CPP) procedures will begin with nicotine. Rats from each pretreatment group will be randomly assigned to receive one of the following doses of nicotine: 0, 0.04, 0.10, 0.40, 2.0 mg/kg (N=8 per dose level). Each animal will receive a total of four nicotine and four saline injections or eight saline injections during this phase of the experiment. The CPP experimental procedures will be identical to those described in the original protocol.) have been approved by the Institutional Animal Care and Use Committee.

Approval Termination: May 1, 2017

[Signature]