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Low-Dose Stimulant Treatment During Periadolescence in a FASD Model: Interactions Among the Catecholamines

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LOW-DOSE STIMULANT TREATMENT DURING PERIADOLESCENCE IN A FASD MODEL: INTERACTIONS AMONG THE CATECHOLAMINES

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ABSTRACT

One of the most common of deficits observed in Fetal Alcohol Spectrum Disorders (FASD) is difficulties with attention. Because attention deficits are commonly treated with stimulants, the impact of *d*-amphetamine (AMPH) treatment during the juvenile period in an animal model of FASD was examined. A dose-response study first assessed the appropriate dose of AMPH to use. In the dose-response study, therapeutic doses of 0.5, 1.0, and 2.0 mg/kg/day of AMPH were chronically administered to female rats between postnatal days (PD) 26-40. Rats were subjected to an open field test on the first and last day of treatment. The dose of 1.0 mg/kg/day was the lowest dose which resulted in significant behavioral sensitization and therefore was selected for the FASD study. In the FASD study, pups were exposed to alcohol between PD 2 and 10. Control groups included an intubated control (IC) and a non-treated control (NC). At PD 26, rats were randomly assigned to either amphetamine or water treatment for twice-daily subcutaneous injections from PD 26 to 41. On PD 26, 27, 40, and 41, an open field test was administered to assess locomotion. On PD 42, the rats were perfused, and the brains were removed and prepped for histological measures. The results indicated that amphetamine increased distance traveled acutely, and that this effect became greater over days of treatment. Amphetamine-treated rats exhibited significantly more rearing behaviors and less grooming behavior than water-treated rats. Both rearing and grooming behavior decreased over test days. There was no effect of alcohol exposure on any open field measures. Dopaminergic and noradrenergic systems were analyzed via

immunohistochemistry for tyrosine hydroxylase (TH), the dopamine transporter (DAT) and dopamine-beta-hydroxylase (DBH). An omnibus ANOVA revealed no impact of alcohol exposure or amphetamine treatment on TH or DAT within the nucleus accumbens core. Although amphetamine treatment caused a small but significant increase in DBH within the medial prefrontal cortex, there was no effect of alcohol exposure on this measure. These results suggest that catecholaminergic neurons are resistant to the developmental impact of alcohol. However, juvenile amphetamine treatment may increase noradrenergic synthesis or innervation within the prefrontal cortex, influencing developmental trajectories.

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List of Abbreviations

ADHD ..... Attention/deficit-hyperactivity disorder

BEC ..... Blood Ethanol Concentrations

DA ..... Dopamine

DAT ..... Dopamine Transporter

DOPAC ..... 3,4-dihydroxyphenylacetic acid

ERP ..... Event-related potential

FAS ..... Fetal Alcohol Syndrome

FASD ..... Fetal Alcohol Spectrum Disorders

HVA ..... homovanillic acid

IQ ..... Intelligence Quotient

mRNA ..... Messenger Ribonucleic acid

NAcc ..... Nucleus Accumbens

NE ..... Norepinephrine

NET ..... Norepinephrine transporter

PD ..... Postnatal Day

PFC ..... Prefrontal cortex

SN ..... Substantia Nigra

5HT ..... Serotonin

5-HTT ..... Serotonin transporter

TH ..... Tyrosine Hydroxylase

VMAT-2 ..... Vesicular monoamine transporter-2

VTA ..... Ventral Tegmental Area

CHAPTER 1

INTRODUCTION

1.1 ALCOHOL IS A TERATOGEN

An alcohol is any compound containing a functional hydroxyl group attached to a saturated hydrocarbon (Pohorecky & Brick, 1990). Although there are multiple types of alcohols, humans typically consume ethyl alcohol, an amphipathic molecule with a negatively charged oxygen "head" and a neutral methyl "tail" (Pohorecky & Brick, 1990). This amphipathic property allows its distribution throughout bodily tissues, including easy passage through the blood brain barrier where it can affect functioning of neural systems (Hanig et al., 1972). The result is a state of intoxication. At low doses, alcohol intoxication is associated with decreased inhibitions, elevated mood, and impaired motor coordination (Davidson et al., 1997). At high levels, intoxication is associated with stupor, coma, and potentially death (Sanap & Chapman, 2003). Despite these risks, alcohol use transcends cultures. It is the oldest psychoactive compound consumed by humans, and vestiges of purposeful distillation can be traced back to the Stone Ages (Gately, 2008).

Ethanol's chemical nature poses special implications for pregnant women as ethanol can diffuse through the placental barrier to interfere with healthy fetal development (Guerri & Sanchis, 1985). Despite the long association between humans

and alcohol consumption, the effects of exposure to alcohol *in utero* weren't delineated in the medical literature until the late 1900s. French pediatrician Paul LeMoine first described alcohol-derived birth defects in 1968 after documenting 127 case studies of children from alcoholic mothers (LeMoine, 1968). These effects included miscarriages, stillbirths, growth retardation, and a constellation of facial malformations. Five years later, American pediatricians Jones and Smith independently categorized the effects of alcohol exposure *in utero* with 8 case studies. This article by Jones and Smith (1973) launched worldwide investigations into the impact of alcohol on the fetus (Koren, 2012). By 1981, the U.S. Surgeon General responded to the emerging body of literature on alcohol's teratogenicity by advising no alcohol consumption during pregnancy (US Surgeon General, 1981). As the bodies of literature on the variable effects of alcohol exposure in utero have grown, classifications of these effects have expanded to encompass this spectrum. These effects are diagnosed as fetal alcohol spectrum disorders (FASD) with fetal alcohol syndrome (FAS) referring to the most severe cases.

Despite awareness of the teratogenic effects, alcohol remains a widely abused substance throughout pregnancy. According to data from prenatal clinics and postnatal studies, up to 30% of pregnant women drink during their pregnancy, and 2% binge drink (CDC, 2012). Shockingly, these estimates have shown no decline in the last two decades. As a result, FASD occurs in approximately 9.1 per 1000 live births with FAS occurring in approximately 2 per 1000 live births. Statistically, this makes FASD the most preventable source of neurological deficits in the United States (Abel, 1995; Sampson et al., 1997).

While the effects of *in utero* exposure to alcohol are devastating at an individual level, society also feels the weight of this burden. Every child born with FAS has a staggering economic cost of $2 million, and the U.S. government spends over $4 billion in annual care for FAS individuals (Lupton et al., 2004). These numbers increase exponentially when economic costs are extended to the entire FASD spectrum. Many of these costs are associated with the multiple cognitive and social secondary disabilities with FASD children. These secondary problems include functional deficits in problem solving, deficits in adaptive function, decreased intelligence quotients, trouble with the law, alcohol and drug problems, executive function deficits, and inappropriate sexual behavior (Streissguth et al., 2004). However, the most prominent secondary deficits associated with FASD are attention deficits.

Forty-one to 94% of children with FASD will later be diagnosed with attention-deficit/hyperactivity disorder (ADHD) (Bhatara, Loudenberg, & Ellis, 2006; Fryer et al., 2007). Comorbidity with ADHD becomes increasingly prevalent with greater exposure to alcohol *in utero*, indicating that alcohol directly interferes with the development of attention networks (Bhatara, Loudenberg, & Ellis, 2006). In fact, neonatal attention deficits may be a more sensitive indicator of potential *in utero* exposure to alcohol than even the characteristic facial dysmorphology (Lee, Mattson, and Riley, 2004).

Not only are attention deficits common in FASD children, but onset of these deficits are one of the earliest symptoms of cognitive impairments. Streissguth, Barr, and Martin (1983) examined approximately 500 one-day-old infants with and without

exposure to alcohol using the Brazelton Scale. The Brazelton Scale assesses neonatal behavior in general, and some of the measures are associated with attention. Socioeconomic status, marital status, age, education, and race were all accounted for to rule out confounding factors. Maternal prenatal alcohol consumption was also assessed to examine alcohol effects on a dose-response scale. Streissguth et al. discerned that neonates from alcoholic mothers had significantly lower states of arousal, poor habituation to environmental stimuli, and basic deficits in operant learning. This finding provided concrete evidence that developmental problems in FASD children were evident even on the first day of life. Early occurring deficits in attention could have a dynamic impact on function, aggravating ontogenesis of cognitive skills that mature later such as learning and social skills. As a result, early treatment options could remediate developmental trajectories in FASD, ameliorating the astronomical impact at both individual and societal levels.

Understanding how alcohol interferes with the development of attentional networks and the phenotype of attention deficits in FASD children will inform treatment strategies for this population. Therefore, this introduction will examine the relationship between prenatal ethanol exposure and one of the most overt behavioral characteristics of FASD: attention deficits. The literature on attention deficits in FASD will be reviewed in the context of neurobehavioral theories on attention with the argument that FASD children exhibit a unique phenotype of attention deficits that is detrimentally masked by the indiscriminate diagnosis of ADHD. The unique phenotype of FASD children is argued to be the result of distinct changes in underlying neural networks – data which has been

elucidated by animal models of FASD. A rationale for the use of animal models of FASD will be provided, followed by a discussion of disruptions in catecholaminergic transmission in FAS in relation to networks of attention. Because attention deficits are primarily treated with stimulant medications, mechanisms of action of the two primary stimulant prescriptions, methylphenidate and *d*-amphetamine, will be compared and contrasted in relation to attention deficits and FASD. This literature review will be followed by a rationale for the current experiments which examined the impact of ethanol and *d*-amphetamine on developing dopaminergic and noradrenergic systems. *The overarching hypothesis is that a chronic therapeutic dose of d-amphetamine normalizes hypo-functioning dopaminergic and noradrenergic systems in a rat model of FASD.*

1.2 Attention Deficits in FASD

Attention is an abstract concept with multiple different subcomponents that collectively facilitate the selection of relevant information and the suppression of irrelevant information. Two models of attention have been used to describe the phenotype of attention deficits in FASD populations: Posner (1980) and Mirsky (1991).

Posner's model (1980) describes attention as a spotlight which highlights a point of interest, then disengages and moves to an alternative point of interest. This analogy describes functional components essential to the attentional process: *alerting, orienting,* and *executive function*. *Alerting* prepares an organism for processing high-priority signals. *Orienting* is the ability to locate a stimulus within a visual or auditory

field. *Executive function* is a heterogeneous term which describes cognitive control mechanisms resulting in top-down decision making for the attentional spotlight. *Alerting* and *executive function* deficits have been extensively documented in FASD.

At a neurological level, alerting is associated with global levels of arousal and is driven by the noradrenergic system. This association has been tested at a physiological level in regards to spatial processing. Noradrenergic afferents increase spatial processing in the prefrontal cortex (PFC) by inhibiting inputs with similar spatial properties, thereby suppressing stimuli that could conflict with the target of interest (Wang et al., 2007). Children with FASD have different problems alerting to stimuli compared to children with ADHD without FASD comorbidity. This distinction was demonstrated by Kooistra et al. (2009) using a Go/No-Go task. In the Go/No-Go task subjects are presented with a predictable stimulus to which they must respond quickly. However, in a small subsection of the trials, subjects must inhibit their response (i.e. "No-Go" trials). Because No-Go trials are rare, response inhibition is challenging. There were two trial rates: a fast rate with short intervals between trials and a slow rate with long intervals. ADHD children without FASD comorbidity are sensitive to the stimulus presentation rate with more errors and variability in slower conditions. In contrast, FASD groups had declining performance in fast-rate conditions. These data indicate that children with FASD have more problems handling overstimulation whereas non-FASD ADHD children have greater problems handling understimulation. Sensitivity to multiple stimuli may reflect changes in noradrenergic arousal systems.

Executive function deficits are identifiable even in the absence of facial dysmorphology with FASD (Mattson et al., 1999; Green et al. 2009). Green et al. (2009) assessed executive function using the Cambridge Neuropsychological Tests Automated Battery (CANTAB) and determined that FASD individuals exhibited deficits in multiple executive function domains including set shifting, general attention, strategy and planning, and spatial working memory. These deficits in executive function were evident even in the absence of facial dysmorphology, which is indicative of the most severe cases of FASD. Linking these behavioral findings with neural substrates would suggest that these deficits are paralleled by deficits in the monoaminergic systems. Indeed, animal literature has demonstrated that alcohol exposure induces many monoaminergic alterations. Similar to Greene et al. (2009), Mattson et al. (1999) used to the Delis-Kaplan Executive Function Scale to evaluate planning, cognitive flexibility, selective inhibition, and concept formation and reasoning in children with FASD compared to children not exposed to alcohol *in utero*. Deficits in executive function were evident in FASD populations even after deficits in intelligence quotients (IQ) were accounted for. Additionally, deficits in executive function were evident in FASD children even when IQ was within normal ranges. Collectively, these results indicate that executive function domains are especially sensitive to the teratogenic effects of alcohol.

Another model of attention was proposed by Mirsky in 1991 (Mirsky, 1991). In this model, attention is comprised of four primary elements: *focus, sustain, encode,* and *shift*. *Focus* describes the ability to select information for further, higher-level processing. *Sustain* describes the capacity to maintain focus over the course of time.

Shift describes flexibility in attention, such as the ability to adaptively change focus. *Encode* describes the ability to use working memory to manipulate information and then transfer that information into long-term memory. According to Mirsky, these elements could be partially functionally localized to discrete brain regions. For example, Mirsky associated *attentional shift* with the prefrontal cortex, *encoding* with the amygdala and hippocampus, *focus* with the inferior parietal lobe, and *sustained* attention with the reticular formation in the brainstem. Using Mirsky's model, clinical diagnoses of attention deficits can be associated with specific brain regions, providing targets for both research and clinical intervention strategies.

In 2006, Mattson, Calarco, & Lang used Mirsky's model to examine attention in FASD populations. Mattson and colleagues demonstrated that FASD children have difficulty with attentional shift, visual focused attention, and sustaining auditory attention, indicating that attention deficits in FASD extend across many measurable domains. Mirsky's model also provided a basis for comparing attention deficits between FASD populations and ADHD populations without FASD comorbidity. These comparisons, which will be discussed in the following section, are part of a movement to distinguish a unique cognitive profile of FASD populations that can inform treatment paradigms.

Both Mirsky and Posner created models that attempted to subdivide attention into specific components and then functionally localize those components to specific neural systems and brain regions. These models have provided useful templates for

examining attention deficits in the FASD literature from a clinical perspective, informing experimental models. However, the phenotype of attentional deficits in FASD populations may change across development.

Hypersensitivity to environmental stimuli and poor habituation are early indicators of attentional problems (Streissguth et al., 1983). These deficits in habituation shift into attention deficits, as measured by classical attention-based tasks, during early school years, and later in life these basic attention deficits become superseded by more complex behavioral problems (Streissguth, Martin, & Barr, 1984; Fryer et al., 2007). Evidence suggests that attention deficits are especially deleterious to development in FASD populations. When examined in conjunction, comorbidity of FASD *and* ADHD has a synergistic effect on future comorbidities (Ware et al., 2013). Specifically, oppositional defiant disorder and conduct disorder had a far greater prevalence in ADHD-FASD comorbid populations than ADHD populations without FASD or FASD populations without ADHD (Ware et al., 2013). Lastly, secondary disabilities increase with age in untreated populations of FASD (O'Malley & Nanson, 2002).

These data suggest that an attentional profile in FASD populations is a developmental issue. Understanding manifestations of attention deficits in FASD and the differentiation of those attention deficits from ADHD populations without FASD comorbidity may provide insight to the impact of different treatments on general prognosis. Therefore, this next section will focus on attention deficits in FASD and how they compare to attention deficits in ADHD.

1.3 The Unique Phenotype of ADHD in FASD Populations

Diagnosis of ADHD in FASD populations reflects different attentional subtypes than ADHD populations without FASD comorbidity. The *Diagnostic Statistical Manual of Mental Disorders* (5th ed.; DSM-5; American Psychiatric Association, 2013) identifies 3 major domains in ADHD: (a) *Inattentive subtype*, (b) *hyperactive and impulsive subtype,* or (c) a *combined subtype*. Diagnosis of ADHD in FASD populations preferentially reflects inattentive subtypes over hyperactive and impulsive subtypes (O'Malley & Nanson, 2002). These results support the idea of a unique attentional profile of FASD children with ADHD. Over the last decade, studies have increased efforts to document distinctions between the progression and manifestation of attention deficits in FASD children and ADHD with the hope that understanding these distinctions can inform treatment of FASD children.

One of the earliest studies comparing attention deficits in FASD versus non-FASD populations with ADHD was performed by Coles et al. in 1997. Coles et al. (1997) demonstrated that while children with ADHD but not FASD are best identified using measures of focused and sustained attention, children with FASD appear to have greater visual and spatial reasoning deficits, encoding deficits, deficits in attentional shift, and impaired flexibility in problem solving tasks.

Crocker et al. (2011) supported differential patterns of cognitive deficits between ADHD and FASD children in a study that matched groups on age, sex, race, and ethnicity, handedness, and socioeconomic status. FASD children had greater difficulty

with encoding of information whereas ADHD children had greater difficulty with retrieval. Burden et al. (2010) supported neural distinctions of FASD and ADHD populations without FASD comorbidity using event-related potentials (ERPs) during a Go/No-Go task. Although children with FASD performed similarly to those with ADHD but no FASD comorbidity, ERPs varied between groups, indicating differences in neural processing. These distinctions likely reflect differences in underlying neural networks, indicating that attention deficits in FASD populations should not be treated the same as ADHD without FASD comorbidity. Indeed, some evidence suggests that children with FASD respond preferentially to *d*-amphetamine compared to methylphenidate stimulant treatment for attention deficits (O'Malley, 2000). This distinction is not reflected in ADHD populations without FASD where methylphenidate is the primary treatment option (Zito et al., 2000; Goldman et al., 1998). The relationship between neural networks, attention deficits, and treatment strategies need to be further examined so neural networks can inform treatment of attention deficits in FASD. Because understanding alcohol's impact on neural networks has primarily been examined using animal models, the next section will examine animal models of FASD.

1.4 Animal Models of FASD

A variety of animal models can be used to assess the impact of alcohol exposure *in utero*. These models include zebrafish, rodents, and non-human primates (Patten, Fontaine, & Christie, 2014). Rodent models are the most frequently utilized as they display high levels of face and construct validity: deficits in rat models of FASD parallel

deficits in human models. These parallels extend from physical to neurocognitive and behavioral domains. Facial dysmorphology in mice is consistent with human facial dysmorphology (Lipinski et al., 2012). Mouse models demonstrated that this characteristic of FASD is dependent upon early gestational exposure (Parnell et al., 2009). Rat models also display impairments in attention, learning impairments, and hyperactivity, demonstrating symmetry between attentional phenotypes in humans and rats (Hausknecht et al., 2005; Melcer et al., 1994; Stoltenburg-Didinger & Spohr, 1983).

Rodent models provide several advantages for studying alcohol exposure compared to human studies of FASD. Studies of fetal alcohol exposure in humans have a number of confounding factors. These factors include but are not limited to socioeconomic status, multidrug use, racial differences, genetic predispositions, unreliable reporting of drinking patterns, and environmental stress. In addition, human studies of fetal alcohol exposure are ethically limited as humans cannot be assigned to drinking and non-drinking pregnancy groups. This makes causal relationships between ethanol and behavior impossible to determine. Animal models enable a randomized and highly controlled examination of how ethanol impacts trajectories of neurodevelopment, but in order to replicate human drinking behaviors, several factors must be addressed.

One major consideration is that human development does not perfectly align with rat development with respect to timing of birth (Bayer et al., 1993; Dobbing &

Sands, 1979). A comparison of human and rat developmental periods can be found in Table 1.1.

Exposure paradigms in animal models vary across gestation. The third trimester of development (PD 1-10) is especially critical as this is considered the brains growth spurt (Dobbing & Sands, 1979; Bonthius & West, 1991). There is evidence from both animal models (Kelly et al., 1988; Maier et al., 1997; West, Kelly, & Pierce, 1986) and also human studies (Rosett, 1981) that the third trimester is a period when the brain is particularly vulnerable to insults including ethanol. The current study used a third-trimester paradigm for alcohol exposure. This exposure paradigm uses intragastric intubation, delivering an alcohol-milk enriched mixture directly into the stomach of the neonatal rat pups (Kelly & Lawrence, 2008). Alcohol exposure in neonatal rats can result in nutritional deficits as the ethanol-exposed pups do not nurse properly. In order to compensate for any malnourishment, a second, vitamin-rich intubation should be performed. This limits neural deficits to alcohol, specifically.

A second consideration for animal models is how to accurately replicate human patterns of drinking behavior. The most deleterious type of drinking for fetal alcohol development is binge drinking as high blood alcohol concentrations increase the severity of neural deficits (Bonthius, Goodlett, & West, 1988). Intubation procedures can replicate this drinking pattern with minimal stress to the rat. This type of administration allows precise oral doses of alcohol with rapid peak blood ethanol concentrations (BEC) which is then eliminated through zero-order effects, as in humans.

Intubated control groups who do not receive an ethanol solution provide comparisons for the stress effect. Therefore, having three exposure groups (i.e. ethanol treated, intubated control, and non-treated control) is essential to examining the effects of alcohol on neurodevelopment and later behaviors.

Rats are well-suited for behavioral studies due to the plethora of behavioral measures that are well established with the species (Cudd, 2005). However, several studies suggest that female rats may be more vulnerable to chronic consequences of prenatal alcohol exposure than males, especially in regards to behavioral dysfunctions (Kelly et al., 1988; Grant et al., 1983). Kelly et al. (1988) demonstrated that spatial navigation was selectively impaired in females during a Morris water maze task. Females also are typically more active in the Open Field Test than males (Blizard, Lippman, & Chen, 1975). The open field test is highly relevant to ADHD literature as it assesses hyperactivity with locomotor behaviors as well as well as general levels of anxiety. Hyperactivity in rat pups exposed to alcohol *in utero* corresponds with human clinical literature (Riley, 1990). Several studies have assessed hyperactivity patterns in rat models of FASD with mixed results. Melcer et al. (1994) demonstrated that males and females exposed to high doses of alcohol during postnatal days 4-9 exhibited hyperactivity by postnatal day 18. In contrast, Grant, Choi, and Samson (1983) demonstrated that male rats exposed to ethanol demonstrated no behavioral differences in open field activity. However, female rats neonatally exposed to ethanol demonstrated more hyperactivity than female controls. This suggests that females may be more behaviorally sensitive to the teratogenic effects of alcohol. Consequently, the

current study focused on the effects of alcohol exposure on female rats in the open field test as a model of an early precursor of attention deficits – hyperactivity.

1.5 Linking the Catecholamines in FASD to Attention Deficits

Attentional processes are intimately linked with the catecholamine neurotransmitters. Catecholamines are a subdivision of monoamine neurotransmitters that consist of a catechol nucleus (i.e. a benzene ring with two hydroxyl groups) and an amine side chain (i.e. NH_2) (Horn, 1973). Within the catecholamine group, dopamine and norepinephrine are particularly essential for regulation and modulation of attention networks (Clark & Noudoost, 2014). These two neurotransmitters work in tandem with subcortical and cortical networks to mediate various aspects of attention and hyperactivity. Some evidence suggests that selective lesions of these neurotransmitter systems impair attentional processes as severely as surgical ablation of the cortex (Brozoski et al., 1979). In FASD populations, hypofunctioning of catecholamine systems may generate similar attention deficits. Conversely, stimulant medications that increase synaptic catecholamine levels can enhance attention at low-doses (Berridge et al., 2006). In FASD populations, stimulant medications may normalize catecholamine levels, improving attention and hyperactivity (Figure 1.1). This section will focus on the impact of dopamine and norepinephrine on attentional networks in relation to FASD. Subsequent sections will then discuss the role of stimulant medications in FASD.

1.5.1 Dopamine and Attention Networks in FASD

Dopamine (DA) neurons originate from two distinct nuclear groups within the brainstem: the ventral tegmental area (VTA) and the substantia nigra (SN) (Swanson, 1982). Neurons in the ventral tegmental area project to the nucleus accumbens (NAcc) and the prefrontal cortex, making up the mesolimbic and mesocortical pathways, respectively (Swanson, 1982). Neurons in the SN innervate the striatum, making up the nigrostriatal pathway (Swanson, 1982). Collectively, these pathways mediate a variety of functions including attention, working memory, reward salience, motivation, movement, and learning (Schultz, 1992; Sawaguchi & Goldman-Rakic, 1991; Wise, 2004). Deficits in dopamine could mediate a wide range of behavioral deficits seen in FASD populations, and the specific deficit may be pathway specific.

1.5.1A Dopamine in Whole Brain

Early models assessing the relationship between dopamine and attention used 6-hydroxydopamine (6-OHDA) to ablate vast areas of subcortical and cortical dopaminergic networks. These lesion studies resulted in broad impairments in cognitive performance, including attention. One of the earliest experiments to associate dopamine with cognitive performance was performed in 1979 by Brozoski and colleagues. In this study, rhesus monkeys depleted of dopamine were tested on a spatial delayed alternation performance task. Dopamine-depleted monkeys performed more poorly than controls on this task. More importantly, performance was rescued with dopamine agonists (e.g. L-DOPA and apomorphine). This experiment was the first to

establish a causal link between dopamine and cognitive performance. Additionally, it demonstrated that pharmacological manipulations aimed at increasing dopamine in dopamine-deficient models can abate cognitive deficits. If dopamine deficiency is an underlying cause of attentional deficits in FASD, this suggests that drug treatment aimed at augmenting dopaminergic networks may be beneficial.

Like lesion studies, the earliest examples of dopamine deficiency in fetal alcohol models analyzed global dopamine neurochemical profiles from whole brain homogenates. These studies used a liquid-diet paradigms of alcohol exposure, which have concerns of malnutrition and dosage (Bonthius & West, 1988). Despite these concerns, liquid-diet studies provided an important precedent for future studies on the interaction between ethanol and developing dopaminergic systems. One of the first studies of dopamine content following a dam-fed liquid diet paradigm during pre and postnatal development was by Detering and colleagues in 1980. Detering et al. (1980) determined that dopamine was significantly reduced in ethanol-treated pups by 21 days of age. However, in this study, Detering et al. suggest that the decrease in dopamine may be linked to malnutrition rather than alcohol effects because the effect was also evident in the isocaloric matched control group. In 1996, Maier et al. reassessed the impact of alcohol on dopamine and its metabolites using a binge-drinking paradigm of prenatal alcohol exposure (Maier et al., 1996). This paradigm more accurately reflects maternal drinking behavior in humans. Maier et al. demonstrated that dopamine and one of its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), were reduced in whole

brain homogenates of ethanol-exposed rat pups following both chronic exposure throughout gestation and a single-dose at gestational day 20.

1.5.1B Dopamine in Striatal and Frontal Circuits

In an attempt to examine brain region-specific changes in dopamine levels, Rathbun & Druse (1985) examined dopamine and its metabolites, DOPAC and homovanillic acid (HVA), within specific brain homogenates: hypothalamus, cerebellum, cortex, brain stem, striatum, and hippocampus. Rathbun & Druse (1985) demonstrated that dopamine levels varied by both age and brain region following a prenatal liquid diet paradigm of alcohol exposure. Developmentally, DA content increased in the hypothalamus and striatum between 19 and 35 days while DOPAC and HVA levels decreased in the striatum and increased in the cortex in controls. Across this same developmental window, DA and HVA levels decreased in the cortex in ethanol-exposed rats. These data provided 3 important pieces of information: 1) ethanol impacts the developmental trajectory of dopaminergic systems, and therefore dopamine levels cannot be assessed as static constructs, 2) the periadolescent developmental window may be an important target for treatment due to the fluctuation in dopaminergic networks during this time, and 3) ethanol may have the strongest impact on mesocortical dopamine content.

Druse, Tajuddin, & Connerty (1990) supported the idea that ethanol impacts dopaminergic systems in a developmental manner. Druse et al. (1990) examined the impact of impact of a liquid ethanol diet during gestation on postnatal development of

dopaminergic networks. Results indicate that ethanol severely impacts striatal and frontal dopaminergic systems. Striatal dopamine exhibited a transient (44%) deficiency during periadolescence. Frontal dopaminergic systems were also impaired in a transient developmental manner: D_1 receptors were reduced by 40% at postnatal day 19 but normalized by postnatal day 37 in ethanol-exposed rats. Cortical D_1 receptors are especially important for attentional processing (Vijayraghavan et al., 2007). Stimulation of D_1 receptors reflects an inverted-U shape on attention with both high and low levels of D_1 stimulation being associated with poor allocation of attention (Vijayraghavan et al., 2007). From a physiological level, low levels of firing of dopaminergic neurons enhance spatial tuning, whereas high levels of firing are overwhelming and impair processing of spatial stimuli. An analogy is that dopamine modulates levels of "noise" within attentional networks (Clark & Noudoost, 2014). Early deficiency in dopaminergic systems in FASD may underlie shifts attention profiles in FASD in an age-dependent manner.

Other studies have examined on the dopamine transporter (DAT) and tyrosine hydroxylase (TH) as opposed to post-synaptic markers. DAT is the primary means of removing dopamine from the synaptic cleft in subcortical circuits, and TH is the rate-limiting step in dopamine synthesis. TH and DAT provide important information on dopamine synthesis and clearance. Barbier et al. (2009) found that perinatal ethanol from a liquid diet paradigm decreased striatal levels of DAT. This study is in agreement with a study by Szot et al. (1999) which demonstrated that prenatal ethanol exposure via a liquid diet paradigm results in a decrease in DAT messenger ribonucleic acid

(mRNA) in the SN and VTA in adult male rats. TH mRNA was also decreased in the VTA (Szot et al., 1999). Collectively, these data suggest that dopamine synthesis and synaptic clearance are hypofunctioning in nigrostriatal and mesolimbocortical circuits in FASD models.

Prenatal ethanol also induces morphological changes in the mesocortical and nigrostriatal dopaminergic system. Shetty, Burrows, & Phillips (1993) reviewed the effects of the perinatal ethanol exposure in rats on dopaminergic structure in the SN using a Golgi-Cox stain. Specifically, Shetty et al. demonstrated that perinatal ethanol exposure in rats resulted in smaller and more densely packed dopaminergic somata, decreased dendritic arborization, decreased dendritic branch length, and dysmorphic development of these dopaminergic neurons. The impact of prenatal alcohol exposure on structure, neurochemistry, and morphology of dopamine neurons is consistent with the hypothesis of a hypofunctioning dopaminergic system underlying attention deficits in FASD. A hypofunctioning dopamine system may underlie symptoms of hyperactivity as well as poor reduction of "noise" when attending specific stimuli.

1.5.1C Dopamine in the Mesolimbic System

Cortical and subcortical dopaminergic pathways interact with each other dynamically. Lesion studies have demonstrated that cortical depletion of dopamine is paralleled by mesolimbic hyperactivity in dopaminergic neurons, indicating a functional link between these two systems (Nieoullon, 2002). In terms of cognitive performance, deficits in dopamine-mediated prefrontal cortical function could reflect poor inhibitory

control of sensory information and a decreased ability to instigate appropriate motor responses (Russell et al., 1995). In conjunction, deficits in dopaminergic function in the nucleus accumbens may distort signals of reward-based stimuli, altering salience values and influencing attention to these stimuli (Sonuga-Barke, 2005). In measurements of attention, alterations within the mesolimbic system results in reductions in response speed and vigor (Robbins & Everitt., 2007).

FASD models have demonstrated structural and functional deficits in mesolimbic dopamine systems. In 1993, Blanchard et al. used in vivo microdialysis to examine mesolimbic dopaminergic response to alcohol following prenatal exposure to alcohol (Blanchard et al., 1993). Ethanol-exposed rats showed a dopamine response equivalent to controls in the nucleus accumbens and striatum following a low dose of alcohol administration. At higher doses, males showed the expected increase in dopamine in response to alcohol in both structures. However, females showed no reaction in the nucleus accumbens and a decrease in dopamine levels in the striatum. These data indicate 1) that neurochemical changes in the dopaminergic system following prenatal alcohol exposure are both sex and brain-region specific, and 2) that neurochemical decrease in dopamine may be more marked in females, further supporting the examination of females in the current study.

Functional differences in stimulus-driven dopamine activity within mesolimbic circuits are supported by a series of electrophysiological studies on dopaminergic firing patterns (Shen, Hannigan, & Chiodo, 1995; Shen, Hannigan, & Kapatos, 1999). In 1999,

Shen, Hannigan, & Kapatos demonstrated that rats exposed to alcohol prenatally via an intragastric intubation binge-drinking paradigm had a reduction in spontaneously active dopaminergic firing within the mesolimbic circuit. Using tyrosine-hydroxylase based cell counts, Shen and colleagues determined that this reduction is not due to dopaminergic cell loss, and this reduction is persistent throughout adulthood. This reduction in spontaneous firing patterns of DA neurons was only seen in ethanol-treated rats and not in intubated controls, indicating that any changes are specific to the ethanol treatment and not due the stress from the intubation procedure.

Collectively, these studies suggest attentional deficits in FASD may reflect impairments in different dopaminergic pathways. Interactions between mesolimbic input and executive regulation of attention in the prefrontal cortex is of special interest in FASD. In FASD populations, hypofunctioning of dopaminergic neurons may result in poor tuning of spatial stimuli, thereby impairing attentional modalities such as "shifting" of attention. Because stimulant treatments acutely increase striatal and mesolimbic dopamine levels, spatial tuning may be normalized following stimulant treatment in FASD populations.

1.5.2 Norepinephrine and Attention Networks

Noradrenergic afferents originate from the locus coeruleus in the brainstem and project to various cortical regions where they modulate a variety of functions. In the prefrontal cortex, noradrenergic afferents influence attention and vigilance by honing neuronal responses to stimuli in attended directions (Rajkowski et al., 2004). Rajkowski

et al. (2004) demonstrated that noradrenergic neurons in the locus coeruleus are phasically activated in response to target stimuli, and that this response occurs prior to any behavioral action, indicating that noradrenergic responses are closely linked to the presentation of sensory stimuli. This suggests that attention towards motivationally driven targets is modulated by phasic noradrenergic activity from the locus coeruleus. Because firing patterns varied with stimuli presentation and behavioral reaction time, Rajkowski et al. postulated that norepinephrine (NE) is an important facilitator of behavioral responses to attended stimuli by rapidly reorganizing neural networks in response to sensory stimuli. In other words, phasic firing of noradrenergic neurons allows adaptation to the changing environment that is imperative to selective attentional processes.

Like DA, the relationship between NE and attention exhibits an inverse-U shape (Figure 1.1) where low and high NE is associated with impaired attentional states (Arnsten & Pliszka, 2011). In relation to FASD populations, low levels of NE in the prefrontal cortex may be associated with low "signals" for motivationally driven stimuli, thereby facilitating inattentive phenotypes of ADHD. Examining markers of dopaminergic and noradrenergic neurotransmitter systems within mesolimbic and cortical regions may help elucidate some of the underlying mechanisms distinguishing attentional profiles in FASD populations.

1.5.4 Noradrenergic Systems in FASD

Norepinephrine (NE) is very similar in structure to DA, and in fact, there is some overlap in receptor binding between these two catecholamines. Noradrenergic neurons use dopamine β-hydroxylase to transform DA into NE. As such, effects on levels of DA content could have downstream effects on NE. In addition, because DA works in tandem with NE to modulate attention, it is important to understand if ethanol differentially affects these neurotransmitter systems.

Effects of prenatal alcohol exposure on noradrenergic neurons have been mixed. Results in animal models appear to be dependent upon windows of exposure to alcohol, brain region, alcohol exposure paradigm, and postnatal treatment. Some of the earliest work on the impact of ethanol on developing catecholamine systems came from Detering and colleagues. Detering et al. (1980) demonstrated that NE levels were significantly lower in whole brain regions of rats prenatally or postnatally exposed to alcohol. This result was replicated by Sari et al. in 2010. Sari et al. demonstrated that ethanol exposure at early embryonic stages (gestational days 7-13) in mice results in a reduction in whole-brain concentrations of norepinephrine via liquid chromatography, indicating that the effects of alcohol on noradrenergic systems are visible even at the earliest developmental windows.

In contrast, Rudeen & Weinberg (1993) did not find any differences of norepinephrine concentrations among liquid-diet ethanol exposed groups compared to pair-fed and *ad libitum*-fed control groups in any brain region during resting behavioral

states. Rather, differences between neurotransmitter systems became apparent after stressors. Following a single exposure of restraint stress, NE concentrations were reduced in the cortex and hypothalamus but elevated in the hippocampus of rats exposed to ethanol relative to both control groups. Following chronic restraint stress, NE levels were decreased in the cortex of ethanol-exposed rats relative to both control groups. Because these effects varied by brain regions, whole brain measures may inadequately discern the more complex effects of alcohol on developing systems. Rudeen & Weinberg also noted sex differences in response to alcohol exposure: females exposed to ethanol exhibited greater reductions in noradrenergic content than males. This indicates that females may be especially sensitive to the effects of ethanol on noradrenergic transmission.

Disagreement between Rudeen & Weinberg (1993), Sari et al. (2010), and Detering (1980) may be due to differences in exposure and brain region. Although all studies used liquid-diet paradigms of alcohol exposure, Detering et al. (1980) examined whole brain homogenates whereas Rudeen & Weinberg (1993) partitioned specific brain regions. One possibility is that global changes in noreprinephrine levels may be due to brain regions not measured by Rudeen & Weinberg. Disagreement between Rudeen & Weinberg (1993) and Sari et al. is likely due to time since exposure. Because Sari et al. (2010) examined norepinephrine levels after shortly after alcohol exposure, the effects from Sari et al. may be transient.

Because early studies often focused on liquid-diet paradigms of alcohol exposure during prenatal periods, Tran and Kelly (1999) examined the effects of early postnatal exposure on neurotransmitter systems using a binge-drinking paradigm. As postnatal exposure corresponds with the brain growth spurt, it is a critical period for the development of neural systems. Early postnatal alcohol exposure was associated with an increased in NE concentrations in the hippocampus. Early postnatal alcohol exposure effects were greater for females than males, again indicating that females may be more susceptible during this developmental window. In 1999, Tran and Kelly sought to connect disjointed literatures with varying exposure timelines. Using high-performance liquid chromatography, Tran and Kelly demonstrated that NE levels were increased in the hippocampus in both males and females following a three-trimester paradigm of alcohol exposure. These data indicate that binge-drinking paradigms during postnatal exposure or during all three trimesters will impact norepinephrine levels in a brain-region and sex-dependent manner.

For attention, noradrenergic systems within the prefrontal cortex are especially critical. Zimmerberg and Brown (1998) examined the effects of prenatal alcohol on plasma concentrations of NE in the prefrontal cortex using a liquid diet paradigm of prenatal exposure. Results indicate that NE is decreased in the prefrontal cortex compared to chow-fed controls, but an increase in NE compared to pair-fed controls. There was no effect of alcohol on NE levels in the NAcc or striatum. Like previous results, these data indicate that alcohol exposure impacts catecholamine levels in a brain-region-dependent manner and that certain brain regions may be more vulnerable

to the effects of alcohol exposure than others. Because noreprinephrine is important for enhancing relevant signals within the prefrontal cortex during attention tasks, decreased norepinephrine levels in this brain region in FASD models may be responsible for inattentive behaviors in FASD populations.

1.6 Treating Attention Deficits: An Examination of Stimulant Medications

Stimulant medications are the most common and effective treatment for attention deficits. Stimulants comprise a class of psychoactive drugs that augment neural activity, increasing mood, awareness, and alertness. Two primary stimulants are methylphenidate (Ritalin) and *d*-amphetamine (Adderall). Although both methylphenidate and amphetamine increase monoamine levels, their mechanisms of action are distinct. Methylphenidate is part of a class of monoamine blockers whereas amphetamine is a monoamine releaser. Blockers obstruct the catecholamine transporters DAT, the noradrenergic transporter (NET), and the serotonin transporter (5-HTT). These transporters are important regulators of extracellular monoamine levels. They bind DA, NE, and serotonin (5-HT) in order to remove them from the synaptic cleft and transport them back into the cytosol of the presynaptic neuron. Blocking this mechanism acutely increases synaptic levels of DA, NE, and 5-HT for postsynaptic receptor binding, resulting in augmented signaling. Because methylphenidate inhibits monoamine reuptake *after* release, its efficacy is dependent upon classic action-potential driven release from vesicular pools.

Unlike methylphenidate, amphetamine is a releasing agent. Therefore, amphetamine is not dependent upon classic action-potential mediated release. Instead, amphetamine acutely reverses the directional functioning of transporters. This includes DAT, NET, 5-HTT, and an intracellular transporter known as the vesicular monoamine transporter-2 (VMAT-2). VMAT-2 transports monoamines from the cytosol into synaptic vesicles where they await action-potential mediated synaptic release (Riddle, Fleckenstein, & Hanson, 2005). These vesicular transporters are dynamic regulators of vesicular load, where their functional effects have direct influences on how much neurotransmitter is released during synaptic transmission (Eiden, 2000). Amphetamine reverses VMAT-2 by disrupting the vesicular pH gradient. As a result, monoamines that are packaged into synaptic vesicles are re-released into the cytosol (Sulzer & Rayport, 1990; Sulzer et al., 1995). Because amphetamine also reverses membrane-bound transporters (i.e. DAT, NET, 5-HTT) by initiating an inward-facing conformation change, the cytosolic monoamines bind to the now-inward facing transporters and are reverse-transported into the synaptic cleft (Chen et al., 2010). This results in the force-released mechanism of monoamines. Therefore, a critical difference between methylphenidate and amphetamine is that methylphenidate efficacy is dependent upon tonic release of dopamine while amphetamine is not (Fleckenstein et al., 2007).

As a result, amphetamine has a much greater effect on extracellular DA, NE, and 5-HT levels than methylphenidate. Using microdialysis, Kuczenski & Segal (1997) demonstrated that a 10 mg/kg dose of methylphenidate increases extracellular DA levels in the caudate/ putamen to a concentration of 100 nM whereas a 2.5 mg/kg dose

of amphetamine increased DA to nearly 500 nM with peak effects at 30 minutes after a subcutaneous injection. Hippocampal levels of NE also significantly increased with amphetamine relative to methylphenidate. 5-HT levels were also more impacted by amphetamine than methylphenidate. However, both drugs exhibited the greatest impact of extracellular levels of DA.

Although both methylphenidate and amphetamine increase synaptic levels of monoamines through similar mechanisms, children diagnosed with FASD+ADHD and ADHD without FASD comorbidity respond differently to these treatments (O'Malley, 2000). Approximately 90% of preschoolers diagnosed with ADHD are prescribed methylphenidate (Goldman et al., 1998; Zito et al., 2000). However, evidence suggests that children co-diagnosed with FASD and ADHD have a more positive clinical reaction to amphetamine (O'Malley, 2000). O'Malley conducted a pilot study of 30 patients with ADHD and FASD. These patients received treatment with either amphetamine or methylphenidate. Nineteen of the 30 patients responded positively to amphetamine whereas only 5 responded well to methylphenidate. In addition, 8 patients who were treated with methylphenidate had negative responses and had to be switched to amphetamine; these patients responded positively to amphetamine. Only one patient responded preferentially to methylphenidate over amphetamine, and 3 patients responded poorly to both methylphenidate and amphetamine. These results indicate an increased efficacy for amphetamine treatment compared to methylphenidate treatment in FASD clinical populations.

Other results on stimulant intervention for attention deficits in FASD are mixed. Oesterheld et al. (1998) examined the effectiveness of methylphenidate in Native American Children with FASD. FASD children were randomly assigned methylphenidate or a placebo for 5 consecutive days. Although methylphenidate significantly improved hyperactivity, attention scores were not improved. Because this study did not compare methylphenidate to amphetamine, it is unclear whether amphetamine would have succeeded where methylphenidate failed.

1.6.2 Developmental Perspectives: Treatment During Windows of Opportunity

The time period for administration of amphetamine is critical to the types of effects that are reported. Juvenile response to pharmacological treatments can be quite different from the adult response. For example, juveniles have a decreased sensitivity to the locomotor effects of stimulants. This is reflective of fluctuating changes in the dopamine transporter during this period. Neurological systems in flux may react differently to pharmacological interventions. In adult animals, drug exposure is followed by a biological compensatory reaction that counteracts the pharmacologically induced state. This process is known as the opponent process. However, when neural systems are still maturing, such as during the juvenile developmental window, pharmacological effects can induce permanent neurodevelopmental changes that persist long after the drug administration has been ceased (Andersen, 2004). This process is known as neuronal imprinting. Neuronal imprinting has exciting implications for pharmacological

treatments. Pharmacotherapy targeted during specific developmental windows may provide an opportunity to shape and normalize aberrant developmental trajectories.

In support of this theory, Choong & Shen (1999) demonstrated a reduction in dopaminergic firing in the VTA following a prenatal paradigm of alcohol exposure. This reduction of dopaminergic firing is not evident until four weeks of age and then persists into adulthood. This suggests the possibility that early intervention paradigms that target the transition period in firing patterns may be effective at shaping developmental trajectories in FASD populations.

1.7 Justification of Study

Noradrenergic and dopaminergic systems both play important and diverse roles in attention. Both systems have been demonstrated to have deficits in FASD populations, and the patterns of these deficits may reflect specific profiles of attention deficits. Although each system has been assessed independently in the literature, studying NE and DA congruently will provide a more holistic picture of attention systems. There is some conflicting evidence on deficits in NE. This may be reflective of variations in patterns of exposure. The current study proposes to use a third-trimester model of exposure to isolate ethanol's teratogenicity during the brain's growth spurt. This controlled paradigm will provide valuable additional information on the impact of alcohol on developing neural networks.

Because catecholamine systems continue developing throughout adolescence, extending studies across this developmental window is important for translation into

clinical FASD literature. Rat models of FASD have suggested that hypofunctioning of catecholamine systems may adversely impact developmental trajectories. Because amphetamine directly increases synaptic NE and DA levels, amphetamine may rectify this trajectory if administered during appropriate developmental windows. However, neurotransmitter systems are still maturing throughout adolescence. This complicates clinical understanding of the impact of chronic pharmacological treatments. Understanding the impact of chronic amphetamine treatment in FASD populations during the periadolescent developmental window will inform treatment paradigms in clinical populations of FASD. Therefore, the current study addresses chronic periadolescent amphetamine exposure in a rat model of FASD.

Lastly, rat models suggest that females may be more sensitive to the teratogenic effects of ethanol, displaying greater hyperactivity than males. However, females are often underrepresented in the animal literature. This study will therefore focus on the impact of ethanol on female hyperactivity. Because hyperactivity is an early precursor to more complex attentional deficits, locomotor behaviors will be assessed in an Open Field Test at multiple developmental time points across periadolescent development.

These aforementioned pieces of information have been inadequately addressed in the current literature. Therefore, the current study had three aims: 1) to investigate the effects of neonatal ethanol exposure on DAT and TH in the NAcc, 2) to investigate the effects of neonatal ethanol exposure on DBH in the prefrontal cortex, and 3) to investigate the effects of chronic therapeutic doses of amphetamine treatment on DAT,

TH, and DBH in the NAcc and PFC during a critical developmental window in a rat model of FASD in females.

It was hypothesized that DAT, TH and DBH would be decreased in ethanol-treated rats compared to controls. Chronic exposure to amphetamine was hypothesized to increase these markers in all groups. These changes were hypothesized to be reflected in behavioral measures of hyperactivity: amphetamine treatment was hypothesized to interact with neonatal treatment so that amphetamine reduces hyperactive locomotor responses in ethanol-treated rats and exacerbates locomotor responses in controls.

Table 1.1

Comparison of human and rat developmental periods.

Human	Rat
First Trimester	Gestational days 1-10
Second Trimester	Gestational Days 11-22
Third Trimester	Postnatal Days (PD) 1-10

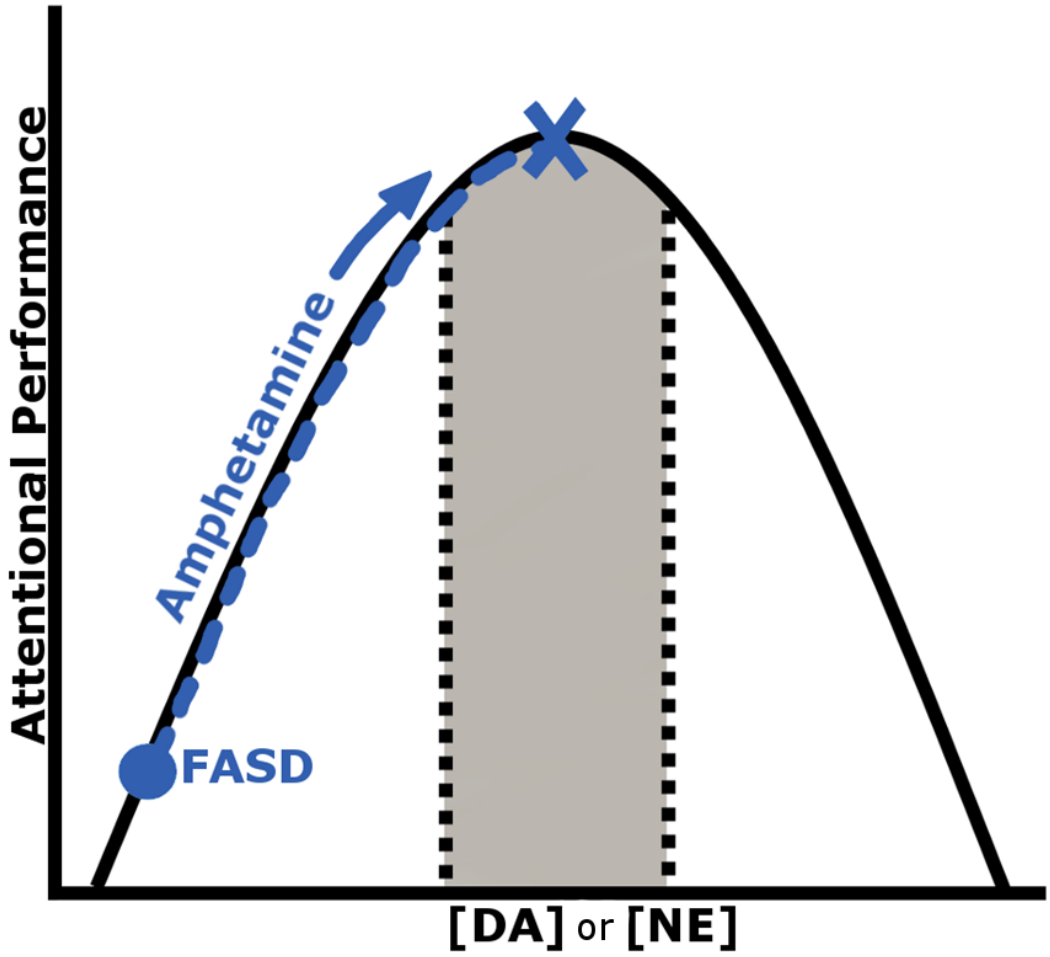

Figure 1.1. Attentional Performance as an Inverted-U. Low or high levels of DA/ NE result in impairments in attention. FASD is associated with hypofunctioning of DA/NE systems and impaired attentional performance. Amphetamine may improve attention in FASD populations by optimally increasing catecholamine levels.

CHAPTER 2

METHODS

2.1 DOSE-RESPONSE STUDY

This study had four levels of amphetamine treatment measured across a repeated measure of day (PD 26 to 40). See Figure 2.1 for a timeline.

2.1.1 ANIMALS

Twenty-eight juvenile female Long-Evans rats were obtained at postnatal day 23 (PD 23) with day of birth counted as PD 1. Thirty-two rats were pair-housed in standard polypropylene cages at the University of South Carolina School of Medicine's animal facility with *ad libitum* access to both standard food and water. Cages were changed twice weekly with new bedding, fresh water, and additional food. The facility was temperature controlled at 22°C and runs on a 12/12 h light-dark cycle with the light cycle beginning at 07:00 h. Rats were handled and weighed daily (09:00 h) in order to keep track of any effects on body weight effects due to the amphetamine treatment as well as to accurate daily treatment dosages. These housing procedures were in accordance to all guidelines and regulations by the University of South Carolina's Animal Care and Use Committee.

2.1.2 Amphetamine Treatment

Each rat was randomly assigned to one of four treatment groups: water (H_2O), 0.5

mg/kg/day *d*-amphetamine, 1 mg/kg/day *d*-amphetamine, and 2 mg/kg/day *d*-amphetamine (Sigman, amphetamine HCl). Each group had a total of eight rats. Amphetamine doses were calculated based on the salt form of *d*-amphetamine. The range of doses were set to accommodate for the full range spanning from doses typically used in children (up to .6 mg/kg/day) (Wolraich & Shubiner, 2009) with the higher dosages (up to 2 mg/kg/day) tested in the animal literature (Koffarnus & Katz, 2010; Bizot, David, & Trovero, 2011).

D-amphetamine was obtained from Sigma Aldrich (catalog number A5880-5G). Solutions were prepared by dissolving with *d*-amphetamine in distilled H_2O for a volume of 1 mL/kg. The rats were injected subcutaneously twice a day at 10:30 h and again at 16:30 h in order to compensate for the short, 1 hour plasma half-life of *d*-amphetamine in juvenile rats (Heijtz, Kolb, & Forssberg, 2003) compared to the longer, 4-6 hour half-life noted in children (Wolraich & Shubiner, 2009). Each injection was half of the total daily dose for the designated treatment condition. Treatment lasted a total of 14 days, beginning at PD 26 and culminating PD 40.

2.1.3 Behavioral Measurements

The rats were given an Open Field Test twice during the treatment paradigm to assess motor hyperactivity. The first test was conducted on day PD 26, 30 minutes after amphetamine treatment on the first day in order to discern baseline levels of activity following acute drug administration. Timing was set in accordance with peak amphetamine-induced dopamine efflux in the NAcc as determined by Schiffer et al. (2006). The second test was conducted on PD 40, 30 minutes after the last treatment. Each rat was transported to the behavioral testing room in an opaque transport cage immediately prior to testing and then placed in a square 60 x 60 x 35 cm, gray Plexiglas chamber for a total of 15 minutes. The floor of the chamber was divided into an inner and an outer section, and an overhead video camera recorded the rat's movements. Lighting averaged 400 lux.

Total distance traveled and proportions of time spent in inner versus outer zones were analyzed using Ethovision 7 (Noldus). Total distance traveled is the summation of distance traveled in centimeters over the entire testing period. The inner zone was a 30×30 cm square zone defined as all areas medial to the perimeter (Figure 2.2). After the testing procedure, the rat was returned to its cage. Between each test, the chamber was wiped down with 5% ammonium hydroxide.

There are two major locomotor considerations due to the time course of this study. The first is the normal developmental changes of locomotor behavior over time.

The second is the potential of locomotor sensitization from chronic amphetamine treatment.

2.1.4 Statistical Analyses

Body weights were tracked throughout the experimental procedure as a decreased body weight is a potentially adverse side-effect to chronic amphetamine treatment. These data were analyzed by a repeated measures ANOVA with the factors of *day* and *treatment*. Results for the Open Field Test were analyzed as a repeated measures ANOVA. The four levels of *treatment* are as follows: H2O, 0.5 mg/kg AMPH, 1 mg/kg AMPH, 2 mg/kg AMPH. The repeated measures are the two levels of the factor *day* (acute, chronic). For all data, outliers were replaced with mean values. Outliers were defined as any value that is either above or below 2 standard deviations from the mean. There were no outliers for the dose-response study. Due to a fire alarm interruption on PD 26, two rats were removed from analyses. One rat was from the medium amphetamine dose group and the other was from the high amphetamine dose group. Statistical significance was set at $\alpha = .05$. To explore statistically significant main effects or interactions, Tukey's HSD *post hoc* tests were run.

2.2 FASD Study

Experiment 2 was a 2×2×3 between-groups design with 2 levels of drug *treatment* (i.e. H_2O, AMPH), 4 levels of *day* (PD 26, PD 27, PD 40, PD 41) and 3 levels of neonatal *exposure* (i.e. NC, IC, ET). See figure 2.3 for a timeline.

2.2.1 Animal Procedures: The FASD Model

Timed-pregnant dams were ordered and individually housed in standard polypropylene cages with *ad libitum* access to standard food and water. Housing and animal maintenance were the same as those in the dose-response study. A total of 68 pups from each dam were randomly assigned in a split litter design to one of three *treatment* groups: non-intubated controls (NC), intubated controls (IC), or ethanol treated (ET). Day of birth for each pup was designated PD 1. The intubation procedure consisted of Intramedic PE 10 tubing dipped in corn oil for lubrication purposes that was lowered down the pup's esophagus. Neonatal ethanol exposure lasted from PD 2 until PD 10. The NC pups did not receive any intubations in order to control for the procedure's stress factor. IC pups were intubated twice daily without any administration of solutions. ET pups received two daily intubations. The first intubation was between 09:00 h and 11:00 h and consisted of 3.0 g/kg ethanol in 27.8 mL/kg of enriched milk. The second intubation was between 11:00 h and 13:00 h and consisted only of the enriched milk in order to compensate for any malnutrition due to the lack of feeding behaviors following the ethanol administration.

All pups were weighed and tattooed for identification on PD 7 (Animal Identification & Machine Systems, Inc.). The pups remained with their dams until PD 21 upon which they were weaned and pair-housed with their same sex littermate. As in the dose-response study, this study consisted only of females. The male pups from these litters were utilized in a different experiment.

2.1.2 Blood Ethanol Concentrations

Blood samples were collected from tail bleeds from IC and ET pups on PD 10 two hours after the first intubation, which is optimal for assessing maximum BEC levels (Kelly & Lawrence, 2008). Blood samples from the IC pups were used to create a standard curve of specific BECs: 0, 50, 100, 200, 300, 400, 500, and 600 ng/mL. Blood was collected into centrifuge tubes with 190 µL of 0.53 N perchloric acid. 200 µL of 0.30 M potassium carbonate was then added to block coagulation. The samples were vortexed, placed on iced, and then centrifuged at 4^{o}C for 20 minutes. The supernatant was removed and stored at -80^{o}C until the assay was run. BECs were analyzed via an enzymatic process using a 96-well plate according to previously established procedures by Dudek & Abbott (1984). Samples and standards were run in duplicate with 50 µL of sample, 400 µL of 1.86 mM Tris-NAD stock, and 50 µL of alcohol dehydrogenase added to each well. The plate was briefly mixed on an orbital shaker and then incubated for one hour prior to absorbance reading (340 nm). BEC values were interpolated from the standard's values.

2.2.3 Amphetamine Treatment

Amphetamine treatment was conducted as described in the dose-response experiment using the middle dose (1 mg/kg/day) as determined by the results from that study. In this study there were two levels of treatment factor: H_2O control versus amphetamine.

2.2.4 Behavioral Analysis

Locomotor behavior was assessed with all 68 animals at four times in this study: PD 26, PD 27, PD 40, PD 41. Procedures are identical to the dose-response experiment with the test performed 30 minutes following the last *d*-amphetamine injection.

Two behaviors were measured in addition to locomotor activity as described in the dose-response study: rearing, grooming. Rearing behavior is defined as both front paws lifted simultaneously from the floor. This is used as a basic assessment of motor stereotypy (Creese & Iversen, 1973). Grooming behavior can be an index of anxiety (Dunn et al., 1987). Grooming was defined as cleaning of the paws or body. All tests were analyzed blindly by the same experimenter, Victoria Macht, who has significant history analyzing behavioral data. Forty-five videos were randomly selected to be analyzed for rearing and grooming behaviors.

2.2.5 Tissue Preparation and Analysis

Twenty-four hours after the last behavioral testing, the rats were anesthetized with isoflurane in a bell jar and subsequently perfused with 0.9% saline followed by ~300mL of a double-filtered 4% paraformaldehyde in 0.1 M phosphate buffered solution, pH 7.4. Following decapitation, the brains were removed and post-fixed in the 4% paraformaldehyde for 24 hours. Then the brains were placed in a 30% sucrose/0.1 M phosphate buffered solution where they incubated for 48-72 hours at 4°C until they sank. After sinking, they were transferred to cryoprotecting solution and then sliced on a freezing microtome at a thickness of 40 µm into coronal sections. Tissue from two

targets were separated during sectioning: the NAcc and the medial PFC. The NAcc was defined anatomically by plates 10-15 from Paxinos and Watson's (1998) rat brain atlas; the medial PFC was defined anatomically by plates 8-10. Sections were collected serially into four separate tubes and then tested for TH, DAT, and DBH content via immunohistochemistry. TH and DAT immunohistochemistry was performed on tissue from the NAcc; DBH immunohistochemistry was performed on tissue from the medial PFC (Figure 2.4). Tissue from 43 rats was analyzed for DBH, tissue from 31 rats was analyzed for DAT, and tissue from 35 rats was analyzed for TH.

Immunohistochemical procedures were performed at room temperature (~25°C) unless otherwise specified. Each section was washed in Tris-buffered saline (0.1 M Tris-HCL with 0.9% NaCl, pH 7.4) and then rinsed with methanolic peroxide to block the endogenous peroxidase enzymes in the tissue. Next, the sections were blocked against nonspecific staining and the membranes were permeabilized by incubating for 30-60 minutes in a solution of 0.3% Triton X-100 (TX), Tris-buffered saline (TBS), and 2% normal goat serum (NGS).

The primary antibody for TH was a rabbit anti-tyrosine-hydroxylase IgG polyclonal antibody from Millipore. Tissue was incubated in the primary antibody for 24 hours at a 1:5000 dilution at room temperature and then another 48 hours in the cold room (4°C). Following the primary incubation, the tissue was rinsed in TBS and incubated with a biotinylated donkey anti-rabbit IgG (1:1000) from Vector Laboratories, Inc. for 1.5 hours at room temperature. Next, the tissue was re-rinsed in TBS and

incubated for one hour in peroxidase-conjugated streptavidin (1:1600). Finally, the tissue was rinsed in TBS, developed in a 0.05%, 3,3' diaminobenzidine-HCl with Nickel Chloride in TBS for 12 minutes, and then mounted on 0.3% gelatin-coated slides. Slides were subsequently dried and dehydrated prior to cover-slipping.

DAT was measured using the same immunohistochemical procedure as TH. The primary antibody was the DAT1 monoclonal rat anti-DAT IgG (1:1000) from Millipore. The secondary antibody was a biotintylated horse anti-rat IgG (1:1000) from Vector Laboratories, Inc. The tertiary remains peroxidase-conjugated streptavidin (1:1600).

DBH was also measured using the same immunohistochemical procedure as described above. The primary antibody was a monoclonal mouse-anti-dopamine-beta-hydroxylase (1:3000). The secondary antibody was a biotintylated horse anti-mouse IgG (1:1000) from Vector Laboratories, Inc. The tertiary remains peroxidase-conjugated streptavidin (1:1600).

2.2.6 Image J Analysis

Each slide was observed under a light microscope with a digital camera attachment. Pictures were saved as a TIFF file and processed in Image J, a free software program from NIH (http://rsb.info.nih.gov/ij). The NAcc was identified on sections from plate 12 as the area medial and adjacent to the anterior commissure. Images from the NAcc were taken at a 20X magnification. The medial PFC was identified as the area medial to the white matter from the corpus callosum. Images targeted neuronal layer II/III, and these images were taken at a 10X magnification. A minimum of two images

per section were processed. For all data, results were averaged so that only one data point was attributed to each animal. Within each picture, immunoreactivity for TH and DAT were assessed as average gray values using optical density via ImageJ. Each image was 1392 ×1040 pixels which corresponds to approximately 0.64×0.48 mm at 10X magnification and 0.32×0.24 mm at 20X magnification, respectively. Immunoreactivity for DBH was assessed using a dichromatization method as described by Iritani et al. (2010). With this method, an index of immunoreactivity is calculated automatically as pixel units within the defined area (Figure 2.5).

2.2.7 Statistical Analysis

Results for the Open Field Test were analysis as a repeated measures ANOVA (RMANOVA). There were two independent variables: *exposure*, *treatment*. *Exposure* refers to the three levels of neonatal exposure: NC, IC, ET. The two levels of *treatment* are H_2O and AMPH. There are also four levels of *time* (i.e. PD 26, PD 27, PD 40, and PD 41). Four dependent variables were assessed: total distance traveled, time spent in the inner zone, rearing, and grooming. Immunohistological data were assessed by 2x3 ANOVAs. The dependent variables in this case were DAT, TH, and DBH.

For all data, statistical significance was set at $\alpha = .05$. Outliers were replaced with mean values. Outliers were defined as any value that is either above or below 2 standard deviations from the mean. There were two outlying data points for total distance traveled in the Open Field Test. On PD 26, one rat from the NC-amphetamine group was identified as an outlier and replaced with a group mean value for that date. On PD 27, one rat from the

IC-water group was identified as an outlier and replaced with the group mean value for that date. For time spent in the inner zone, there were five total outliers. One was from a NC-amphetamine on PD 40, 1 from an IC-amphetamine on PD 26, 1 from an IC-amphetamine on PD 41, and 1 from the ET-amphetamine on PD 40 and 41. Following statistically significant main effects or interactions, Tukey's HSD *post hoc* tests or simple effects tests were run as appropriate.

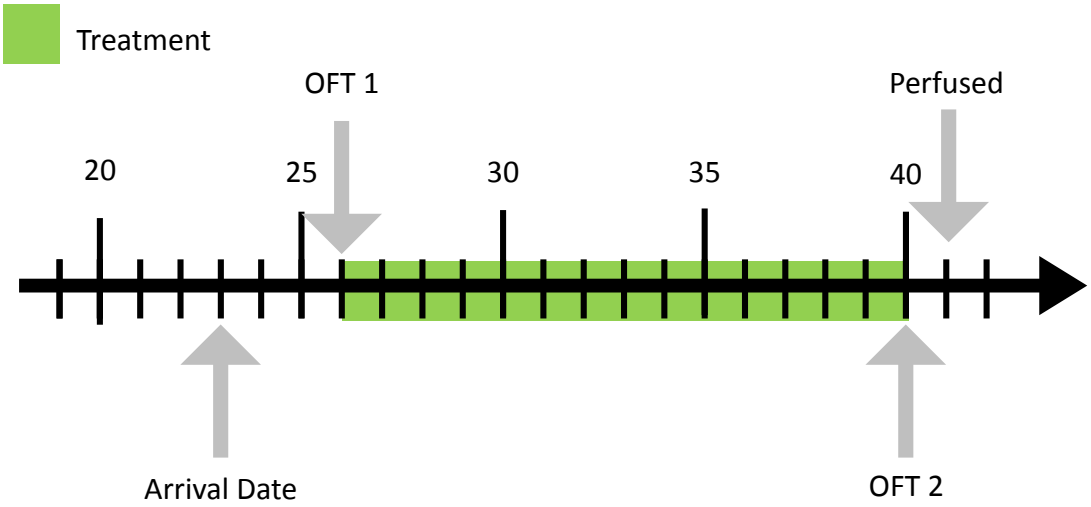

Figure 2.1. Timeline for Dose-Response Study.

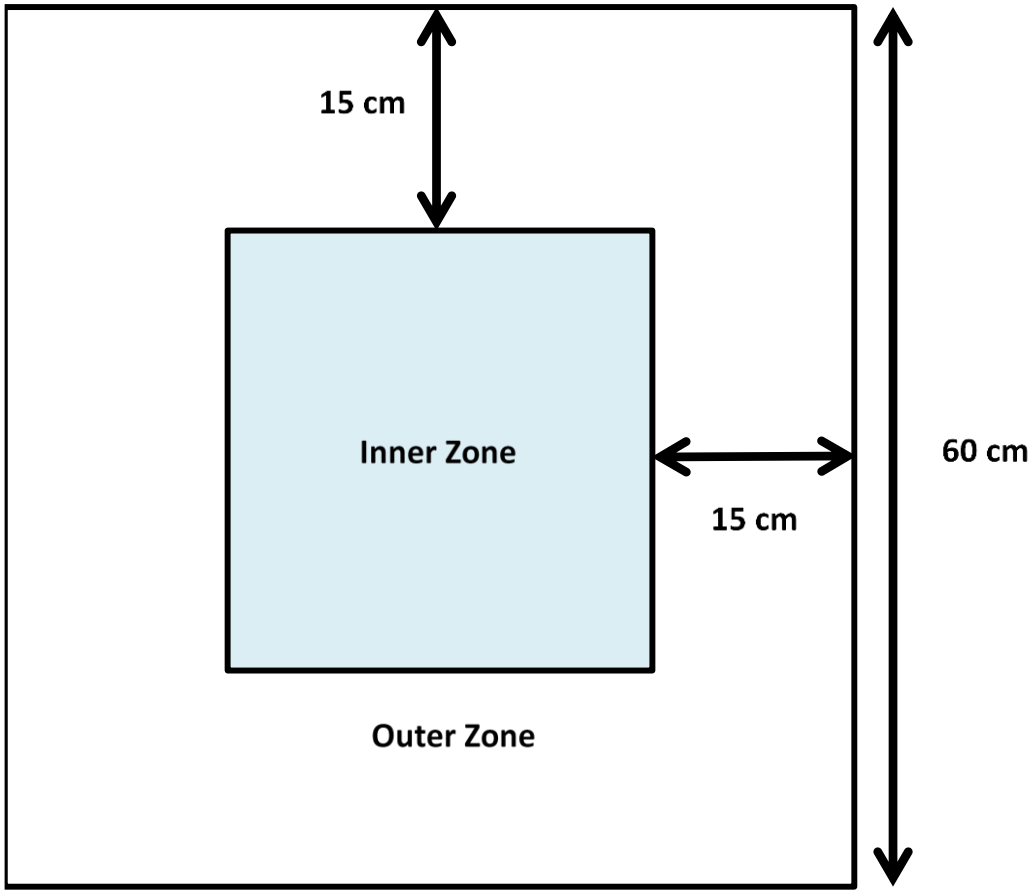

Figure 2.2. Open Field Test Apparatus.This Figure depicts the square, plexiglass chamber used in the Open Field Test. Inner versus outer zones were measured accordingly.

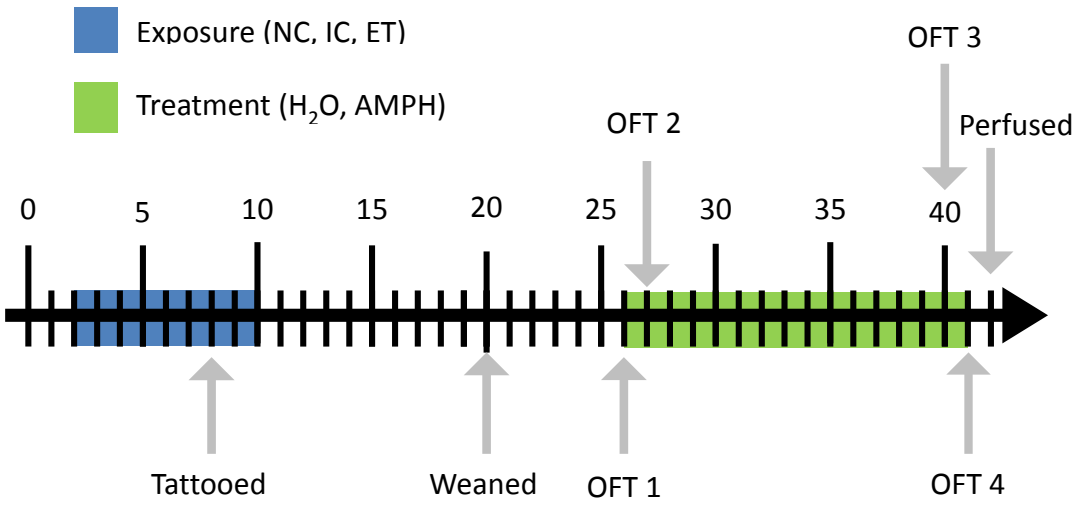

Figure 2.3. Timeline for the FASD study.

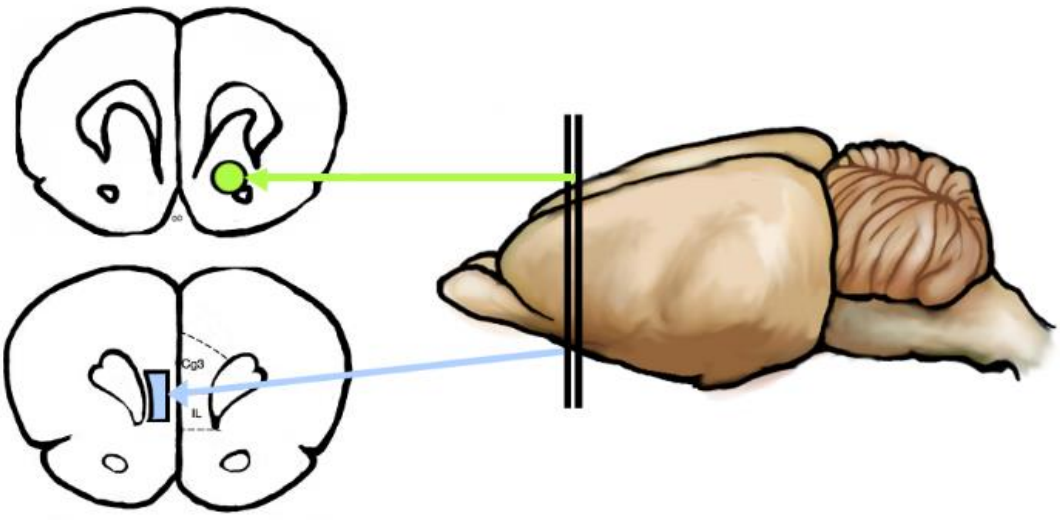

Figure 2.4. Targets from coronal sections. The top target is the NAcc. The bottom target is the medial PFC.

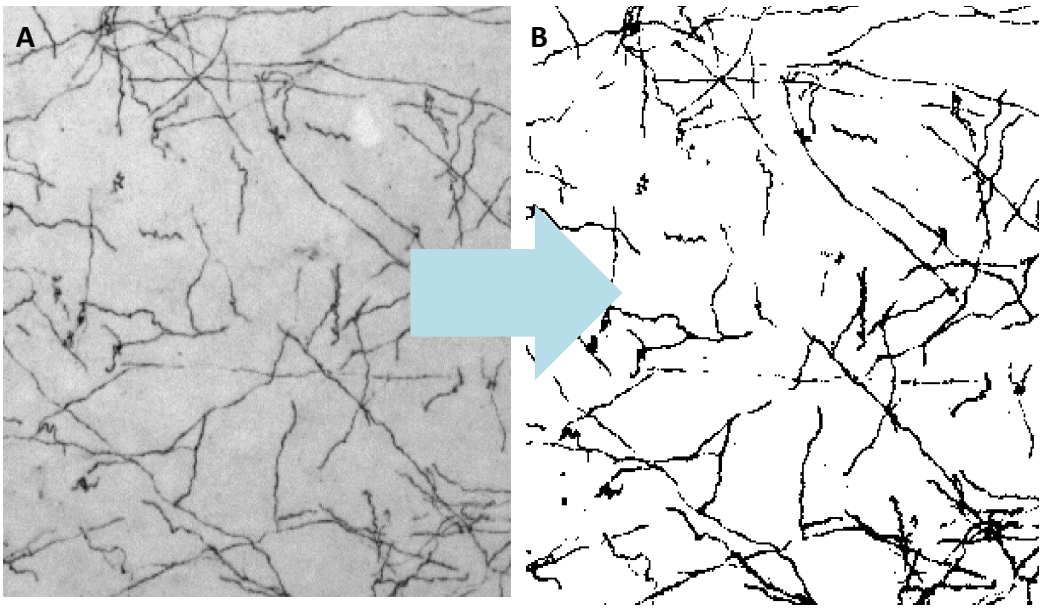

Figure 2.5.A. DBH staining in medial prefrontal cortex. B. ImageJ dichromatization of DBH staining. Analysis is expressed as total immunopositive pixels.

CHAPTER 3

RESULTS

3.1 DOSE-RESPONSE STUDY

This study examined the acute and chronic dose-response effects of therapeutic doses of amphetamine treatment across the periadolescent developmental window.

3.1.1 BODY WEIGHTS

A repeated measures ANOVA revealed no interaction between *day* and drug *treatment*, $F(3, 14)=1.26$, $p > 0.05$, nor was there a main effect of drug *treatment* on body weight, $F(3, 14) = 0.410$, $p > 0.05$. Body weight was significantly impacted by *day* which was expected across development, $F(3, 14)=179.61$, $p < 0.05$. As *day* increased, so did body weight. This indicated that all animals grew appropriately, and amphetamine treatment did not result in attrition which is a potential negative side effect with higher doses of stimulants. These results are summarized in Table 3.1.

3.1.2 BEHAVIORAL MEASURES

A repeated-measures ANOVA demonstrated that total distance traveled exhibited a main effect of *day*, $F(1, 26)=38.34$, a main effect of *treatment*, $F(3, 26)=22.92$, and a *day***treatment* interaction, $F(3, 26)=4.85$, $p < 0.05$. Tests for simple effects revealed that on the first day of testing, rats given medium (1.0 mg/kg/day) and high (2 mg/kg/day) doses of amphetamine moved significantly more than rats given

water or a low (0.5 mg/kg/day) dose of amphetamine, $p < 0.05$. At the last day of testing, all doses of amphetamine significantly increased locomotion over water, $p < 0.05$. This indicates that locomotor effects of amphetamine were sensitized over time. However, there was no difference between rats given the medium and high doses of amphetamine on the last day of testing, $p > 0.05$. In summary, the low dose of amphetamine was not sufficient to induce a change in locomotion acutely, and there was no difference between the medium and high dose. These data are summarized in Figure 3.1.

Time spent in the inner zone demonstrated a main effect of *treatment*, $F(3, 26)=5.44$, $p < 0.05$. Post-hoc tests revealed that a medium or high dose of amphetamine significantly increased time spent in the inner zone when compared to water treatment, $p < 0.05$. In addition, while 1.0 mg/kg/day amphetamine significantly increased time spent in the inner zone when compared to 0.5 mg/kg/day amphetamine, $p < 0.05$, mean time spent in the inner zone decreased in the 2.0 mg/dg/day amphetamine group. Therefore 2.0 mg/kg/day amphetamine was not significantly different from either the 0.5 mg/kg/day dose or the 1 mg/kg/day dose, $p > 0.05$. Anxious rats have a tendency to remain close to the walls and explore the peripheral zone in an open field test, a behavior termed thigmotaxis. Decreased thigmotaxis with the 1 mg/kg/day dose of amphetamine can be indicative of decreased anxiety at this dose. There was also a main effect of *day*, $F(3, 26)=4.64$, $p < 0.05$, but no *day*treatment* interaction, $p > 0.05$. Time spent in the inner zone increased from acute (PD 26) to chronic (PD 40) time points,

indicating an effect of age on exploratory behavior. These results are summarized in Table 3.2.

The medium dose of amphetamine was selected for use in the FASD study for two reasons: 1) this dose resulted in both the greatest acute effects as well as the greatest sensitization of behavioral effects over time, and 2) the high dose of amphetamine exhibited significantly higher measures on anxiety than the medium dose.

3.2 FASD Study

This study examined the effect of amphetamine treatment across the periadolescent developmental window using a model of FASD. This study used the medium dose of amphetamine, as determined by the results in the dose-response study.

3.2.1 BECs & Body Weights

BECs for ethanol-exposed animals were 418.8±87.4 (SEM). Body weight significantly increased from PD 2 to PD 10, which is expected during development, $F(8, 35) = 939.47$, $p < .05$. Ethanol treatment did not significantly impact body weight in neonatal rats, $F(2, 27) = 0.41$, $p > 0.05$. This indicates than any group differences with ethanol treatment are not due to malnutrition. These data are summarized in Table 3.3.

There was no main effect of amphetamine on body weight, $F(1, 35) = 0.04$, $p > 0.05$. Neither was there an interaction between amphetamine and neonatal ethanol exposure, $F(2, 35) = 0.60$, $p > .05$. This indicates that amphetamine did not induce

weight loss in these groups, and ethanol treated rats were not more sensitive to weight loss effects of amphetamine than controls. These data are also summarized in Table 3.3.

3.2.2 Behavioral Measures

Analyses of total distance traveled revealed two main effects and an interaction. There was a main effect of *day*, $F(3, 49) = 17.59$, $p < .05$, and drug *treatment*, $F(1, 51) = 95.87$, $p < 0.05$. There was also a significant *day*treatment* interaction, $F(3, 49) = 6.60$, $p < 0.05$. Analyses of simple main effects revealed that within the amphetamine treatment group, total distance traveled significantly increased an average of 2096.19±475.79 cm from PD 26 to PD 27, $p < 0.05$, an average of 2890.82±504.75 cm from PD 26 to PD 40, $p < 0.05$, an average of 3881.48±474.44 from PD 26 to PD 41, $p < 0.05$, an average of 1785.29±474.44 from PD 27 to PD 41, $p < 0.05$, and average of 990.66±331.70 cm from PD 40 to PD 41, $p < 0.05$. These data are represented as mean ± SEM. Total distance traveled did not significantly increase across any days in the water treatment groups. These results indicate that amphetamine induced hyperactivity acutely and that rats became sensitized to the locomotor effects of amphetamine chronically, irrespective of neonatal ethanol exposure condition. These results are summarized in Figure 3.2.

Analyses of time spent in the inner zone revealed a main effect of *day,* $F(3, 51) = 8.39$, *treatment*, $F(1, 53) = 41.22$, and a *day*treatment* interaction, $F(3, 51) = 12.75$, $p < 0.05$. Analyses of simple main effects revealed that within the amphetamine treatment condition, time spent in the inner zone increased an average of 31.41±7.23 seconds from PD 26 to PD 27, $p < 0.05$, an average of 42.06±5.1 seconds from PD 26 to PD 40, $p <$

0.05, and an average of 42.41±6.01 seconds from PD 26 to PD 41 $p < 0.05$. Time spent in the inner zone did not change within water treatment groups. These data are represented as mean ± SEM with results summarized in Figure 3.3.

Rearing behaviors were also analyzed as a measure of stereotypy. Rearing behaviors exhibited a main effect of *day,* $F(3, 37) = 6.04$, $p < 0.05$, and *treatment,* $F(1, 39) = 36.75$, $p < 0.05$. Rearing behaviors decreased as *day* increased when collapsed across all conditions (Figure 3.4). Specifically, rearing behaviors decreased from PD 26 to PD 40 by an average of 18.71±7.67 counts. As expected, rats receiving amphetamine treatment exhibited more rearing behaviors than rats receiving water treatment. These data indicate that amphetamine did not differentially induce rearing by treatment groups, suggesting that ethanol-exposed groups were not more sensitive to this form of stereotypy.

Grooming exhibited main effects of *day,* $F(3, 36) = 8.51$, $p < 0.05$, and *treatment*, $F(1, 38) = 49.91$, $p < 0.05$. Amphetamine significantly reduced grooming behavior from an average of 92.70±5.61 seconds±SEM within water treated groups to 38.47±5.59 seconds±SEM. Grooming behavior also significantly decreased from acute (79.64±5.43) to chronic (54.42±5.28) sessions, indicating a developmental impact on grooming behavior.

3.2.3 Immunohistochemistry

An omnibus ANOVA revealed no impact of ethanol exposure or amphetamine treatment on DAT or TH, $p > 0.05$. Data are provided in Table 3.4.

There was a significant effect of *treatment* on DBH staining within the prefrontal cortex, $F(1, 37) = 27.04$, $p < 0.05$, but no effect on *exposure,* and no interaction. DBH was significantly increased following amphetamine treatment. DBH results and representative pictures of staining are displayed in Figure 3.5.

Table 3.1

Change in Body weights (g) During Amphetamine Treatment

Dose-Response Study	
Treatment	Change in Body Weight (g)
H_2O	71.16±2.99
0.5 mg/kg/day AMPH	72.01±1.98
1 mg/kg/day AMPH	74.86±2.92
2 mg/kg/day AMPH	74.86±2.92

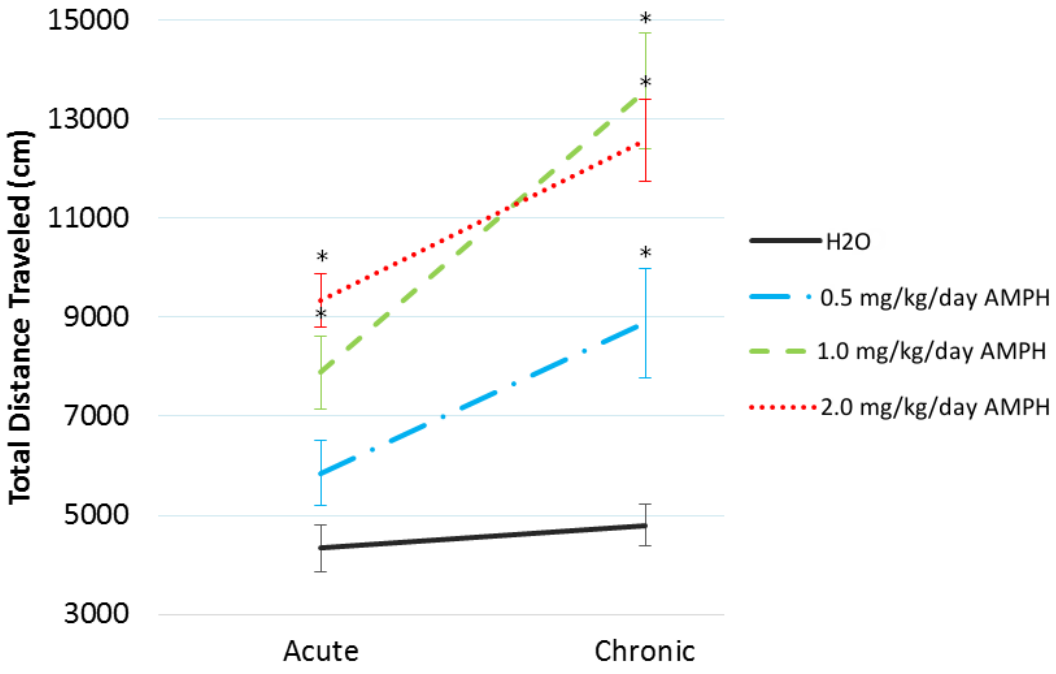

Figure 3.1. Total Distance Traveled in Dose-Response Study. Data are represented at both acute (PD 26, 27) and chronic (PD 40, 41) time points. Data are expressed as the mean ± SEM. Amphetamine significantly increases total distance traveled in a dose response fashion. Asterisks represent data that is significantly different from the water treatment group, $p < 0.05$.

Table 3.2.

Behavioral Data for the Dose-Response Study

Treatment	Distance Traveled (cm)		Time in Inner Zone (sec)	
	Acute	Chronic	Acute	Chronic
H_2O	4332 ± 474	4802 ± 423	15 ± 9	20 ± 9
0.5 mg/kg/day AMPH	5855 ± 658	8886 ± 1105	24 ± 7	54 ± 20
1.0 mg/kg/day AMPH	7887 ± 731	13571 ± 1170	57 ± 16	73 ± 13
2.0 mg/kg/day AMPH	9336 ± 544	12569 ± 828	34 ± 11	67 ± 16

Table 3.3.

Exposure and Treatment Impact on Body Weights (g)

		Non-treated Control	Intubated Control	Ethanol Treated
PD 2-10		11.29±0.37	11.24±0.41	10.56±0.36
PD 26-40	H_2O	70.99±3.07	69.25±2.43	71.20±2.19
	Amphetamine	66.48±2.76	67.22±1.47	67.44±2.45

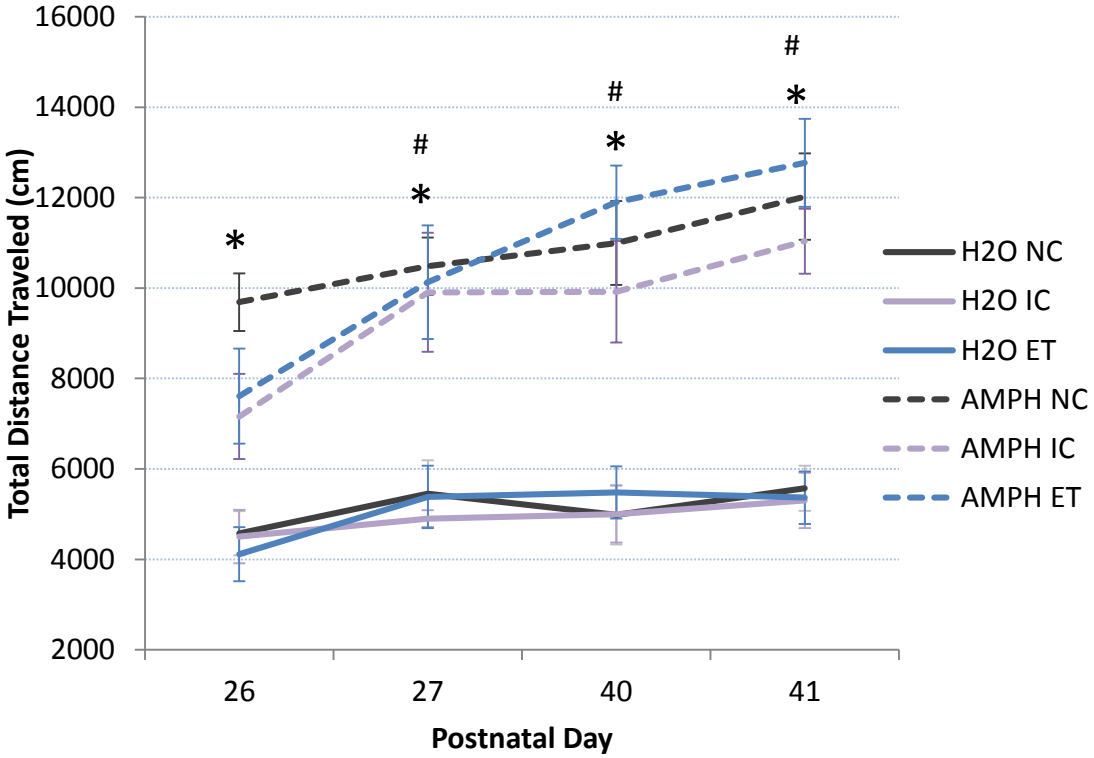

Figure 3.2. Total distance traveled (cm) in the Open Field Test for the FASD Study. Acute testing consists of postnatal days 26 and 27. Chronic testing consists of postnatal days 40 and 41. Habituated testing is the second test day during the acute and chronic conditions. The data are expressed as the mean ± SEM. Asterisks represent data significant from H_2O, $p < 0.05$. Pound symbols represent data significant from PD 26.

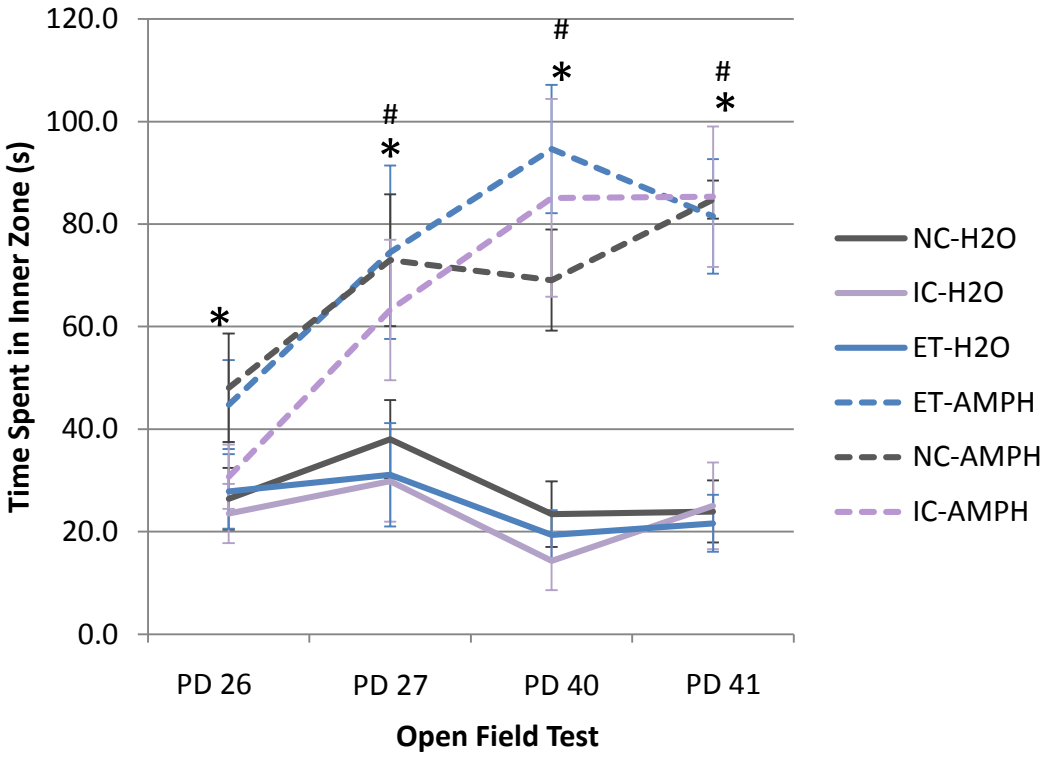

Figure 3.3. Time in Inner Zone in FASD Study. Data represent time spent in the inner zone at PD 26, 27, 40, and 41 respectively. Time was evaluated as total seconds in inner zone. The data are expressed as the mean ± SEM. Asterisks represent data significant from H_2O, $p < 0.05$. Pound symbols represent data significant from PD 26.

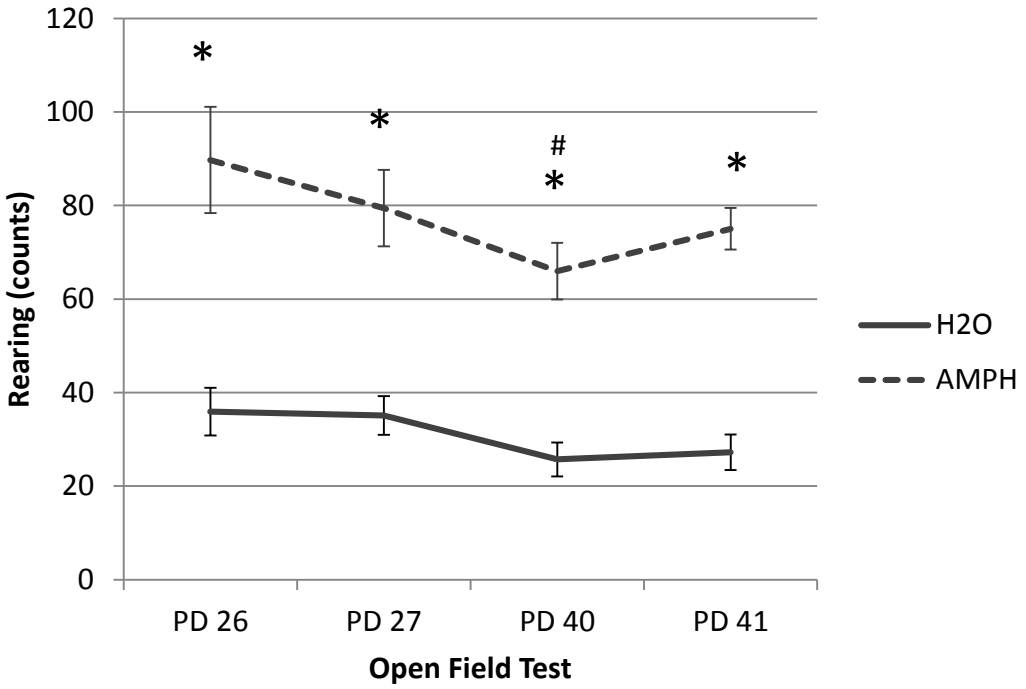

Figure 3.4. Rearing in FASD Study. Counts of rearing are expressed across all four Open Field Tests. Ethanol exposure groups have been collapsed. The data are expressed as the mean ± SEM. Asterisks represent data significant from H_2O, $p < 0.05$. Asterisks represent data significant from H_2O, $p < 0.05$. Pound symbols represent data significant from PD 26.

Table 3.4.

Immunohistological Analysis via Optical Density

	Tyrosine Hydroxylase		Dopamine Transporter	
	H_2O	AMPH	H_2O	AMPH
ET	2898 ± 433	3166 ± 320	3371 ± 61	3844 ± 167
IC	3092 ± 371	3325 ± 247	3859 ± 189	3943 ± 159
NC	3001 ± 361	3298 ± 261	3775 ± 132	4064 ± 122

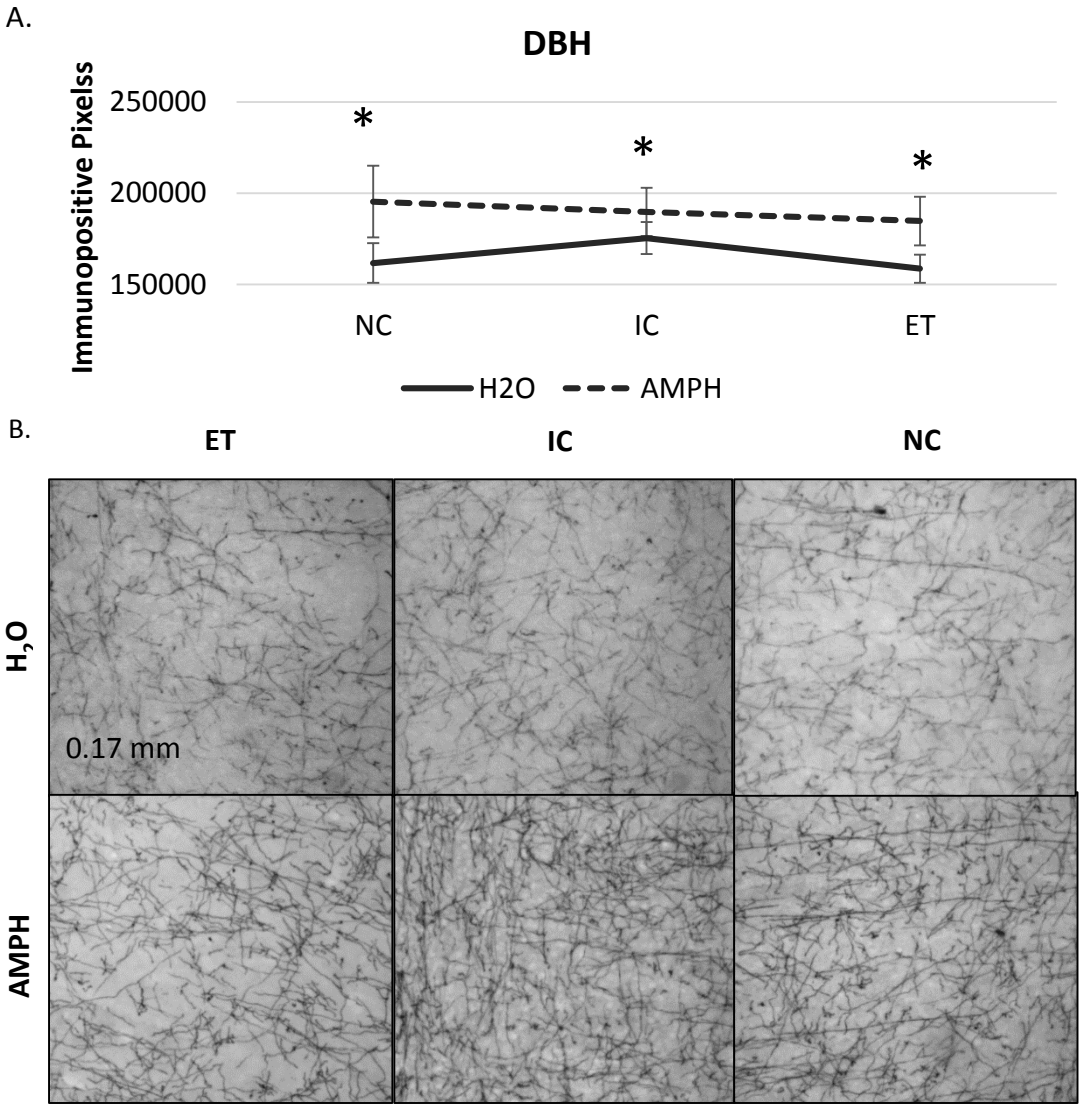

Figure 3.5. A. Immunopositive staining for DBH in the medial prefrontal cortex, as analyzed by ImageJ. The data are expressed as the mean ± SEM. B. Representative pictures of DBH staining by exposure group and treatment. Images represent staining within layers II/III from the medial PFC.

CHAPTER 4

DISCUSSION

The current studies support previous literature indicating that amphetamine increases locomotor activity acutely (Dandiya & Kulkarni, 1974), and that this activity becomes sensitized chronically (Lynch, Kennny, & Leonard, 1978). In the dose-response study, amphetamine increased total distance traveled acutely, and these effects were sensitized over time. Sensitization to amphetamine-induced locomotor hyperactivity was replicated in the FASD study. However, contrary to hypotheses, ethanol-treated rats did not exhibit any behavioral differences in the open field test, nor did they exhibit any differential response to amphetamine. These behavioral findings are paralleled by neurochemical data. DBH staining in the medial prefrontal cortex was increased in amphetamine treated rats, but there were no differences DBH, DAT, or TH staining in any neonatal treatment groups for the FASD paradigm.

4.1 DOSE-RESPONSE STUDY

The dose-response study examined the acute and chronic dose-response effect of multiple therapeutic doses of amphetamine on locomotor behavior in female rats. In accord with previous literature, amphetamine induced a dose-response increase in total distance traveled (i.e. locomotor activity) when it is first administered on PD 26.

Chronically (PD 40), amphetamine-induced locomotor hyperactivity increased further, indicating sensitization. However, there were no significant differences between the medium and high doses chronically, suggesting that 1 mg/kg/day of amphetamine is sufficient to induced changes in locomotor behavior but that increasing that dose is not necessary.

In addition to increasing locomotor behavior, amphetamine treatment impacted time spent in the inner zone both after one administration and chronically. Unlike the effect on locomotion, amphetamine did not exhibit a linear dose-response effect on time spent in the inner zone. Rather, time spent in the inner zone increased with a medium (1 mg/kg/day) but not a not high (2 mg/kg/day) or low dose of amphetamine (0.5 mg/kg.day). Although crude, more time spent in the inner zone can be indicative of an anxiolytic response as time spent in the inner zone tends to increase with anxiolytic drugs (Prut & Belzung, 2003; Wallace et al., 2008). In contrast, wall hugging (i.e. thigmotaxis) can be considered an anxiogenic behavior (Wallace et al., 2008). Time spent in the inner zone may reflect evolutionary mechanisms modulating necessary risk taking in new environments for exploration to find food and mates. Low anxiety and high exploration in novel environments are also important during adolescence as these behaviors are thought to help develop independence (Spear, 2000). The anxiogenic response to higher doses of amphetamine is well-substantiated in the literature (Biala & Kruk, 2007; Cancela et al., 2001; Pellow et al., 1985). In contrast, the anxiolytic response of the medium dose of amphetamine is not well documented. These results could indicate a curvilinear relationship between the pull of environmental exploration and

risk-assessment responses from non-specific threats: moderate activation of catecholamine networks enhance exploratory behavior but higher activation induces anxiogenic responses (Prut & Belzung, 2003).

Importantly, time spent in the inner zone is not correlated with the distance traveled: higher doses of amphetamine resulted in increased distance traveled but decreased time spent in the inner zone compared to the medium dose. Underdevelopment of the prefrontal-limbic circuits in juveniles may have contributed to the medium dose's interesting effect (Lewis, 1997). 1 mg/kg/day of amphetamine may provide optimal activation within the prefrontal cortex without provoking an anxiogenic response with over-activation at prefrontal glutamatergic inputs.

These results suggest two things: 1) a medium dose is sufficient to induce a behavioral change in locomotion, and 2) increasing the dose beyond this point is unnecessary. Collectively, the dose-response data supported the use of 1mg/kg/day of amphetamine for the FASD study.

4.2 FASD Study

This study examined the impact of a third-trimester binge paradigm of ethanol-exposure followed by chronic amphetamine administration on behavioral hyperactivity and catecholamine networks. Although amphetamine induced several behavioral effects and one neurochemical effect, ethanol-exposed animals were not differentially impacted on any measures.

4.2.2A Behavioral Data

In the FASD study, amphetamine increased distance traveled in all groups. Like the dose-response study, this effect on locomotion became sensitized over time. In addition, amphetamine increased time spent in the inner zone acutely, and this effect became sensitized chronically within the study. This is in agreement with the dose-response study regarding data on the medium dose but not the high dose of amphetamine, indicating that behavioral effects are dose-dependent.

Two additional behavioral measures were added in the FASD study: rearing and grooming. Rearing behaviors were used as a marker of amphetamine-induced stereotypy. Unsurprisingly, rats treated with 1 mg/kg/day amphetamine exhibited more rearing behaviors than their water-treated counterparts. However, all rats demonstrated decreased rearing across each testing day, and there was no interaction between amphetamine and testing days on rearing behavior. One explanation is that at this dose, amphetamine does not induce sensitization to stereotypy. Alternatively, rearing behavior may have decreased over time due to progression to minute, oral stereotypies. However, Scholl et al. (2009) demonstrated that even at 6 consecutive intraperitoneal injections of 2.5 mg/kg amphetamine, intense oral stereotypies were minimal. This suggests that the current dose did not result in sensitization of stereotypy, which would have been a negative consequence for a chronic therapeutic dose.

Unlike rearing, grooming behavior was inversely related to amphetamine treatment. Because grooming is sometimes taken as a measure of anxiety, this indicates

that amphetamine increased exploratory behavior without increasing anxious behavior (Dunn et al., 1987). This parallels that finding that amphetamine increased time spent in the inner zone compared to controls. Collectively, these results argue that chronic administration of 1 mg/kg/day dose of amphetamine does not have anxiogenic effects. In contrast, Cancela et al. (2001) demonstrated that 2 mg/kg intraperitoneal injections of amphetamine for 9 consecutive days did induce anxiogenic responses in an elevated plus maze. Because the 2 mg/kg/day dose of amphetamine in the current dose-response study increased distance traveled but decreased time spent in the inner zone when compared to the 1 mg/kg/day dosage, the current study supports the proposal of a threshold dose for anxiogenic effects of stimulants. Many studies utilize 2 mg/kg doses of amphetamine as equivalent to a therapeutic dose used in humans. The current study does not support these results. Rather, these data suggest that doses should not exceed 1 mg/kg/day in order to avoid negative behavioral and potentially neurological consequences.

Contrary to the hypotheses that ethanol-treated rats would exhibit hyperactivity, ethanol-treated rats did not exhibit increases in locomotion nor did they show differential responses to amphetamine compared to control rats. Similarly, there were no differences among groups with respect to time spent in the inner zone. Generally, hyperactivity is the result of either insufficient inhibition of locomotion during exploration or deficits in habituation to the environment (Riley, 1990). Associations with locomotor hyperactivity have yielded mixed results in animal models. These results appear to vary by three factors: age, exposure paradigm, and sex.

In regards to age, young rats show enhanced hyperactivity compared to adolescent and young-adult rats. An early study by Means et al. (1984) demonstrated hyperactivity in male rats exposed to ethanol prenatally via a liquid diet paradigm at postnatal day 26. Means et al. (1984) also demonstrated that ethanol-exposed rats were hypersensitive to the locomotor-inducing effects of methylphenidate at 100+ days of age. Kelly, Hulsether, and West (1987) demonstrated that hyperactivity peaks around postnatal day 20 and declines by postnatal day 30. This hyperactivity re-emerges in aged rats (Abel &Dintcheff, 1986). These studies suggest that locomotor activity in ethanol-exposed rats exhibits a curvilinear function.

Although locomotor hyperactivity is not a direct measure of attention, the relationship between locomotor hyperactivity and attention is extremely important in FASD. Hyperactivity is frequently reported in children with FASD, although it is replaced by attentional deficits during pre-adolescent development (Driscoll et al., 1990). This developmental shift between hyperactivity and attention deficits in human studies parallels the curvilinear function of hyperactivity demonstrated in animal models. Therefore one possibility for the lack of hyperactivity in ethanol-exposed rats is that the tested ages (PD 26, 27, 40, and 41) fell within the developmental depression of hyperactivity demonstrated in animal models. Comparison with human studies suggests that this depression in hyperactivity may be due to developmental shifts toward attentional deficits. This possibility necessitates further studies examining attentional capacities during this age range.

Variation in locomotor activity by age was also reported by Marche, Danel, and Bordet (2011). However, in contrast with previous studies, Marche, Danel, and Bordet demonstrated decreased locomotion (i.e. hypoactivity) in rats exposed to ethanol prenatally at three weeks (~postnatal day 21), increased locomotion (i.e. hyperactivity) of those same rats at 5 weeks (~postnatal day 35), and then a return to hypoactivity at 10 weeks (~postnatal day 70). These effects contradict previous research. One major difference between these studies is that Marche, Danel, and Bordet (2010) used a much more extensive exposure paradigm. Dams were exposed to alcohol for four weeks before breeding, throughout gestation, and then for 3 weeks during lactation. Because the last period of exposure extended past the human equivalent of the third trimester (Bayer et al., 1993), these data may not generalize to the human condition. However, these data do suggest that the relationship of ethanol-exposure on activity can vary substantially and potentially be inverted, depending on exposure paradigm.

The most compelling differences in activity are found in examining sex differences. Most studies (Means et al., 1984; Marche, Danel, & Bordet, 2011) exclusively examine males whereas the current study examined females. Wilcoxon et al. (2005) demonstrated that locomotor activity varies by sex. Using a liquid diet paradigm of alcohol exposure, Wilcoxon et al. (2005) demonstrated hypoactivity in females in an open field test at postnatal day 100. This decrease in locomotor activity was not evident in males. Similarly, Gilbertson and Barron (2005) documented hyperactivity following neonatal ethanol exposure during postnatal days 19-21. However, hyperactivity during this period was limited to males. Females were not significantly different from controls.

Because males are more commonly used in animal research, the current study supports the need to include females in future studies, especially in regards to the developmental effects of ethanol.

In conclusion, hyperactivity is most frequently reported in males during preadolescent (PD ~21) and adult (PD 100+) ages following prenatal exposure using prenatal exposure and liquid diet paradigms. Because the current study used females and a third-trimester exposure paradigm, and because activity was monitored between PD 26 and 41, hyperactivity in exploratory behavior may not have been evident.

An alternative explanation for failure to demonstrate hyperactivity in ethanol-exposed rats is that hyperactivity was masked by an augmented stress response. Animal models of FASD are robustly associated with a hyper-reactive hypothalamic-pituitary-adrenal axis and behavioral responses to stress (Taylor et al., 1982; Weinberg, 1992; Hellemans et al., 2010). This stress-response could also have been elicited due to variations in testing conditions between studies. Rats habitually prefer dark or dim-light settings (8-13 lux). Bouwknecht et al. (2007) demonstrated that high-light conditions (400 lux) in an open field test significantly reduced locomotor activity and increased anxiety responses when compared to low-light conditions, indicating that low-light conditions reduce anxiety behaviors. Thomas et al. (1998) demonstrated hyperactivity in rats neonatally exposed to alcohol, but testing was conducted in dark conditions. Similarly, Wilcoxon et al. (2005) demonstrated age and sex effects of a liquid diet paradigm of alcohol exposure, but testing conditions were performed at 160 lux. As the

current experiment was tested at high-light settings (400 lux), sex and age may have also impacted sensitivity to lighting-induced stress effects.

Weinberg (1992) demonstrated that the stress response, as indicated by corticosterone reactivity, of ethanol-treated rats following four-hours of restraint stress is greater in females than males. Since the current study used only females, light-induced stress may have had a greater impact on locomotion than in previous experiments. In sum, stress from lighting conditions in the current study could have increased anxiogenic behavior which would result in decreased locomotion, masking any hyperactivity in ethanol-treated rats.

The lack of developmental impact of ethanol on rearing behaviors in the current study is discordant with previous studies. Using a liquid diet paradigm of ethanol exposure, Wilcoxon et al. (2005) demonstrated that rearing behaviors are impacted by ethanol exposure in a sex-dependent manner: females reared more than males, but females exposed to ethanol reared less than controls. This finding was not replicated in the current study. Females exposed to alcohol showed no differences in rearing when compared to intubated and non-treated controls across any testing day. This could be due to paradigm differences as the current study used a binge-drinking paradigm whereas Wilcoxon et al. used a liquid-diet paradigm for FASD. Alternatively, differences in ethanol's impact on rearing behaviors could be due to age-differences. The current study tested rats at PD 40 whereas Wilcoxon et al. performed the open field test at PD 100.The developmental impact of ethanol on rearing may only be noticeable in older

rats. This hypothesis is supported by the study by Marche, Danel, & Bordet (2011). Marche, Danel, & Bordet (2011) demonstrated that rearing behaviors decrease in an age-dependent manner. At 3 weeks of age, ethanol-exposed rats exhibited similar rearing behaviors to their controls. However, by 10 weeks of age, ethanol-exposed rats reared less than controls. Because the current study only examined rats just over 5 weeks of age, ethanol's impact on rearing may have been obscured by the juvenile drive for exploration.

In conclusion, ethanol-treated rats did not exhibit any behavioral differences in the open field test. Ethanol-treated rats also did not exhibit any differential response to amphetamine. Although multiple studies have suggested that ethanol-treated rats should exhibit hyperactivity, failure to replicate this finding could be due to 1) lighting conditions in the open field environment, or 2) an attentional task would have been more suitable measure considering the developmental age at which the open field tests were conducted. The current study did support well established results in the field that amphetamine induces locomotion in a dose-response fashion. Importantly, the current study supports the idea that in order for animal studies to exhibit human translational validity for therapeutic uses of amphetamine, dosages should not exceed 1 mg/kg/day. This finding encourages re-evaluation of dosing procedures for future studies in these fields.

4.2.2B Neuroanatomical Data

Although amphetamine mechanistically impacts both dopaminergic and noradrenergic neurons, only DBH staining in the prefrontal cortex was impacted by amphetamine in the current studies. DBH staining increased in the medial prefrontal cortex in rats chronically treated with amphetamine during the periadolescent window, meaning that there was an increase in either noradrenergic innervation or synthesis. Amphetamine did not impact DAT or TH, nor was there an effect of alcohol treatment on any markers.

An increase in DBH may indicate an increase in innervation by noradrenergic axons. Innervation may be due to either 1) amphetamine-induced plasticity of the prefrontal cortex during the periadolescent developmental window, or 2) amphetamine-induced plasticity that is independent of developmental period. Because the adolescent prefrontal cortex is undergoing rapid maturation and reorganization of neural networks, drug exposure during this time window can change developmental trajectories (Andersen, 2004). The former hypothesis parallels a similar finding by Reynolds et al. (2014) that chronic amphetamine treatment during adolescence (PD 22-31) but not adulthood (PD 75-84) increases dopaminergic innervation in the medial prefrontal cortex in mice. Conversely, this increase in innervation was paralleled by a reduction of presynaptic sites on dopaminergic axons. Reynolds et al. (2014) suggest that drug exposure during adolescence has an increased ability to alter neural networks. Unlike the current study, Reynolds et al. (2014) examined dopaminergic and not

noradrenergic networks. Reynolds et al. (2014) also used a much larger (4 mg/kg) dose of amphetamine as a means of studying adolescent exposure to drugs of abuse. However, the same principles apply to the current study: the adolescent window may be sensitive to changes in noradrenergic innervation within the medial prefrontal cortex following repeated amphetamine exposure.

Within the medial prefrontal cortex, noradrenergic networks may be more sensitive to chronic amphetamine than dopaminergic networks. Berridge and Stalnaker (2002) demonstrated that a 0.5 mg/kg subcutaneous injection of amphetamine increased extracellular norepinephrine levels 175% above baseline and extracellular dopamine levels by only 125% above baseline. Baseline concentrations of DA and NE were both approximately at 1 pg/20 uL of cerebral spinal fluid. Amphetamine's ability to preferentially increase extracellular levels of NE in the medial prefrontal cortex may be due to anatomical distinctions between DAT and the noradrenergic transporter (NET). DAT has higher concentrations and clearance capacity within the infralimbic cortex whereas NET has higher concentrations within the medial prefrontal cortex (Heidbreder & Groenewegen, 2003). Because amphetamine reverses both DAT and NET to force release of presynaptic NE and DA, amphetamine may have a greater impact on noradrenergic levels due to increased NET concentration within this region.

The ability of a therapeutic dose of amphetamine to change developing neurocircuitry could have significant behavioral and cognitive effects for adolescents using stimulant medication. Within the prefrontal cortex, noradrenergic neurons act as

neuromodulators of glutamatergic pyramidal afferents (Ji et al., 2008; Market & Aghajanian, 1999; Steketee, 2003). Therefore, changes in noradrenergic innervation could have significant impacts on afferent projects of other neurotransmitter systems. This, in turn, could impact functions dependent upon the medial prefrontal cortex such as arousal, attention, and working memory (Dalley, Cardinal, & Robbins, 2004).

In addition, noradrenergic release within the prefrontal cortex modulates amphetamine-induced hyperactivity (Darracq et al., 1998). Darracq et al. (1998) demonstrated that locally administering an α-1 noradrenergic antagonist into the rat prefrontal cortex in conjunction with an intraperitoneal injection of 2.0 mg/kg amphetamine blocked amphetamine-induced locomotor hyperactivity without impacting amphetamine induced dopamine release in the nucleus accumbens. This suggests that stimulation of noradrenergic postsynaptic receptors are critical for amphetamine's effect on locomotion. Changes in noradrenergic innervation within the prefrontal cortex may partially explain sensitization to locomotion following chronic amphetamine administration. This would also explain sensitization to amphetamine-induced locomotion following chronic treatment in the absence of changes in TH or DAT content in the current study.

Contrary to predictions, neither dopaminergic nor noradrenergic markers varied by alcohol exposure. One possibility is that synthesis markers like TH and DBH may be resistant to neonatal ethanol exposure. Alternatively, physiological changes in these systems may not be reflected in immunohistological measures. The latter is supported

by electrophysiological studies by Shen, Hannigan, & Kapatos (1999). Shen et al. (1999) demonstrated that mesolimbic dopaminergic neurons had reductions in spontaneous firing patterns. However, there were no differences in tyrosine-hydroxylase based cell counts. Because the current study also examined TH staining, dopaminergic neurons may have exhibited electrophysiological alterations that were not detectable with the current methods.

Although dopamine-beta hydroxylase specifically has not been investigated previously, literature regarding noradrenergic content has yielded mixed results. Although some of these differences can be explained by variations in alcohol exposure paradigms, Rudeen & Weinberg (1993) suggested that even when no baseline differences in noradrenergic content are detected, deficiencies in noradrenergic systems become evident following stressors. Therefore one explanation of the current data is that immunohistological measures did not capture functional differences in ethanol-mediated effects on noradrenergic neurons. This explanation would be in accord with previous studies regarding dopamine which suggests that synthesis enzymes may not be good markers for ethanol's developmental impact on catecholaminergic networks.

In summary, chronic amphetamine increased noradrenergic innervation within the prefrontal cortex, but not dopaminergic markers within the nucleus accumbens. This increase in innervation could partially explain sensitization to amphetamine-induced locomotor hyperactivity. Ethanol did not impact any neurological measures. This may be

due to functional rather than structural differences in catecholaminergic transmitter systems.

CHAPTER 5

FUTURE DIRECTIONS

The current study had many limitations which may have impacted results. These limitations pertain to both behavioral measures as well as immunohistological measures.

5.1 LIMITATIONS OF BEHAVIORAL MEASURES

The greatest limitation of the current study is that attention was not directly measured. Rather, hyperactivity was used as a correlation of attentional indexes. Human studies have suggested that hyperactivity diminishes during the ages tested in this study in favor of attentional deficits (Driscoll et al., 1990). Hence, assessing attention directly using a five choice-serial reaction test could provide a more sensitive measure of cognitive deficits than the open field test (Robbins, 2002).

Although the open field test has been used as a measure of locomotor activity for decades, examining locomotion in the open field test has many limitations and confounding variables (Walsh & Cummins, 1976). Testing conditions, age, sex, habituation to the testing chamber, and transport to the testing chamber all impact locomotion (Walsh & Cummins, 1976). Because the current study used high-lighting conditions, interactions with light-induced stress response and ambulation may have confounded ethanol effects. Lighting conditions in an open field test could be lowered

for future studies, which would hopefully decrease the anxiogenic response to the test. Alternatively, using an elevated plus maze in conjunction to an open field chamber would help separate anxiolytic behavior from locomotor behavior (Pellow et al., 1985). Anxious rats spend more time in the closed arms of an elevated plus maze (Hogg, 1996). Variations in time spent in the closed arms could then be used as a covariate for locomotor activity, thus separating decreased activity due to anxiety from any locomotor hyperactivity evident in the open field.

An additional limitation of the current study is that both experiments focused on effects of alcohol and amphetamine in female rats. Due to the sex differences in locomotor activity, development, vulnerability to ethanol-mediated damage, and dopaminergic tone, examination of sex differences is an important future measure. Success of pharmacological intervention for hyperactivity and attentional deficits may be sex-dependent. Therefore, a future direction would be to investigate males and females using the proposed measures.

A final behavioral point would be to investigate whether the potentially beneficial effects of chronic amphetamine treatment in hyperactivity and attention translate into the social realm in animal models. Deficits in social functioning are a core feature of FASD children and provide far-extending repercussions due to the social nature of educational development (Thomas et al., 1998). Non-FASD ADHD children that have been treated with stimulants show a variety of benefits, including improved social skills (Greene et al., 1999). Future studies could examine the impact of early chronic

exposure to amphetamine followed by observations of social play. Activity in both the striatum and medial prefrontal cortex are integral to the mediation of social play (van Kerkhof et al., 2013). Because chronic amphetamine increased noradrenergic innervation within the medial prefrontal cortex, these changes could also impact social play behavior.

In sum, more elaborate behavior studies on attentional measures will be useful future directions to rectify limitations in the current study. Future studies should include both males and females as the current study suggests supports previous literature suggesting that females exhibit different behaviors following developmental exposure to alcohol than males.

5.1 Limitations of Neurological Measures

Although DBH, DAT, and TH are integral to understanding the chronic effects of amphetamine treatment for models of fetal alcohol syndrome, they are only the preliminary steps in investigating the dopaminergic and noradrenergic systems with regards to influence their influence on attention and hyperactive behavior in FASD. This study was selective in examining the dopaminergic markers within the nucleus accumbens and noradrenergic markers within the medial prefrontal cortex. In the future, dopaminergic and noradrenergic markers should be examined within the same brain region to provide a more cohesive picture of network integration. Markers for acetylcholine would provide additional support on neural networks for attention as acetylcholine is also robustly associated with modulation of attention networks. Post-

synaptic receptors for dopamine, norepinephrine, and acetylcholine may also provide insight as to how enhanced dopaminergic release impacts post-synaptic structure. Examination of these postsynaptic markers within the prefrontal cortex is the next step in this project.

Future studies should also assess phosphorylated TH and DBH to examine activation of this enzyme rather than quantification of TH and DBH presence in general. This presents a general limitation of all of these immunohistological measures: they do not represent functional differences, only structural differences. An additional potential future step would be to examine DAT, TH and DBH activity using functional assays. A microdialysis study would also enable examination of real-time tonic and phasic dopaminergic release providing indications of total neuronal load using a high K+ flush and then recovery. This could provide more direct correlations between neurotransmitter content and synthesis enzymes.

In conjunction to examining different areas in attention circuits, tissue from this study should be examined in relation to glutamatergic morphology. Glutamatergic neurons project from the nucleus accumbens to the prefrontal cortex, and dopaminergic and noradrenergic modulation of these projections also may underlie differences in attention. By integrating immunohistochemistry between these measures as a multivariate analysis, neural networks of attention may be examined at a holistic level.

Also, although d-amphetamine is a classic stimulant drug used in ADHD treatment, novel pharmacotherapies for ADHD involve non-stimulant drugs. Atomoxetine holds particular promise as it is designed to target inattentive features of ADHD – a symptom particularly prevalent in FASD populations. Atomoxetine targets norepinephrine. Because noradrenergic networks demonstrated the greatest plasticity in the current study, drugs targeting this system may have the greatest impact on neurodevelopment within the prefrontal cortex during the periadolescent developmental window.

This study aimed to provide further evidence towards dopaminergic and noradrenergic impairments and the mechanisms behind common pharmacological treatments in FASD. Thus far, amphetamine appears to have a selective impact on DBH within the prefrontal cortex. However, examining interactions between different neurotransmitter systems could provide a better explanation of networks influencing attention and hyperactivity. Understanding the etiology behind FASD and its relationship with symptomology and treatment will help guide future therapeutic directions for this extremely prevalent and detrimental syndrome.

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