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EFFECTS OF NEONATAL ETHANOL EXPOSURE IN NORMAL AND DOPAMINE DEFICIENT RATS

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EFFECTS OF NEONATAL ETHANOL EXPOSURE IN NORMAL AND DOPAMINE DEFICIENT RATS

A Thesis

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ABSTRACT

Attention deficit hyperactivity disorder (ADHD) is a common developmental disorder characterized by impulsivity, inattention, and hyperactivity. In rodents, neonatal 6-OHDA lesions is an established model of ADHD because it produces symptoms of hyperactivity and cognitive deficits that improve following psychostimulant treatment. Recently, early alcohol exposure in rodents has also been suggested as a model of ADHD due to the high prevalence of ADHD in children exposed prenatally to alcohol. However, children exposed to prenatal alcohol differ in symptoms from children with idiopathic ADHD, suggesting that ADHD caused by prenatal alcohol exposure may be a special subtype of ADHD or a completely different disorder. The aim of the present study was to compare the 6-OHDA lesion model with the effects of early alcohol exposure on hyperactivity, psychostimulant response, and passive avoidance learning in preweanling rats. It was hypothesized that alcohol exposure and the combined effects of a 6-OHDA lesion and exposure to alcohol would result in increased locomotor hyperactivity and poorer performance on the passive avoidance task. A total of 1,053 male and female Sprague-Dawley rats were lesioned with 6-OHDA or placed in a lesion control group on postnatal day (PD 3). Beginning on PD 4, rats were exposed to alcohol (0, 0.3, or 3 g/kg) for six consecutive days or were unhandled. On PD 19, rats were habituated to a locomotor chamber for 60 min. On the following day (PD 20), the rats were injected with amphetamine (1 mg/kg), methylphenidate (2.5 mg/kg) or saline and

locomotor activity was measured for 60 min. On PD 23, a separate group of rats were trained on a passive avoidance task and retention was tested for three consecutive days. Our results demonstrated that exposure to a low dose of alcohol can cause an increase in the locomotor activity of female rats, while exposure to a high dose of alcohol can disrupt memory. In addition, our results indicated that 6-OHDA lesions and alcohol exposure do not have an additive effect. In summary, these data suggest neonatal alcohol can cause a dose dependent increase in ADHD-like symptoms.

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CHAPTER ONE:

ADHD

Attention Deficit Hyperactivity Disorder (ADHD) is a highly prevalent disorder affecting children regardless of socio-economic background or ethnicity. ADHD was initially known as “hyperkinetic disorder of childhood” and is found predominantly in males (Curatolo, D'Agati, & Moavero, 2010; Kiely, 2015). A recent systematic review and meta-analysis estimated that approximately 7% of children worldwide, under the age of 18, meet the diagnostic criteria for ADHD (Thomas, Sanders, Doust, Beller, & Glasziou, 2015).

The three core symptoms of ADHD, inattention, hyperactivity, and impulsivity, correspond with the clinical presentations of the disorder: Inattentive, hyperactive-impulsive, and combined type (Baumeister, Henderson, Pow, & Advokat, 2012). Symptomology of ADHD can vary across settings, such as in the home, school, and workplace (Kiely, 2015; Russell, 2011). In the inattentive presentation, the individual has difficulty staying focused, is disorganized, has difficulty following instructions or conversations and is forgetful. The symptoms of hyperactive-impulsive presentation include fidgeting, impulsivity, and disruption of others. In the combined presentation the reported symptoms are a blend of inattentive and hyperactive-impulsive (American Psychiatric Association, 2013).

ADHD is defined as a disruptive neurobehavioral disorder, because it is associated with academic struggles, socialization issues, and increased criminal

behavior (Punja et al., 2016; Watts, 2018). The symptoms of ADHD are excessive, pervasive, and persistent in that the behaviors are extreme, show up in multiple settings, and can be long-term. ADHD is often comorbid with other disorders, such as oppositional defiant disorder, major depressive disorder, and anxiety (Sharma & Couture, 2014; Watts, 2018).

Currently, there is no screening test available to detect ADHD nor is there a cure; thus, only treatment is available for individuals with ADHD. Treatments for ADHD include behavioral therapy, dietary restrictions, and pharmacological intervention (Shier, Reichenbacher, Ghuman, & Ghuman, 2013). Psychostimulant compounds, such as methylphenidate and amphetamine, are recommended as the first-line of treatment for ADHD (Chung, Tchaconas, Meryash, & Adesman, 2016; Shier et al., 2013). For the 30% of ADHD patients not suitable for psychostimulants there are alternative options, such as atomoxetine and α-2 agonists (Curatolo et al., 2010; Kiely, 2005). Stimulant and non-stimulant treatments are effective; however, a number of concerns involving long-term effects are still unanswered.

Neurobiology of ADHD

The etiology and pathophysiology of ADHD are not yet completely understood, but a number of structural, functional, neurochemical, and genetic abnormalities in the brains of individuals with ADHD have been identified. Magnetic resonance imaging (MRI) studies have reported smaller anatomical

areas and volumes in patients with ADHD (Castellanos et al., 2002). There is a reduction in total brain size in children with ADHD that persists into adolescence and their brain volumes are decreased by 3% when compared to controls (Castellanos et al., 2002; Tripp & Wickens, 2009). Cortical thinning is a robust neuroanatomical marker for ADHD in childhood and adolescence (Narr et al., 2009; Shaw et al., 2006; Tripp & Wickens, 2009) and the rate at which cortical thinning occurs is correlated with the severity of hyperactivity and impulsivity (Shaw et al., 2011).

A review of neuroimaging studies involving individuals with ADHD indicates that the prefrontal cortex (PFC), caudate, and cerebellum are the primary brain regions showing reduced volumes (Sharma & Couture, 2014). These reductions may be of importance, because these regions are involved in cognitive processing, attention, emotion, and behavior regulation (Sharma & Couture, 2014). In addition, neuroimaging studies on children with ADHD found reductions of the amygdala, thalamus, hippocampus, cerebellar vermis, splenium of the corpus callosum, as well as abnormal asymmetry of the caudate nucleus and the pallidum (Kasparek, Theiner, & Filova, 2015; Tripp & Wickens, 2009). Asymmetry of the caudate is related to severity of attention deficits (Schrimsher, Billingsley, Jackson, & Moore, 2002).

A recent meta-analysis of functional magnetic resonance imaging (fMRI) studies showed consistent differences between individuals with and without ADHD in two distinct domains: inhibition and attention. Abnormalities of inhibition

were related to the right hemispheric fronto-basal ganglia networks, including the inferior frontal cortex, supplementary motor area, and anterior cingulate cortex (Hart, Radua, Nakao, Mataix-Cols, & Rubia, 2013). In the attention domain, the areas exhibiting reduced activity were the dorsolateral prefrontal cortex (DLPFC) and cerebellar areas (Hart, et al., 2013; Kasparek et al., 2015). Furthermore, Hart et al. (2013) reported a different pattern of brain dysfunction during inhibition tasks in children and adults with ADHD. Specifically, hypoactivation of the supplementary motor area and basal ganglia was present in children, while hypoactivation of the inferior frontal cortex and thalamus was detected in adults.

Neurochemical theories of ADHD have been around since at least 1970 (Baumeister et al., 2012). Dysfunction of monoamine systems is suspected, because the fronto-subcortical circuits associated with ADHD are rich in catecholamines, and the most effective drugs used to treat ADHD are psychostimulants that block dopamine (DA) and norepinephrine (NE) transporters (Curatolo et al., 2010; Sagvolden, Russell, Aase, Johansen, & Farshbaf, 2005). Furthermore, functional activation studies on individuals with ADHD show that long-term treatment with stimulant medication is associated with normalization of caudate activity (Hart et al., 2013).

ADHD has a complex etiology, and multiple genetic factors are thought to play an important role. Numerous studies have found a strong familial genetic contribution, with the heritability rate estimated at 76% (Curatolo et al., 2010; Shier et al., 2013; Tripp & Wickens, 2009). Twin studies have also demonstrated

high heritability of approximately 0.80 (Kieling, Goncalves, Tannock, & Castellanos, 2008). No single gene yet discovered plays a major role, but gene associations have been found (Shier et al., 2013; Tripp & Wickens, 2009). The genes that may be involved are those coding for D4 (DRD4) and D5 receptors (DRD5), the DA transporter (DAT1), the norepinephrine transporter (NET1), synaptosomal-associated protein 25 (SNAP-25), the serotonin transporter (5HTT), and the serotonin 1B receptor (HTR1B) (Russell, 2011; Sharma & Couture, 2014; Tripp & Wickens, 2009). In rodent brains, the SRY gene is highly expressed in areas that contain dense populations of dopaminergic neurons, which have been implicated in the pathophysiology of ADHD (Kiely, 2015).

CHAPTER TWO:

ADHD PHARMACOTHERAPIES

ADHD can be treated effectively with pharmacological agents, behavioral therapies, and a combination of both. Psychostimulants are the most widely used treatment for ADHD and have been used for over 50 years. Psychostimulant treatment is associated with improved academic achievement in school and improved quality of life (Shier et al., 2013). Currently, an estimated 2.8 million children in the United States take psychostimulants for ADHD (Baumeister et al., 2012; Shier et al., 2013).

The two most commonly prescribed psychostimulants for the treatment of ADHD are methylphenidate (MPH) and amphetamine (AMP). These psychostimulants work by increasing synaptic levels of NE and DA, are available in immediate or extended release forms, and are efficacious for short- and long-term use (Punja et al., 2016; Shier et al., 2013). Common adverse effects of stimulant medications include loss of appetite, stomachache, insomnia, and headaches (Shier et al., 2013). Unusual side-effects include tics, irritability, and increased heart rate and blood pressure. A majority of long-term follow-up studies on stimulant medications did not find an increased risk for substance use, abuse, or dependence in adulthood (Shier et al., 2013).

Amphetamine increases the availability of NE and DA in the synaptic cleft by stimulating release and inhibiting re-uptake and metabolism (Shier et al.,

2013). A common formulation of AMP is Adderall (Sharma & Couture, 2014). In contrast, MPH works by blocking the DAT and NET transporters (Curatolo et al., 2010). MPH increases extracellular levels of DA in the prefrontal cortex, nucleus accumbens, and caudate nucleus (Kasparek et al., 2015). Some of the brand names for MPH include Ritalin, Concerta, and Methylin (Sharma & Couture, 2014). Although their clinical effects are qualitatively similar, MPH has milder side effects than AMP. MPH has a half-life of about 2 hours versus AMP's half-life of 7 to 30 hours (Baumeister et al., 2012). Furthermore, MPH is less likely to produce psychosis and other side effects (Baumeister et al., 2012).

Because of their efficacy, psychostimulants are considered first-line agents for the treatment of ADHD (Shier et al., 2013). However, non-stimulant medications are an important second option for treating ADHD. Typically, these compounds are only used after the first-line agents have exhibited a poor response or cannot be used (Sharma & Couture, 2014; Shier et al., 2013). Non-stimulants possess inferior efficacy compared to psychostimulants, but their side effects are generally milder (Shier et al., 2013). Importantly, non-stimulants do not have the same abuse potential as stimulants. The second-line medications for the treatment of ADHD include atomoxetine, bupropion, α-2 agonists, and tricyclic antidepressants (Sharma & Couture, 2014; Shier et al., 2013). Since most of these second-line medications are seldom prescribed there is inadequate empirical information regarding efficacy and safety of higher doses (Shier et al., 2013).

Atomoxetine (ATX) is a potent selective norepinephrine reuptake inhibitor that works by enhancing DA and NE transmission in cortical and subcortical areas, but has limited actions in the striatum (Curatolo et al., 2010; Sharma & Couture, 2014; Shier et al., 2013). ATX promotes attention and executive functioning in ADHD individuals (Curatolo et al., 2010). ATX is the most favorable of the non-stimulants because of its safety and efficacy (Himpel, Banaschewski, Heise, & Rothenberger, 2005). ATX reduces tics and anxiety, thus it can serve as a good alternative for ADHD individuals with these comorbidities (Sharma & Couture, 2014).

Bupropion has a similar mechanism of action as both psychostimulants and ATX, as bupropion inhibits the reuptake of DA and NE (Sharma & Couture, 2014). Bupropion is available in immediate and extended release form, but due to its short half-life a twice daily administration is recommended (Sharma & Couture, 2014). This pharmacological agent improves the ADHD symptom of hyperactivity (Shier et al., 2013). FDA warnings of increased risk of suicidal thoughts and behaviors are applicable to bupropion, since it is classified as an antidepressant (Waxmonsky, 2005).

The immediate release α-2 agonists, guanfacine and clonidine, were initially used off-label, until the FDA approved their use for the treatment of ADHD in an extended release form (Sharma & Couture, 2014). These α-2 agonists stimulate presynaptic and postsynaptic α_2 receptors resulting in improved cognitive functioning in individuals with ADHD (Sharma & Couture,

2014). Guanfacine, a selective α-2 agonist, strengthens the functional connectivity of prefrontal cortex networks, resulting in improved working memory (Curatolo et al., 2010). Clonidine, a nonspecific α-2 agonist, is more effective at reducing symptoms of hyperactivity and impulsivity than symptoms of inattentiveness (Waxmonsky, 2005). A common side-effect of clonidine is sedation, which limits its daytime use (Waxmonsky, 2005). Unlike the previously mentioned pharmacological agents, gradual withdrawal from α-2 agonists is suggested to prevent potential risks associated with their actions as antihypertensive agents (Shier et al., 2013).

The least preferred non-stimulant treatment for ADHD are the tricyclic antidepressants (TCAs), because they have a wide range of negative side-effects and drug interactions (Pliszka, 2003; Sharma & Couture, 2014). Until concerns surfaced involving sudden death, TCAs were the primary second-line treatments for ADHD (Pliszka, 2003). TCAs, like imipramine, are effective for treating hyperactivity, but are only used when stimulants or alternative agents prove ineffective (Himpel et al., 2005). TCAs may also be a reasonable alternative for ADHD individuals who experience tics (Pliszka, 2003). Gradual tapering off from these non-stimulants is recommended, due to the potential of side-effects (Waxmonsky, 2005).

A fairly new, but promising, stimulant is modafinil, which was initially approved for the treatment of narcolepsy (Shier et al., 2013; Waxmonsky, 2005). The mechanism of action of this drug is not yet fully understood, but it is known

to affect DA, NE and histamine (Shier et al., 2013). Modafinil has been used off-label to treat ADHD symptoms and has been found to be effective in a few studies (Biederman & Pliszka, 2008). The common side-effects of modafinil are mild, including insomnia, headaches, and decreased appetite (Shier et al., 2013; Waxmonsky, 2005).

CHAPTER THREE:

RODENT MODELS OF ADHD

Animal models have proven useful for understanding the cause of human disorders and in developing treatments. To be useful, these models must possess face validity, construct validity, or predictive validity (Russell, 2011). Face validity refers to the animal model being able to mimic the fundamental symptoms of the human disorder. Construct validity refers to the model having similar etiology and underlying pathophysiological mechanisms. Lastly, models that have predictive validity should display symptom to the same treatments given to the clinical population, provide insight into the underlying mechanisms of the disorder, as well as predict biological and behavioral aspects of the disorder that are yet to be observed in clinical evaluations (Russell, 2011). In short, animal models that are similar in terms of etiology, biochemistry, symptomatology, and treatment are the most useful (Sagvolden et al., 2005).

The most thoroughly studied animal model of ADHD is the spontaneously hypertensive rat (SHR) bred from the Wistar Kyoto control rat strain (WKY) (Russell, 2011; Sagvolden et al., 2005). SHRs exhibit poor performance in visual discrimination tasks that require sustained attention, they display impulsivity during the extinction phase of an operant task, and hyperactivity is evident by their increased response rates in free operant tasks and increased locomotor activity in an open field (Russell, 2011; Sagvolden et al., 2005; Stanford & Tannock, 2011). Similar to the neuropathology of children with ADHD, SHR rats

have reduced brain volumes in the prefrontal cortex, occipital cortex, and hippocampus. Moreover, there are fewer neurons in these brain areas when compared to controls rats (Russell, 2011; Sagvolden et al., 2005). Dysfunction of the DA and NE neurotransmitter systems are observed in this model (Sagvolden et al., 2005). The SHR model has predictive validity, because medications used to treat ADHD, such as MPH, AMP, and guanfacine improve behavioral deficits (Stanford & Tannock, 2011). Even though this model displays numerous behavioral characteristics similar to those observed in humans with ADHD, the usefulness of the model has been criticized by a confounding factor of hypertension, however hypertension does not develop until adulthood (Russell, 2011; Sagvolden et al., 2005).

A transgenic rodent model of ADHD, the DAT knock-out (DAT-KO) mouse model, is also extensively studied. While the genetic basis of ADHD is not fully understood, neurobiological evidence on the etiology of ADHD provides insight into plausible genes that are involved. This rodent model is used to study the absence of the gene that codes for DAT-1, which is responsible for synaptic DA uptake (Sagvolden et al., 2005). The DAT-KO model provides useful information on the neurobiological consequences of reduced midbrain DAT (Russell, 2011). DAT-KO mice exhibit impulsivity and impairments on learning and memory tasks (Stanford & Tannock, 2011; Sagvolden et al., 2005). DAT-KO mice are three to five times more hyperactive than controls, possibly due to elevated extracellular DA levels within the striatum (Sagvolden et al., 2005; Stanford & Tannock, 2011).

Psychostimulants (e.g., MPH and AMP) attenuate hyperactivity in DAT-KO mice, but extracellular DA concentrations are unchanged (Gainetdinov, Caron, & Lombroso, 2001). These results suggest that non-dopaminergic systems are involved in the modulation of locomotor activity in the DAT-KO mice model. While a selective NE transporter inhibitor, nisoxetine, has no effect on the hyperactivity of DAT-KO mice, the selective serotonin reuptake transporter (SERT) inhibitor, fluoxetine, does reduce hyperactivity in this ADHD model (Gainetdinov et al., 2001; Stanford & Tannock, 2011). DAT-KO mice provide convincing evidence that the hyperactivity induced by elevated DA levels can be reduced by drugs that activate the serotonergic system (Gainetdinov et al., 2001).

Environmentally-induced models of ADHD involve the application of an exogenous manipulation, such as a toxin or trauma, to induce ADHD-like phenotypes. In neonatal rats, anoxia causes permanent neurochemical abnormalities in monoamine systems, along with hyperactivity and spatial memory impairment (Puumala et al., 1996; Russell, 2011; Sagvolden et al., 2005). Furthermore, amphetamine attenuated hyperactivity in the anoxia model, thus supporting its use as an animal model of ADHD (Kostrzewa et al., 2008). ADHD resulting from anoxia lacks construct validity and, hence, has not gained popularity for the study of ADHD.

One of the most commonly-utilized ADHD models is the neonatal 6-hydroxydopamine (6-OHDA) rat model. Administering an intracranial injection of 6-OHDA in neonatal rats causes selective and permanent DA depletion (Stanford

& Tannock, 2011). This selective chemical lesion model has a phenotypic resemblance to ADHD, because rats express hyperactivity and inattention, but not impulsivity (Russell, 2011; Sagvolden et al., 2005; Stanford & Tannock, 2011). Neonatal 6-OHDA lesioned rats also display impaired learning in spatial discrimination tasks (Russell, 2011). The neonatal 6-OHDA-lesioned rat has face and predictive validity (Sagvolden et al., 2005; Stanford & Tannock, 2011). This model has permitted the evaluation and prediction of the efficacy of new ADHD therapies (Caballero et al., 2011). For example, both AMP and MPH reduce the hyperactivity of neonatal 6-OHDA lesioned rats. Findings from these studies suggest that the therapeutic effects of AMP and MPH may not be mediated through the DA system, but rather through NE and SE transmission (Stanford & Tannock, 2011; Russell, 2011).

A comparatively new pharmacological model of ADHD involves prenatal alcohol exposure (PAE). In humans, PAE has been linked to impairments in learning and memory, with ADHD being a common diagnosis (Marquardt & Brigman, 2016; Patten, Fontaine & Christie, 2014; Rojas-Mayorquin et al., 2016). Although prenatal alcohol treatment is not the typical method for modeling ADHD, it is still representative because it has face and predictive validity (Kostrzewa et al., 2008). The PAE model has face validity because rats exhibit impulsivity, hyperactivity, and cognitive deficits, such as delays in learning and memory, that are comparable to humans with ADHD (Atalar, Uzbay & Karakaş, 2016; Kostrzewa et al., 2008; Russell, 2011). PAE rats demonstrate reduced cell

numbers in layers II and V of the medial prefrontal cortex and cerebellar cortex, which likely results in the learning deficits. PAE rats also have neurochemical deficits involving catecholamine, indolamine, and amino acid neurotransmitters, similar to those observed in ADHD (Atalar et al., 2016; Rojas-Mayorquin et al., 2016). The PAE model has predictive validity because the psychostimulant MPH normalizes VTA DA neuron activity (Choong & Shen, 2004). In all, this animal model mimics the behavioral, neuroanatomical, and neuropsychopharmacological aspects of ADHD, thus making it a promising model.

CHAPTER FOUR:

ALCOHOL

Ethanol is the alcohol found in commonly consumed alcoholic beverages. When ingested, alcohol acts as a central nervous system (CNS) depressant (Roberto & Varodayan, 2017). Excessive alcohol use can result in a wide range of behavioral and health problems, such as cardiovascular diseases, liver cirrhosis, cancer, depression, and motor vehicle accidents (Liang & Olsen, 2014; Tan et al., 2015). The consumption of alcohol in excess is the third leading cause of preventable death in the United States, as reported by the Centers for Disease Control and Prevention (CDC) (Liang & Olsen, 2014). An estimated 18 million Americans over the age of 18 suffer from alcohol use disorders (AUD), creating a substantial public health problem (Liang & Olsen, 2014; Roberto & Varodayan, 2017).

Alcohol Pharmacology

The pharmacodynamics of alcohol are complex, due to the various neurotransmitter systems involved (Koob, 2004). Some of the systems known to be affected by alcohol consumption include serotonin, GABA, and glutamate. There is well-established evidence that alcohol alters 5-HT function with the most consistent finding being potentiation of 5-HT_3 receptor function (Banerjee, 2014; Lovinger, 1999). Alcohol acts pre-synaptically at the GABA neuron to increase GABA release and acts post-synaptically to enhance GABA receptor action

(Banerjee, 2014; Koob, 2004). Alcohol increases GABA receptor action by allowing more CI- to enter at the $GABA_A$ receptor, this results in inhibitory postsynaptic potential (Davies, 2003; Koob, 2004). Alcohol also inhibits glutamate activity in the brain. This decrease in glutamate transmission is thought to be mediated via NMDA receptors (Banerjee, 2014; Koob, 2004).

Most of the alcohol consumed is metabolized in the liver (Zakhari, 2006). The most common pathway involves alcohol dehydrogenase (ADH). ADH metabolizes alcohol to acetaldehyde, which in turn, is converted to acetic acid in the presence of aldehyde dehydrogenase (ALDH). Acetic acid is then oxidated to carbon dioxide and water (Eberhart & Parnell, 2016; Zakhari, 2006). Although ADH mediates the majority of the biotransformation of alcohol to acetaldehyde, cytochrome P450 2E1 (CYP2E1) and catalase can also metabolize alcohol (Eberhart & Parnell, 2016; Zakhari, 2006).

Prenatal Alcohol Exposure

The consumption of alcohol by pregnant women is particularly problematic. A nationwide telephone survey by the Behavioral Risk Factor Surveillance System (BRFSS) revealed that among women alcohol use is at 53.6% (Tan et al., 2015). The same poll indicated that the prevalence of alcohol use by pregnant women was 10.2% (Tan et al., 2015). Interestingly, pregnant women aged 35-44 and those with college degrees reported a higher rate of alcohol use than other age groups and less educated women (Tan et al., 2015).

Prenatal alcohol exposure (PAE) occurs when a woman drinks alcohol while pregnant. The teratogenic effects of maternal ingestion of alcohol during pregnancy has been observed in both human and animal populations (Cronise et al., 2001). Numerous developmental, cognitive, and behavioral problems, ranging from mild to severe, can occur from PAE (Gupta et al., 2016; Hausknecht et al., 2005). The first disorder recognized to result from prenatal alcohol use was fetal alcohol syndrome (FAS). This syndrome is characterized by facial abnormalities, growth deficits, both prenatally and postnatally, as well as CNS dysfunction (Thomas, Warren, & Hewitt, 2010). Because PAE produces such a wide range of effects, and not all cases meet the diagnostic criteria for FAS, there is now the umbrella term of fetal alcohol spectrum disorder (FASD) in addition to FAS (Thomas et al., 2010).

FASD encompasses the broad range of impairments that can occur from in utero alcohol exposure, such as deficits in intellectual performance, executive function, learning and memory, language, sensory function, motor function, behavior, and secondary disabilities including depression and anxiety (Hellemans et al., 2010; Mattson et al., 2011; Schneider, Moore, & Adkins, 2011; Wetherill et al., 2018). FASD includes partial FAS, which describes some but not all signs and symptoms of FAS. FASD also includes alcohol-related birth defects (ARBD), this being PAE-induced physical abnormalities. Lastly, FASD also includes alcohol-related neurodevelopmental disorder (ARND), which incorporates alcohol-induced impairments of growth and development of the CNS as well as

cognitive and behavioral problems without facial or growth deformities (Thomas et al., 2010). A recent meta-analysis of FASD reported a prevalence rate of 33.5 per 1,000 births in the United States (Wetherill et al., 2018). Because of the lifelong consequences of PAE, it is considered to be a major social and economic burden (Wetherill et al., 2018).

Rodent models are ideal for studying the effects of alcohol on development, because rodent studies examining PAE show similar effects to those observed in humans. In an MRI study, the brain images of mice and humans exposed to comparable amounts of prenatal correspond well with each other. For example, mice given a moderate dose of alcohol showed thinning of the corpus collosum, similar to a child with partial FAS, while mice exposed to a large dose of alcohol exhibited a severe reduction of the corpus collosum and damage to the hippocampal commissure (O'Leary-Moore et al., 2011). Rodent studies assessing the effects of PAE have also demonstrated impairments in behavioral and cognitive function, including learning and memory deficits, hyperactivity, hyper-responsivity to stressors, as well as deficits in both response inhibition and the appropriate use of environmental cues, which are similar to those observed in children with FASD (Cronise et al., 2001; Hellemans et al., 2010; Schneider et al., 2011).

In humans and animals, a number of risk factors influence the teratogenicity of alcohol and the probability of fetal alcohol-related effects. These

risk factors are exposure pattern, dose, stress, environmental influences and certain genetic variants (Schneider et al., 2011; Sulik, 2014). Due to the delicate process of neurogenesis, the timing of alcohol exposure in relation to the developing neural system can profoundly affect neuronal outcomes (Schneider et al., 2011). FASD research conducted in rodents has determined that the first and third trimesters are the most vulnerable periods for alcohol-induced neuroteratogenesis (Dursun et al., 2006; Schneider et al. 2011; Sulik, 2014). Environmental factors that may interact with and exacerbate the effects of alcohol exposure include exposure to other drugs, maternal nutrition, and obstetric complications (Russell, 2011).

The gene-alcohol interactions underlying FASD are not yet well understood. Genes from the alcohol dehydrogenase (ADH) family have been investigated for a potential genetic link with FASD. Investigations of human gene alcohol interactions have focused on the major alcohol metabolizing enzyme, ADH1 (Eberhart & Parnell, 2016; Gupta et al., 2016). This family has 3 loci: ADH1A, ADH1B, and ADH1C. Data from these investigations have produced contradictory results as to whether the ADH1B*3 allele works as a protective mechanism or induces susceptibility to FASD. The ADH1B*3 allele clears alcohol rapidly suggesting a protective mechanism (Neumark et al., 2004). Additionally, maternal genotypes with at least one ADH1B*3 allele correlated with a lower rate of FASD (Gupta et al., 2016). In contrast, mothers with an ADH1B*1/ADH1B*3

genotype have a greater chance of bearing children with FASD (Eberhart & Parnell, 2016).

CHAPTER FIVE:

GABA & GLUTAMATE

The amino acid γ-aminobutyric acid (GABA) is an important neurotransmitter in the regulation of brain neuronal activity. GABA is one of the earliest expressed neurotransmitters during ontogeny. It is detectable during the embryonic stage and is present throughout the lifespan (Wang & Kriegstein, 2009). During embryonic development, GABA acts in an excitatory manner and is implicated in neurogenesis (Allen et al., 2015; Wang & Kriegstein, 2009; Wu & Sun, 2015). As the brain matures, GABA's function switches from excitatory to inhibitory (Allen et al., 2015; Wang & Kriegstein, 2009; Wu & Sun, 2015). GABA and the excitatory neurotransmitter glutamate modulate the inhibitory-excitatory balance necessary for proper brain function (Allen et al., 2015; Wu & Sun, 2015). The imbalance of either GABA or glutamate can result in several pathologies, including anxiety, depression, and schizophrenia (Allen et al., 2015; Wu & Sun, 2015).

GABA Synthesis, Release, and Catabolism

GABA is synthesized from glutamate via the enzyme glutamate decarboxylase (GAD) (Wong, Bottiglieri & Snead, 2003). Once synthesized, GABA is packaged into vesicles by vesicular GABA transporters (VGAT). When the presynaptic neuron is depolarized it releases GABA into the synaptic cleft via calcium dependent exocytosis (Wong et al., 2003; Wu & Sun, 2015). After

release, GABA is removed from the cleft by GABA transporter proteins (GATs) (Wu & Sun, 2015). GABA is metabolized by GABA transaminase to form succinic semialdehyde (Wong et al., 2003). Succinic semialdehyde is then oxidized by succinic semialdehyde dehydrogenase to succinate (Ravasz et al., 2017).

Glutamate Synthesis, Release, and Catabolism

Glutamate is a non-essential amino acid that serves as the major excitatory neurotransmitter of the nervous system (Yelamanchi et al., 2016). Within the presynaptic terminal, glutamate is synthesized from glutamine by glutaminase (Hertz, 2011; Meyer & Quenzer, 2005; Yelamanchi et al., 2016). Three different vesicular glutamate transporters move glutamate into synaptic vesicles: VGLUT1, VGLUT2, and VGLUT3 (Zhou & Danbolt, 2014). Synaptic release of glutamate occurs from nerve terminals by exocytosis in synaptic vesicles (Zhou & Danbolt, 2014). Once released into the synaptic cleft, glutamate activity can be terminated by uptake via high affinity transporters located in glial cells and presynaptic terminals (Meyer & Quenzer, 2005). The glutamate taken back into glial cells is converted into glutamine via glutamine synthetase and reintroduced in the glutamine–glutamate cycle (Hertz, 2013; Meyer & Quenzer, 2005).

GABA Receptors

GABA stimulates two receptor subtypes $GABA_A$ and $GABA_B$, both of which contribute to the long-term inhibition of synaptic transmission (Banerjee, 2014).

These receptors differ from one another in structure, function, and sequence. $GABA_A$ has 13 ionotropic receptor subunits (α1-6, β1-3, γ1-3 and δ) and is considered a fast-synaptic inhibitory receptor (Allen et al., 2015; Sigel & Steinmann, 2012; Wong et al., 2003; Wu & Sun, 2015). When activated, $GABA_A$ receptors allow CI- into the cell membrane (Allen et al., 2015). High densities of $GABA_A$ receptors are found in the limbic system, retina, and spinal cord (Allen et al., 2015). In contrast, $GABA_B$ receptors are metabotropic (Sigel & Steinmann, 2012; Wong et al., 2003; Wu & Sun, 2015). $GABA_B$ receptors have two subtypes: $GABA_{B1}$ and $GABA_{B2}$ (Wu & Sun, 2015). $GABA_B$ receptors are slow synaptic inhibitors that regulate K^+ and Ca^{2+} channels via a G-protein mediated mechanism (Allen et al., 2015; Koob, 2004; Wong et al., 2003; Wu & Sun, 2015). $GABA_B$ receptors are primarily found in the thalamus, hippocampus, and cerebellum (Allen et al., 2015; Padgett & Slesinger, 2010; Wu & Sun, 2015).

Ontogeny of GABA Receptors

$GABA_A$ receptors have a complex pattern of development, in situ hybridization of tissue sections in rodents reveals differences in the pattern of expression across ontogeny. In Purkinje cells, α1, β2, β3 and γ2 are persistently expressed from birth to adulthood (Laurie, Wisden, & Seeburg, 1992; Ma et al., 1993; Zdilar et al., 1992). In the cortex and thalamus, expression of subunits α2, α3, α5 and β3 is noted in both embryonic and early postnatal period (Laurie et al., 1992; Simeone, Donevan, & Rho, 2003). In the spinal cord, subunits α4 and

γ1 are expressed during the embryonic period, weakly detected after birth, and are almost absent in adulthood (Ma et al., 1993). In the mantle zone of the spinal cord, subunit γ3 is expressed transiently during the embryonic period and postnatally (Ma et al., 1993). In the cerebellar cortex, subunit β1 demonstrates low expression in the first postnatal week, decreased expression in the second postnatal week and is minimally expressed in the third week (Zdilar et al., 1992). Peak expression of subunits α2, α3, β3, and γ2 occurs between the late embryonic and early postnatal period (Ma et al., 1993; Zdilar et al., 1992).

In situ hybridization revealed a differential expression of $GABA_B$ receptor subtypes in the developing rat spinal cord. Both GABA subtypes were present at birth, but $GABA_{B1}$ receptors exhibited increased expression across postnatal development, while $GABA_{B2}$ receptors decreased in number (Sands et al., 2003). Expression of $GABA_{B2}$ receptors was less robust than $GABA_{B1}$ across all ages (Sands et al., 2003).

Glutamate Receptors

Glutamate exerts its effects through two classes of receptors: ionotropic and metabotropic receptors. The three glutamate ionotropic receptors are α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), N-methyl-D-aspartate (NMDA), and kainate (Roberto & Varodayan, 2017). The ionotropic receptors are nonselective cation channels allowing the passage of Na^+ and K^+ and, in some instances, Ca^{2+} (Meyer & Quenzer, 2005). AMPA receptors can be

found in the hippocampus, cerebral neocortex, cerebellum, retina and thalamic reticular nucleus, while NMDA receptors are detected in the substantia nigra, brain stem, and spinal cord (Gereau & Swanson, 2008). Kainate receptors are predominantly found in the spinal cord, but also mediate responses in the amygdala, thalamus, and the mossy fibers of the hippocampus (Gereau & Swanson, 2008)

Glutamate has eight metabotropic receptors (mGluR 1-8). These receptors are G-protein coupled and are classified into three groups: group one includes mGluR 1 and 5, group two consists of mGluR 2 and 3, and group three is made up of mGluR 4, 6, 7, and 8 (Gereau & Swanson, 2008). MGluR 1 and 5 are coupled to G_q, and are primarily postsynaptic; whereas, mGluR 2-4, 6, and 7 are coupled to G_i/G_o and are generally presynaptic (Gereau & Swanson, 2008). Through their coupling to G proteins MGluR 1 and 5 increase the excitability of postsynaptic cells, whereas mGluR 2-4, 6, and 7, are inhibitory (Conn & Pin, 1997).

Similar to the glutamate ionotropic receptors, mGluRs have a heterogenous distribution throughout the CNS. MGluR 1 is expressed in the globus pallidus, olfactory bulb, thalamus, basal ganglia, substantia nigra, amygdala, hypothalamus, medulla, cerebellum, and the CA1 and CA3 regions of the hippocampus (Catania et al., 1994; Gereau & Swanson, 2008). MGluR 2 is expressed in the hippocampus and thalamus. MGluR 3 is distributed in the retina, thalamus, basal ganglia, corpus collosum, trigeminal nerve, and spinal

cord (Catania et al., 1994; Gereau & Swanson, 2008). MGluR 4 has a patchy distribution, with receptors in the thalamus, striatum, and cortex (Catania et al., 1994). MGluR 5 can be found in the olfactory bulb, caudate, cortex, hippocampus, globus pallidus, and nucleus accumbens (Catania et al., 1994; Gereau & Swanson, 2008). MGluR 6 has the most restrictive expression, being found in only the retina (Gereau & Swanson, 2008). MGluR 7 is expressed in the hippocampus, ventral pallidum, thalamus, hypothalamus, basal ganglia, brain stem, and spinal cord (Gereau & Swanson, 2008). Lastly, mGluR 8 can be found in the olfactory bulb, neocortex, hippocampus, amygdala and cerebellum (Gereau & Swanson, 2008).

Ontogeny of Glutamate Receptors

AMPA receptors are not common in early embryonic days, but increase in numbers postnatally (Brennan, et al., 1997; Cristóvão, Oliveira, & Carvalho, 2002), while NMDA receptors are abundant in early postnatal development and decrease in adulthood (Gereau & Swanson, 2008). There is a notable developmental shift in the expression of the glutamate metabotropic receptors. Catania et al. (1994) reported that mGluR1, mGluR2 and mGluR4 receptors are low in expression at birth, but increase gradually with maturation, while mGluR3 and mGluR5 receptors are expressed at birth in high levels but decline with increasing age (Catania et al., 1994; Gereau & Swanson, 2008).

CHAPTER SIX:

MONOAMINE NEUROTRANSMITTERS

Monoamines are small molecular weight neurotransmitters that mediate a variety of physiological and homeostatic functions (Jaber et al.,1996; Kopin, 1968). Monoamines contain an amino group that is connected to an aromatic ring by a two-carbon chain (Kopin, 1968). There are different types of monoamine neurotransmitters: catecholamines and indolamines. Catecholamines contain a catechol group and a side chain amine and are derived from the amino acid tyrosine. This group of neurotransmitters includes DA, NE and epinephrine (Kopin, 1968). The indoleamine, serotonin, derives from the amino acid L-tryptophan (Fidalgo, Ivanov, & Wood, 2013; Coulombe & Sharma, 1986). These monoamine neurotransmitter systems are relevant to the present study; thus, they will be further discussed in the sections to follow.

The Dopamine System

DA was once thought of as only a precursor for epinephrine and norepinephrine (Goldstein, 2010; Kopin, 1968), but DA is now recognized as an important catecholamine neurotransmitter (Jaber et al.,1996). DA is involved in a variety of functions, including locomotion, emotions, memory, and neuroendocrine secretion (Jaber et al.,1996). DA imbalance and dysfunction are associated with neurological and psychiatric disorders, such as attention-deficit hyperactivity disorder, schizophrenia, and drug addiction (Jaber et al.,1996;

Kobayashi, 2001), while degeneration of DA neurons can lead to the neurodegenerative disorder Parkinson's disease (Jaber et al.,1996). DA was first synthesized in 1910 by George Barger and James Ewen at Wellcome Laboratories (Goldstein, 2010). DA was identified as a neurotransmitter by Arvid Carlsson and Nils-Ake Hillarp in 1958 (Goldstein, 2010).

Dopamine Synthesis, Release, and Catabolism

DA is synthesized in the terminal of the presynaptic neuron, in a two-step process. The biosynthesis of DA begins with the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the cytosolic enzyme tyrosine hydroxylase. In turn, L-DOPA is converted into DA by aromatic amino acid decarboxylase (AADC) (Fernstrom & Fernstrom, 2007; Nagatsu, Levitt, & Udenfriend, 1964). Tyrosine hydroxylase is the rate limiting step in the synthesis of DA and controls the rate of synthesis due to its availability (Fernstrom & Fernstrom, 2007). DA signaling and distribution are dynamically regulated by several factors. Following synthesis, VMAT2 transports DA from the cytoplasmic space into synaptic vesicles (German et al., 2015). DA is released into the synaptic cleft via calcium-dependent exocytosis (Tritsch & Sabatini, 2012). Once released, DA can bind to and activate both pre- and postsynaptic DA receptors (German et al., 2015). The termination of DA neurotransmission is initiated by the reuptake of DA back into the terminal via DAT (German et al., 2015; Meiser, Weindl, & Hiller, 2013). DA can then be metabolized by monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Meiser et al., 2013). DOPAL can be

further oxidized into carboxylic acid 3,4-dihydroxyphenylacetic acid (DOPAC) via ALDH (Meiser et al., 2013). DOPAC is converted to homovanilic acid (HVA) via catechol-*O*-methyl transferase (COMT) (Meiser et al., 2013).

Dopamine Innervation

In the CNS, cell bodies of DA neurons are found in the mesencephalon, diencephalon, olfactory bulb, and retina (Binder et al., 2001). The neuronal projections from these brain areas give rise to four major dopaminergic pathways: the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular pathways. The nigrostriatal pathway originates in the substantia nigra pars compacta and ascends to the striatum where it terminates. The nigrostriatal pathway plays a crucial role in motor control, and damage to this pathway results in Parkinson's disease (Binder et al., 2001). The pathway that mediates reward and motivation is referred to as the mesolimbic pathway, which encompasses neurons projecting from the ventral tegmental area to the nucleus accumbens, amygdala, and olfactory tubercule (Binder et al., 2001; Opland, Leinninger, & Myers, 2010). The mesocortical DA pathway projects from the ventral tegmental area to the cerebral cortex, particularly the prefrontal cortex (Compton & Hudzik, 2015; Kobayashi, 2001). The mesocortical pathway is important for motivation, emotion, and memory formation (Kobayashi, 2001). The last of the major pathways is the tuberoinfundibular pathway. This pathway projects from the hypothalamus to the posterior pituitary (Kobayashi, 2001). DA neurons in the tuberoinfundibular pathway are involved in the development and maintenance of

the pituitary gland, as well as in gene expression and metabolism of pituitary peptide hormones (Kobayashi, 2001).

Dopamine Receptors

DA receptors are members of a large G-protein coupled receptor family (Jaber et al.,1996; Niznik & Van Tol, 1992; Tritsch & Sabatini, 2012). The various actions of DA are mediated by five receptor subtypes, which are divided into two major subclasses: D1-like and D2-like receptors. Dopaminergic receptors are classified on the basis of physiological, pharmacological and biochemical criteria (Jaber et al., 1996; Kebabian & Calne, 1979). D_1 and D_5 receptors are part of the D1-like receptor family due to their high sequence homology (Jaber et al., 1996). These receptors are excitatory, because receptor activation stimulate G_s, thereby increasing adenylyl cyclase activity and subsequently activating cyclic adenosine monophosphate (cAMP) dependent protein kinases (Niznik & Van Tol, 1992). D1-like receptors are mostly found post-synaptically and are more abundant than D2-like receptors (Jaber et al., 1996). The D2-like family is made up of the D_2, D_3 and D_4 receptor subtypes (Gerfen et al., 1990). In contrast to D1-like receptors, D2-like receptors are inhibitory. D2-like receptors were first discovered in the pituitary gland and they inhibit adenylyl cyclase (Niznik & Van Tol, 1992; Jaber et al., 1996). D2-like receptors bind to the inhibitory G-proteins, G_i and G_o, and decrease cAMP formation. While D1-like receptors are only found on the post-synaptic side, D2-like receptors are localized both pre- and post synaptically (Niznik & Van Tol, 1992).

Ontogeny of Dopamine Receptors

Research on D1-like and D2-like receptors in the mammalian rat brain has revealed that both of these receptor subtypes are present at birth, but their pattern of development differs (McDougall et al., 2014; Moody & Spear, 1992; Rao, Molinoff, & Joyce, 1991). Throughout postnatal development the density of D1-like receptors is consistently higher than D2-like receptors (Gelbard et al.,1989; Rao et al., 1991). The density of D1-like receptors increases linearly from PD 1 to 10 followed by a dramatic increase in receptor density from PD 10 to PD 16 (Rao et al.,1991). After this increase, comes a gradual decline until adult-like levels are reached between PD 28 and PD 40 (Andersen, 2003; Andersen et al., 2000). On the other hand, D2-like receptor expression increases linearly between the first and fourth postnatal weeks, they then peak around PD 30 (Rao et al., 1991). Both D1 and D2-like receptors demonstrate peak expression at PD 40, this being the onset of puberty (Andersen et al., 2000). Additionally, both subtypes of DA receptors prune by PD 120 (Andersen et al., 2000).

Norepinephrine

The catecholamine NE is involved in a broad range of functions, such as attention, memory, mood, endocrine function, and response to stressors (Goldstein, 2010; Maletic et al., 2017). In 1946, Ulf von Euler was the first to identify NE as a neurotransmitter (Yamamoto et al., 2014). Dysfunction of NE systems has been linked to various psychiatric disorders, such as ADHD,

depression, anxiety, schizophrenia, and post-traumatic stress disorder (Biederman & Spencer, 1999; Koob, 1999; Yamamoto et al., 2014).

Norepinephrine Synthesis and Inactivation

NE is synthesized from DA in the presence of the enzyme DA β-hydroxylase, with this conversion occuring within vesicles (Fernstrom & Fernstrom, 2007; Glowinski & Baldessarini, 1966; Goldstein, 2010; Kopin, 1968; Ressler & Nemeroff, 1999). Once released, NE can bind to pre- or postsynaptic receptors, or it can be removed via reuptake by its transporter protein NET (Goldstein, 2010; Ressler & Nemeroff, 1999; Wassall, Teramoto, & Cunnane, 2009). NE can be metabolized in two ways, first, NE that has been transported back into the axon terminal is catabolized via MAO, which converts NE into either 3,4-dihydroxyphenylglycol (DHPG) or 3,4-dihydroxymandelic acid (DHMA). Second, NE can go through enzymatic degradation by COMT, which catabolizes NE into its metabolite normetanephrine (Ressler & Nemeroff, 1999; Wassall et al., 2009).

Norepinephrine Innervation

Noradrenergic pathways originate in the locus coeruleus (LC) and project to many parts of the brain, including the frontal cortex, cerebellum, amygdala, hippocampus, basal ganglia, thalamus, and hypothalamus (Maletic et al., 2017; Ressler & Nemeroff, 1999; Sara, 2009).

Classification of Norepinephrine Receptors

Adrenergic receptors are found in both the central and peripheral nervous system (Bylund et al., 1994). There are two types of adrenergic receptors, α and β, both of which are G-protein coupled receptors (Bylund et al., 1994; Cotecchia et al.,1990; Maletic et. al., 2017). Each receptor type has its own family. The α receptor family includes the α1 and α2 subtypes (Bylund et al., 1994), while the α1 and α2 receptors each have three subtypes: α1A, α1B, and α1D; α2A, α2B, and α2C (Bylund et al., 1994; Kobilka, 2011; Maletic et al., 2017). The second major class of adrenergic receptors, β, has three subtypes: β1, β2, and β3 (Bylund et al., 1994; Maletic et. al., 2017). Adrenergic α1 and β receptors have a stimulatory effect on cAMP, while adrenergic α2 receptors have an inhibitory effect on cAMP signaling by interacting with the G_i/G_o proteins (Happe et al., 1999; Maletic et al., 2017; Sara, 2009).

Ontogeny of Norepinephrine Receptors

Research has revealed differences in the developmental pattern of adrenergic receptors. α2 Adrenergic receptors in rat brain are widely expressed at birth, even in brain regions that have low expression in adulthood (e.g., white matter, cerebellum and the brainstem) (Happe et al., 2004). α2 Adrenergic receptors reach their peak expression in many brain regions around PD 15, while in other regions they mature later at PD 28 (basomedial amygdala, lateral

septum and hippocampal formation) (Happe et al., 1999, 2004). Unlike $\alpha 2$ adrenergic receptor development, levels of β_1 and β_2 adrenergic receptors are low at PD 1 and increase over time (Pittman, Minneman, & Molinoff, 1980). In the cerebral cortex of the rat brain, β adrenergic receptor density increases rapidly between PD 7-21, after which receptor density remains constant and then begins to decline at PD 42 (Harden et al., 1977; Pittman et al., 1980). In the cerebellum, β adrenergic receptor density increases slowly and steadily from PD 5-42, and the density of receptors remains constant until approximately PD 180 (Pittman et al., 1980).

Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT), is found in both the CNS and peripheral nervous system. This monoamine plays a key role in sleep, sexual behavior, mood, and cognition (Fidalgo et al., 2013; Żmudzka et al., 2018). The majority of 5-HT, an estimated 95%, is produced in the digestive tract (Fidalgo et. al., 2013; McCorvy & Roth, 2015), while the 5-HT found in the brain is produced by tryptophan hydroxylase 2 (TPH2) in the raphe nucleus (Muller, Anacker, & Veenstra-VanderWeele, 2016). The 5-HT system is linked to a variety of disorders, including Parkinson's disease, Alzheimer's disease, depression, anxiety, schizophrenia, obsessive-compulsive disorder, bipolar disorder, and, more recently, autism spectrum disorder (López-Figueroa et al., 2004; Muller et al., 2016; Żmudzka et al., 2018).

Serotonin Synthesis and Reuptake

Serotonin is synthesized in two steps. The initial step is the conversion of L-tryptophan to 5-hydroxy-L-tryptophan (5-HTP) by the rate-limiting enzyme tryptophan 5-hydroxylase (Fidalgo et al., 2013). Tryptophan 5-hydroxylase (Tph) has two isoforms: Tph1 and Tph2 (Fidalgo et al., 2013; Muller et al., 2016). The second and final step is the conversion of 5-HTP to 5-HT via AADC. AADC is also involved in DA synthesis (Fidalgo et al., 2013). 5-HT is released from its vesicles through Ca^{2+}-dependent exocytosis; once released, it can bind to pre- or postsynaptic 5-HT receptors (Fidalgo et al., 2013; Filip & Bader, 2009). 5-HT is rapidly removed from the synaptic cleft by a reuptake process that involves the 5-HT transporter (SERT) (Fidalgo et al., 2013). 5-HT is catalyzed by monoamine oxidase A (MAOA) to yield 5-hydroxyindoleacetic acid (5-HIAA) (Fidalgo et al., 2013; Muller et al., 2016; Squires et al., 2007).

Serotonin Innervation

The 5-HT system is widespread, with the majority of cell bodies located in the dorsal and median raphe nuclei of the caudal midbrain (Carolyn et al., 1998; Fidalgo et al., 2013). 5-HT axons project to the thalamus, hypothalamus, striatal regions, cortical regions, medulla, pons, midbrain, cerebellum, and spinal cord (Carolyn et al., 1998; Fidalgo et al., 2013).

Classification of Serotonin Receptors

5-HT has a large family of receptors, with a total of seven types (5-HT_{1-7}) (Żmudzka et al., 2018). The majority of these receptors are G-protein coupled receptors, with the exception of 5-HT_3, which is a ligand-gated ion channel (Filip & Bader, 2009; McCorvy & Roth, 2015). 5-HT_1 and 5-HT_5 receptors are couple to the G_i protein. The 5-HT_1 receptor type has five subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}), while the 5-HT_5 receptor has two subtypes (5-HT_{5A} and 5-HT_{5B}) (McCorvy & Roth, 2015). The protein $G_{q/11}$ is coupled to 5-HT_2 receptors, which includes three subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. These 5-HT_2 receptors activate phospholipase C, thereby producing inositol triphosphate (IP3) and diacylglycerol (DAG), which increases intracellular calcium (McCorvy & Roth, 2015). The G_s coupled receptors include 5-HT_4, 5-HT_6, and 5-HT_7. Stimulation of the G_s coupled receptors increases cAMP levels (McCorvy & Roth, 2015). The 5-HT_3 receptor family includes the isoforms 5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} (Filip & Bader, 2009).

Ontogeny of the Serotonin System

In rats, 5-HT levels are low at birth, peak around PD 21–30 and then decline to adult levels (Murrin, Sanders, & Bylund, 2007). Similar to age-dependent decreases in 5-HT levels (Fidalgo et al., 2013), there is evidence of altered levels of 5-HT receptors across ontogeny. In the cerebellum, 5-HT_1 and 5-HT_3, receptors reach peak expression levels between PD 7 and PD 12, while 5-HT_2 receptors peak two weeks after birth and sustain maximal expression until ten weeks postnatally (Oostland & van Hooft, 2013). In the brain stem, 5-HT_1

receptors are expressed at higher than adult levels at birth, but decrease to adult levels by PD 15 (Murrin et al., 2007). As a whole, 5-HT_2 receptors exhibit peak expression around PD 13 and then decline to adult levels (Murrin et al., 2007).

CHAPTER SEVEN:

SUMMARY AND HYPOTHESIS

ADHD is a highly prevalent developmental disorder characterized by impulsiveness, inattention, and hyperactivity across multiple settings (Baumeister et al., 2012; Kiely, 2015; Russell, 2011). The etiology of this neurodevelopmental disorder is unclear, but is thought to have genetic, neurochemical, and environmental origins. Dysregulation of the central monoamine neurotransmitters has long been suspected to underlie the pathophysiology of ADHD, primarily because of the efficacy of AMP and MPH (Curatolo et al., 2010; Oades, 1987). In addition, a number of allelic variations involving the dopaminergic system, such as DAT, DRD4, and DRD5 are associated with a diagnosis of ADHD (Curatolo et al., 2010; Peadon & Elliott, 2010). More recently, prenatal exposure to alcohol has been strongly associated with a later diagnosis of ADHD (O'Malley & Nanson, 2002).

Interestingly, while there is a strong link between prenatal alchohol and ADHD, clinical observations of children with ADHD suggest that the neurobehavioral disorder caused by prenatal alcohol differs from idiopathic ADHD (Peadon & Elliot, 2010). Specifically, children exposed to prenatal alcohol have an earlier onset of ADHD symptoms, higher rates of inattention symptoms, lower rates of hyperactivity, different neuroanatomical changes, and less of a clinical response to MPH than AMP (Doig et al., 2008; Glass et al., 2014; O'Neill et al., 2019; Peadon & Elliott, 2010). These findings suggest that ADHD caused

by prenatal alcohol exposure may be a special subtype of ADHD or is a completely different disorder.

In summary, clinical and pre-clinical studies suggest that dopaminergic dysfunction and alcohol exposure both lead to ADHD-like symptoms, such as hyperactivity, impulsivity and deficits in executive function. While many children with fetal alcohol spectrum disorder (FASD) have ADHD symptoms, the relationship between the two is not well understood. In particular, the effects of pre-existing DA dysfunction in combination with early alcohol exposure is unknown. To gain insight on this relationship, this thesis assessed the effects of early alcohol exposure on normal and DA-deficient rats.

To this end, we measured DA content, basal and psychostimulant-induced locomotor activity, and passive avoidance learning in DA-deficient rats, alcohol exposed rats, and a combined group that consisted of rats with both a DA deficiency and alcohol exposure. Rats were assessed during preadolescence (PD 20-26), since previous reports have found more pronounced deficits during this age span (Barron & Riley, 1990; Dursun et al., 2006; Schneider et al., 2011). Due to the age of the animals, we do not expect to see sex differences. A total of 12 groups were used in order to precisely determine whether a DA deficiency in combination with alcohol exposure enhances hyperactivity and impulsivity.

Overall, we had two primary hypotheses. First, we looked at locomotor hyperactivity by measuring the activity of the rats. We predicted that the alcohol exposed rats would display locomotor hyperactivity, because this has been

reported in children and in preweaning and juvenile rats after perinatal exposure to ethanol (Barron & Riley, 1990; Dursun et al., 2006). We predicted that the DA-deficient rats would show more locomotor hyperactivity when compared to the alcohol exposed rats. This hypothesis was based on reports from clinical studies showing that children with prenatal alcohol exposure exhibit lower rates of locomotor hyperactivity than children with ADHD (Glass et al., 2014). Lastly, we hypothesized that the combined group would exhibit increased locomotor hyperactivity, relative to the other two groups. Additionally, AMP- and MPH-induced changes in locomotion were compared. The purpose of this experiment was to determine whether findings using this rodent model were consistent with results obtained in clinical populations with this subtype of ADHD. We predicted that DA-deficient rats would have a similar response to AMP and MPH. We anticipated that the alcohol exposed group and the combined group would show less of a response to MPH than AMP. This hypothesis was based on clinical evidence demonstrating that humans exposed to alcohol at an early age responded better to AMP than to MPH (Peadon & Elliott, 2010).

Second, this thesis assessed learning impairments and impulsivity via the passive avoidance learning task. The passive avoidance task is used to assess aspects of executive function, such as learning and memory (Hausknecht et al., 2005; Schneider et al., 2011) as well as inhibitory control (Abel, 1982; Barron & Riley, 1990; Bizot & Thiébot, 1996; Cronise et al., 2001; Dursun et al., 2006). In this task, animals learn to avoid an aversive stimulus by inhibiting a previously

punished response (Abdel-Mouttalib, 2015). We predicted that DA-deficient rats would show poor passive avoidance learning. This hypothesis was based on past findings demonstrating that 6-OHDA lesioned rats exhibit learning impairments in similar tasks (Russell, 2011; Sagvolden et al., 2005; Stanford & Tannock, 2011). We expected alcohol exposed rats to show more profound deficits on the passive avoidance task than 6-OHDA rats. This hypothesis was based on evidence from previous studies showing that alcohol exposure during the neonatal period results in a lack of response inhibition during early adolescence (Abel, 1982; Barron & Riley, 1990; Cronise et al., 2001; Dursun et al., 2006). Lastly, it was hypothesized that a 6-OHDA lesion in combination with neonatal alcohol treatment would result in a greater inability to withhold responding when compared to alcohol exposed rats or DA-deficient rats.

CHAPTER EIGHT:

METHODS

Subjects

Subjects were 1,053 male and female rats (Charles River, Hollister, CA) of Sprague-Dawley descent. All subjects were born and raised at California State University, San Bernardino (CSUSB). The day of parturition was considered PD 0. Beginning on PD 3, each rat was given a distinctive tail mark using colored nontoxic markers. The subjects were given unlimited access to both food and water throughout the study. Pups were kept with the dam until PD 26 in a climate-controlled vivarium maintained at 22-24°C and kept under a 12:12h light/dark cycle. All subjects were cared for according to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 2010) under a research protocol approved by the Institutional Animal Care and Use Committee of CSUSB.

Drugs

Desipramine hydrochloride, 6-hydroxydopmaine (6-OHDA), methylphenidate, and amphetamine were obtained from Sigma-Aldrich (St. Louis, MO). 6-OHDA was mixed in a sterile saline solution containing 0.1% ascorbic acid. Methylphenidate and amphetamine were dissolved in saline at a volume of 2.5 ml/kg. Ethanol (Decon Labs, Inc., 100% USP certified) was mixed in a commercially available milk solution (Enfamil Premium Infant Formula, Mead

Johnson). Powder Enfamil was mixed with distilled water according to the manufacturer's directions to make 60 ml each day (8.9 g powder in 60 ml of water). Ethanol was diluted with the milk solution to make 0.3 g/kg and 3 g/kg solutions and was stored in the refrigerator until used. Ethanol and milk solutions were given orally, at a volume of 19 ml/kg. Unused ethanol and milk solutions were discarded at the end of each day.

Apparatus

Locomotor Activity

Behavioral testing was done in activity monitoring chambers (26 x 26 x 41 cm) that consist of a plastic floor, acrylic walls, and an open top. Distance traveled was measured.

Passive Avoidance

Passive-avoidance testing occurred in clear Plexiglas shuttle boxes divided into two equal compartments (14 x 7 x 12cm) separated by a guillotine door. One of the chambers was transparent and the other was dark and opaque. The flooring of the boxes was composed of stainless-steels bars. A Coulbourn solid-state shock generator delivered a 0.5-mA pulse of distributed shock to the floor of the black compartment.

Procedure

6-OHDA Microinjection

On PD 3, culled rats were given an injection of desipramine (25 mg/kg, IP) to preserve noradrenergic neurons. After 45 min, pups received a topical anesthetic (4% lidocaine solution) and were injected (IC) with 10 µL of 6-OHDA or vehicle at a depth of 0.4 cm using a 30-gauge needle attached to a 25 µL Hamilton microsyringe. The needle was inserted into the foramen magnum between the occipital bone and the first cervical vertebra. Following the 6-OHDA microinjection procedure, pups were returned to their home cages.

Intragastric Intubation Feeding

Daily intragastric intubations occurred from PD 4-9. Weight was recorded on each day of feeding. Pups were placed on a heating pad for the duration of the intubation procedure. Intubations were administered using PE 10 Intramedic tubing connected to a syringe (1 mL with a 23-guage needle) via a small piece of PE 50 Intramedic tubing. The PE 10 tubing was marked prior to the start of intubation, in order to indicate the distance necessary to reach the stomach. Rat pups were held securely so their esophagus was in linear plane. The PE 10 tubing was dipped in corn oil to ease the stress of intubation. The tubing was inserted so that it moved over the tongue and follows the roof of the mouth, to the throat, and down to the stomach.

Dopamine Content Assay

On PD 21, 6-OHDA and alcohol treated animals were euthanized by rapid decapitation and their striatum was removed and stored for future analysis of DA levels. The striatum of each subject was stored at -80 °C. During the DA content

assay, frozen tissue was sonicated and dissolved in 10 volumes of 0.1 N $HClO_4$ and centrifuged at 20,000 x g for 30 min at 4 °C. Resulting supernatant was filtered through a 0.22 µm centrifugation apparatus at 2,000 x g for 5 min at 4 °C. The resulting extracts (20 µL) were assayed for DA content using high performance liquid chromatography with electrochemical detection (Coulochem II; ESA). The mobile phase was comprised of 75 mM NaH_2PO_4, 1.4 mM 1-octane sulfonic acid, 10 mM EDTA, and 10% acetonitrile [(pH 3.0) MD-TM Mobile Phase; ESA] and pumped at a rate of 0.5 mL per min.

Experiment 1: Comparison of Dopamine Depletion, Early Alcohol Exposure, or Combined Treatment on Basal and Psychostimulant-Induced Locomotor Activity

Male and female animals were randomly assigned to one of 36 groups (n= 9). The first independent variable was lesion, with three levels (no lesion, sham, or 6-OHDA). The second independent variable was intubation, with four levels (no intubation, 0, 0.3, or 3 g/kg ethanol). The third independent variable was psychostimulant treatment with three levels (saline, methylphenidate or amphetamine).

On PD 19, male and female rats were habituated to the locomotor activity chambers and then returned to their home cage. The following day (PD 20) male and female rats were injected intraperitoneally (ip) with saline, methylphenidate (2.5 mg/kg) or amphetamine (1 mg/kg). On both days, locomotor activity was measured for 60 min. The design of Experiment 1 is shown in Table 1.

Table 1. Design of Experiment 1

LESION	INTUBATION	DRUG
No lesion	No Intubation	Saline, MPH, or AMP
No lesion	0 g/kg ethanol	Saline, MPH, or AMP
No lesion	0.3 g/kg ethanol	Saline, MPH, or AMP
No lesion	3 g/kg ethanol	Saline, MPH, or AMP
Sham	No Intubation	Saline, MPH, or AMP
Sham	0 g/kg ethanol	Saline, MPH, or AMP
Sham	0.3 g/kg ethanol	Saline, MPH, or AMP
Sham	3 g/kg ethanol	Saline, MPH, or AMP
6-OHDA	No Intubation	Saline, MPH, or AMP
6-OHDA	0 g/kg ethanol	Saline, MPH, or AMP
6-OHDA	0.3 g/kg ethanol	Saline, MPH, or AMP
6-OHDA	3 g/kg ethanol	Saline, MPH, or AMP

Experiment 2: Comparison of Dopamine Depletion, Early Alcohol Exposure, or Combined Treatment on Passive Avoidance Learning

Male and female animals were randomly assigned to one of twelve groups (n= 10). On PD 23, subjects began training on a step-through passive avoidance task. Acquisition occurred on PD 23 and retention of the passive avoidance was

assessed on the following three days. On the acquisition day, the rat was placed in the clear illuminated compartment with the guillotine door closed. After 30 s, the door opened. Movement to the adjoining chamber resulted in closure of the dividing door and delivery of a brief (3 s) foot shock (0.5-mA). Latency for the rat to cross from the clear to the dark compartment was recorded. After 30 s, the rat was removed from the testing chamber and returned to the home cage. Retention was assessed after an interval of 24 h (PD 24), 48 h (PD 25), and 72 h (PD 26). Specifically, rats were placed in the clear illuminated compartment with the door open and the latency to leave the illuminated chamber was measured. No shock was administered on retention trials. Retention trials were discontinued after 600 s if the rat did not move into the dark compartment.

Table 2. Design of Experiment 2

LESION	**INTUBATION**
No lesion	No Intubation
No lesion	0 g/kg ethanol
No lesion	0.3 g/kg ethanol
No lesion	3 g/kg ethanol
Sham	No Intubation
Sham	0 g/kg ethanol

Sham	0.3 g/kg ethanol
Sham	3 g/kg ethanol
6-OHDA	No Intubation
6-OHDA	0 g/kg ethanol
6-OHDA	0.3 g/kg ethanol
6-OHDA	3 g/kg ethanol

Data Analysis

DA Content

DA and DOPAC were analyzed by separate 3 x 4 x 2 (lesion x alcohol x sex) three-way ANOVAs. Post hoc analysis of the neurochemical data was made using Tukey tests (p <.05).

Experiment 1

Basal locomotor activity (distance traveled) was analyzed by a 2 x 3 x 4 (sex x lesion x alcohol) three-way ANOVA. Significant higher-order interactions were further analyzed using one- or two- way ANOVAs. Post hoc analysis of the behavioral data was made using Tukey tests (p <.05)

Psychostimulant-induced locomotor activity (distance traveled) was analyzed by a 2 x 3 x 4 x 3 (sex x lesion x alcohol x drug) four-way ANOVA.

Significant higher-order interactions were further analyzed using one- or two- way ANOVAs. Post hoc analysis of the behavioral data was made using Tukey tests (p <.05). Litter effects were controlled by assigning no more than one rat from each litter to a particular group.

Experiment 2

Passive avoidance data were analyzed using a 2 x 3 x 4 x 4 (sex x lesion × alcohol x day) mixed factors ANOVA, with sex, lesion, and alcohol being between-subject factors and day being a within-subject repeated measures factor. Mauchly's test was used to detect violations of the sphericity assumption. When violations of sphericity were detected, the Huynh-Feldt Epsilon statistic was used to make corrections. When appropriate, post-hoc analyses were conducted using Tukey tests (p <.05).

CHAPTER NINE:

RESULTS

Experiment One

Habituation Day

On the habituation day, lesion condition significantly impacted locomotor activity because rats that received 6-OHDA lesions had larger distance traveled scores than rats in the sham or no lesion groups [Lesion main effect, $F(2,614) = 57.38$, $p<.001$] (see Figure 1). The 6-OHDA lesion ($M = 6035.63$, $SEM = 186.72$) significantly increased the distance traveled scores of rats when compared to sham lesion ($M = 3592.21$, $SEM = 181.34$) and no lesion rats ($M = 3629.98$, $SEM = 180.53$). Neither sex nor early exposure to alcohol altered locomotor activity (see Figure 2). In addition, sex, lesion and alcohol did not interact to affect basal locomotor activity.

Further analyses showed that there were no significant differences between the two lesion control groups (i.e., sham and no lesion) and the two alcohol control groups (no intubation and only milk). Thus, the separate control groups were combined to form a single lesion control group and a single alcohol control group for subsequent analyses.

Test Day

On the test day, locomotor activity was greatly increased by the psychostimulants (amphetamine or methylphenidate) [Drug main effect, $F(2,557) = 410.52$, $p<.001$ (see Figure 3). The effects of amphetamine (1mg/kg) ($M =$

20661.70, *SEM* = 474.40) and methylphenidate (2.5 mg/kg) (*M* = 22169.99, *SEM* = 487.52) did not significantly differ from one another. The effects of saline (*M* = 4736.14, *SEM* = 473.77) were significantly different than that of amphetamine and methylphenidate.

The effect of psychostimulant administration on locomotor activity was altered by sex, lesion condition, and alcohol exposure [Sex × Lesion × Alcohol × Drug interaction, F(4,593) = 2.46, $p<.05$]. To interpret the meaning of the four-way interaction, separate ANOVAs were conducted for each drug treatment.

Saline Treatment. Similar to the habituation day, rats given 6-OHDA lesions exhibited greater locomotor activity than lesion controls [Lesion main effect, $F(1,202) = 31.55$, $p<.001$] (see Figure 4). The 6-OHDA lesion (*M* = 6518.14, *SEM* = 3831.95) significantly increased the distance traveled scores of rats when compared to lesion controls (*M* = 3818.7007, *SEM* = 2590.26). This effect of lesion was affected by both sex and alcohol condition [Sex × Lesion × Alcohol interaction, $F(2,202) = 3.40$, $p<.05$] (see Figure 4). Specifically, when only female rats were analyzed, non-lesioned rats treated with the low dose of alcohol (0.3 g/kg), had increased distance traveled scores as compared to female rats in the alcohol control or high dose alcohol groups, [Alcohol effect, $F(2,72) = 10.64$, $p<.001$, and Tukey tests, $p<.05$] (see Figure 4).

Amphetamine Treatment. Sex significantly impacted the rat's response to AMP [Sex main effect, $F(1,201) = 4.95$, $p<.05$] (see Figure 5). Male rats (*M* = 22341.32, *SEM* = 900.62) given AMP had greater distance traveled scores than

female rats (*M* = 19502.98, *SEM* = 904.38). In contrast to my predictions, the effect of sex was not significantly affected by lesion or alcohol condition [Sex × Lesion × Alcohol interaction, $F(2,201) = 2.70$, $p=.07$, ns] (see Figure 5).

Methylphenidate Treatment. Male rats (*M* = 23154.18, *SEM* = 872.46) that received MPH on the test day had higher levels of locomotor activity than female rats (*M* = 21233.07, *SEM* = 749.38), [Sex main effect, $F(1,190) = 6.77$, $p<.05$] (see Figure 6). This increased locomotor activity in male rats was most pronounced in rats that received 6-OHDA lesions [Sex × Lesion interaction, $F(1,190) = 4.16$, $p<.05$] (see Figure 6), because further analyses showed MPH-treated male rats with 6-OHDA lesions had greater distance traveled scores than MPH-treated female rats with 6-OHDA lesions [Sex main effect, $F(1,57) = 6.99$, $p<.05$] (see Figure 6).

Monoamine Assays

DA Levels. As expected, there were significant differences in striatal DA levels as a result of 6-OHDA lesions [Lesion main effect, $F(2,190) = 375.23$, $p<.001$ and Tukey tests $p<.001$] (see Figure 7). Specifically, the 6-OHDA lesion group (*M* = 1.68, *SEM* = .20) had significantly lower levels of DA than both the sham lesion (*M* = 6.95, *SEM* = .17) and the no lesion group (*M* = 6.82, *SEM* = .15). Alcohol did not affect DA levels, [Alcohol main effect, $F(3,190) = 2.59$, $p=0.054$, ns] (see Figure 7). While not significant, the low dose of alcohol (0.3 mg/kg) appeared to produce a small reduction in DA levels, when compared to the control and high alcohol groups.

DOPAC Levels. Rats given 6-OHDA lesions (*M* = .39, *SEM* = .03) had reduced striatal DOPAC levels when compared to the sham (*M* = 1.36, *SEM* = .05) and no lesion group (*M* = 1.29, *SEM* = .04), [Lesion main effect, $F(2,190) = 208.58$, $p<.001$, and Tukey tests, $p<.001$] (see Figure 8). Alcohol treatment did not significantly impact striatal DOPAC levels [$p=.29$].

Experiment Two

Passive Avoidance Training

All rats responded similarly on the conditioning trial, since the time to enter the dark chamber was unaffected by sex, lesion treatment or alcohol exposure. Furthermore, rats in all groups had significantly greater latencies to enter the dark chamber after the conditioning trial, thus demonstrating that the rats learned and retained the passive avoidance task on all four retention test days [Day main effect, $F(3, 621) = 53.719$, $p<0.001$] (see Figure 9). Because the latency times were significantly affected by day, further analyses were conducted separately for each test day.

Test Day 1. On the first test day, step-through latencies were significantly shorter for rats lesioned with 6-OHDA (*M* = 72.07, *SEM* = 12.72), as compared to the no lesion control group (*M* = 191.13, *SEM* = 21.75), [Lesion main effect, $F(2, 207) = 10.357$, $p<0.001$, Tukey tests, $p<0.05$] (see Figure 10). Sham lesioned rats (*M* = 119.80, *SEM* = 17.66) had marginally longer latencies when compared to 6-OHDA lesioned rats, but this difference was not significant. There was also a non-significant trend for both sex and alcohol to alter the effects of lesion on

step-through latencies [Sex × Lesion × Alcohol interaction, $F(6, 207) = 2.108$, $p=0.054$]. Specifically, alcohol-exposed male rats in the no lesion condition had shorter step-through latencies than non-exposed rats in the same lesion condition.

Test Day 2. Similar to the first test day, 6-OHDA lesioned rats ($M = 78.44$, $SEM = 15.45$) on Test Day 2 had shorter step-through latencies when compared to rats in the control lesion groups, no lesion ($M = 184.86$, $SEM = 24.43$) and sham lesion ($M = 142.23$, $SEM = 20.95$), [Lesion main effect, $F(2, 207) = 6.609$, $p<0.01$, Tukey tests, $p<0.05$] (see Figure 11). Alcohol exposure and sex did not affect step-through latencies on Test Day 2.

Test Day 3. On the last test day, once again the 6-OHDA lesion rats ($M = 58.49$, $SEM = 12.74$) showed a decrease in latency to move to the dark chamber when compared to both no lesion ($M = 175.01$, $SEM = 24.35$) and sham lesion ($M = 141.00$, $SEM = 23.47$) controls [Lesion main effect, $F(2, 207) = 8.484$, $p<0.001$, Tukey tests, $p<0.05$] (see Figure 12). This difference in step-through latencies in the lesion condition was moderated by alcohol treatment [Lesion × Alcohol interaction, $F(6, 209) = 2.648$, $p<0.05$] (see Figure 12). Specifically, both male and female rats in the no lesion condition exposed to the high dose of alcohol (3 g/kg) had shorter latencies than rats in the same lesion condition exposed to milk only (0 g/kg) [Tukey tests, $p<0.05$]. Moreover, males in the sham condition that were exposed to a high dose of alcohol (3 g/kg) had longer step-through latencies than male rats in the same lesion condition but not exposed to

alcohol [Sex × Lesion × Alcohol interaction, $F(6, 207) = 2.154$, $p<0.05$, Tukey tests, $p<0.05$] (see Figure 12).

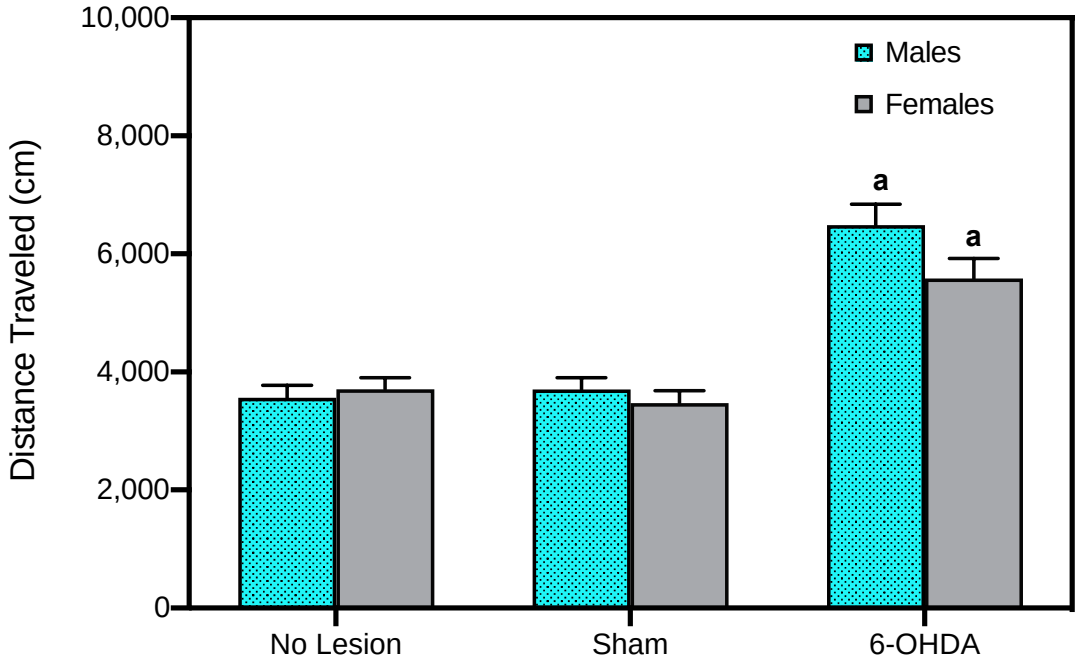

Figure 1. Experiment 1 Habituation Day
Mean distance traveled scores (± SEM) of male and female rat pups on the habituation day (PD 19). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). Starting on PD 4, rats received alcohol infusions (0, 0.3 or 3 g/kg) daily or were unhandled (no intubation) until PD 9. Distance traveled testing occurred on PD 19. 'a' Indicates a significant difference relative to the sham and no lesion groups.

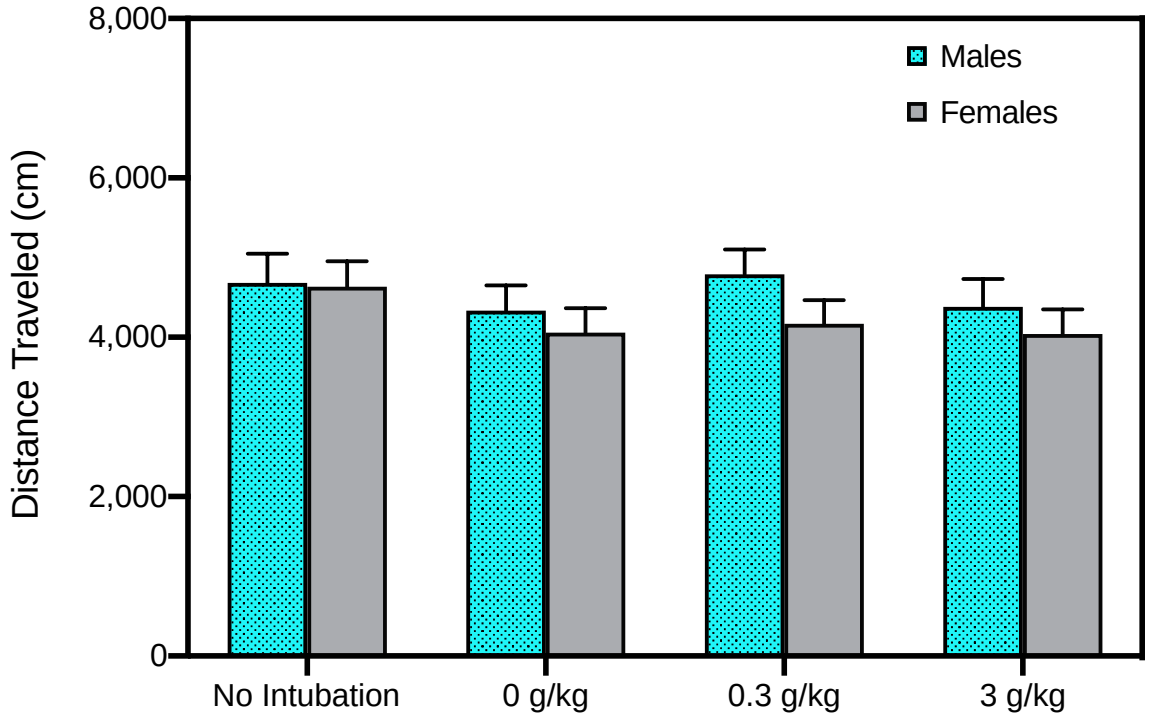

Figure 2. Experiment 1 Habituation Day
Mean distance traveled scores (± SEM) of male and female rat pups on the habituation day (PD 19). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). Starting on PD 4, rats also received ethanol infusions (0, 0.3 or 3 g/kg) daily or were unhandled (no intubation) until PD 9.

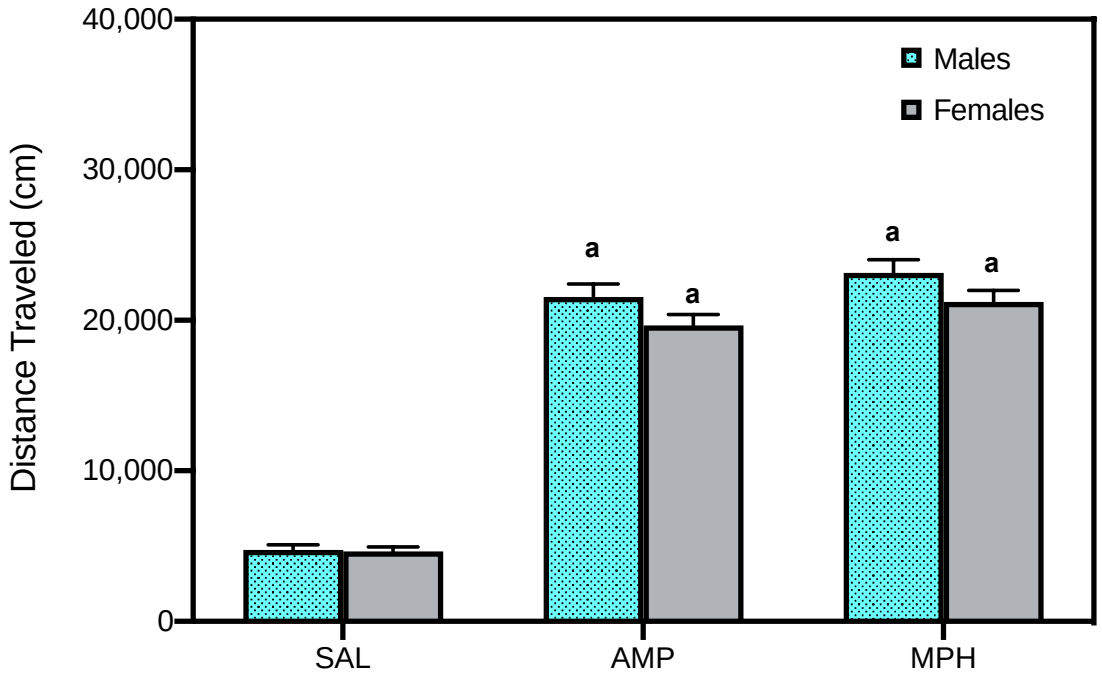

Figure 3. Experiment 1 Test Day
Mean distance traveled scores (± SEM) of male and female rat pups on the test day (PD 20). On PD 3, Rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). Starting on PD 4, rats also received alcohol infusions (0, 0.3 or 3 g/kg) daily or were unhandled (no intubation) until PD 9. On the test day, rats received saline, AMP (1 mg/kg, ip), or MPH (2.5 mg/kg, ip) before distance traveled testing.
'a' Indicates a significant difference compared to the AMP or MPH groups.

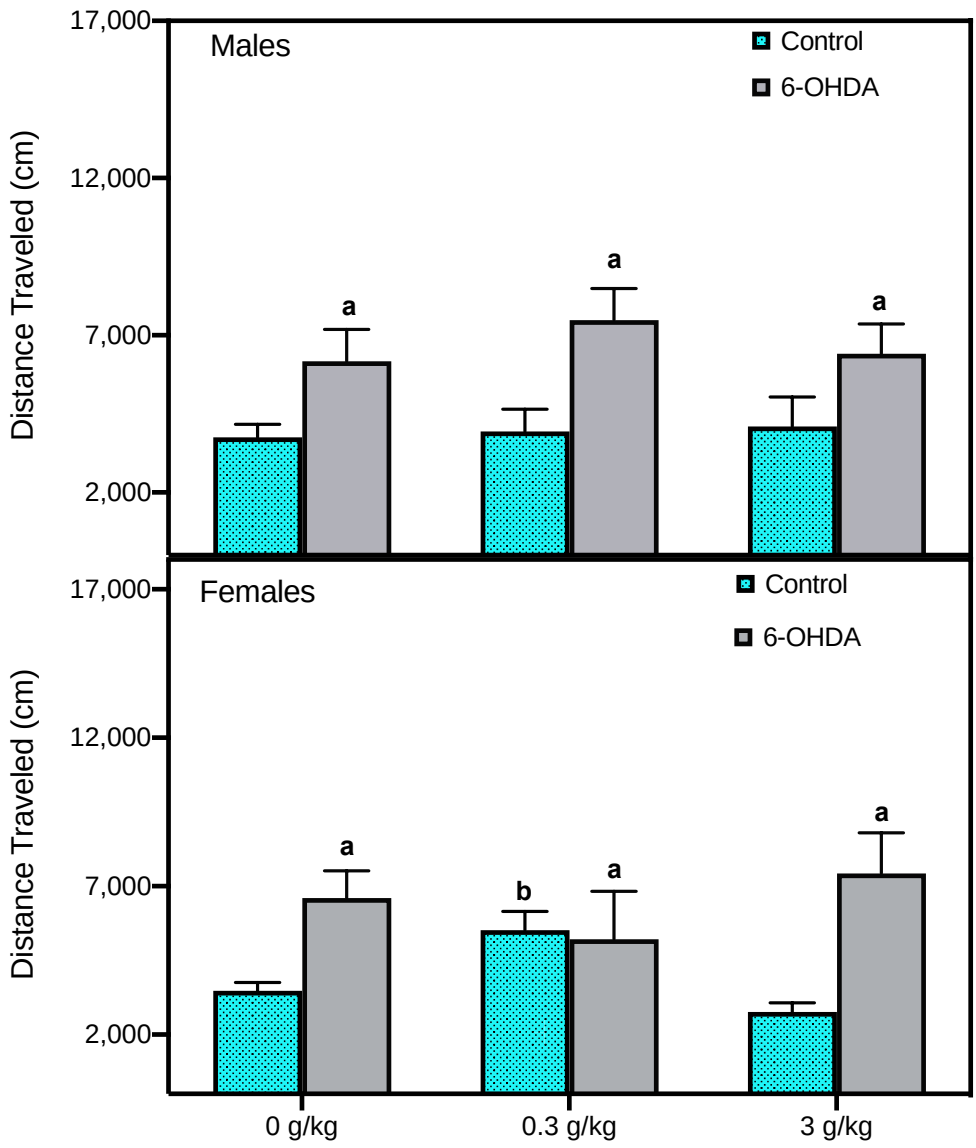

Figure 4. Experiment 1 Test Day
Mean distance traveled scores (± SEM) of male and female rat pups in the saline condition on the test day (PD 20). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to ethanol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 20, rats had been injected with saline, AMP (1 mg/kg, ip), or MPH (2.5 mg/kg, ip) before being placed in the activity chambers. 'a' Indicates a significant difference from the control lesion rats (i.e., no lesion and sham). 'b' Indicates a significant difference from the no alcohol and high alcohol (3 g/kg) group.

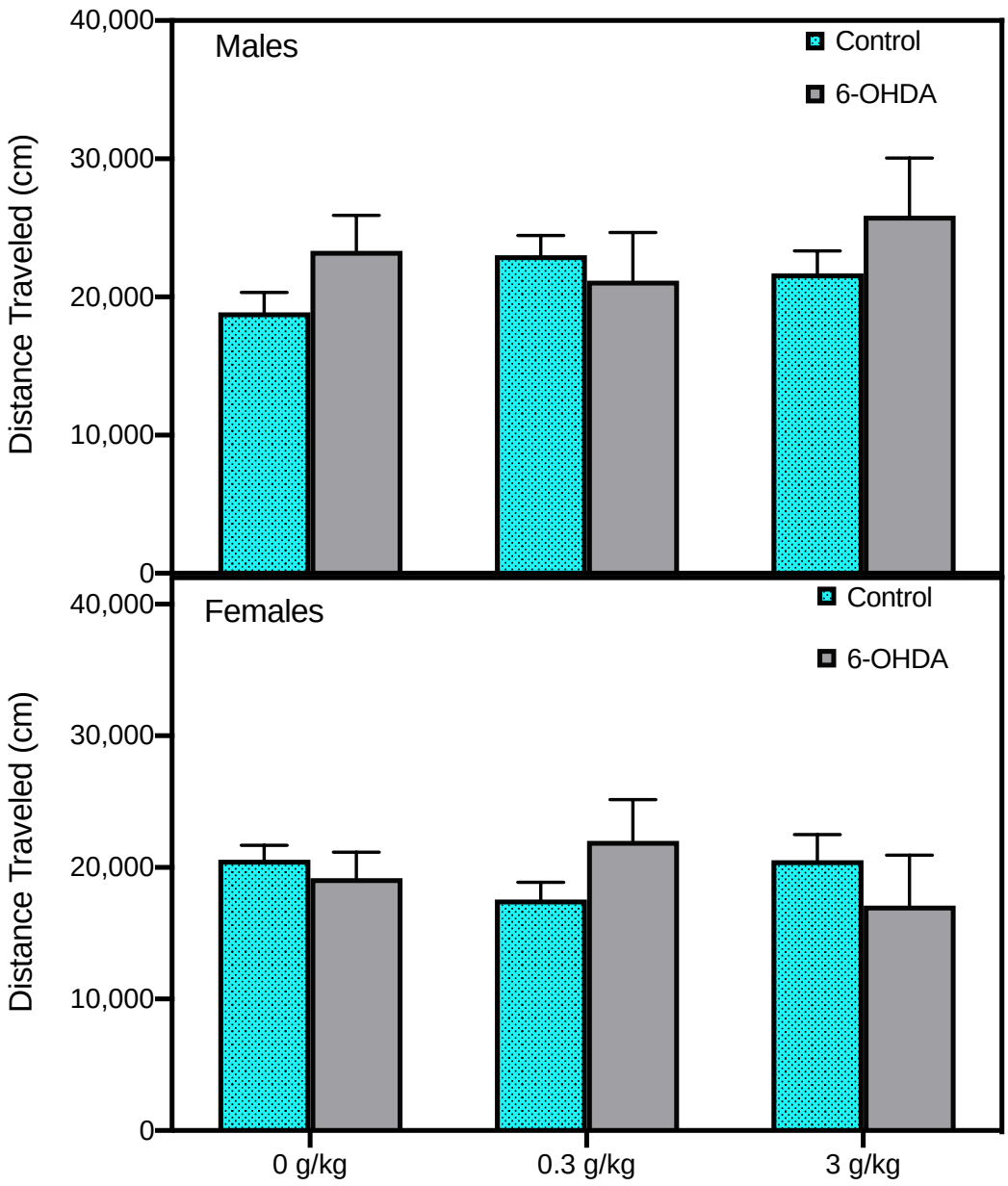

Figure 5. Experiment 1 Test Day
Mean distance traveled scores (± SEM) of rats in the amphetamine condition on test day (PD 20). On PD 3, male and female rats were untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to ethanol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 20, rats were injected with saline, AMP (1 mg/kg, ip), or MPH (2.5 mg/kg, ip) before being placed in the activity chambers.

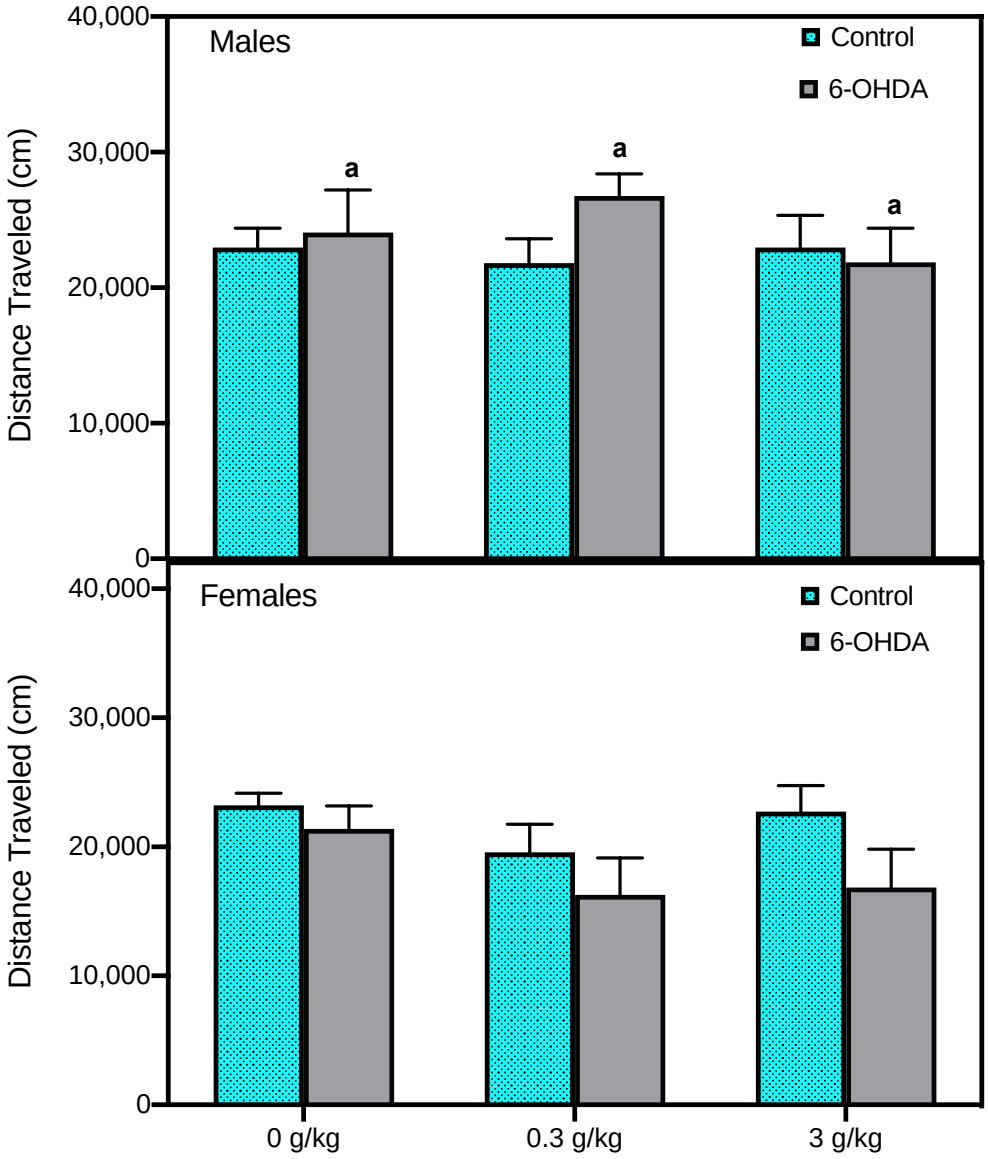

Figure 6. Experiment 1 Test Day
Mean distance traveled scores (± SEM) of male and female rat pups in the MPH condition on the test day (PD 20). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to ethanol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 20, rats were injected with saline, AMP (1 mg/kg, ip), or MPH (2.5 mg/kg, ip) before being placed in the activity chambers ‘a’ Indicates a significant difference from female 6-OHDA rats treated with MPH (2.5 mg/kg, ip).

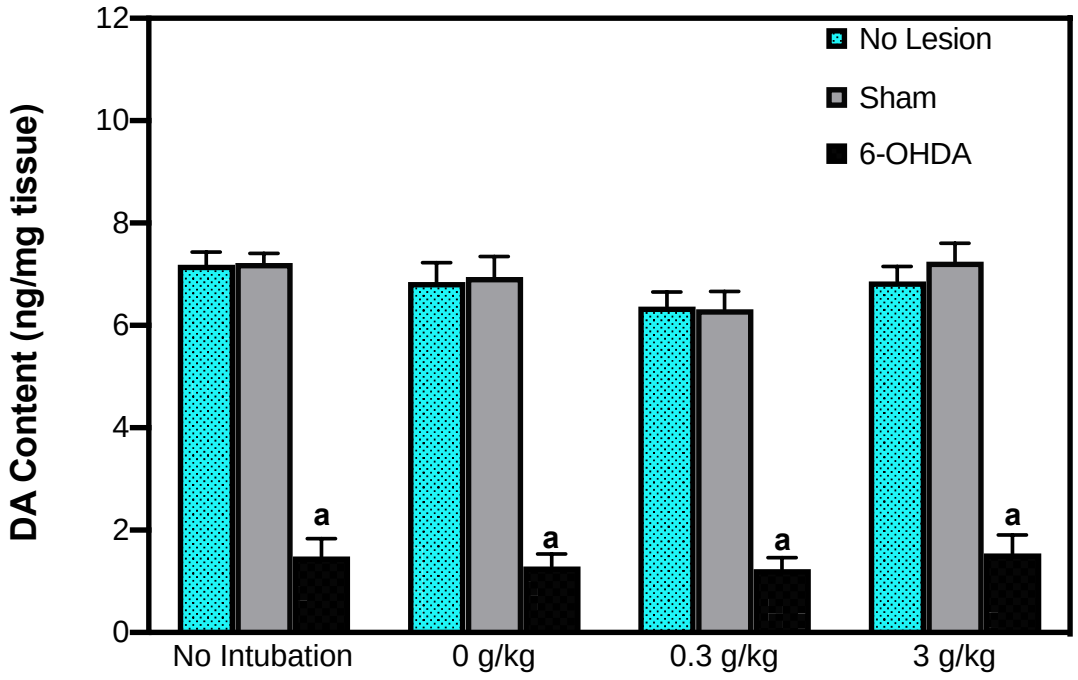

Figure 7. Striatal DA Content
Mean (±SEM) striatal DA content of male and female rat pups on PD 21. On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to ethanol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation).
'a' Indicates a significant difference from no lesion and sham lesioned rats.

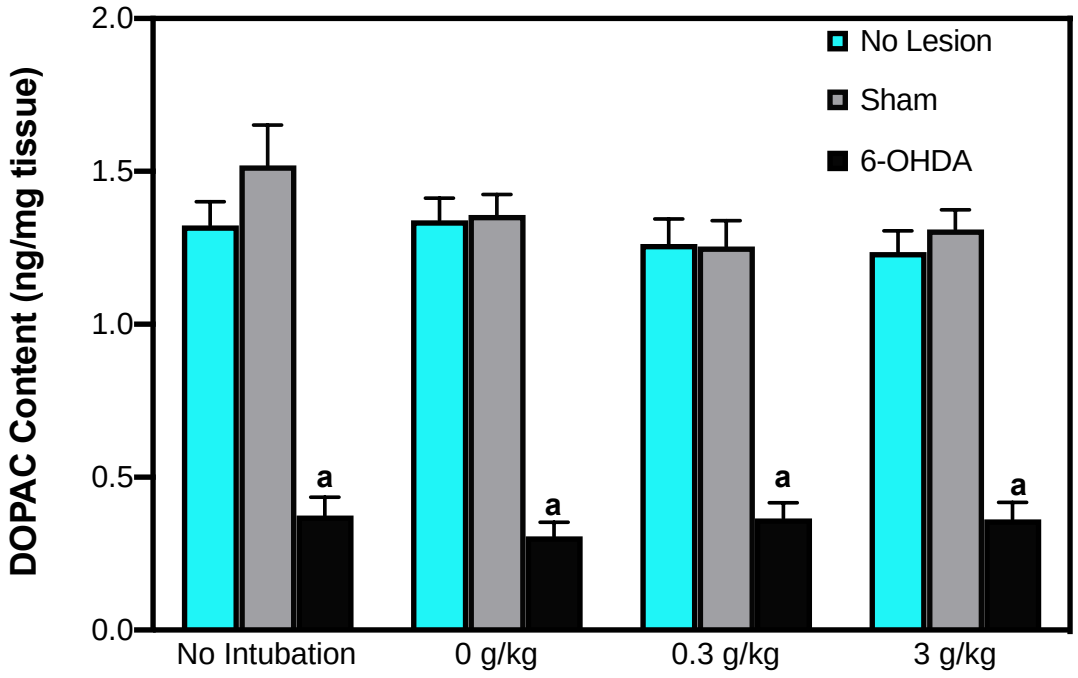

Figure 8. Striatal DOPAC Content
Mean (±SEM) striatal DOPAC content of male and female rat pups on PD 21. On PD 3, rats were untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). Starting on PD 4, rats received ethanol infusions (0, 0.3 or 3 g/kg) daily or were unhandled (no intubation) until PD 9. 'a' Indicates a significant difference from no lesion and sham lesioned rats.

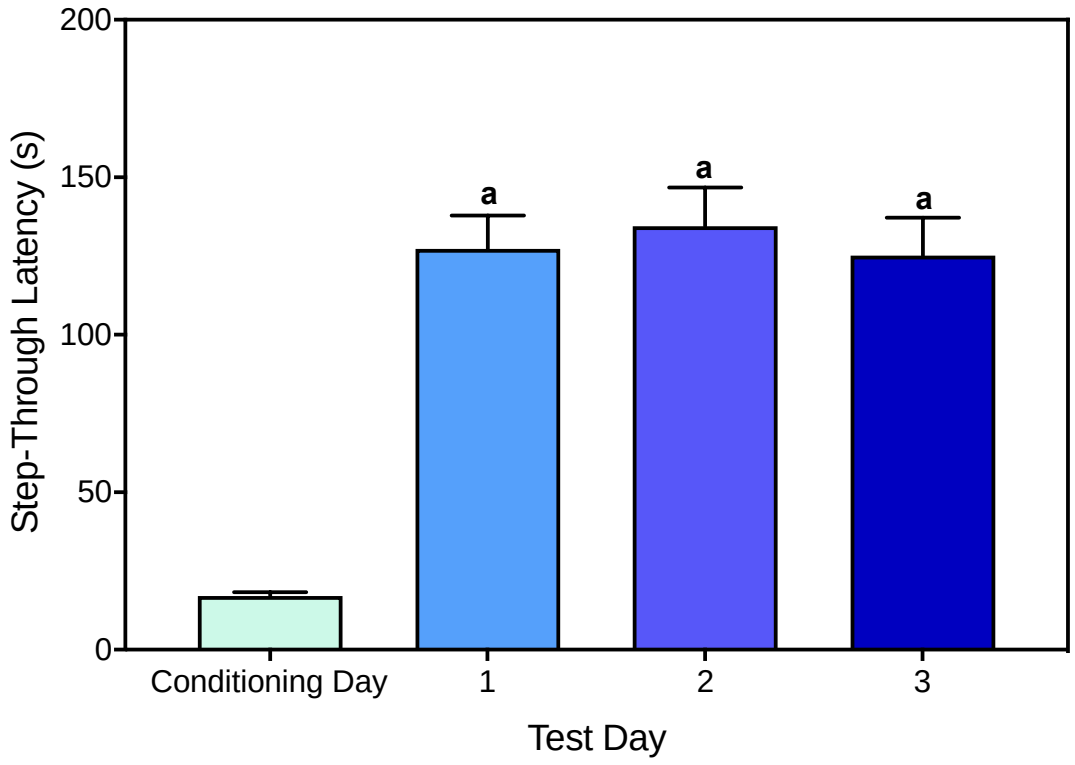

Figure 9. Step-through Latency on Conditioning Day
Mean step-through latency (± SEM) of male and female rat pups on the conditioning day (PD 23) and test days 1-3 (PD 24-26). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to alcohol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 23, rats were conditioned by receiving a shock when they entered the dark chamber. Retention was tested for three consecutive days (PD 24-26). 'a' Indicates a significant difference relative to the conditioning day.

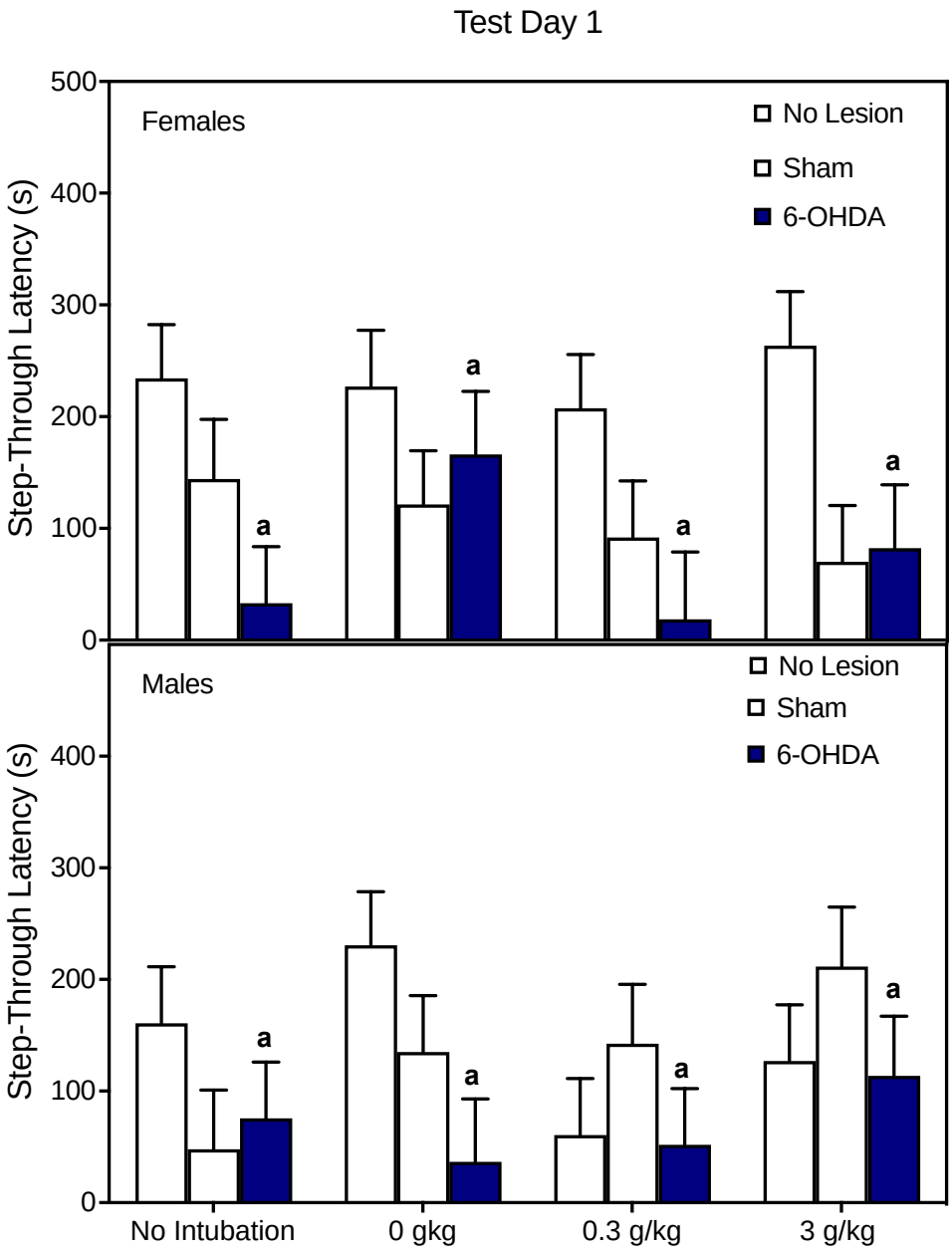

Figure 10. Step-through Latency on Test Day 1
Mean step-through latency (± SEM) of male and female rat pups on test day 1 (PD 24). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to alcohol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 23, rats were conditioned by receiving a shock when they entered the dark chamber. Retention was tested for three consecutive days (PD 24-26). 'a' Indicates a significant difference relative to the no lesion group.

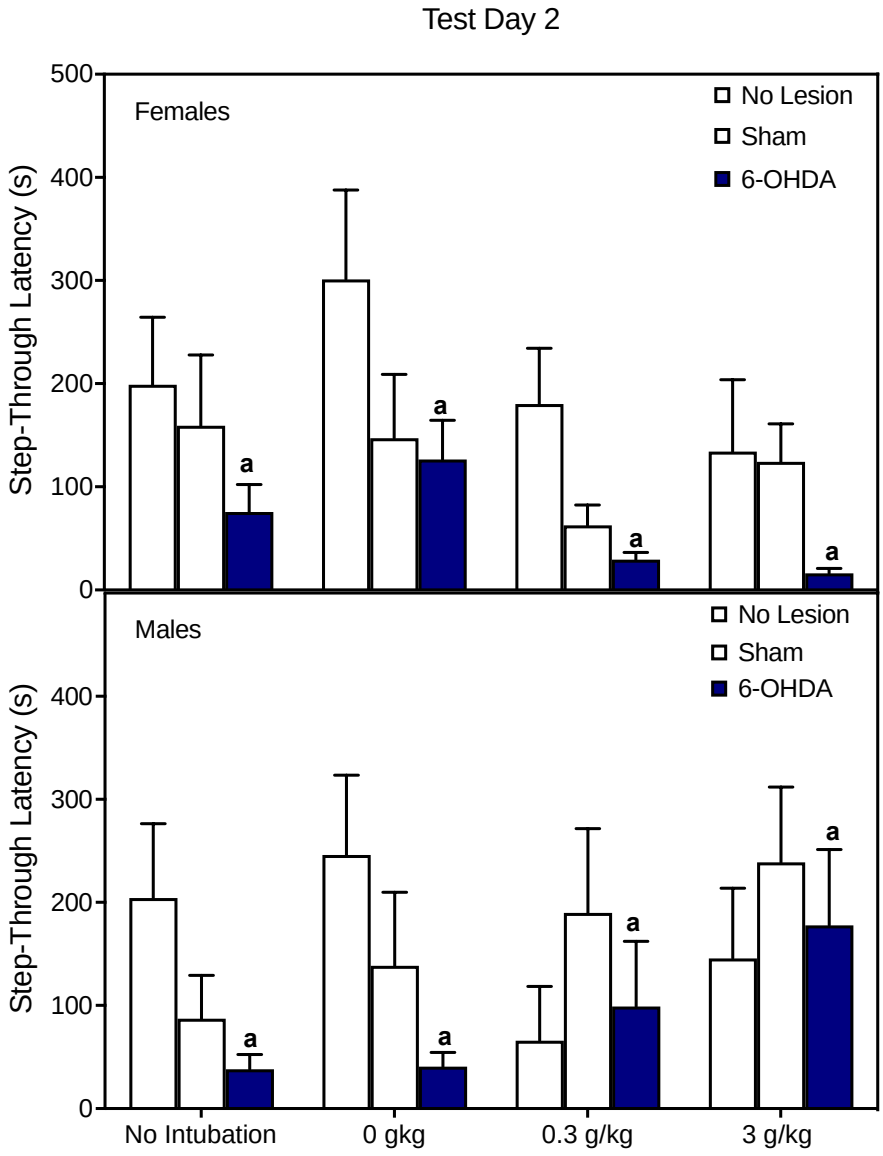

Figure 11. Step-through Latency on Test Day 2
Mean step-through latency (± SEM) of male and female rat pups on test day 2 (PD 25). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to alcohol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 23, rats were conditioned by receiving a shock when they entered the dark chamber. Retention was tested for three consecutive days (PD 24-26). 'a' Indicates a significant difference relative to the no lesion group.

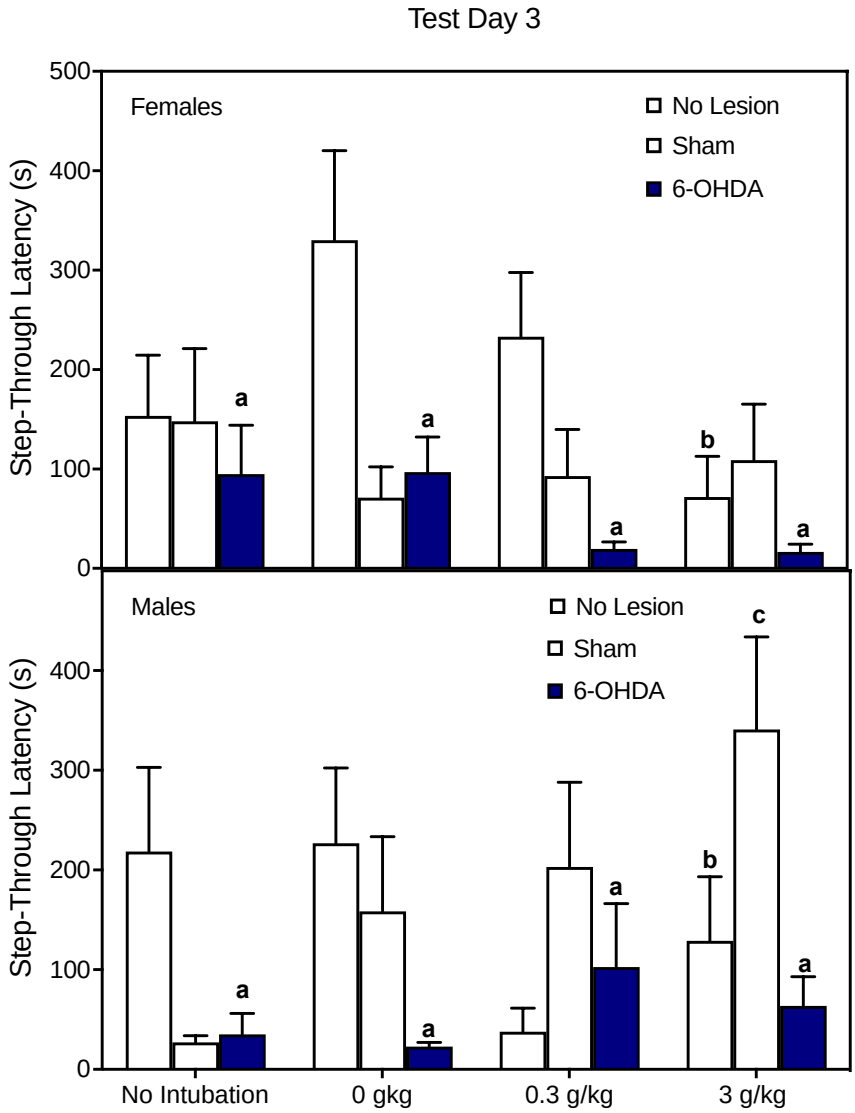

Figure 12. Step-through Latency on Test Day 3
Mean step-through latency (± SEM) of male and female rat pups on test day 3 (PD 26). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to alcohol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 23, rats were conditioned by receiving a shock when they entered the dark chamber. Retention was tested for three consecutive days (PD 24-26). 'a' Indicates a significant difference relative to the no lesion and sham group. 'b' Indicates a significant difference from the no alcohol group (0 g/kg). 'c' Indicates a significant difference from the sham lesion male rats in the unhandled group.

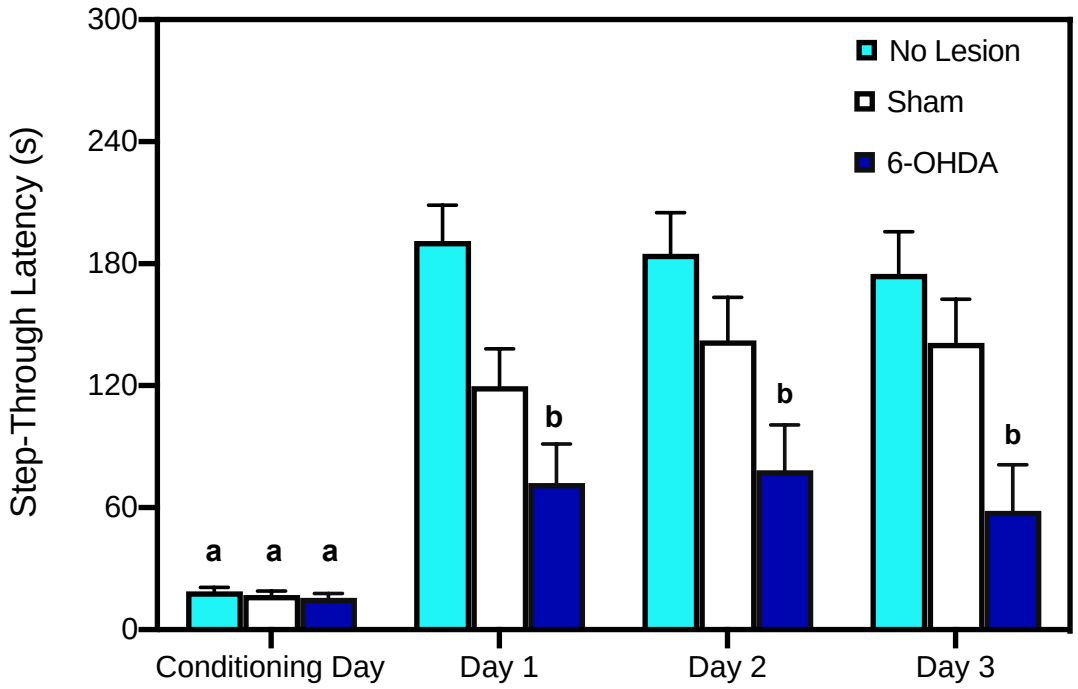

Figure 13. Step-through Latency by Lesion
Mean step-through latency (± SEM) of 6-OHDA lesioned male and female rat pups on the conditioning day (PD 23) and test days 1-3 (PD 24-26). On PD 3, rats were either untreated (no lesion), given a sham lesion (sham), or lesioned with 6-ODHA infusions (100 µg/10 µl, ic). From PD 4-9, rats were exposed to alcohol (0, 0.3, or 3 g/kg) daily or were unhandled (no intubation). On PD 23, rats were conditioned by receiving a shock when they entered the dark chamber. Retention was tested for three consecutive days (PD 24-26). 'a' Indicates a significant difference relative to test days (1-3). 'b' Indicates a significant difference from the no lesion rats.

CHAPTER TEN:

DISCUSSION

Overview

The present thesis examined the individual and combined ability of neonatal 6-OHDA lesions and early alcohol exposure to induce ADHD-like symptoms in preweanling rats. In the first experiment, we assessed the aforementioned treatments on basal and psychostimulant-induced locomotor activity. We had three hypotheses about the effects of our treatment conditions on basal locomotion. First, we predicted that alcohol exposed rats, similar to the DA-deficient rats, would display an increase in locomotor activity, because rats treated prenatally with alcohol show higher activity than control rats (Barron & Riley, 1990; Dursun et al., 2006; Hausknecht et al., 2005). Second, we hypothesized that alcohol exposed rats would exhibit less locomotor activity than DA-deficient rats. This prediction was based on past research demonstrating that children with prenatal alcohol exposure exhibit less hyperactivity than children with ADHD (Glass et al., 2014). Lastly, we hypothesized that rats given the combined treatment would exhibit greater locomotor hyperactivity than the other two groups. Additionally, we made predictions about differential responses to psychostimulant administration after DA depletion and alcohol exposure in rats. Specifically, we hypothesized that DA-deficient rats would have similar reduced responses to both AMP and MPH, but that both the alcohol exposed and

combined group would show less of a response to AMP than to MPH, this prediction was based on clinical literature (Peadon & Elliott, 2010).

In the second experiment, we assessed the effects of neonatal 6-OHDA lesions and early alcohol exposure in combination and individually on passive avoidance learning. We predicted that DA-deficient rats would perform worse than lesion controls on the passive avoidance task in concordance with past studies using 6-OHDA lesioned rats (Russell, 2011; Sagvolden et al., 2005; Stanford & Tannock, 2011). It was also predicted that alcohol exposed rats would show a more profound deficit on the passive avoidance task when compared to 6-OHDA lesioned rats. This hypothesis was based on past research demonstrating that alcohol exposure results in a lack of response inhibition (Abel, 1982; Barron & Riley, 1990; Cronise et al., 2001; Dursun et al., 2006). Lastly, it was hypothesized that combined treatment would produce the greatest deficit on this task.

Basal Locomotion

The locomotor data revealed that the 6-OHDA lesion resulted in greater distance traveled in both male and female rats. Additionally, the low dose of alcohol increased locomotor activity in female rats after habituation to the chamber. In contrast to our predictions, the combined effects of lesion and alcohol did not result in any significant increases in locomotor activity.

It is well established that 6-OHDA lesions result in locomotor hyperactivity in rats (Russell, 2011; Sagvolden et al., 2005; Stanford & Tannock, 2011). Thus,

the present findings were not unexpected because both male and female rats lesioned with 6-OHDA had greater distance traveled scores than lesion controls.

On the test day, non-lesioned female rats exposed to the low dose (0.3 g/kg), but not the high dose (3 g/kg), of alcohol, exhibited an increase in locomotor activity. This finding is novel, as the dose range in alcohol exposure studies typically falls between 1 and 6 g/kg (Brocardo et al., 2012; Hamilton et al., 2011; Hausknecht et al., 2017; Juárez & Guerrero-Álvarez, 2015; Patten et al., 2014; Vaglenova & Petkov, 1998). To our knowledge, no published studies report alterations in locomotor activity after low dose alcohol treatment. Data on the effects of early alcohol exposure on locomotor activity is limited and the data that do exist provide mixed results. For example, some studies report higher locomotor activity after moderate-dose alcohol exposure (Juárez & Guerrero-Álvarez, 2015), while other studies do not find changes in locomotion (Atalar et al., 2016; Dursun et al., 2006). The present findings suggest that low-dose alcohol exposure during the postnatal period may have more impact on activity levels than higher doses.

Psychostimulant-Induced Locomotion

Overall, treatment with AMP (1 mg/kg) and MPH (2.5 mg/kg) produced nearly identical levels of locomotor activity. These findings are consistent with our hypothesis that rats would have a similar response to AMP and MPH. It was also hypothesized that psychostimulant treatment would reduce locomotor hyperactivity in the alcohol exposed rats and in the combined group; however,

our results did not support this hypothesis. Overall, alcohol treatment did not differentially alter locomotor activity on test day, yet sex and dopamine depletion did alter the response to the two drugs.

Amphetamine administration produced more locomotor activity in male rats than female rats. Although not statistically significant, rats exposed to alcohol showed a trend towards an increased locomotor activity on the test day when compared to the habituation day. Currently, only a few studies have measured the effects of psychostimulants on alcohol exposed rats and those studies report mixed results. One study reported that treatment with AMP resulted in no differential effect on hyperactivity (Bond, 1985), while another study reported an increase in activity following AMP treatment (Blanchard, Hannigan, & Riley, 1987). These findings suggest that AMP may enhance activity in alcohol-treated rats.

Similar to the findings with AMP, male rats treated with MPH were more active than female rats. 6-OHDA depletion of dopamine enhanced this difference, as MPH-treated male lesioned rats were more active than MPH-treated female lesioned rats. Alcohol exposure did not significantly alter locomotor activity in MPH-treated rats; however, when compared to the habituation day, there was an increase in MPH-induced locomotor activity. Consistent with our findings, Abel (1993), reported that MPH treatment enhances the locomotor activity of alcohol exposed rats. Conversely, Juárez and Guerrero-Álvarez (2015) report that MPH has no effect on hyperactivity. Taken together

these findings suggest that treatment with MPH may not be effective at reducing hyperactivity in alcohol treated rats.

Our hypothesis that the alcohol exposed group and the combined group would show a lessened response to MPH, relative to AMP, was not supported. These findings contrast with clinical evidence showing that humans exposed to alcohol at an early age respond better to AMP than to MPH (Peadon & Elliott, 2010).

Monoamine Assays

Because decreases in DA content are reliably associated with ADHD as well as deficits in learning and memory, we hypothesized that alcohol exposure would induce declines in DA levels similar to 6-OHDA lesions (Jaber et al.,1996; Kobayashi, 2001). However, our hypothesis was not supported because alcohol treated rats did not exhibit a decline in DA or DOPAC levels. In contrast, 6-OHDA lesions resulted in the expected decrease in DA and DOPAC levels in preweanling rats (Stanford & Tannock, 2011). In brief, the 6-OHDA lesion reliably caused a significant decrease in DA and DOPAC levels, whereas the alcohol treatment did not cause a reduction.

Passive Avoidance

Consistent with past studies, decreasing DA content with 6-OHDA lesions resulted in impaired performance on the learning task. Specifically, 6-OHDA lesioned male and female rats had significantly shorter latencies when compared

to control rats on the step-through passive avoidance task, suggesting that the 6-OHDA lesion had an impact on learning inhibitory control and memory. While 6-OHDA lesions impair performance on some tasks, only one other study reported that 6-OHDA lesioned rats exhibited deficits on the passive avoidance task (Pearson et al., 1980). Like us, Pearson et al. (1980) injected their pups with desipramine prior to lesioning with 6-OHDA and their injection volumes and age (PD 27) were similar to our own (Pearson et al., 1980). In contrast several other studies found that 6-OHDA lesions did not affect passive avoidance performance (Anderson et al., 1986; Cooper, 1973; Takasuna & Iwasaki, 1996); however, there were clear methodological differences that could account for the disparate results, including the dose of 6-OHDA used and age at testing. The current study used a modest dose of 6-OHDA (100 µg/10 µl), while Cooper (1973) used twice this dose (240 µg) and also tested the rats at a younger age (PD 14). Takasuna and Iwasaki (1996) also used a low dose of 6-OHDA but tested the rats at PD 90. Future research is necessary to determine the relationship between 6-OHDA dose and age at testing on passive avoidance learning.

The results relating to the effects of alcohol exposure on passive avoidance learning were in partial support of our hypothesis. Specifically, when given a high dose of alcohol (3 g/kg) both non-lesioned male and female rats as well as sham lesioned male rats exhibited memory deficits on the third retention day. Interestingly alcohol did not affect the performance of non-lesioned or sham lesioned rats on the earlier test days.

The current study demonstrated memory deficits in both sexes, while a past investigations only saw deficits in female rats (Abel,1982; Barron & Riley, 1990). The Barron and Riley (1990) study, like the current investigation, specifically assessed sex differences, but had a number of important methodological differences from our study. First, was the mode of alcohol exposure. The current study used intragastric intubation, while Barron and Riley (1990) used artificial rearing. Second, the alcohol concentration differed. Our study used a dose of 3 g/kg, while their dose (6.0 g/kg) was twice the amount of the current study (Barron & Riley, 1990). It is unclear whether these methodological differences played a role in the differing results found in the present thesis and in the study of Barron and Riley (1990), but direct comparisons between the two modes of exposure and level of alcohol are warranted and could provide clarity on this issue.

Taken together, findings from the present thesis and past research demonstrate that alcohol exposure during the neonatal period does affect performance in male and female rats on the passive avoidance task. Further research will be needed to determine how this alcohol effect differs by sex and dose. Researchers may consider using doses between 3.0 to 6.0 g/kg, because evidence suggests that during the neonatal period a higher dose of alcohol is more effective in causing impairments in behavioral measures (Brocardo et al., 2012; Hamilton et al., 2011).

Conclusion

The major findings from the present thesis are fourfold: 1) 6-OHDA lesions resulted in both a DA-deficiency and hyperactivity in male and female rats. Moreover, psychostimulant administration increased locomotor activity instead of attenuating this behavior. 6-OHDA lesions also resulted in an impairment in inhibitory control and memory on the passive avoidance task. 2) Exposure to a low dose of alcohol (0.3 g/kg) did not alter DA levels, but did cause an increase in locomotor activity in female rats. 3) Exposure to a high dose of alcohol (3 g/kg) did not alter DA levels or acquisition of a passive avoidance task, but did impair memory. 4) Lastly, the combined effects of 6-OHDA lesions and alcohol did not have an additive or synergistic effect on locomotor activity, DA depletion, or passive avoidance performance. Taken together findings from the present thesis and past research suggest that neonatal alcohol exposure can produce hyperactivity and deficits in memory, but this effect differs by sex, time of exposure, as well as a dose of alcohol. Additionally, the neurochemistry findings suggest that DA system dysfunction may not be related to the ADHD-like symptoms observed in alcohol-treated rats.

Because the results of previous preclinical work are inconsistent, more research needs to be conducted in this area. Researchers may consider investigating sex differences using various behavioral procedures and alcohol concentrations as there is very limited data on early alcohol exposure and ADHD. Future research on the effects of alcohol should also investigate the

noradrenergic system as our study was not able to detect a dysfunction in the DA system and a recent study found that atomoxetine was effective at reducing the ADHD-like symptoms seen in alcohol treated rats (Juárez & Guerrero-Álvarez, 2015).

In conclusion, the current study provides evidence that low doses of alcohol can have long lasting effects. This information is important as there has been a substantial increase in alcohol consumption in women of childbearing age as gender roles have changed (Tan et al., 2015). The results from this study may be informative for pregnant women who consider low to moderate doses of alcohol consumption to be acceptable or safe.

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