PRENATAL ALCOHOL EXPOSURE AS A RISK FACTOR FOR
INCREASED VULNERABILITY OF THE ADOLESCENT BRAIN TO
ALCOHOL INDUCED DAMAGE

A Thesis
Presented to the
Faculty of
San Diego State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
in
Psychology

by
Rashmi Risbud
Fall 2014
SAN DIEGO STATE UNIVERSITY

The Undersigned Faculty Committee Approves the

Thesis of Rashmi Risbud:

Prenatal Alcohol Exposure as a Risk Factor for Increased Vulnerability of the

Adolescent Brain to Alcohol Induced Damage

Jennifer Thomas, Chair
Department of Psychology

Susan Brasser
Department of Psychology

Greg Harris
Department of Biology

Carmen Pulido
Department of Psychiatry, UCSD

October 21, 2014
Approval Date
DEDICATION

This thesis is dedicated to my subjects – the Sprague Dawley lab rats, without whom this project (as well as a vast base of scientific knowledge) would not exist. These misunderstood creatures are exemplary mothers, problem solvers, and social beings. They are more curious and intelligent than we give them credit for.
ABSTRACT OF THE THESIS

Prenatal Alcohol Exposure as a Risk Factor for Increased Vulnerability of the Adolescent Brain to Alcohol Induced Damage

by

Rashmi Risbud

Master of Arts in Psychology
San Diego State University, 2014

Alcohol is a teratogen that causes damage to the developing fetus. Prenatal alcohol exposure can cause physical deformities, neuropathology, and behavioral alterations. In addition to hyperactivity and impairments in motor, social, attention and learning domains, prenatal alcohol exposure may lead to increased alcohol preference later in life. Thus, the same children exposed to alcohol during fetal life may experiment with alcohol during adolescence - a period of development when the brain is also particularly vulnerable to alcohol. It is not known, however, whether prenatal alcohol exposure influences the vulnerability of the adolescent brain to alcohol-induced pathology and subsequent behavioral alterations. The present study used a 2 (neonatal alcohol exposure, control) x 2 (adolescent alcohol exposure, control) x 2 (male, female) design. Sprague-Dawley rats received ethanol exposure (2.5 g/kg/day) or sham intubations from postnatal days (PD) 4-9, a period of development equivalent to the third trimester. Subjects were then exposed to ethanol (4.0 g/kg/day) or sham intubations during adolescence from PD 28-42 on a 2 day on / 1 day off schedule. Mean blood alcohol concentrations (BAC) were around 200 mg/dl during both developmental periods. During early adulthood, subjects were tested on tasks that depend on the functional integrity of the hippocampus and prefrontal cortex, areas of the brain that are sensitive to alcohol exposure. Spatial learning was measured in a Morris water maze task on PD 52-60, and contextual and conditioned stimulus learning was measured with a trace fear-conditioning task on PD 63-64. Alcohol exposure during each developmental time period alone was not expected to robustly impair behavior, but it was hypothesized that subjects exposed to alcohol during both developmental periods would show significant impairments on behavioral tasks. Unexpectedly, neonatal alcohol exposure produced significant neuropathology, reducing both forebrain and cerebellar weight. Consistent with gross neuropathology, neonatal alcohol significantly slowed acquisition of trace fear conditioning in both males and females and slowed acquisition in spatial learning in males. In contrast, adolescent alcohol exposure did not significantly induce gross brain pathology or disrupt learning. These data suggest that 200 mg/dl/day ethanol is sufficient to disrupt cognitive development during the 3rd trimester equivalent but not during adolescence. Females exposed to alcohol during adolescence did, however, exhibit an increased conditioned fear response to the conditioned stimulus once the response was learned, suggesting alterations in emotional responding. Finally, the combination of neonatal and adolescent alcohol exposure did not alter these patterns with one exception. Male subjects exposed to alcohol during both developmental periods spent less time in the periphery of the Morris water maze tank (thigmotaxis), which may indicate that combined alcohol exposure during the prenatal and
adolescent periods reduced anxiety or increased risk taking in a sex-dependent manner. Thus, these data suggest that prenatal alcohol exposure does not increase risk for cognitive deficits, but may affect emotional consequences of adolescent alcohol exposure, at least with the alcohol exposure parameters used in the present study. Future studies should more specifically examine the effects of combined prenatal and adolescent alcohol exposure on stress and emotional development.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>Effects of Prenatal Alcohol Exposure / FASD</td>
<td>2</td>
</tr>
<tr>
<td>Adolescent Alcohol Exposure</td>
<td>4</td>
</tr>
<tr>
<td>Purpose of Present Study</td>
<td>8</td>
</tr>
<tr>
<td>CHAPTER 2 METHODS</td>
<td>9</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>9</td>
</tr>
<tr>
<td>Neonatal Alcohol Exposure</td>
<td>9</td>
</tr>
<tr>
<td>Adolescent Alcohol Exposure</td>
<td>10</td>
</tr>
<tr>
<td>Blood Alcohol Concentrations (BACs)</td>
<td>10</td>
</tr>
<tr>
<td>Behavioral Testing</td>
<td>10</td>
</tr>
<tr>
<td>Morris Water Maze</td>
<td>10</td>
</tr>
<tr>
<td>Trace Fear Conditioning</td>
<td>11</td>
</tr>
<tr>
<td>Measuring Intoxication in Neonatal Subjects</td>
<td>11</td>
</tr>
<tr>
<td>Data Analyses</td>
<td>12</td>
</tr>
<tr>
<td>Expected Results</td>
<td>12</td>
</tr>
<tr>
<td>CHAPTER 3 RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>Neonatal Body Weights</td>
<td>13</td>
</tr>
<tr>
<td>Adolescent Body Weights</td>
<td>13</td>
</tr>
<tr>
<td>Blood Alcohol Concentration (BAC)</td>
<td>15</td>
</tr>
<tr>
<td>Morris Water Maze</td>
<td>16</td>
</tr>
<tr>
<td>Visible Platform</td>
<td>26</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Trace Fear Conditioning</td>
<td>28</td>
</tr>
<tr>
<td>Brain Weights</td>
<td>32</td>
</tr>
<tr>
<td>Righting Reflex</td>
<td>34</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>36</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Number of Subjects per Sex per Exposure Group .................................................................13
Table 2. Blood Alcohol Concentrations by Exposure Group .................................................................16
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Male and female body weight growth by neonatal exposure group PD 4-9.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Male and female body weight growth by neonatal exposure group PD 12-21.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Body weight growth of male and female subjects through the adolescent period.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Male subject path lengths to platform decrease over acquisition days of Morris water maze.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Female subject path lengths to platform decrease over acquisition days of Morris water maze.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Male subject latencies to platform decrease over acquisition days of Morris water maze.</td>
<td>17</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Female subject latencies to platform decrease over acquisition days of Morris water maze.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Male subject swimming speeds decrease over acquisition days of Morris water maze.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Female subject swimming speeds decrease over acquisition days of Morris water maze.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Changes in male subject heading angles over acquisition days of Morris water maze.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Female subject heading angles do not change much over acquisition days of Morris water maze.</td>
<td>20</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Male subjects in the combination exposure group show greater reductions in thigmotaxis behavior over acquisition days than male subjects in the neonatal exposure only group.</td>
<td>21</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Female subjects show small reductions in thigmotaxis over acquisition days of Morris water maze.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Average reduction in percent time spent in thigmotaxis (day 6 in comparison to day 1) in male subjects.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Time spent in each quadrant during the probe trial.</td>
<td>24</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Time spent in target area during probe trial.</td>
<td>24</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Average passes through the target during probe testing.</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 18. Average heading angle during the probe trial ..............................................25
Figure 19. Average percent time spent in thigmotaxis during the probe trial .................26
Figure 20. Subject path lengths were lower on the second day of visible platform ..........26
Figure 21. Subject latencies were lower on the second day of visible platform ..............27
Figure 22. Subject speeds increase by the second day of visible platform ....................27
Figure 23. Subject heading angles during visible platform ...........................................28
Figure 24. Changes in male subject freezing levels during each CS (tone), TR (trace), and ITI (Inter-trial interval) during the trace training paradigm .........................................29
Figure 25. Changes in female subject freezing levels during each CS (tone), TR (trace), and ITI (Inter-trial interval) during the trace training paradigm. ...................29
Figure 26. Changes in male subject freezing levels over the five minutes of the context test .........................................................................................................................31
Figure 27. Changes in female subject freezing levels over the five minutes of the context test .........................................................................................................................31
Figure 28. Changes in freezing levels of male subjects in response to events during the CS Test ..........................................................................................................................32
Figure 29. Changes in freezing levels of female subjects in response to events during the CS Test ..........................................................................................................................33
Figure 30. Male and female total brain weights ..............................................................33
Figure 31. Male and female forebrain weights ...............................................................34
Figure 32. Male and female cerebellum weights ............................................................34
Figure 33. Latency to right during the righting reflex test in ethanol exposed and control subjects ......................................................................................................................35
ACKNOWLEDGEMENTS

I would like to say thank you first and foremost to Dr. Jennifer Thomas for her unwavering support, academic and personal guidance, and dedication to being an amazing mentor over the past two years. From her, I have learned compassion, patience, attention to detail, time management, and the understanding that curiosity will always be the crux of science. I am truly lucky to have been her student. I would also like to thank my thesis committee – Dr. Susan Brasser, Dr. Greg Harris, and Dr. Carmen Pulido for their guidance and support. Thank you to the Psychology Department faculty for the enthusiasm, humor, and interest with which they taught the research skills necessary to conduct a thesis project. I want to specifically mention Dr. Georg Matt, who showed me that I was capable of understanding and using advanced statistics, and for always providing encouragement with pretzels and gummy bears.

This project would not have been possible without the constant, daily oversight of Dr. Nathen Murawski, who was instrumental in my training for laboratory and behavioral testing skills, who was always there for moral support and for late hours in the lab, and who taught me that “if you want to make an omelet, you have to break a couple eggs”. The laboratory and data management skills acquired were practiced and perfected under the watchful eye of Dr. Nirelia Idrus, whose detailed scrutiny at each step has made me a better scientist. And of course, thank you to all of the laboratory members at the Center for Behavioral Teratology who have been supportive through the best and worst of this project, and to my fellow classmates whose companionship has made the past two years enjoyable.

Last but certainly not least, I must acknowledge the loving support of my family members, who have made frequent trips to San Diego, who have been constantly accessible via phone or internet, and who have endured this project and all that it entails alongside me.
CHAPTER 1

INTRODUCTION

BACKGROUND

Alcohol is a known teratogen that can cause severe brain damage, cognitive impairments, and behavioral problems in the developing fetus. Prenatal alcohol exposure remains a pervasive problem in today’s society despite extensive research highlighting the detrimental effects of alcohol consumption during pregnancy. Prenatal alcohol exposure is the leading known cause of intellectual disability in the United States today (Riley, Infante, & Warren, 2011). It is estimated that 2-7 of 1,000 children are born with fetal alcohol syndrome (FAS), and that 2-5% of children in the United States are born with fetal alcohol spectrum disorders (FASD) (May et al., 2009).

Unfortunately, prenatal alcohol exposure is not an isolated problem. The same children who suffer disabilities due to maternal drinking are often reintroduced to alcohol during adolescence. First, prenatal alcohol exposure may result in increased alcohol consumption during adolescence, in part by affecting development of the dopaminergic reward system (Brown et al., 2008; Chotro & Arias, 2003). Thus, prenatally exposed individuals may be at a greater risk of developing alcohol use disorders (Alati et al., 2006). In addition, early exposure to alcohol may make the brain more vulnerable to damaging effects of alcohol during subsequent exposures (Maldonado-Devincci, Badanich, & Kirstein, 2010). Alcohol exposure during adolescence can damage the prefrontal cortex and hippocampus, areas of the brain that are sensitive and still developing throughout adolescence (Nixon & McClain, 2010). Adolescents who have been prenatally exposed to alcohol may have a different vulnerability to the damaging effects of alcohol compared to adolescents who have not been prenatally exposed. Using an animal model, the present study examines whether performance on hippocampal and prefrontal cortex dependent behavioral tasks following alcohol exposure during adolescence is affected by a history of early developmental alcohol exposure. A history of developmental exposure may exaggerate sensitivity to alcohol’s damaging effects. Conversely, a history of exposure may increase tolerance, rendering the
adolescent brain more resilient to an alcohol insult. Examining the combined effect of early developmental alcohol exposure in addition to subsequent adolescent alcohol exposure on learning and memory will shed light on whether alcohol’s actions over multiple developmental periods affect the developing brain and behavior.

**Effects of Prenatal Alcohol Exposure / FASD**

The teratogenic effects of alcohol have been well documented. Prenatal alcohol exposure can lead to a wide range of developmental problems including physical deformities, functional and structural damage to the developing brain, and cognitive and behavioral impairments (Riley et al., 2011). Severe cases of alcohol exposure during pregnancy can result in fetal alcohol syndrome (FAS) in the child (Jones, Smith, Ulleland, & Streissguth, 1973; O'Leary, 2004). Children with FAS exhibit growth deficiencies as well as facial abnormalities such as short palpebral fissures, flattened philtrum, and a thin upper lip (Jones et al., 1973). Children with FAS also exhibit central nervous system damage/dysfunction such as microencephaly, intellectual problems, and developmental delays (Streissguth, Herman, & Smith, 1978). Often, these children exhibit structural and/or functional impairments in several brain regions, including the hippocampus and cerebellum. Other brain regions such as the prefrontal cortex, basal ganglia, corpus collosum and other white matter tracts are also compromised (Mattson, Schoenfeld, & Riley, 2001). In addition, children diagnosed with FAS experience behavioral alterations including, but not limited to, impulsivity, hyperactivity, mood disorders, and problems with motor coordination, social processing, attention, learning and memory (Riley et al., 2011).

However, not all children who have been prenatally exposed to alcohol meet the criteria to be diagnosed with FAS. The behavioral and cognitive development of such individuals can still be affected by prenatal alcohol exposure even if they do not exhibit the physical features common in FAS patients (Mattson, Riley, Gramling, Delis, & Jones, 1998). Therefore, a broader term than FAS was necessary to describe the wide range of outcomes following prenatal alcohol exposure. Fetal alcohol spectrum disorders (FASD) is an umbrella term used to describe the continuum of effects caused by maternal intake of alcohol (Sokol, Delaney-Black, & Nordstrom, 2003).
Variability in outcomes resulting from prenatal alcohol exposure may be due to differences in maternal drinking patterns as well as genetics, nutrition, timing of exposure, and other environmental factors (May et al., 2004). For example, there is a lot of variability in patterns of maternal drinking. Some women drink low levels of alcohol consistently over a long period of time while others engage in binge drinking episodes (several drinks over a short period of time). In animal models, it has been shown that binge drinking can cause more damage to the developing fetus than greater volumes of alcohol spread over time (Maier & West, 2001). Binge drinking achieves a higher blood alcohol concentration (BAC) and results in prolonged fetal alcohol exposure. Binge drinking also results in periods of withdrawal for both the mother and her fetus, which may play a critical role in the damaging effects of alcohol (Maier & West, 2001; Thomas & Riley, 1997).

In the United States, 14 g of alcohol a day (one drink) is considered moderate drinking for non-pregnant women and government guidelines state that no known level of alcohol is safe to drink during pregnancy (International Center for Alcohol Policies, 2010). It is possible that even a single drink during a period of rapid brain development can cause neuronal cell death (Eckstrand et al., 2012). Eckstrand et al. (2012) showed that young adults (18-20 years of age) who had been exposed to low levels of alcohol (the mothers consumed one drink a day on average) in utero exhibit lower grey matter volume in several areas of the brain, despite no overall differences in brain size between alcohol-exposed participants and controls. The research suggests that any alcohol exposure during pregnancy may impact fetal brain development in permanent ways, and that effects can last through adolescence.

Additionally, prenatal alcohol exposure causes hippocampal dependent learning and memory impairments. It can also hinder adult neurogenesis and alter development of dopaminergic and serotonergic neurotransmitter systems (Valenzuela, Morton, Diaz, & Topper, 2012). These alterations may increase vulnerability to ethanol and other substances of abuse. One consequence of prenatal alcohol exposure is an increased preference for alcohol and increased alcohol consumption later in life (Brown et al., 2008; Chotro & Arias, 2003). This suggests that prenatal alcohol exposure may increase the likelihood that an individual will consume alcohol during adolescence.

A number of animal studies suggest that prenatal alcohol exposure is linked to higher incidences of behavioral and cognitive problems that persist through early adulthood.
(Valenzuela et al., 2012). However, deficits in the functional integrity of brain areas caused by prenatal alcohol exposure may not always be evident unless the individual is challenged. For example, the exposure may not impair behavioral performance unless the task is more difficult, or the subject is challenged with pharmacological or environmental factors. Thus, the brain may be compromised even if gross behavioral alterations are not immediately evident.

Although it is well known that alcohol intake during pregnancy causes damage to the fetus, women continue to drink during pregnancy. The continued drinking of pregnant women may be due to lack of awareness, inability to abstain from alcohol intake, or unplanned pregnancies - women continue to drink in the first few months because they do not know they are pregnant (Ethen et al., 2009; Warren & Foudin, 2001). Maternal drinking is of concern because the developing brain may be adversely affected in children who have experienced alcohol exposure, even if they do not exhibit the physical malformations or severe behavioral and cognitive impairments characteristic of FAS (Mattson et al., 1998). It is unclear how prenatal alcohol exposure affects vulnerability to insults sustained later in life.

**ADOLESCENT ALCOHOL EXPOSURE**

Children who have been prenatally exposed to alcohol are often socially reintroduced to alcohol intake during adolescence along with their peers. The drinking pattern of teenagers in the United States is problematic, as a significant portion of the adolescent population drinks alcohol and most binge drink to achieve high levels of intoxication (Johnston, O’Malley, Bachman, & Schulenberg, 2006). Binge drinking is defined as drinking in excess of 4 drinks on one occasion, resulting in a blood alcohol concentration of .08 or higher (14g = one standard drink). An estimated 70% of high school aged students drink, and more than half of them exhibit binge-drinking patterns (Johnston et al., 2006). Previous research has shown that those who start drinking at an earlier age are twice as likely to develop alcohol use disorders. Approximately 40% of people diagnosed with alcohol use disorders developed symptoms during high school (Brown et al., 2008).

Adolescence is a period of development during which the brain is especially sensitive to the detrimental effects of alcohol. For example, the adolescent brain responds uniquely to acute effects of alcohol. Adolescents exhibit decreased sensitivity to some “negative” effects
of alcohol such as decreased intoxication, motor problems, hangover and withdrawal levels when compared to adults with the same blood alcohol content (Brown et al., 2008; Nixon & McClain, 2010; Spear & Varlinskaya, 2005). This decreased sensitivity to negative effects can result in heavier drinking because adolescents may underestimate their level of intoxication. Evidence also suggests that during adolescence, increased dopamine release causes hyperactivation of the striatum during reward anticipation, resulting in stronger reward-seeking behaviors (Bava & Tapert, 2010). Alcohol exposure in adolescents causes a marked increase in dopamine release in the nucleus accumbens (Nixon & McClain, 2010). A combination of increased reward and decreased negative effects leads to a higher level of alcohol intake (Nixon & McClain, 2010) and it has been shown that in an animal model, alcohol-induced changes to dopaminergic system during adolescence leads to increased alcohol seeking behavior in adult rats (Maldonado- Devincci et al., 2010).

Increased alcohol intake is particularly problematic as adolescents exhibit enhanced sensitivity to neurodegenerative effects of alcohol, which can change behavioral control systems and eventually lead to addiction (Doremus-Fitzwater, Varlinskaya, & Spear, 2010). The prefrontal cortex, hippocampus, and several neurotransmitter systems are still developing during adolescence and are especially vulnerable to damage by alcohol (Witt, 2010). In humans, repeated binge-like alcohol exposure (4 or more drinks on one occasion resulting in blood alcohol concentrations of .08 or higher) causes more severe memory impairments in adolescents when compared to adults and leads to long term changes in GABA, glutamate, serotonin and dopamine neurotransmitter systems (Witt, 2010). Alcohol binge exposure during adolescence causes atrophy of white matter throughout the brain but especially in the hippocampus and prefrontal cortex, as shown by diffusion tensor imaging studies in clinical populations (McQueeny et al., 2009).

These results are consistent with animal research that shows the vulnerability of the hippocampus and prefrontal cortex to alcohol during adolescence (Nixon, Morris, Liput, & Kelso, 2010). For example, animal models have also shown that adolescent alcohol exposure may disrupt normal life-span neuroplasticity in the hippocampus. Adolescent alcohol exposure impairs hippocampal neurogenesis through alcohol inhibition of neural stem cell proliferation and decreased survival of new cells (Morris, Eaves, Smith, & Nixon, 2010). Ehlers, Liu, Wills, and Crews (2013) showed that intermittent ethanol exposure by vapor
(14h on/10h off/day) from PD 23 to PD 58 (average blood ethanol concentration: 163mg/dl) results in reduced neurogenesis and proliferation in the rat hippocampus. The study also showed increased markers of cell death in the hippocampus. Similarly, adolescent rats exposed to ethanol (3.0 g/kg) via intraperitoneal injection intermittently from PD 25-39 exhibit cell death in the hippocampus, as well as in the neocortex and cerebellum (Pascual, Blanco, Cauli, Minarro, & Guerri, 2007).

Changes in the brain contribute to behavioral alterations. Rats exposed to alcohol during adolescence in exhibited hypoactivation on locomotor tasks as well as lowered anxiety in an open field behavioral task during adulthood (Ehlers et al., 2013). Given the vulnerability of the hippocampus, it is not surprising that learning and memory are adversely affected by adolescent alcohol exposure. Animals exposed to alcohol during adolescence show persistent deficits in conditional discrimination learning and object recognition tasks, deficits that are consistent with alcohol-induced hippocampal damage (Pascual et al., 2007). Sircar and Sircar (2005) also found that deficits in spatial learning associated with adolescent alcohol exposure (2.0 g/kg/day for 5 consecutive days) may be long-lasting. Consistent with the animal studies, Weissenborn and Duka (2003) showed that adolescent binge drinkers performed poorly on pattern recognition memory and spatial working memory tasks than non-binge drinkers. Of relevance to the current project, intermittent ethanol binges during adolescence that produce blood alcohol levels > 200 mg/dl also decrease memory retention and accelerate forgetting in Morris Water maze – a spatial working memory task dependent on the hippocampus (Schulteis, Archer, Tapert, & Frank, 2008).

The prefrontal cortex is another brain region that undergoes structural and functional changes during adolescence and is especially vulnerable to damage caused by alcohol during this time (Crews & Boettiger, 2009; Oscar-Berman & Marinkovic, 2007). In fact, adolescents with an alcohol use disorder have smaller overall prefrontal cortex and prefrontal cortex white matter volumes than controls (De Bellis et al., 2005). These results are consistent with those of Medina et al. (2008) who also showed lowered prefrontal cortex volumes in adolescents with alcohol use disorders. The prefrontal cortex is responsible for executive function – abstract thinking, complex cognition, inhibition, attention, and the ability to carry out planned or goal-directed behaviors (Crews & Boettiger, 2009). Parada et al. (2012) showed that human adolescents with a history of binge drinking perform worse on tests of
executive functioning than controls, as measured by the Backward Digit Span Test and the Self Ordered Pointing Test (SOPT). Both these tests require manipulation of information in working memory, which is dependent on prefrontal cortex function (Parada et al., 2012). Adolescent binge drinkers are also impaired in sustained attention tasks and memory recall when compared to controls (Hartley, Elsabagh, & File, 2004). Similarly, rats exposed to intermittent ethanol (5.0 g/kg, 2-day on/2-day off schedule) from PD 25 – PD 55 and tested in the Barnes maze for spatial and reversal learning during early adulthood (PD 64-75) (Vetreno & Crews, 2012) exhibit deficits in reversal learning, suggesting prefrontal cortex damage.

Alcohol exposed animals also show impairments in both contextual (hippocampus-dependent) and trace fear (hippocampus and prefrontal cortex dependent) conditioning. Contextual fear conditioning is a paradigm in which subjects learn that a certain context is associated with an aversive stimulus. Research has shown that animals exposed to an alcohol dose of 4 g/kg/day from PD 4-9 or above exhibit deficits in context conditioning (Murawski & Stanton, 2011). However, animals exposed to an alcohol dose of 2.75 g/kg/day do not exhibit deficits in context conditioning. Yttri, Burk and Hunt (2004) demonstrated that rats administered ethanol on PD 28, 30, 32 and 34 showed deficits in trace fear conditioning during adulthood if the ethanol dose was equal to or exceeded 2.5 g/kg/day. Animals exposed to lower levels of ethanol on the same days did not show trace conditioning deficits. However, there was no effect of any level of adolescent ethanol exposure on delay conditioned responding, showing that the animals did not have trouble engaging in freezing behavior or trouble associating a US (unconditioned stimulus) with a CS (conditioned stimulus). Unlike delay conditioning, trace fear conditioning specifically requires use of the hippocampus and prefrontal cortex. An impairment in trace conditioning, but not delay conditioning, suggests that the hippocampus and prefrontal cortex in particular may be especially sensitive to alcohol insult during adolescence (Yttri et al., 2004).

Collective evidence suggests that long-lasting damage to the hippocampus and prefrontal cortex related deficits in associated behaviors depend on the combination of alcohol level and duration of exposure. In animal models, alterations in hippocampal-dependent behaviors can be seen following high levels of alcohol administration (3-5 g/kg/day) over short periods of time (4-5 days), or lower levels of alcohol administration
(less than 2.5 g/kg/day) if administered over a longer duration. Lower levels of alcohol administered over a short period of time (less than 2.5 g/kg/day) during adolescence do not seem to result in long-term behavioral and cognitive deficits.

**PURPOSE OF PRESENT STUDY**

The present study will examine whether a model of prenatal alcohol exposure influences the effects of adolescent alcohol exposure on behaviors that depend on the functional integrity of the hippocampus and prefrontal cortex. In this study we will examine how prenatal alcohol exposure affects alcohol’s neuropathological effects on the adolescent brain. In other words, do rats that are administered alcohol in adolescence after previous exposure during early development perform worse on behavioral tasks in adulthood than rats that were administered alcohol during adolescence without prior history of exposure? Are there differences between the two groups in neuropathology after both exposures? These questions are important because the children of mothers who drink during pregnancy may eventually be exposed to alcohol in high school and college. It would be important to know if a history of prenatal alcohol exposure alters the consequences of adolescent alcohol exposure, as understanding risk factors that influence the vulnerability of these brain areas has important implications for adolescents who binge drink.

It is hypothesized that the combination of early alcohol exposure followed by binge-like alcohol exposure during adolescence will result in behavioral deficits that persist through adulthood. The model of prenatal alcohol exposure used in the present study is not expected to result in hippocampus based cognitive deficits. The level of adolescent alcohol exposure is expected to produce only mild learning deficits. However, it is predicted that rats with a history of early alcohol exposure will be more sensitive to alcohol insult to the brain during adolescence, and will exhibit hippocampus and prefrontal cortex -dependent behavioral deficits in early adulthood.
CHAPTER 2

METHODS

EXPERIMENTAL DESIGN

The present study includes a total of 90 Sprague-Dawley rats (approximately 10 per sex/group) in a 2 (neonatal alcohol exposure, control) x 2 (adolescent alcohol exposure, control) x 2 (male, female) design. This design results in a total of 4 treatment groups: (1) no neonatal or adolescent exposure, (2) neonatal exposure, no adolescent exposure, (3) no neonatal exposure adolescent exposure, and (4) both neonatal exposure and adolescent exposure.

NEONATAL ALCOHOL EXPOSURE

All litters were generated at the animal facilities at the Center for Behavioral Teratology. Pairs of adult females and males were placed in separate cages overnight. Seminal plugs indicated pregnancy and gestational day (GD) 0. The pregnant females were each singly housed until pups were born, typically GD 22 (which is also denoted as postnatal day (PD) 0). On PD 1, litters were culled to 8 animals and subjects were randomly assigned to treatment groups resulting in one subject per sex per treatment in each litter. Subjects that received neonatal alcohol exposure were treated daily from PD 4-9, a period of brain development that parallels the third trimester period of development in humans. Subjects that received alcohol were administered 2.5 g/kg/day ethanol (EtOH) (11.39% v/v ethanol concentration in water) in a milk formula via intragastric intubation once a day, followed by one milk feeding 2 hours later. This pattern of exposure was expected to result in BAC’s between 160 – 200 mg/dl (Murawski & Stanton, 2011). Control subjects received sham intubations. Between intubations, pups remained with the dam. On PD 7, each pup was given a paw tattoo, indicating pup identification number and allowing the investigator to be blind to treatment condition during behavioral testing.
ADOLESCENT ALCOHOL EXPOSURE

Subjects that received a subsequent “binge-like” alcohol exposure during adolescence (PD 28 – 42) were administered 4.0 g/kg/day ethanol once a day on a 2 day on / 1 day off schedule throughout adolescence (18.23% v/v ethanol concentration in water) via intubation. This dose was expected to result in blood alcohol concentrations between 200-250 mg / dl. Control subjects received sham intubations of tap water.

BLOOD ALCOHOL CONCENTRATIONS (BACs)

To determine blood alcohol levels, 20 uL of blood were collected via a tail clip 1.5 hours after the second ethanol treatment on PD 6 for determination of peak BACs (mg/dl) during the neonatal exposure period. The same procedure was repeated 1.5 hours after ethanol treatment on PD 35 to determine peak BAC following adolescent exposure. Peak BAC was determined using an Analox analyzer. Blood samples were collected from all subjects, but samples from sham-intubated subjects were discarded.

BEHAVIORAL TESTING

All subjects underwent Morris water maze spatial learning, and trace fear conditioning behavioral tests following the adolescent exposure time period.

MORRIS WATER MAZE

Starting on PD 52, subjects were trained on a spatial learning task, the Morris Water Maze, which is a behavioral task that requires the hippocampus. Examining performance in the water maze allows comparison of hippocampus-based spatial learning and memory between treatment groups. Subjects were placed in a water tank 1.5 meters in diameter (water at 26 C°) in a room equipped with video recording. During the acquisition phase, subjects used visuospatial cues in the room to find a circular escape platform 4 inches in diameter that was submerged underwater. The location of the platform remained the same across training for each subject. Each subject was trained for 4 trials per day (from different start positions) with an inter-trial interval 4-5 minutes for 6 consecutive days. Each trial lasted no more than 60 seconds. If the subject did not find the platform in 60 seconds, they were led to the platform by the experimenter. Subjects were given 10 seconds on the platform after each trial to study its spatial location. Path-length (distance to platform), latency (time to platform),
swimming speed, and heading angle (accuracy in orientation toward the platform) were measured. On day 7, subjects were tested for memory of platform location using a probe trial. The escape platform was removed from the tank and each subject was placed in the water tank for one 60-second trial. The number of passes through the target quadrant (where the platform used to be) and amount of time spent in the target quadrant, and time spent in the target area (the area 3 times the platform diameter where the platform used to be) were measured as an assessment of memory. After probe day, subjects underwent trials with a visible platform for 2 days to test for performance variables. During this testing phase, the platform protruded from the water and was made visible, and visual cues around the room were covered. Each subject was tested for 4 trials per day from the same start position, but the platform position changed for each trial (the order of platform rotation remained the same between days).

**TRACE FEAR CONDITIONING**

Both context and trace fear conditioning occurred over a period of 2 days (PD 63-PD 64). On day 1, subjects were trained to associate a conditioned stimulus (CS), a 10-second, 80-decibel white noise, with an unconditioned stimulus (US), a 2-second 0.6 mA foot shock. The CS and US were separated by a 10 second trace interval. Training occurred in a novel context (Context A). Following a 120-second baseline period, subjects experienced 5 CS-US pairings, with an average inter-trial interval of 120 seconds. On Day 2, subjects were returned to Context A and tested for freezing over a 300-second stimulus free test. This measure served as an indication of the contextual learning. Two hours later, subjects were placed in an alternate context (Context B) to test freezing specific to the CS. Onset of the CS (10-second, 80-decibel white noise) occurred after a 120-second baseline period. The 10-second CS was followed by 120 seconds of stimulus free testing (trace and inter trial interval), and then by a 2-minute continuous CS. This paradigm measured freezing during the baseline period in context B, freezing at onset of CS, freezing to the trace period after the CS, and freezing to the CS itself.

**MEASURING INTOXICATION IN NEONATAL SUBJECTS**

A separate set of subjects was generated to determine level of intoxication in neonatal subjects. This sub-experiment had a 2 (alcohol exposure, control) x 2 (male, female) design.
The righting reflex was conducted an hour and a half following a single acute dose of 2.5g/kg on PD 8. Each subject was placed on a heating pad and allowed to acclimate for one minute. Following acclimation, each subject was flipped over into a supine position. Latency to right was measured using a stopwatch. Subjects were given a total of 2 minutes to successfully right themselves, and each subject was given two righting reflex trials (Thomas, Abou, & Dominguez, 2009).

**DATA ANALYSES**

SPSS software was used to analyze all body weight, blood alcohol level, and behavioral task data. Data for each behavioral task were analyzed separately. Data were analyzed with three between-subjects factors: neonatal ethanol exposure (ethanol or sham), adolescent ethanol exposure (ethanol or sham), and sex (male or female). For Morris water maze, days and trials served as within-subject repeated measures. A univariate ANOVA was used to analyze probe day trials of the Morris Water Maze task. For fear conditioning, 5 training trials on day 1 served as within-subject repeated measures. Context testing data was analyzed using repeated measures ANOVA, and CS Test data was analyzed using a univariate ANOVA.

**EXPECTED RESULTS**

It was predicted that the neonatal exposure paradigm in this study, by itself, should not result in behavioral or cognitive impairments. Subjects receiving only the neonatal exposure were expected to perform similar to controls on all behavioral tasks. However, the adolescent exposure paradigm was expected to result in mild alcohol impairments on all behavioral tasks. Subjects receiving a combination of both exposures were expected to show behavioral task impairments above and beyond any of the other treatment groups.
CHAPTER 3

RESULTS

A total of six subjects were lost over the course of the study due to incorrect intubations. Additional litters were generated to adjust for the loss of subjects. This resulted in an overall N=90. Table 1 shows the final number of subjects per sex per treatment group that were included in the present study.

Table 1. Number of Subjects per Sex per Exposure Group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th># Male Subjects</th>
<th># Female Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Neonatal Exposure Only</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Adolescent Exposure Only</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Neonatal Exposure + Adolescent Exposure</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

NEONATAL BODY WEIGHTS

Neonatal alcohol exposure did not significantly affect body growth. All subjects gained weight throughout the neonatal period, producing a main effect of day (F(5,88)=6600, p<.01). Females weighed less than males during the neonatal period, producing a main effect of sex (F(1,88)=8.6, p<.01). There was also an interaction of day*sex (F(5,88)=3.0, p<.05). Simple effects by day were conducted as follow up analyses, but did not yield any significant effects. Figure 1 shows differences in male and female body weights from PD 4-9. These trajectories continued past the neonatal period from PD 12-21 (see Figure 2). Males gained more weight than females over consecutive days, producing a main effect of sex (F(1,88)=7.3, p<.01), and a day*sex interaction (F(3,88)=4.3, p<.01).

adolescent body weights

All animals gained weight throughout the adolescent period, producing a main effect of day (F(9,747)=6212, p<.01), shown in Figures 3 and 4. Females weighed less than males,
producing a main effect of sex ($F(1,83)=127.9, p<.01$), and the difference between male and female body weights increased with age, resulting in an interaction of day*sex ($F(9,747)=263.1, p<.01$). A day*adolescent exposure interaction was also found ($F(9,747)=4.9, p<.01$). Simple effects analyses by day revealed a significant effect of adolescent ethanol on PD 41 ($F(1,83)=4.0, p<.05$). Figure 3 shows that this effect was likely driven by female subjects. A closer look at the data revealed that two outliers in the female adolescent ethanol group lowered the average for the entire group.
Figure 3. Body weight growth of male and female subjects through the adolescent period.

Figure 4. Male subject path lengths to platform decrease over acquisition days of Morris water maze.

**Blood Alcohol Concentration (BAC)**

There were no differences in BAC between male and female subjects at either developmental age. When collapsed across groups, the average BAC on PD 6 (during neonatal exposure) was $207.0 \pm 4.9$ mg/dl at a dose of 2.5 g/kg/day, and the average BAC on PD 35 (during adolescent exposure) was $207.8 \pm 5.2$ mg/dl at a dose of 4.0 g/kg/day. BACs on PD 35 were analyzed as a 2 (male, female) x 2 (neonatal exposure, sham) to examine whether ethanol exposure during the neonatal period affected BACs during the adolescent period, but no significant effects were found. Thus, a history of ethanol treatment during the
neonatal period did not significantly affect BACs during adolescence. Table 2 shows BACs by exposure group at each exposure period.

**Table 2. Blood Alcohol Concentrations by Exposure Group**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>PD 6</th>
<th>PD 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Neonatal Only</td>
<td>203.0 ± 5.8 mg/dl</td>
<td>N/A</td>
</tr>
<tr>
<td>Adolescent Only</td>
<td>N/A</td>
<td>215.6 ± 7.0 mg/dl</td>
</tr>
<tr>
<td>Neo+Adol</td>
<td>211.0 ± 8.0 mg/dl</td>
<td>200.3 ± 7.6 mg/dl</td>
</tr>
</tbody>
</table>

**MORRIS WATER MAZE**

Subjects underwent training and testing in the Morris water maze as a measure of spatial learning and memory following the adolescent exposure period. Performance over the six acquisition days indicates that the neonatal ethanol exposure alone impaired learning in male, but not female, subjects.

Path length data were collected as a measure of distance to examine how much subjects had to swim before they found the hidden platform. Shorter path lengths to platform over consecutive days indicate learning of platform location. Overall, subjects reduced their path length over successive acquisition days, producing main effects of day (F(5,410)=113.1, p<.01), trial (F(3,246)=44.5, p<.01), and a day*trial interaction (F(15,1230)=2.1, p<.01). Male subjects had shorter path lengths to platform compared to female subjects, producing a main effect of sex (F(1,82)=11.8, p<.01). Among the males, the neonatally alcohol-exposed group had longer path lengths than the adolescent alcohol-exposed groups during the latter half of testing, contributing to a day*trial*adolescent exposure*sex interaction that approached, but failed to reach, significance (F(15,1230) = 1.6, p=.07). Figures 4 and 5 show male and female path length over acquisition.

Latency data were also collected to examine how quickly subjects could find the hidden platform. A similar pattern as path length was seen. Overall, latency to find the platform declined over training days, producing a main effect of day (F(5,410)=93.5, p<.01) and trial (F(3,246) = 51.0, p<.01) and a day*trial interaction (F(15,1230)=2.2, p<.01). Males had shorter latency to platform than females on average, resulting in a main effect of sex.
Figure 5. Female subject path lengths to platform decrease over acquisition days of Morris water maze.

(F(1,82)=12.3, p<.01). A day*trial*sex*adolescent exposure interaction (F(15,1230)=1.9, p<.01) was also found. Due to this interaction, separate analyses were conducted for males and females. An effect of adolescent alcohol exposure reached significance for male subjects (F(1,41)=3.8, p=.05) (see Figure 6), but no significant effects were found in female subjects (see Figure 7). Although the interaction of neonatal and adolescent alcohol exposure failed to reach significance, the main effect of adolescent alcohol exposure was due to the slower latencies of subjects that received alcohol during the neonatal period only. In fact, when the neonatal only group was removed from the analyses, there were no significant differences in acquisition among the adolescent alcohol-exposed subjects and controls.

Because swimming speed can affect subjects’ latency to platform, speed data were also analyzed. Figures 8 and 9 show male and female changes in speed over acquisition days. Overall, subjects decreased their swimming speed over training days, producing a main effect of day (F(5,410)=30.8, p<.01) and trial (F(3,246)=14.1, p<.01) and a day*trial interaction (F(15,1230)=2.6, p<.01), as shown in Figures 8 and 9. Males exposed to alcohol neonatally were slower than females exposed to alcohol neonatally, producing a sex*neonatal exposure interaction (F(1,82)=6.3, p<.05). There were also significant trial *sex (F(3,246)=4.6, p<.01), and day*trial*sex*neonatal exposure interactions (F(15,1230)=1.8, p<.05). These interactions were due to the steeper decline in swimming speed of male subjects exposed to
neonatal alcohol compared to female subjects. In contrast, there was a steeper decline in swimming speed among female subjects that did not receive neonatal alcohol in comparison to male subjects. When daily swimming speeds were analyzed for males and females separately, the only significant effect of neonatal alcohol exposure was on day 4 within male...
Figure 8. Male subject swimming speeds decrease over acquisition days of Morris water maze.

Figure 9. Female subject swimming speeds decrease over acquisition days of Morris water maze.

Subjects that received neonatal alcohol (the neonatal exposure only group and the combination group) swam slower than subjects that received sham intubations neonatally.

Heading angle was measured throughout acquisition as a measure of accuracy in orientation toward the platform, an indication of subjects’ knowledge of platform location.
Analyses of heading angle revealed a main effect of day (F(5,410)=7.0, p<.01) and trial (F(3,246)=9.3, p<.01), and a day*trial interaction (F(15,1230)=3.1, p<.01). Males improved in accuracy over consecutive acquisition days but females did not, producing a main effect of sex (F(1,82)=8.0, p<.01) and a day*sex interaction (F(5,410)=6.3, p<.01) (see Figures 10 and 11). A main effect of adolescent exposure (F(1,82)=4.8, p<.05), a sex*neonatal*adolescent exposure interaction (F(1,82)=5.7, p<.05) and a trial*sex*neonatal exposure interaction (F(3,246)=2.8, p<.05) were also found. Because of these interactions, follow up analyses were conducted for males and females separately. No effects were seen for female subjects (seen in Figure 11). Among male subjects, a main effect of adolescent exposure (F(1,41)=4.5, p<.05) as well as a neonatal exposure*adolescent exposure interaction (F(1,41)=7.7, p<.01) and a trial*neonatal exposure interaction (F(3,123)=2.9, p<.05) were found. In male subjects, the combination exposure group had significantly smaller heading angles than the neonatal exposure only group, as confirmed by a Student-Newman Keul’s post hoc test (p<.05). However, neither the neonatal only group nor the combined exposure group performed significantly different from controls. The adolescent alcohol exposure only group performed similarly to control subjects in heading angle throughout acquisition.

![Figure 10. Changes in male subject heading angles over acquisition days of Morris water maze.](image-url)
Figure 11. Female subject heading angles do not change much over acquisition days of Morris water maze.

Accuracy and path length to find the platform can be affected by the subjects’ search strategy. Initially, when subjects are first placed into the tank, they swim around the perimeter close to the wall of the maze, a behavior referred to as thigmotaxis. Thigmotaxis can also indicate anxiety levels, as subjects that are stressed stay close to the maze wall whereas others may be more likely to venture into the center of the water tank.

Analysis of thigmotaxis data revealed that subjects spent the highest percentage of time in thigmotaxis over the first few days of training. Subjects spent less time in thigmotaxis over subsequent training days, producing a main effect of day (F(5,410)=41.2, p<.01), a main effect of trial (F(3,246)=91.0, p<.01), and a day*trial interaction (F(15,1230)=16.7, p<.01), as shown in Figures 12 and 13. Females spent more percent time swimming around the perimeter than males and showed less reduction in thigmotaxis over training days, resulting in a main effect of sex (F(1,82)=13.4, p<.01), a day*sex interaction (F(5,410)=5.1, p<.01), and a day*trial*sex interaction (F(15,1230)=1.9, p<.05). Although there were no effects of ethanol exposure in the overall analysis, males in the neonatal exposure only group did not show much of a decrease in thigmotaxis behavior, whereas males in the combination exposure group did. This pattern is similar to differences in male subject heading angles. This can be more easily seen when a difference in percent time spent in thigmotaxis on day 6 in compared to day 1 is presented (see Figure 14). An analysis of thigmotaxis data from
Figure 12. Male subjects in the combination exposure group show greater reductions in thigmotaxis behavior over acquisition days than male subjects in the neonatal exposure only group.

Figure 13. Female subjects show small reductions in thigmotaxis over acquisition days of Morris water maze.
days 3-6 was conducted for male subjects to investigate this divergence, and a neonatal exposure*adolescent exposure interaction was found (F(1,41)=4.5, p<.05). A Student-Newman Keul’s post-hoc analysis confirmed that this interaction was due to the maintenance of higher thigmotaxis behavior in the neonatal only group and the marked decrease in thigmotaxis behavior in the combination exposure group (p<.05). Thigmotaxis of control and adolescent exposure only subjects was intermediate, and did not differ significantly from other groups. Similar effects were found when data for percent of path length traveled close to the perimeter were analyzed.

A probe trial is a way to test spatial memory, as the platform is removed and subjects are allowed to swim for a 60 sec trial. On probe day, there were no differences by sex or treatment group on time spent in target quadrant, time spent in target area, or number of passes through the target (see Figures 15, 16, and 17). Analysis of heading angle on probe day also did not reveal differences between sex or exposure group. Figure 18 indicates that the neonatal ethanol group showed poorer accuracy on probe day when compared to other groups, but this effect did not reach statistical significance. The combination exposure group performed similarly to controls and the adolescent ethanol only group in accuracy on probe day. Thigmotaxis behavior on the probe trial did not differ by exposure group or sex (Figure 19).
Figure 15. Time spent in each quadrant during the probe trial.

Figure 16. Time spent in target area during the probe trial.
Figure 17. Average passes through the target during probe testing.

Figure 18. Average heading angle during the probe trial.
Figure 19. Average percent time spent in thigmotaxis during the probe trial.

VISIBLE PLATFORM

During visible platform, path length, latency, speed, and heading angle were measured. Subjects improved their path length over days and trials, producing a main effect of day (F(1, 82) = 74.8, p < .01) and trial (F(2, 346) = 2.7, p < .05) and a day*trial interaction (F(3, 246) = 2.7, p < .05). Female subjects had longer path lengths to platform on average, resulting in a main effect of sex (F(1, 82) = 11.0, p < .01). Figure 20 shows these data.

Similar to path length, subjects improved in latency over days and trials, producing a main effect of day (F(1, 82) = 116.3, p < .01) and trial (F(3, 246) = 8.8, p < .01) and a day*trial interaction.
interaction ($F(3,246)=3.4, p<.05$). Female subjects had longer latencies than male subjects on average, producing a main effect of sex ($F(1,82)=6.9, p<.05$). Figure 21 shows these data.

![Visible Platform Latency: Males](image1)

**Figure 21.** Subject latencies were lower on the second day of visible platform.

Subjects also increased their speed on average over visible platform, resulting in a main effect of day ($F(1,82)=47.3, p<.01$) and trial ($F(3,246)=18.3, p<.01$) and a day*trial interaction ($F(3,246)=3.0, p<.05$), as seen in Figure 22. A sex*neonatal exposure interaction was also found ($F(1,82)=4.7, p<.05$). Follow up analyses were conducted for males and females separately. Male subjects that received ethanol during the neonatal period (the neonatal exposure only group and the combination group) swam slower than subjects that received sham intubations neonatally (see Figure 22), producing a main effect of neonatal alcohol exposure ($F(1,41)=4.0, p=.05$).

![Visible Platform Speed: Males](image2)

**Figure 22.** Subject speeds increase by the second day of visible platform.

Subjects showed changes in heading angle over days and trials, producing a main effect of day ($F(1,82)=8.8, p<.01$) and trial ($F(3,246)=11.0, p<.01$), as seen in Figure 23. A day*sex interaction ($F(1,82)=6.4, p<.05$) and a day*sex*adolescent exposure interaction
Simple effects analyses of day were subsequently conducted, revealing an adolescent exposure effect in male subjects on day two ($F(1,41)=4.8$, $p<.05$). Male subjects that received alcohol during adolescence (the adolescent ethanol only group and the combination group) showed improved heading angle on day two of visible platform in comparison to subjects that received sham intubations during adolescence (see Figure 23).

**TRACE FEAR CONDITIONING**

Subjects showed the lowest levels of freezing during the baseline period of the training session. Males were less active than females during the baseline, producing a main effect of sex ($F(1,82)=5.8$, $p<.05$), as shown in Figures 24 and 25. Subjects froze more over subsequent trials during training (each set of CS, Trace, and ITI is a trial) and had the lowest freezing during each CS and highest freezing during each trace period, producing a main effect of trial ($F(4,328)=53.1$, $p<.01$) and event ($F(2,164)=121.1$, $p<.01$) and a trial*event interaction ($F(8,656)=10.6$, $p<.01$). Subjects that received neonatal alcohol exposure (the neonatal only group and the combination group) froze less than others over subsequent trials, producing a trial*neonatal exposure interaction ($F(4,328)=3.2$, $p<.05$). Simple effects analyses were conducted for each trial separately as a follow-up, revealing that, overall, neonatally alcohol-exposed groups exhibited slower acquisition, with lower freezing during trial 3 ($F(1,82)=4.5$, $p<.05$) and trial 4 ($F(1,82)=3.9$, $p=.05$). Additionally, an event*neonatal exposure*sex interaction ($F(2,164)=4.5$, $p<.05$) and an event*neonatal exposure*adolescent exposure interaction ($F(2,164)=3.9$, $p<.05$) were found in the overall analysis. Analyses of
Figure 24. Changes in male subject freezing levels during each CS (tone), TR (trace), and ITI (Inter-trial interval) during the trace training paradigm.

Figure 25. Changes in female subject freezing levels during each CS (tone), TR (trace), and ITI (Inter-trial interval) during the trace training paradigm.
CS trials, trace trials, and ITI trials were conducted separately to further examine the event*neonatal*adolescent exposure interaction found in the overall analysis. Subject freezing levels increased over five CS administrations, producing a main effect of CS trial (F(4,328)=13.1, p<.01). A CS trial* sex*adolescent exposure interaction also approached significance (F(4,328)=2.2, p=.06), as female subjects that received alcohol during the adolescent period had lower levels of freezing than other subjects during CS trial 4 (see Figure 31, pg. 34). Similar to CS trial freezing, subjects also increased freezing level through subsequent trace trials, producing a main effect of trace trial (F(4,328)=38.1, p<.01). A trace trial* neonatal exposure effect was also significant (F(4,328)=2.4, p=.05). Simple effect analyses of each trace trial were conducted as follow up due to this interaction. Subjects that received alcohol during the neonatal period froze less than subjects that received sham intubations during the neonatal period. The neonatal exposure effect reached statistical significance during trace trial 3 (F(1,82)=4.3, p<.05). Subjects also increased freezing levels over subsequent inter-trial intervals, producing a main effect of inter-trial interval (F(4,328)=28.4, p<.01). Although subjects that received alcohol neonatally had lower freezing during the ITI, this effect failed to reach statistical significance (F(1,82)=3.5, p=.07). Figures 24 and 25 show male and female freezing over the entire training session. In sum, the slower acquisition of a learned fear response following neonatal exposure was most robust during the trace period, producing the higher order interactions.

During the context test, subjects showed increasing levels of freezing over the first 4 minutes and decreased freezing in minute 5, producing a main effect of time (F(4,328)=13.94, p<.01). Male subjects had higher levels of freezing on average than female subjects throughout the session, producing a main effect of sex (F(1,82)=9.3, p<.01). Figure 26 shows male subject freezing during the context test. Notably, female subjects exposed to alcohol during the neonatal period showed no increase in freezing during the context test (see Figure 27). The difference in freezing between minute 4 and minute was analyzed, but no statistically significant group effects were found.

During the CS test, subjects had the lowest levels of freezing during the baseline period. Male subjects froze more on average than female subjects during the baseline, producing a main effect of sex (F(1,82)=8.5, p<.01). Subject freezing levels remained low during the discrete CS, but increased sharply during the trace period. During the trace period,
Figure 26. Changes in male subject freezing levels over the five minutes of the context test.

Figure 27. Changes in female subject freezing levels over the five minutes of the context test.
a sex*adolescent exposure interaction was found (F(1,82)=5.6, p<.01). Analyses were conducted separately for males and females as follow-up. Female subjects that received alcohol during the adolescent time period (adolescent only and combination group), froze significantly more than other groups during the trace period, producing a main effect of adolescent exposure in female subjects (F(1,41)=8.4, p<.01). Subject freezing continued to increase during the inter-trial interval, but dropped sharply during the continuous tone presentation. During the continuous tone presentation, male subjects had higher freezing levels on average than female subjects, producing a main effect of sex (F(1,82)=8.3, p<.01). Figures 28 and 29 show male and female freezing during the CS Test.

![Figure 28. Changes in freezing levels of male subjects in response to events during the CS Test.](image)

**BRAIN WEIGHTS**

Total brain weights, forebrain weights, cerebellum weights, and brain stem weights were collected and analyzed. Data were not collected from 5 subjects due to problems with equipment. There were no differences in body weights among exposure groups on the day of brain collection. Male subjects weighed more than female subjects on the day of brain collection, producing a main effect of sex (F(1,78)=434.7, p<.01).

Subjects that received alcohol during the neonatal time period had lower total brain weights than subjects that received sham intubations during the neonatal time period.
producing a main effect of neonatal treatment ($F(1,78)=8.2, p<.01$). A main effect of sex ($F(1,78)=70.1, p<.01$) was also seen. Male subjects had greater average total brain weights in comparison to female subjects (see Figure 30).

Subjects that received alcohol during the neonatal time period had lower forebrain weight on average than subjects that sham intubations during the neonatal time period, producing an effect of neonatal treatment ($F(1,78)=8.9, p<.01$). A main effect of sex ($F(1,78)=63.6, p<.01$) was also found, confirming that male subjects had greater average forebrain weights than females (see Figure 31).
Similar to total brain weight and forebrain weights, subjects that received alcohol during the neonatal time period had lower cerebellum weights on average than subjects that received sham intubations during the neonatal time period, producing an effect of neonatal treatment \((F(1,77)=8.3, p<.01)\). Again, a main effect of sex \((F(1,77)=19.3, p<.01)\) was also seen, confirming that male subjects had higher average cerebellum weights than female subjects (see Figure 32).

To determine the level of intoxication in neonatal pups, a righting reflex test was conducted an hour and a half following a single 2.5 g/kg dose of ethanol. Analysis of the righting reflex test revealed that subjects that received ethanol had much longer righting latencies on average than sham intubated subjects \((F(1,29)=31.1, p<.01)\). Subjects had an average BAC of 177.1 ± 7.9 mg/dl. Two blood samples were excluded as they were inaccurate due to Analox calibration problems. Out of 17 ethanol exposed subjects, 10 subjects failed to right themselves in the allotted 2 minutes, and 7 subjects righted themselves.
in under a minute. Two outliers were able to right themselves in less than 10 seconds. Average righting reflex time for alcohol exposed subjects was 78.3 ± 12.6 seconds. In contrast, subjects that received sham intubations were consistently able to right themselves in 5 seconds or less, with an average righting reflex latency of 3.3 ± 0.4 seconds. These data are shown in Figure 33.

![Righting Reflex: Latency to Right](image_url)

**Figure 33.** Latency to right during the righting reflex test in ethanol exposed and control subjects.
CHAPTER 4

DISCUSSION

The mechanisms by which alcohol can affect the developing brain are varied and complex. Alcohol is known to have widespread impact on neuronal migration, neurogenesis, neural connectivity, and receptor function (Eckstrand et al., 2012; Valenzuela et al., 2012). Research has consistently shown that neonatal alcohol exposure can be particularly damaging to the developing brain, resulting in a range of problems including impairments in learning and memory, and disruptions in emotional regulation (Riley et al., 2011; Streissguth et al., 1978). Additionally, research has suggested that alcohol exposure during adolescence may cause further damage to brain areas that are sensitive during development (Nixon & McClain, 2010). However, there has also been evidence showing that the adolescent brain can be quite resilient to negative effects of alcohol (Brown et al., 2008; Nixon & McClain, 2010; Spear & Varlinskaya, 2005). It remains unclear how a history of early alcohol exposure would change the effects of alcohol exposure during adolescence. The present study examined effects of both exposure periods alone along with the effects of a combination of exposures. The present results show that the effects of alcohol on learning and memory task performance depend on the developmental time period at which it is administered. Additionally, many of the alcohol-related effects were sex-specific.

In the present study, the neonatal ethanol group showed impairments in most learning and memory domains. Male subjects exposed to alcohol during the neonatal period showed subtle increases in latency and longer path lengths to platform during the acquisition phase of Morris water maze, as well as decreased accuracy during both training and testing. They also spent the most percent time and percent path length displaying thigmotaxis behavior during acquisition, indicating a difference in search strategy in comparison to the combination group, though they did not differ from controls. High levels of thigmotaxis may also indicate heightened anxiety levels, which could lead to poorer performance. Research has shown that, in addition to cognitive deficits, prenatal alcohol exposure results in long-term changes in the stress response through alteration of the hypothalamic pituitary axis (HPA-axis), although the
types of change are often sexually dimorphic (Weinberg, 1992, 1998; Weinberg, Siliowska, Lan, & Hellemans, 2008).

In fear conditioning, neonatally alcohol-exposed subjects (the combination and neonatal only group) showed slower learning during training. Females within the neonatal only alcohol group also showed low contextual freezing during fear conditioning, though this effect did not reach statistical significance. However, the neonatal exposure group showed freezing levels comparable to the control group during the CS Test, indicating that they did form an associative memory between the tone and the shock. The present results suggest that neonatally alcohol-exposed subjects are slower to learn associations. The pattern of behavior observed is consistent with research showing contextual learning impairments in neonatally alcohol exposed rats. Contextual impairment has been shown following high neonatal alcohol doses (5.25 g/kg/day resulting in BACs of 414±13.4) but not at lower doses comparable to the present study (2.75g/kg/day resulting in BACs of) (Dokovna, Jablonski, & Stanton, 2013; Jablonski & Stanton, 2014; Murawski & Stanton, 2011). Lower doses and BACs in the present study probably account for failure of a contextual learning impairment to reach significance in this study.

It is notable that the adolescent alcohol exposure group performed similarly to control subjects in both behavioral tasks. This was particularly apparent in Morris water maze, where acquisition and probe testing of the adolescent only exposure group was similar to that of controls. In fear conditioning, the adolescent alcohol group displayed freezing levels that were very similar to the control group during training as well as during the context test. During the CS test however, the adolescent alcohol group, like the combination group, actually displayed significantly higher levels of freezing in comparison to the control group in female subjects. Collectively, the data indicate that alcohol exposure during the adolescent time period alone (average BAC of 200 mg/dl) did not cause impairments in learning and memory performance in the tasks used in this study. Animal studies and clinical research have showed that binge-like adolescent alcohol exposure causes damage to the hippocampus and prefrontal cortex, often resulting in learning and memory deficits (Ehlers et al., 2013; Sircar & Sircar, 2005; Weissenborn & Duka, 2003; Yttri et al., 2004). However, in comparison to previous studies that tested spatial learning, the adolescent alcohol dose given in this study was lower, and only mild impairments were originally expected.
Crews (2012) showed that a dose of 5 g/kg/day (heavier than the dose given in the present study) over a longer duration (PD 25-55) resulting in BACs of 165±17 mg/dl did not cause spatial learning deficits. Schulteis et al. (2008) showed that Morris water maze deficits were seen in subjects with BACs of 241±7.5 mg/dl (greater than the BACs reached in the present study). Given that the adolescent BACs in the present study reached just about 200 mg/dl on average, it is not surprising that the adolescent exposure did not produce spatial learning impairments. Yttri et al. (2004) demonstrated that rats administered ethanol during adolescence showed deficits in trace fear conditioning during adulthood if the ethanol dose was equal to or exceeded 2.5 g/kg/day. However, this study did not report BACs, and it is unclear why the adolescent exposure alone in the present study did not cause trace conditioning impairments.

Most strikingly, with one exception, the combination of alcohol exposure during both developmental periods did not significantly alter the pattern of effects of either alcohol exposure period alone. Like the neonatal alcohol-exposed group, the combination group also showed learning impairments in fear conditioning acquisition, but displayed unique behaviors in the Morris water maze. Male subjects in this group displayed enhanced accuracy in heading angle over consecutive training days when compared to the neonatal exposure only group. Although enhanced accuracy might suggest improved spatial memory, this group did not show shorter path lengths or latencies during acquisition or improved performance on probe day and, in fact, performed similarly to controls on these measures. Interestingly, the combined alcohol group also displayed the lowest levels of thigmotaxis through acquisition (they were more willing to enter the center of the tank), suggesting that their improved accuracy may be related to their greater willingness to enter the center of the Morris water maze. The pattern of lowered anxiety is supported by research showing that animals exposed to alcohol during adolescence exhibit reduced anxiety and increased exploratory behavior in the elevated plus maze (Gass et al., 2014) and in an open field task (Ehlers et al., 2013). Like prenatal alcohol exposure, adolescent alcohol exposure has also been shown to alter stress regulation both through actions on the HPA axis and through interactions with hormones (Logrip et al., 2013; Ogilvie & Rivier, 1997). It is possible that changes caused by the neonatal alcohol exposure somehow facilitate decreases in stress response following adolescent exposure.
The behavioral alterations are related to brain pathology. While spatial learning is mostly hippocampus dependent, trace fear conditioning requires both the hippocampus and the prefrontal cortex. Neonatal alcohol exposure produced more robust impairments in trace conditioning compared to spatial learning, suggesting that neonatal alcohol impacted prefrontal cortex functioning more than the hippocampus. It is possible that alcohol exposure is also affecting performance on learning and memory by altering stress response or emotional regulation and that alcohol-related dysfunction in the amygdala or HPA axis contribute to the cognitive deficits.

The present study only examined gross brain changes, but shows that neonatal ethanol exposure results in lowered total brain, forebrain, and cerebellum weights (evident in both the neonatal alcohol group and the combination group). Thus, although the alcohol exposure level was selected to not produce severe pathology, it is evident that blood alcohol levels of 200 mg/dl during the neonatal period can produce gross neuropathology. The present study focused on behaviors that depend on the functional integrity of the prefrontal cortex and hippocampus. Lowered forebrain volume may reflect prefrontal cortex dysfunction. The existing literature has shown that both neonatal alcohol exposure and adolescent alcohol exposure result in lower volume of various brain regions (Eckstrand et al., 2012; Mattson et al., 2001; Pascual et al., 2007). In the present study, adolescent alcohol exposure alone did not reduce total brain weight or frontal cortex weight. Research on adolescents has shown cell death and reduced neurogenesis specifically in the hippocampus (Ehlers et al., 2013; Morris et al., 2010; Pascual et al., 2007) and the prefrontal cortex (De Bellis et al., 2005; Medina et al., 2008). However, the brain weight data collected in the present study did not examine specific brain regions known to be affected by alcohol exposure (such as the hippocampus and prefrontal cortex), and thus may have failed to capture differences.

Throughout the study, alcohol exposure seems to have different effects on male and female subjects. This may be due to sex differences in development. For example, differing hormonal changes during the adolescent period affect brain development, and may also affect alcohol’s actions on the brain. Ogilvie and Rivier (1997) showed that in adult animals (approximately 60 days of age) androgens inhibited stress response in male subjects, but estrogen elevated stress response in female subjects. Research has also shown that the estrus
cycle in female rats can have a significant impact on anxiety levels (which may affect performance in learning and memory tasks) (Sayin, Derinöz, Yüksel, Şahin, & Bolay, 2014). Thus, hormonal differences between males and females may have produced sex-specific behavioral patterns observed in the present study.

Considering the results from the present study, it may be important to examine whether exposure during different developmental periods results in physical changes in the hippocampus and prefrontal cortex that could explain the observed behaviors. Measures of anxiety and stress following alcohol exposure after both the neonatal period and the adolescent period would also be of interest, as stress can impact performance on learning and memory tasks. For example, measuring cortisol levels in conjunction with behavioral tests could shed light on how stress levels affect performance. Testing in the elevated plus maze task could show differences in anxiety and exploratory behavior between exposure groups. Tracking the estrous cycle in female subjects during adolescence may also be informative, as hormones can affect performance on behavioral tasks. Additionally, it is possible that changing the doses given neonatally and during adolescence might change the pattern of behavior. Future studies will be necessary to answer some of these questions and shed more light on how alcohol exposure affects behavior.

It is important to note that it may be challenging to directly relate the findings of an animal study to clinical populations. Nevertheless, animal studies can provide insight about different domains of functioning that can be impacted by alcohol exposure. Overall, the present study indicates that alcohol exposure at 200 mg/dl/day during the 3rd trimester equivalent, but not during the adolescent period, produces gross brain pathology and results in impairments in learning and memory, particularly in trace fear conditioning. Although it was hypothesized that neonatal alcohol exposure would also exacerbate the cognitive effects of subsequent adolescent alcohol exposure, this hypothesis was not supported - at least not with the alcohol exposure parameters used in the current study. However, prenatal alcohol exposure may affect emotional consequences of adolescent alcohol exposure. Given that emotional regulation affects many behavioral domains and is a critical aspect of leading a functional life, it is imperative that we fully understand the effects of drinking alcohol during adolescence among individuals with a history of prenatal alcohol exposure.
REFERENCES


McQueeny, T., Schweinsburg, B. C., Schweinsburg, A. D., Jacobus, J., Bava, S., Frank, L. R., & Tapert, S. F. (2009). Altered white matter integrity in adolescent binge drinkers.


