Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

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Entitled
Effects of Coadministration of D-Napvsipq [NAP] and D-Sallrsipa [SAL] on Spatial Learning After Developmental Alcohol Exposure

For the degree of Doctor of Philosophy

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EFFECTS OF COADMINISTRATION OF D-NAPVSIPQ [NAP] AND D-SALLRSIPA [SAL] ON SPATIAL LEARNING AFTER DEVELOPMENTAL ALCOHOL EXPOSURE

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Jennifer L. Wagner

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

December 2013

Purdue University

West Lafayette, Indiana

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ABSTRACT

Wagner, Jennifer L. Ph.D., Purdue University, December 2013. Effects of Coadministration of D-Napvsipq [NAP] and D-Sallrsipa [SAL] on Spatial Learning After Developmental Alcohol Exposure. Major Professor: Charles Goodlett.

Despite warnings about the dangers of drinking during pregnancy, little progress has been made in reducing alcohol drinking among women of childbearing age. Even after the recognition of pregnancy, 15% of women continue to drink, 3% of which admit to binge drinking. Because we cannot stop women from drinking during pregnancy, and many children with fetal alcohol spectrum disorders (FASD) are adopted, there is a significant need to develop postnatal interventions that can improve the long-term outcome of children adversely affected by prenatal alcohol exposure. This thesis aims to evaluate one promising new treatment in the rehabilitation or rescue of specific learning deficits long after the damage has occurred.

The treatment evaluated herein (40µg D-NAP + 40µg D-SAL) has long been used in the prevention of the detrimental effects of long-term and binge-like alcohol exposures in rodent models of fetal alcohol syndrome and FASD. Until recently this peptide treatment had only been shown to be effective in preventing some of the consequences of alcohol exposure when administered concurrently with the prenatal alcohol exposure. A recent report by Incerti and colleagues (2010c), however, reported that these peptides could

completely reverse a profound spatial learning deficit induced by one episode of a heavy binge-like alcohol exposure (5.9g.kg in a single intraperitoneal injection) on gestational day 8 (G8) in C57BL/6 mice. In that report, the peptide treatment was administered starting in late adolescence, beginning three days prior to and throughout water maze training, and the profound deficits in their alcohol-placebo group were completely eliminated in the alcohol-peptide group. There are currently no FDA-approved treatments for FASD. An effective treatment for the cognitive and behavioral dysfunctions suffered by the 1% of people born today could potentially improve the lives of millions of children and adults.

The first aim of this thesis was to determine whether the peptide treatment could reverse the significant spatial learning deficits we have demonstrated in adult C57BL/6 mice given high-dose binge-like alcohol exposure (2.5 g/kg in each of two intraperitoneal injections separated by two hours) on postnatal day (P)7. When administered three days prior to and throughout water maze testing (P67-76), the peptide treatment had no effect on spatial learning.

The second aim sought to determine whether the same peptide treatment could reverse water maze spatial learning deficits in G8 binge-like exposure models, as reported by Incerti et al. (2010c). For this analysis, the first study used a different binge-like alcohol exposure model that is more commonly used than that employed by the Incerti et al. (2010c) study, namely administration of 2.8g/kg in each of two intraperitoneal injections separated by four hours (Sulik et al., 1981). This model has been shown to produce high

peak blood alcohol concentrations and neuroanatomical aberrations in the hippocampal formation and septal regions (Parnell et al., 2009), which have been implicated in learning and memory. Surprisingly, this G8 binge-like alcohol exposure failed to produce a spatial learning deficit, undermining the usefulness of this model in evaluating the peptide effects. In direct contrast to the outcomes of Incerti et al. (2010c), the G8 Webster alcohol exposure was also unable to produce any deficits in acquisition of spatial learning in the Morris water maze.

Surprisingly, neither of the heavy binge-like alcohol exposures on G8 were able to produce spatial learning deficits in the Morris water maze. The binge-like alcohol exposure on P7 did yield the expected spatial learning deficit, but the peptide treatment was unsuccessful in recovering water maze learning. These findings fail to support oral administration of 40µg D-NAP and 40 µg D-SAL as a potential therapy for postnatal alcohol-induced spatial learning deficits in adult mice.

CHAPTER 1 INTRODUCTION

1.1 Introduction

Despite 50 years of research documenting the consequences of gestational alcohol exposure, there are currently no FDA-approved treatments for fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorder (FASD). Therefore, the search for therapeutic interventions for the cognitive and behavioral deficits associated with gestational alcohol exposure is a priority (Warren and Foudin, 1994).

In animal model studies, the neuropeptides D-NAPVSIPQ and D-SALLRSIPA, referred to hereafter as D-NAP and D-SAL, respectively, have been shown to be effective in both the prevention and recovery of gestational alcohol-induced impairments in fetal growth and development when administered concurrently (Zhou et al., 2008, Incerti et al., 2010a, Spong, 2006, Spong et al., 2001). In addition, offspring given heavy binge alcohol on gestational day 8 (G8) then given oral peptide treatment administered three days prior to and overlapped with behavioral testing in late adolescence completely recovered spatial learning deficits (Incerti et al., 2010c). Despite the growing reports on the efficacy of the combined peptide treatment on different aspects of alcohol-induced perturbations of neural development (Sari et al., 2013, Sari et al., 2011, Sari, 2009), the mechanisms by which these treatments benefit alcohol-affected offspring remains elusive. The goal of the

present study was to determine whether the efficacy of the peptide treatment was specific to the particular timing of the alcohol insult (e.g. gestational vs. neonatal exposure).

The studies outlined in this thesis will attempt to answer whether or not combined administration of the peptides D-NAP and D-SAL can reverse spatial learning deficits in the adult mouse induced by developmental alcohol insults. Specifically, two separate time periods in human gestation were targeted; the fourth week of human gestation (G8 in mice) and the later portion of the third trimester (roughly 34 weeks in humans or P7 in mice).

1.2 Fetal Alcohol Spectrum Disorder

1.2.1 Background

Maternal alcohol use has long been associated with negative neurobehavioral deviations from normal human development. One historical account of the evils of drinking during pregnancy comes from the Old Testament, "[T]hou shalt conceive and bear a son. Now therefore beware, I pray thee, and drink not wine nor strong drink and eat not any unclean thing (Judges, 13:3-4)." Fetal alcohol syndrome (FAS) was first coined as a term in 1973 when patterns of malformations were noted in 8 unrelated children of alcoholic mothers spanning three different ethnic groups (Jones et al., 1973). However, these same patterns of anomalies were actually published five years earlier in an obscure French journal by a French pediatrician who had compiled over a hundred of cases of children born to alcoholics (Lemoine et al., 1968). Outside the clinic, references to the dangers of consuming alcohol during pregnancy have become commonplace in today's society, with

warnings posted on bottles, in bars, liquor stores and anywhere alcohol is sold across the nation. However, despite significant efforts in publicizing the damage of alcohol drinking during pregnancy and the expansion of public awareness, nearly 15% of women continue to drink after recognition of pregnancy, 3% of which binge drink (NIAAA, 2008).

1.2.2 Fetal Alcohol Spectrum Disorder Epidemiology

For decades, researchers have documented the long-term irreversible neuroanatomical, behavioral and cognitive dysfunctions in children exposed to alcohol during gestation. Yet, it is the distinctive craniofacial dysmorphology (smooth or indistinct philtrum, thin vermillion border, and small palpebral fissures), microcephaly or other central nervous system (CNS) abnormalities, and growth retardation that are required for the clinical diagnosis of FAS, the most severe diagnosis, along the spectrum of FASD (Hoyme et al., 2005). The prevalence of FAS has been estimated to be as high as 0.33 to 2.2 per 1,000 live births in the United States (Abel and Sokol, 1991, May and Gossage, 2001, Stratton et al., 1996), reaching as high as 2-7 per 1,000 within populations of mixed racial and socioeconomic status (May et al., 2009). Lacking the facial dysmorphology required for a diagnosis of FAS does not preclude cognitive and behavioral dysfunction associated with gestational alcohol exposure. Therefore, individuals who were exposed to alcohol during gestation but who fail to meet the criteria FAS, usually because they do not have full facial dysmorphology, may be classified as partial FAS (PFAS), alcohol-related neurodevelopmental disorders (ARND) and alcohol related birth defects (ARBD), which together fall under the umbrella term FASD. As shown in Figure 1, classification as PFAS requires a history of heavy maternal alcohol consumption during pregnancy, two

of the three previously mentioned facial characteristics, small head circumference, and growth retardation or cognitive/behavioral dysfunction. The term ARND is reserved for children with significant cognitive and/or behavioral impairment, but lacking the distinctive craniofacial abnormalities necessary for a classification as FAS or PFAS.

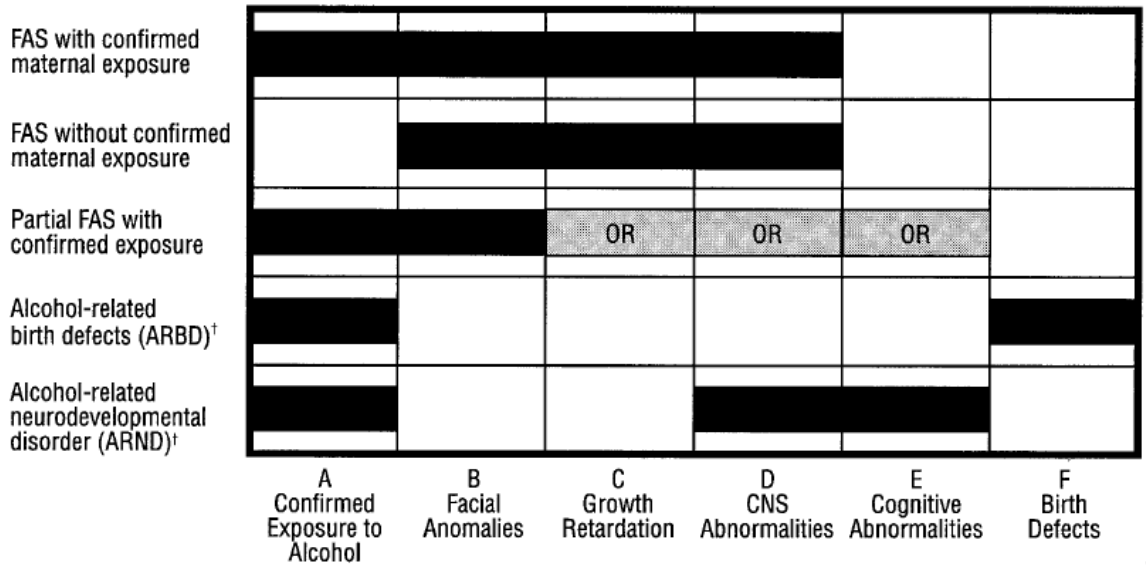

Figure 1.2.1 Graphical representation of the differential diagnostic criteria for Fetal Alcohol Spectrum Disorders. (adapted from Stratton et al., 1996)

The term fetal alcohol spectrum disorder (FASD) was coined as an umbrella term to define the continuum of consequences in children exposed to alcohol during gestation. Many children exposed to alcohol prenatally suffer similar neuropsychological and behavioral deficits, without the facial features necessary for a diagnosis of FAS (Mattson et al., 1998b). As such, the incidence of this more comprehensive category of gestational alcohol-induced impairments was estimated at 9-10 per 1,000 live births here in the US (May and Gossage, 2001, Sampson et al., 1997), but was recently updated to be as high

as 2-5% of younger school children in the US and Western Europe using active case ascertainment methods with in-school populations (May et al., 2009).

1.2.3 Economic Impact of FASD

Roughly 4 million people are born in the US each year (Ventura et al., 2003), and, of those, approximately 40,000 suffer from the neurobehavioral consequences of maternal alcohol consumption during pregnancy. The enormous economic impact caring for an additional 40,000 cognitively impaired individuals each year can have on society is calculated using estimates that factor in health impacts and associated care and services, criminal justice costs, and loss of productivity, as well as indirect factors, such as administrative, policy and research costs to name a few. The annual cost of FAS in the US has been estimated to be between $3.6 and $4 billion (Lupton et al., 2004, Olson et al., 2009). In a Canadian study, Stade and colleagues (2009) reported that the average annual cost per individual with FASD is $21,642, with an annual cost for all individuals with FASD estimated to be $5.3 billion.

1.2.4 Peripheral Clinical Issues Associated with Gestational Alcohol Exposure

Toxicity of alcohol to the developing fetus has effects on other organ systems beyond the central nervous system. Alcohol exposure during the periconceptional period has been associated with an increased risk of skeletal, urogenital and congenital heart defects, specifically conotruncal heart defects (Grewal et al., 2008, Burd et al., 2007, Tsukahara and Kajii, 1988, Pauli and Feldman, 1986), which typically involve the improper separation of the chambers of the heart, or the major blood vessels leaving the heart.

Numerous studies report hepatic, renal and gastrointestinal abnormalities in children exposed to alcohol in utero (for review see: Stratton et al., 1996); however, consistent abnormalities in either of the organ systems have not been identified (Hofer and Burd, 2009). In addition to the distinctive patterns of craniofacial dysmorphology of FAS, children prenatally exposed to alcohol have been found to have an increased incidence of cleft lip, with or without cleft palate (Grewal et al., 2008). While these defects are substantial and detrimental, their incidence is vastly overshadowed by the neuropsychological and behavioral dysfunctions of FASD.

1.2.5 Behavioral Dysfunction Associated with FASD

Gestational alcohol exposure is considered the leading cause of preventable mental retardation. While diminished intellectual capacity is a pronounced sequela of FASD, attentional deficits (Fernandez et al., 1983, Shaywitz et al., 1980, Caul et al., 1979, Landesman-Dwyer et al., 1978, Nanson and Hiscock, 1990, Richardson et al., 2002, Streissguth et al., 1986) and hyperactivity (Mattson and Riley, 1998, Shaywitz et al., 1980, Caul et al., 1979, Streissguth et al., 1978, Kelly et al., 1987b) are also prominent. Hyperactivity is present with such consistency that elevated activity levels have been suggested to be a more accurate phenotypic index of alcohol-induced teratogenicity than the craniofacial dysmorphology required for an FAS diagnosis (Shaywitz et al., 1980, Landesman-Dwyer et al., 1981). Hyperactivity and attentional deficits have been described as being “Hallmark Features of Prenatal Alcohol Exposure” (Mattson and Riley, 1998) and can occur in the absence of obvious intellectual deficits. Thus, it is possible that children diagnosed with disorders of hyperactivity and attention may

actually represent a subset of children with symptoms of FASD that may be under diagnosed (Steinhausen, 1996, Mattson et al., 2013, Ware et al., 2013). Coles (2001) suggests that this misclassification could be due to the conventional attention deficit hyperactivity disorder (ADHD) diagnostic model which utilizes behavioral checklists and parent and teacher ratings to infer attentional dysfunction. However, using standardized tests to evaluate the four factors of attention: sustain, shift, encode and focus; children with FAS or PFAS show a much different behavioral profile than those with ADHD without gestational alcohol exposure (Coles et al., 1997). While ADHD children have severe deficits in focusing and sustaining attention, children with gestational alcohol exposure show deficits in encoding and shifting attention (Coles et al., 1997).

1.2.6 Executive Functioning Deficits Associated with FASD

Children with FASD go mostly unrecognized until challenged in school (Streissguth et al., 1990, Brown et al., 1991). Some deficits, including spatial learning and memory, response inhibition, dyscalcula and psychosocial functioning, have been noted to be more severe in individuals with greater reported prenatal alcohol exposure (O'Connor et al., 1986, Coles et al., 1991, Streissguth et al., 1989, Jacobson et al., 2008, Coles et al., 2009) and have been shown to become more apparent with age (Streissguth et al., 1991, Streissguth et al., 1994). The behavioral profile of children and adults with FAS or FASD includes impairments on experimental tests of executive function such as the Wisconsin Card Sorting and Stroop tasks, Controlled Oral Word Association, Word Context tests and spatial learning and memory as measured by the Trail Making task (Connor et al., 2000, Kodituwakku et al., 1995, Mattson et al., 1999). Deficits in abstract thinking,

cognitive flexibility and working memory measured by the Progressive Planning Test and California Tower Task have further alluded to executive impairment. Verbal learning and auditory memory are often reported to be impaired in children with FASD (Mattson et al., 1999, Olson et al., 1998, Burden et al., 2005, Mattson et al., 1996, Mattson et al., 1998b, Uecker and Nadel, 1998, Hamilton et al., 2003, Kodituwakku et al., 1995, Mattson et al., 1998a, Aragon et al., 2008, Schonfeld et al., 2001). The Delis-Kaplan Executive Function System (DKEFS) is a set of performance-based tasks designed to measure the executive functioning of individuals between the ages of 8 and 89 (Delis et al., 2001). Children with confirmed prenatal alcohol exposure, 60% of which met Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for ADHD, were found to be impaired on all measures of executive function measured by the DKEFS, including the Verbal Fluency-Switching, Design Fluency-Switching, Trail Making Test-Switching, and Color-Word Interference-Inhibition/Switching tasks. Interestingly, subjects with confirmed alcohol exposure were not found to be different than children with ADHD on these tasks (Ware et al., 2012). Deficits in executive functioning tasks, collectively, illustrate impairments in higher-order cognitive functioning governed by the prefrontal cortex. Impairments in attentional set shifting, planning and visual motor integration often lead to difficulty processing complex, hierarchical stimuli impairing higher-order cognitive functioning especially in this population (Kodituwakku, 2007). This underscores the importance of advancing the understanding of the influence of developmental alcohol exposure on higher-order executive functions that impact the quality of daily life in those with FASD.

A common tool used to evaluate domains of daily functioning, communication, social and motor skills is the Vineland Adaptive Behavioral Scale (VABS). The VABS is a parent/caregiver rating form in which behavioral questions are assessed as never, sometimes or partially, and usually occurring; and scored as 0-2 respectively. Adults with FAS assessed with the VABS have been found to show impairment in normal maturation of social skills (Streissguth et al., 1991, LaDue et al., 1992). Adolescent children with FASD have also been found to display more aggression and have more externalizing problems as reported by teachers, an effect that was more pronounced in children exposed throughout gestation rather than those whose mothers stopped drinking in the third trimester of pregnancy (Brown et al., 1991).

In contrast to measures of executive functioning, adolescents with confirmed alcohol exposure are reported to be significantly more impaired than children with an ADHD diagnosis, but no confirmed alcohol exposure, on the VABS. Some studies have found younger children with confirmed alcohol exposure to be less or not at all impaired on the VABS (Coles et al., 1991), while other studies have found severe deficits in social skills of FAS children using the same scales (Thomas et al., 1998). However, there were major differences in the populations evaluated in these two studies. The study by Thomas and colleagues (1998) evaluated those with confirmed alcohol exposure, but more typical IQ scores, where as Coles and colleagues (1991) evaluated lower socioeconomically-challenged, alcohol-abusing population. Despite the disparate findings using the VABS, anecdotal reports within the literature base lend validity to the idea that social deficits

may become more reliably apparent or more disruptive with the increased social interaction that comes with age.

Children with FAS have been known to make bad decisions resulting in increased probability of delinquency, criminality, and combative behavior, have difficulties in work performance, and demonstrate deficits in response inhibition (Streissguth et al., 1997). Taken together, this information provides insights into social disturbances and response inhibition in children with FASD, suggesting that those affected by FASD may have an underlying increase in impulsivity that may be mediating attentional deficits and learning disabilities.

Despite clinical findings suggesting fetal alcohol exposure leads to problems with impulse control, systematic preclinical studies of the mechanisms of gestational alcohol exposure on impulsive choice behavior have yet to be conducted. These faulty decision-making strategies can lead to losses of productivity in ones working life and add more direct economic costs such as criminal activity and processing through the justice department.

Spatial Learning and Memory

Decades of animal research have led to an extensive literature base describing the alcohol-induced damage to the hippocampal formation and correlated spatial learning deficits (Gianoulakis, 1990, Zimmerberg et al., 1991, Goodlett and Peterson, 1995, Johnson and Goodlett, 2002, Blanchard et al., 1987, Goodlett et al., 1989b). Similarly,

children with FAS are found to be deficient on tasks that involve spatial recall. For example, children shown a book of pictures in sets of four can correctly identify pictures previously seen versus new pictures, but can not remember the spatial relationship of missing pictures to others that were previously shown on the same page (Uecker and Nadel, 1998).

Another task that has been shown to be one of the more sensitive to the effects of prenatal alcohol exposure is the stepping stone task. This task requires children to navigate a matrix of stones to find an invisible path through both a short and long version. Adolescents with FAS have also shown deficits in this more complex spatial learning task that requires both short-term memory and spatial learning. Specifically, children exposed to alcohol gestationally require significantly more trials to reach their first success and make more errors than unexposed controls (Streissguth et al., 1994).

More recently, researchers have adapted a commonly used task to evaluate spatial learning in rodents for use in children and adults. Using a computerized version of the Morris water maze, children are asked to navigate a virtual environment to locate a hidden platform (Hamilton et al., 2002). Over trials, humans learn to navigate direct paths to the hidden platform using distal cues. However, patients with compromised neural circuitry involving the hippocampus or neighboring substrates show deficits in task performance (Astur et al., 2002, Maguire et al., 1999). Likewise, children with a history of gestational alcohol exposure show a significant impairment in place learning in the virtual water maze (Hamilton et al., 2003).

1.2.7 Summary

FASDs affect nearly one percent of live births in the Western world. Despite years of research documenting the damage caused by gestational alcohol exposure and the great efforts used to curb alcohol drinking among pregnant women, no measurable progress has been made in reducing risky drinking in this population. As such, work to reduce the behavioral deficits like hyperactivity and attention deficits, as well as the cognitive effects like impulsivity, planning difficulties and spatial learning deficits of children with FASD will be necessary for years to come (Warren and Foudin, 1994).

1.3Models of FASD

The effects of alcohol during brain development have been extensively studied using animal models, and this large literature reveals the complexity of alcohol's effects on the developing nervous system. Several themes have emerged. There are many mechanisms by which alcohol exerts its teratogenic effects and patterns of damage depend on the developmental timing, dose and pattern of alcohol administration (West et al., 1989, West and Hamre, 1985, Bonthius et al., 1988, Bonthius and West, 1990, West et al., 1990, Goodlett and Eilers, 1997, Goodlett and Johnson, 1997, Goodlett and Lundahl, 1996, Marcussen et al., 1994, Goodlett et al., 1989a, Olney et al., 2000, Olney et al., 2002a, Ikonomidou et al., 2000).

1.3.1 Critical Periods of Vulnerability

Toxicant exposure at different points in development can be harmful to an organ system. However, exposure that coincides with the emergence of cascades of developmental

processes are more likely to have adverse effects by altering delicately timed downstream processes (for review see: Rice and Barone, 2000). Development of the brain begins early in embryogenesis when the single-layered blastula develops the primitive streak, which establishes bilateral symmetry and the site of gastrulation. During gastrulation, the blastula reorganizes into the ectoderm, mesoderm and endoderm. Each of the three germ layers develops into specific tissues and organ systems in the embryo. The musculature, skeletal and circulatory system as well as the dermis and notochord are developed from the mesoderm. The digestive, respiratory system and epithelial tissues are formed from the endoderm. The ectoderm gives rise to the epidermis, neural crest and other cells that will eventually become the nervous system (for review see: Arnold and Robertson, 2009).

At approximately two weeks human gestation, the notochord induces the overlying ectoderm to form the neural plate, which will become the brain and spinal cord. This process is known as neurulation. At approximately 18 days of gestation, the neural plate invaginates to form the neural groove with neural folds on either side. By the end of the third week, the neural folds are fusing together to form the neural tube. At this point, populations of cells have separated and begin to form the neural crest. The neural crest will become the sensory ganglia of cranial and spinal nerves, Schwann cells, as well as the meninges and muscular and skeletal components of the head. Neural tube formation is complete by gestational day 10.5 – 11 in rodents, which corresponds to the end of the first month of human gestation, a time when most pregnancies are first detected.

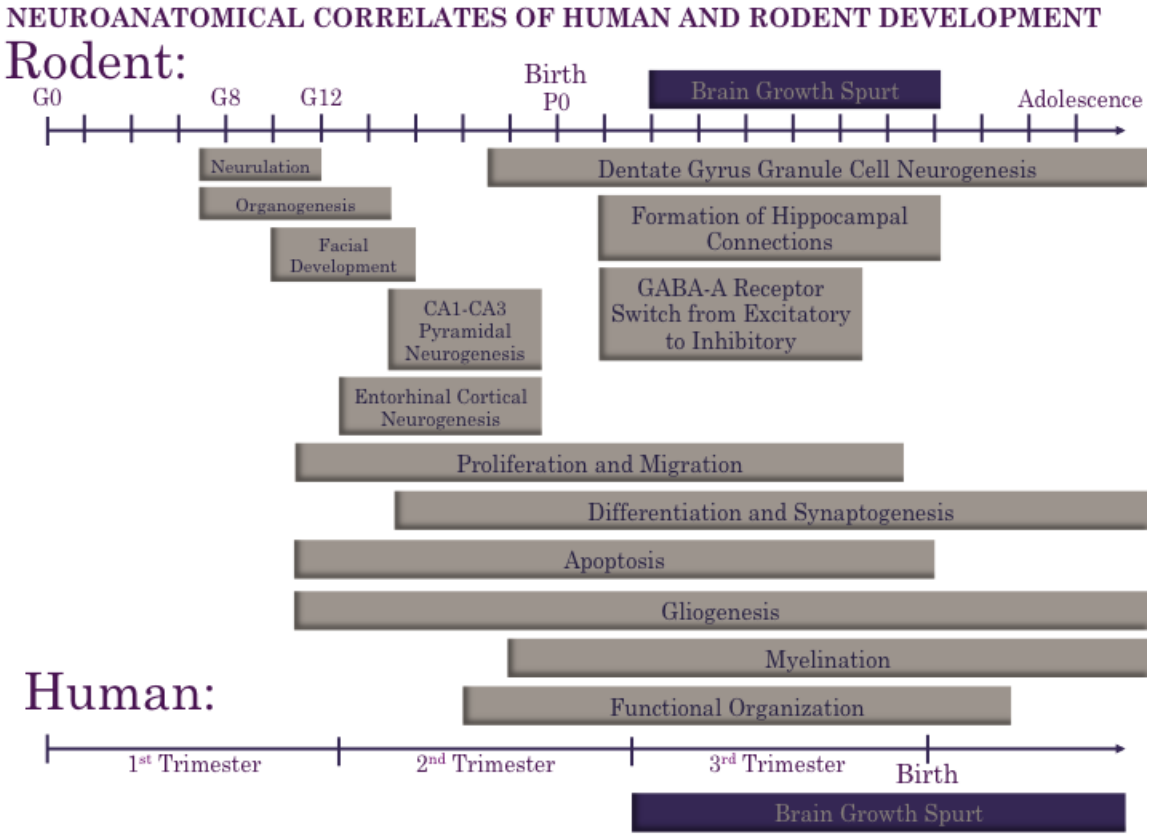

Figure 1.3.1 Comparative Brain Development Between Rodents and Humans. (Figure adapted from: Rice and Barone, 2000, and Winzer-Serhan, 2008)

1.3.2 Alcohol Exposure During Early Development

Alcohol exposure during gastrulation (G7 in mice; week three of human gestation) reliably induces holoprosencephaly, a condition in which the brain fails to form two distinct hemispheres, as well as ocular and midfacial deformities that mimic those necessary for a FAS diagnosis in children (Sulik and Johnston, 1982, Godin et al., 2010, Cook et al., 1987, Lipinski et al., 2012). Further study using this high-dose, binge alcohol exposure on G8 revealed increased incidence of hypoplasia or dysgenesis of the corpus callosum (Sulik, 1984), a finding often reported in human neuroimaging studies of those

exposed to alcohol gestationally (Mattson et al., 1996, Riley and McGee, 2005). These effects result from a very specific exposure on G7 within a three-hour time period in the mouse. The next several days of embryogenesis are associated with other developmental time point-specific effects corresponding with the end of the second through the sixth week of human fetal development.

Alcohol exposure during the 8th day of gestation in mice (fourth gestational week in humans) was associated with extensive apoptosis along the neural crest, a portion of migratory cells forming most of the autonomic and peripheral nervous system. As this process occurs simultaneously with the development of the ectodermal formation, alcohol is likely altering craniofacial formation at this time (Dunty et al., 2001, Zhou et al., 2003). For this reason, midline craniofacial malformation has been proposed to be a reliable endophenotype of severe neurological dysfunction. Alcohol has also been reported to disrupt the retinoic acid signaling cascade, which plays a critical role in the morphogenetic processes of the embryo. As reviewed in Duester (1991), retinal is irreversibly oxidized by aldehyde dehydrogenase to retinoic acid, which in turn disrupts typical development by depleting retinoic acid stores.

Alcohol has been shown to disrupt neurulation in a variety of ways. During the early stages of neurulation, G10-12, the neural plate thickens as a result of proliferation and migration of neuroepithelial cells. However, alcohol delays this process, resulting in increased perforation in and decreased size of the neural tube (Zhou et al., 2003). This disruption in normal neural tube development is likely directly related to the increased

ventricular size, decreased emergence of the serotonergic system and reduced neuronal cell number in humans and animals exposed to alcohol during gestation. As this perforation occurs along the midline of the neural tube, many midline neuroanatomical structures and facial features are affected by alcohol exposure during neurulation.

1.3.3 Critical Periods of Vulnerability for Learning and Memory

The last critical period of development is often referred to as the "brain growth spurt" which occurs from the 18th gestational day through the 9th day of rodent postnatal life, which neurodevelopmentally corresponds to the third trimester of human gestation (27-40 weeks). During this time, the brain undergoes rapid weight gain and proliferation of astroglia oligodendrocytes (Wiggins, 1982, Royland et al., 1993, Wiggins and Fuller, 1978), synaptogenesis (Jacobson, 1991) and dendritic arborization (Rice and Barone, 2000). During this the brain growth spurt, the hippocampus undergoes significant increases in proliferation of dentate gyrus granule cells, maturation of pyramidal cells of the Cornu Ammonis (CA)1 and CA3 fields, as well as increases in dendritic arborization, synaptogenesis and mylination leading to the formation of the forebrain circuitry necessary for learning and memory. One of the main principles of teratology is that organ systems are most vulnerable during the period of their most rapid growth and development.

1.3.4 Postnatal Alcohol Exposure Models

Alcohol exposure during the brain growth spurt results in reduced brain/body weight ratio (Tran et al., 2000), aberrant synaptogenesis, reactive gliosis (Goodlett et al., 1993,

Goodlett et al., 1997), delayed myelination (Lancaster et al., 1984), and cell loss in the hippocampus (Bonthius and West, 1990, Bonthius and West, 1991, Bonthius et al., 2001, Tran and Kelly, 2003, West and Hamre, 1985, West et al., 1986, West and Hodges-Savola, 1983) and cerebellum (Borges and Lewis, 1983, Tran et al., 2005, Goodlett and Eilers, 1997, Goodlett and Lundahl, 1996, Goodlett et al., 1990, Goodlett et al., 1998, Goodlett et al., 1991, Green et al., 2006). Heavy binge-like alcohol exposure on P7 has been shown to produce dose-dependent apoptosis and enduring reductions in neuronal populations in several brain regions, including the cerebral cortex, hippocampal formation, anterior thalamus, mammillary bodies and cerebellum (Ikonomidou et al., 2000, Tran and Kelly, 2003, West et al., 1990, Wozniak et al., 2004, Livy et al., 2003, Greene et al., 1992, Light et al., 2002a, Light et al., 2002b, Light et al., 1998).

Olney and colleagues have extensively studied a rodent model of a single episode of binge alcohol exposure during the 3rd trimester equivalent using two subcutaneous injections of 2.5 g/kg alcohol, given two hours apart, producing peak blood alcohol concentrations (BACs) greater than 500 mg/dl and that remained above 200 mg/dl for more than six hours after the second injection (Ikonomidou et al., 2000, Olney et al., 2002a, Wozniak et al., 2004). This alcohol exposure has been shown to induce epigenetic changes, increasing H3K9 and H3K27 dimethylation, by activating the histone methyltransferase G9a. Enhanced dimethylation of H3K9 and H3K27 leads to increases in neurodegeneration. Treatment with Bix, a G9a inhibitor, inhibited ethanol-induced enhancement of G9a expression and reversed associated neurodegeneration (Subbanna et al., 2013). In a number of studies since 1999, this model of extreme binge exposure has

been shown to cause a wave of apoptotic neurodegeneration, including activation of caspase-3, the appearance of TUNEL-positive cells and positive DeOlmos silver staining, in the cerebral cortex, hippocampal formation, retrosplenial cortex, mamillary bodies, anterior thalamic nuclei and basal ganglia (Wozniak et al., 2004, Ikonomidou et al., 2000, Olney et al., 2002a, Coleman et al., 2012, Ikonomidou et al., 1999, Wilson et al., 2011), with the largest effects elicited by treatment on P7. In addition, the same alcohol-treatment on P7 altered hippocampal functional connectivity in three-month-old adult C57BL/6 mice (Wilson et al., 2011), mimicking a hyperexcitability-like profile (Cortese et al., 1997, Slawecki et al., 2004) that may contribute to impaired spatial learning and memory. Binge alcohol exposure during this period also has been shown to result in a number of neurobehavioral deficits, including deficits in spatial learning and memory that can persist into adulthood in rats (Clements et al., 2005, Girard et al., 2000, Goodlett et al., 1989b, Johnson and Goodlett, 2002, Kelly et al., 1988, Pauli et al., 1995, Tomlinson et al., 1998, Wilson et al., 2011) and in C57BL/6 mice (Wozniak et al., 2004) and ddY outbred albino mice (Furumiya and Hashimoto, 2011).

1.3.5 Modeling and Assessing Ethanol-Induced Changes in Spatial Learning

Morris Water Maze

The Morris water maze, developed in 1981 by Richard Morris, measures spatial learning and memory by allowing animals to locate a hidden platform using only distal room cues. Briefly, the procedure involves placing an animal into a large circular pool of water containing a submerged platform, obscured by opaque water. The animal is placed in the

pool facing the wall and allowed to swim to escape using the platform. The animal escapes the water by first swimming semi-randomly throughout the pool until the platform location is discovered. Over repeated trials, animals learn to use visuospatial cues to formulate a search strategy that allows them to find the platform more quickly (i.e., with reduced latency to escape) and directly (e.g., shorter path lengths to the platform; Eichenbaum et al., 1990, Morris, 1984, Morris et al., 1982, Morris, 1981).

Developmental Alcohol Exposure and Spatial Learning

Many brain regions are known to be important components of the neural systems underlying spatial navigation, learning and memory (e.g., hippocampal formation and entorhinal cortex; frontal, cingulate and parietal cortex; Morris et al., 1988, Morris et al., 1982, Olton and Papas, 1979, Whitlock et al., 2008, Woolley et al., 2013, Kolb et al., 1983). Ethanol exposures targeting the period of enhanced hippocampal vulnerability by administering alcohol from P7-9 (220-265 mg/dl) resulted in spatial learning deficits in juvenile Long-Evans rats (Goodlett and Johnson, 1997). As little as a single day of high-dose alcohol exposure during the brain growth spurt has been shown to induce apoptotic neurodegeneration in the forebrain (Wozniak et al., 2004, Kumral et al., 2005, Ikonomidou et al., 2000) and produce massive spatial learning deficits in adolescent Wistar rats (Pauli et al., 1995) and C57BL/6 mice (Wozniak et al., 2004). In general, studies from several labs indicate that deficits on spatial learning resulting from alcohol exposure during the neonatal brain growth spurt require peak BACs that exceed 200

mg/dl (Ikonomidou et al., 2000, Tomlinson et al., 1998, Pauli et al., 1995, Wozniak et al., 2004, Goodlett and Johnson, 1997).

1.3.6 Summary

Studying children with FASD can be informative, but with the variable exposures experienced by each exposed child, coupled with often-incomplete records of alcohol exposure, animal models are necessary to elucidate different mechanisms of alcohol-induced damage to the fetus. Animal models of FASD have allowed the identification of critical periods of vulnerability during which some neural substrates are more susceptible to alcohol-induced damage. Understanding how alcohol influences development helps to design therapeutic intervention strategies to prevent damage or recovering functioning after the insult has occurred.

1.4 Treatments Used to Ameliorate the Effects of Developmental Alcohol

As with many neurodevelopmental disorders, treatments aimed at ameliorating the deficits seen as a function of fetal alcohol exposure can be behavioral or pharmacological in nature (Paley and O'Connor, 2011). There are currently no treatments for FASD approved by the Food and Drug Administration (FDA). In addition, the safety of pharmacological interventions aimed at preventing FASD during pregnancy has not been established. Therefore, all pharmacological interventions for these disorders are aimed at attenuating the symptoms typically discovered years after the initial alcohol exposure and don't necessarily treat the underlying neuropsychological issues.

1.4.1 Behavioral Interventions

There have been many cognitive and behavioral interventions in schoolchildren with FASD that have shown promise. Kable and colleagues (2007) used a directed math intervention to significantly improve scores on math and the Archenbach child behavioral checklist. Similarly, Adnams and colleagues (2007) used a cognitive control therapy to increase academic performance as well as student confidence, motivation and efficacy. Rehearsal strategies have also been used to help children with FAS increase scores on digit span task (Loomes et al., 2008). Interestingly, these same strategies did not improve performance of control children. Other behavioral interventions directed at improving the motor dysfunction associated with FASD are only in their preliminary stages, but have shown promise. Behavioral interventions showing promise in the amelioration of the effects of fetal alcohol exposure in animals typically fall under the following three categories: environmental enrichment, voluntary exercise and complex motor learning.

Environmental enrichment in animal models typically involves complex, stimulating housing conducive to locomotion, exploration and increases in social interactions among conspecifics (Reeves et al., 2007). Environmental enrichment has been shown to improve alcohol-induced spatial learning deficits (Wainwright et al., 1993, Hannigan et al., 1993), and normalize both alcohol-induced motor coordination deficits (Hannigan et al., 1993) and behavior in the open field (Zhou et al., 2007) without the typical neuroanatomical alterations associated with exposure to voluntary exercise (Berman et al., 1996, for review see: Markham and Greenough, 2004). Voluntary exercise on running wheels has been shown to improve alcohol-induced spatial learning deficits (Thomas et al., 2008,

Christie et al., 2005), normalize alcohol-induced hyperactivity and emotionality in the open field (Thomas et al., 2008), and enhance hippocampal capacity for sustained long-term potentiation (Christie et al., 2005). Unlike environmental enrichment which failed to increase synaptic plasticity in alcohol-treated animals, wheel running promotes plasticity in rats given developmental alcohol exposure, namely increasing the proliferation and survival of newly generated neurons in the dentate gyrus (Redila et al., 2006, Boehme et al., 2011).

In contrast to enriched environments and voluntary exercise paradigms, which involve varied exposure to sensory stimuli, motor activity and social interaction, complex motor learning requires mastery of specific sequences of complex movements to navigate obstacles designed to challenge cerebellar circuitry. Repeatedly forcing rats to traverse a set of ten elevated obstacles has been shown to stimulate cerebellar synaptogenesis in rats, specifically increasing parallel fiber-Purkinje cell synapses in the paramedian lobule of the cerebellum 25% in alcohol exposed offspring and normal controls (Klintsova et al., 1997, Klintsova et al., 2002, Klintsova et al., 2000, Black et al., 1990). Complex motor learning was shown to normalize neonatal alcohol-induced performance deficits in tasks that challenge motor synchronization, increasing rope climbing ability and decreasing falls on the increasing speed version of the rotorod and parallel bars task (Klintsova et al., 1998) and short-delay eyeblink classical conditioning (Wagner et al., 2013). These studies demonstrated that complex motor learning can stimulate synaptic morphological plasticity of surviving cerebellar neurons throughout adulthood and rehabilitate cerebellar-, but not yet tasks that depend on hippocampal integrity.

1.4.2 Pharmacological Manipulations

To date, many pharmacological interventions have been designed to interfere with proposed mechanisms of alcohol's teratogenesis (for review see: Goodlett et al., 2005, and Riley et al., 2001). This section outlines nutritional or otherwise pharmacological manipulations that have shown some success in alleviating or preventing alcohol-induced teratogenesis.

Antioxidants and Dietary Supplements

Work evaluating the effectiveness of dietary supplementation began in the 1980s after reports identified zinc deficiency in pregnant alcoholic women (Flynn et al., 1981). Shortly thereafter, dietary supplementation of zinc, glucose, nicotinamide adenine dinucleotide (NAD, or vitamin B_3/niacin) in animal models of fetal alcohol exposure showed attenuation in alcohol-induced reductions in fetus and brain weight as well as protein content (Tanaka et al., 1982b, Tanaka et al., 1982a, Tanaka et al., 1983). Nicotinamide has been shown to successfully prevent P7 alcohol-induced caspase-3 activation and cytochrome-c release and attenuated performance deficits in fear conditioning and normalized elevated plus maze and open field behavior (Ieraci and Herrera, 2006).

Another antioxidant that has been evaluated for its potential to attenuate alcohol's neurotoxicity during development is Vitamin C (ascorbic acid). Vitamin C has been shown to attenuate growth deficits and NF-kappa B activation in a Xenopus model of fetal alcohol exposure (Peng et al., 2005). Vitamin C also increased cell viability in part

by increasing the programed cell death inhibitor Bcl-2 and decreasing alcohol-induced increases in levels of proapoptitic Bax, which in turn decreased levels of cleaved caspase-3 of cultured prenatal hippocampal neurons (Naseer et al., 2011).

Vitamin E (α-Tocopheral) is known to function in the rat brain by reducing oxidative stress and protecting cell membranes from alcohol-induced lipid peroxidation by free radicals (Altura and Gebrewold, 1996, Halliwell, 1996) and influencing translocation of the proapoptotic protein Bax (Heaton et al., 2011). Vitamin E has been shown to increase viability of gestational hippocampal cells in culture (Mitchell et al., 1999) and protect against third-trimester equivalent alcohol-induced reductions in CA1 pyramidal cells, but not protect against the associated spatial learning impairments in rats (Marino et al., 2004) or cerebellar-mediated eyeblink conditioning deficits (Tran et al., 2005).

Nearly 30 years of research has documented developmental alcohol-induced disruptions on the cholinergic system. In fact, high-dose, binge-like alcohol exposure on G7 in some cases completely ablated the septal region, which contains neurons that provide the primary cholinergic input to the hippocampus (Lewis et al., 1967). This treatment severely reduced the choline acetyltransferase immunostaining when examined one week later or just before birth (Schambra et al., 1990), or two weeks after birth, cholinergic immunostaining was shown to recover normal activity levels by the third week of postnatal life (Swanson et al., 1995). Conversely, alcohol-induced reductions in hippocampal muscarinic receptor number reductions remain present up to at least 90 days of postnatal life (Black et al., 1995). Choline supplementation during the first three

weeks of life significantly improves discrimination learning with and without delay (Thomas et al., 2000). This improvement in functioning was not specific to the alcohol treatment group, but more of a general improvement in performance. However, this effect was so great in the alcohol-treated animals that there was no longer a significant difference between animals given alcohol and controls. Subsequent studies illustrated that postnatal choline, administered concurrently with alcohol exposure from P4-9 and continuing to adolescence ending on P30, significantly reversed neonatal alcohol-induced reductions in muscarinic M2/4, but not M1 receptor density in the hippocampus. This effect was associated with postnatal choline-attenuated alcohol-induced hyperactivity in the open field (Monk et al., 2012). Another exciting finding was that cholinergic supplementation was able to mitigate alcohol-induced deficits in the eyeblink conditioning. Interestingly, these improvements in this associative learning task were seen in the more complex, forebrain-dependent trace variant of this task, but not the more simple cerebellar-dependent short-delay training (Thomas and Tran, 2012). These findings, coupled with the lack of effects on alcohol-induced motor impairments (Thomas et al., 2004b), suggest that the choline supplementation is perhaps not having a global performance improvement, but is having a specific effect on the hippocampal cholinergic system. Clinical studies are currently underway to investigate the effects of choline supplementation on children with FASD.

"Ethanol Antagonists"

Blocking Withdrawal-Evoked Excitotoxicity

Alcohol is known to interact with the NMDA receptor (NMDAR), inhibiting its typical excitatory neurotransmission. Repeated or prolonged exposure can lead to adaptations such as increased number or sensitivity of receptors, leading to a state of hyperexcitability during alcohol withdrawal. This rebound hyperexcitability of NMDARs can contribute to seizure-like behavior and excitotoxic cell death (Lovinger, 1993). The NR2B subunit of the NMDAR-2 family are highly sensitive to alcohol and polyamines which are ubiquitious in development and enhance NMDAR activity (Williams et al., 1994, Williams et al., 1991) and potentiate the alcohol withdrawal-induced cell death in vitro (Barron et al., 2008, Gibson et al., 2003). Blockade of NMDA receptors during alcohol withdrawal reduces alcohol-induced excitotoxic cell damage in culture (Lewis et al., 2012) and attenuates cognitive (Thomas et al., 2004a) and motor coordination (Lewis et al., 2007, Lewis et al., 2012, Idrus et al., 2011b, Idrus et al., 2011a) deficits as well as alcohol-induced hyperactivity (Lewis et al., 2012) in animal models of third trimester alcohol exposure.

Serotonin Receptor Agonists

Serotonin is an essential neurotrophic factor important for the modulation of growth cone elongation and neurite outgrowth, differentiation and synaptogenesis during brain development (Lauder, 1990). Alcohol exposure via a 25% alcohol-derived calorie diet beginning just prior to neurulation increased incidences of neural tube midline defects, including retardation of serotonergic (5-hydroxytriptamine [5-HT] synthesizing) neurite

outgrowth (Zhou et al., 2002, Zhou et al., 2005, Zhou et al., 2001, Zhou et al., 2003, Sari et al., 2001), compromising forebrain development along serotonergic pathways, and reducing the number of 5-HT reuptake sites in adult offspring (Kim and Druse, 1996). Concurrent treatment with 5-HT1A agonists prevented midline serotonergic neuronal depletion (Druse et al., 2004) and attenuated alcohol-induced reductions in pro-survival gene expression (Druse et al., 2006).

Lithium

Lithium has been shown to inhibit several pro-apoptotic pathways by directly inhibiting GSK3β and inositol monophosphatase in Xenopus oocytes (Subbanna et al., 2013). Lithium was subsequently found to be protective against P7 alcohol-induced apoptosis in C57BL/6 mice by preventing alcohol-induced inhibition of phosphorylation of ERK (Young et al., 2008), a pro-survival factor, and prevented alcohol induced down-regulation of phosphorylated-AKT and –AMPK (Chakraborty et al., 2008), which can inhibit proapoptotic capacity of the protein BAD.

Activity-Dependent Neuroprotective Peptides

The neuroactive peptides NAPVSIPQ (NAP) and SALLRSIPA (SAL) are endogenous peptide fragments of activity dependent neurotrophic protein and activity dependent neurotrophic factor respectively. NAP and SAL are released from glia upon stimulation of the hop2 PAC-1 receptor on astrocytic membranes (Ashur-Fabian et al., 1997). These peptides are expressed in both the human and mouse central nervous system, predominantly in the cerebellum, hippocampus and cerebral cortex (Bassan et al., 1999) and have been shown to be neuroprotective against a variety of cellular stresses and

toxins in cell culture. Despite the widespread preclinical and clinical use of these peptides, data on bioavailability exists on relatively few routes of administration. For example, the bioavailability of NAP has been published in only intranasal and intravenous administration. When administered intranasally, NAP and SAL reach detecectable levels in the rat cortex at 30 and 60 minutes (Gozes et al., 2000). Intravenous NAP reaches the cortex and cerebellum of rats within 15 minutes and remains elevated for 60 minutes (Leker et al., 2002). NAP is vital for typical brain development. ADNP levels increase and remain elevated throughout the time of neural tube closure, G7.5-9. ADNP knockout mouse embryos exhibit failure of neural tube closure and die between G8.5 and 9 (Pinhasov et al., 2003).

In vivo studies have confirmed the neuroprotective properties of these peptides demonstrated in culture. For example, developmental NAP exposure prevents spatial learning deficits in adult apolipoprotein E-deficient mice (Brenneman et al., 2004, Bassan et al., 1999). Similarly, gestational treatment with NAP and SAL on G8-12 prevented spatial learning deficits (Incerti et al., 2012) and prevented some motor and sensory developmental delays (Toso et al., 2008) in Ts65Dn mice, a model of Down Syndrome. This treatment effect was found to be associated with normalized NR2A, NR2B and $GABA_A\beta3$ expression in 40-week-old adults (Vink et al., 2009). Gestational NAP and SAL have also been shown to protect against adult spatial learning deficits associated with alcohol-induced toxicity in animal models of FASD (Spong et al., 2001).

NAP has also been shown to prevent spatial learning deficits in adult rats resulting from hypoxic ischemia when administered up to 24 hours after the P7 insult (Greggio et al., 2011). Likewise, NAP reduced mortality and neurological impairments in motor coordination, balance and alertness as early as 24 hours after trauma in adult mice when given subcutaneously within 15 minutes of traumatic brain injury (Beni-Adani et al., 2001). Further, this NAP treatment regimen decreased expression of the adhesion molecule Mac-1, elevations of which are associated with severe neurological impairments in the same motor coordination battery (Romano et al., 2002, Gozes et al., 2005).

Chronic intranasal administration of NAP has been shown to rescue both spatial learning and novel object recognition deficits, and normalized hyperactivity resulting from the stable tubule-only polypeptide knock-out mouse model of schizophrenia (Gozes, 2011) and spatial learning deficits in choline-deficient adult rats (Gozes et al., 2000), an animal model of Alzheimer's disease. Oral administration of NAP and SAL have been shown to be effective in completely reversing spatial learning deficits in mice given G8 heavy binge alcohol exposure when treatment was given in adulthood three days prior to and throughout water maze training (Incerti et al., 2010c). There are a number of purported mechanisms by which NAP and SAL are thought to have their protective effects against alcohol-induced toxicity.

One mechanism is by antagonism of alcohol interactions with immunoglobulin L1 cell adhesion molecules (Wilkemeyer et al., 2000, Wilkemeyer et al., 2002). L1 cell adhesion molecules are transmembrane proteins that interact with neighboring cells to regulate

growth cone elongation, axonal pathfinding and fasciculation and neuronal migration through homophilic binding (Crossin and Krushel, 2000, Schmid et al., 2000). One theory was that perhaps NAP was able to induce neurite outgrowth in cerebellar granular neurons and antagonize alcohol-induced teratogenesis in mouse embryos by increasing L1 protein expression. However, NAP treatment failed to increase levels of L1 mRNA or protein expression in cerebellar granular neurons or astrocytes (Fitzgerald et al., 2011).

NAP is typically shown to be more potent than SAL in neuroprotection assays in vivo (Bassan et al., 1999, Gozes et al., 2000, Spong et al., 2001). In fact, NAP has been shown to be significantly more potent than SAL at antagonizing the alcohol-induced inhibition of L1 cell adhesion molecules in human L1-transfected NIH/3T3 cells, although both NAP and SAL were effective at femtomolar and picomolar ranges, respectively, and have an unusually broad dose-response curve (Wilkemeyer et al., 2003). Additionally, the protective effects of SAL are reversible with increasing concentrations of alcohol (Wilkemeyer et al., 2002). NAP alone was able to prevent alcohol-antagonism of L1 cell adhesion molecules in 2A2-L1 cells, which are alcohol-sensitive subclones of NIH 3T3 cells transfected with human L1 cDNA, at femtomolar doses and exert neuroprotection from TTX-induced cell death in astrocyte/neuronal co-cultures even in the picomolar range (Wilkemeyer et al., 2003). Even in whole embryo culture harvested at G8, 100pM NAP was sufficient to prevent 100mM alcohol-induced growth retardation (Wilkemeyer et al., 2003). Subsequent studies determined that co-incubating G8 embryos with 100mM alcohol and 100pM D-NAP, L-NAP or SAL could prevent neural tube closure defects (Chen et al., 2005).

Co-cultures of astrocytes and neurons from prenatal alcohol-exposed fetuses exhibit significantly reduced neuronal growth and differentiation as well as fewer synaptic contacts compared to control cultures. In addition, these prenatal alcohol-exposed cultures show marked reductions in levels of NAP mRNA (Pascual and Guerri, 2007). Adding NAP to the culture media was able to increase neuronal growth and stimulate synaptic connections, an effect that was reversed by inhibition of the mitogen-activated protein kinase. Inhibition of extracellular signal-related protein kinase (MAP/ERK), phosphatidylinositol-3-kinase (PI-3K)/Akt pathways and cAMP response element-binding proteins (CREB) aborted the NAP-induced rescue of these cellular changes (Pascual and Guerri, 2007), revealing one mechanism by which NAP may be exerting its effects. Similarly, inhibiting sarcoma (Src) family kinases (SFK) and phosphorylation of Fyn kinase abolished NAP-induced axonal outgrowth in cultured cerebellar granule neurons (Chen and Charness, 2008). In fact, NAP was found to significantly increase phosphorylated Fyn kinase within 30 minutes of application. These increases of phosphorylated Fyn kinase lead to increases in phosphorylated Cas, a scaffolding protein necessary for axonal elongation. Ethanol potently inhibits NAP-mediated axonal elongation in these cell cultures by disrupting the Fyn kinase-Cas signaling cascade (Chen and Charness, 2008).

SAL has been shown to increase nuclear factor-κB DNA binding in the hippocampal neurons (Glazner et al., 2000) and neurite extension via increases in CREB phosphorylation in dorsal root ganglia cultures (White et al., 2000). Prenatal administration of the less potent L-isoform of SAL has been shown to reduce alcohol-induced, region-specific forebrain damage; particularly relevant to the current project are

the treatment-related increases in hippocampal and amygdalar volume in alcohol-treated offspring (Zhou et al., 2004). While some studies have shown these potent neuropeptides do not show chiral-specificity in antagonizing alcohol-inhibition of L1 cell adhesion molecules (Wilkemeyer et al., 2004), others have shown that the D-enantiomers of the peptides do not show the typical stereoisomeric ligand-receptor interaction, displaying greater potency in combating alcohol–induced growth restrictions and fetal demise than do their L-counterparts (Brenneman et al., 2004). Dietary D-SAL administration, concomitant with alcohol, significantly reduced alcohol-induced ocular abnormalities in C57Bl/6J mouse offspring (Parnell et al., 2007). One mechanism for this effect that has not yet been discussed are the interactions between Pax 6, a protein critical for ocular development, and L1 cell adhesion molecules (Meech et al., 1999, Chalepakis et al., 1994). It is possible that by eliminating alcohol-inhibition of L1 cell adhesion, Pax 6 signaling cascades proceed unmolested. This could account for the specificity in preventing alcohol-induced ocular abnormalities, while not affecting typical congenital ocular defects (Parnell et al., 2007).

When given simultaneously with developmental alcohol exposure, the peptides (D-NAP and D-SAL) have been shown to antagonize alcohol-induced oxidative stress and inhibition of L1-mediated cell-cell adhesion molecules, important for proper neural tube closure (Spong et al., 2001, Wilkemeyer et al., 2002, Zhou et al., 2008). Combined treatment with D-NAP and D-SAL has proven to be most effective in the prevention of alcohol-induced fetal demise (Spong et al., 2001) and reduction of alcohol-induced deficits in spatial learning and memory in young adult offspring (Vink et al., 2005).

Particularly exciting were the recent findings by Incerti and colleagues (2010c) who showed that combined treatment with D-NAP and D-SAL, given orally beginning in late adolescence, three days prior to the initiation of and throughout the 7 days of water maze training, was able to completely reverse profound G8 alcohol-induced spatial learning deficits. Not only was this the first study to report profound spatial learning deficits following a single binge exposure to alcohol on G8, but it was the first study to show a peptide treatment effect well after the alcohol-induced insult had occurred. Other studies had shown the ability to reduce alcohol-induced weight restrictions and fetal demise when administered intraperitoneally three hours after alcohol administration, but this study administered 40µg of D-NAP and 40µg D-SAL more than one month after alcohol exposure to reverse a behavioral deficit.

1.4.3 Summary

Many treatments have been designed to prevent the damage caused by prenatal alcohol exposure. These preventative therapies have been successful in vitro and in animal models, and help us better understand mechanisms of alcohol-induced damage. However, given that FASD is 100% preventable and caused by alcohol use during pregnancy, treatments relying on compliance of an alcohol-abusing population may not always be effective. In addition, many children with FASD go unrecognized until well after the damage has occurred, when they reach school age. Treatments that can improve the lives of children and adults with FASD should be an important priority. Behavioral therapies like environmental enrichment, voluntary exercise and complex motor learning have

successfully ameliorated many of the deficits associated with FASD when administered in adolescence or adulthood. Historically, pharmacological therapies have targeted preventing alcohol-induced neurodegeneration, but over the past few years, treatments such as dietary choline supplementation and the activity-dependent neuropeptides have been shown to recover alcohol-induced learning deficits in animal models.

1.5 Rationale

While some pharmacological interventions given during pregnancy and aimed at reducing damage incurred during or shortly after alcohol exposure have shown promise (Incerti et al., 2010a, Incerti et al., 2010b, Toso et al., 2007, Wilkemeyer et al., 2003, Zhou et al., 2008, Zhou et al., 2004, Spong et al., 2001), treatment compliance among alcohol-abusing pregnant women is expected to be difficult (NIAAA, 2008) and pharmacotherapies during pregnancy may themselves carry risk for the fetus or the mother. Many children exposed to alcohol prenatally may have received inadequate prenatal care, and often are adopted into families that may not recognize the child's brain damage and learning disabilities until they reach school age (Coles et al., 1991). Therefore, it is important to investigate treatments with potential to improve outcomes in children and adolescents already affected by prenatal alcohol-induced brain damage. Studies by Olney and colleagues using a P7 alcohol exposure in C57B/6 mice have provided neuropathological evidence of significant apoptosis and degeneration of post-mitotic neuronal populations in specific layers of the cerebral cortex (frontal, cingulate, parietal, temporal, and retrosplenial cortex), hippocampal formation (CA1 and subiculum), striatum, anterior thalamus, and mammillary bodies (Olney et al., 2002b,

Wozniak et al., 2004). Previous work in our lab has confirmed that the profound spatial learning deficits shown in adolescent mice in the 2004 study by Wozniak and colleagues persist into adulthood (Wagner et al., submitted). Therefore, the significant spatial learning deficits produced by the P7 alcohol exposure in C57BL/6J mice yield an ideal model to extend the therapeutic benefit of oral administration of D-NAP and D-SAL shown to reverse spatial learning deficits when administered just three days prior to and throughout water maze training (Incerti et al., 2010c).

1.5.1 Hypothesis

Previous research dictates that high-dose, binge-like alcohol exposure (5g/kg) on P7 would significantly impair spatial learning and memory in adult C57Bl/6J mice (Wagner et al., submitted). Based on the work of Incerti et al. (2010c), administration of 40μg D-NAP with 40μg of D-SAL three days prior to and throughout water maze training (P67-76) was hypothesized to significantly improve acquisition of place learning in the Morris water maze.

Based on the work of Parnell et al. (2009) showing significant volumetric reductions in brain areas necessary for intact spatial learning and memory after two 2.8g/kg intraperitoneal alcohol injections separated by four hours on G8, and work by Incerti et al. (2010c) showing profound spatial learning deficits in late adolescent mice after a 5.8g/kg intraperitoneal alcohol injection on G8, the hypothesis was that the G8 alcohol administration protocol would induce significant spatial learning deficits in adult animals. Further, these spatial learning deficits could be reversed by oral administration of 40μg

D-NAP with 40μg of D-SAL three days prior to and throughout water maze training (P67-76).

CHAPTER 2 METHODS

2.1 Subjects

Eight-week old female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) for use as breeders and housed in the Indiana University School of Medicine Biotechnology Research and Training Center vivarium. The vivarium was maintained on a 12h/12h light/dark cycle, lights on at 0900 hrs. For studies involving postnatal alcohol exposure, three cohorts of 12 female mice were ordered in waves approximately 3 weeks apart. Three male sires were also purchased from Jackson Laboratories with the original cohort of females. These sires were re-mated with all three cohorts of female mice until the postnatal studies were complete. Three cohorts of 16 female mice were ordered in waves approximately 3 weeks apart. Four male sires were purchased for repeated matings with the 48 females used in the prenatal alcohol exposure studies.

2.2 Animal Breeding

Upon arrival, female mice were weighed, handled, and housed in breeding harems of four females. Animals were handled during the next 5-7 days during habituation to the facility. On mating days, female mice were placed into the cage of their designated male sire at

0700 hrs. Two hours later (0900 hrs; initiation of the light cycle) females were removed from the sire's cage and examined for a sperm plug. Female mice without evidence of a sperm plug were retuned to their original cage to be re-mated the following morning at 0700 hrs. Once determined to be sperm plug positive, designated G0, female mice were separated from their breeding harem and individually housed until her pups were weaned or until she was determined not to be pregnant. Mating was typically done over four to five consecutive days with three separate cohorts of mice, separated by approximately three weeks. Females failing to mate successfully within the four-day window were transferred to the subsequent cohort.

2.3 Animal Husbandry

Once dams were determined to be sperm plug positive, they were single housed, handled and monitored for body weight on G0, 6, 8 10 and 15. Mice failing to exhibit significant weight gain by G15 were considered not pregnant and transferred to the next breeding cohort. Dams determined to be pregnant on G15 were placed in clean cages and started on wet feed to prepare for delivery on G19, also designated Postnatal Day (P) 0. Birth occurred on G19 for all litters used in these studies. Pregnant dams were not disturbed between G15 and 19 with the exception of the daily addition of wet feed.

After pups were born, they were left undisturbed until P6 at which point all litters were culled to 6 pups, 3 male and 3 female when possible. Pups were given subcutaneous injections of very small quantities of non-toxic India ink into one or more paws to uniquely identify each pup in the litter. Litters of fewer than four pups were considered

non-viable, sacrificed and their dam returned to the pool of breeders for the next mating. Growth was monitored by measuring body weights of offspring on P6, 7, 10, 15, 21, and when weaned on P25. Pups were then allowed to mature until P64 at which point the offspring began daily handling and gavage habituation procedures to prepare for daily peptide intubations and behavioral testing.

2.4 Alcohol Treatment

2.4.1 Prenatal Alcohol Treatment

Sulik Model

Pregnant dams were randomly assigned to be given either intraperitoneal injections of alcohol in Ringer's solution or only Ringer's solution. Following the methods of Sulik et al. (1981), two intraperitoneal injections were administered on gestational day (G) 8, 4 hours apart, of 2.9 g/kg alcohol (total dose of 5.8 g/kg) delivered as a 23.7% (v/v) alcohol solution in Ringer's solution. Injections were given in volumes of 0.015 ml/g of body weight on G8. Control dams were intraperitoneally injected with 0.015ml/g of 0.9% Ringer's solution on the same schedule as alcohol-treated dams to account for the effects of injection stress on behavior. For this procedure pregnant dams were weighed for calculation of their alcohol injection volume and given their first injection (0900 hrs) and left undisturbed until their second injection (4 hours later).

Webster Model

Pregnant dams were randomly assigned to be given either intraperitoneal injections of alcohol in saline solution or only saline solution. Following the methods of Webster et al.

(1980) and Incerti et al. (2010c), 5.8 g/kg alcohol was delivered as a 25% (v/v) alcohol solution in 0.9% saline solution. Injections were given in volumes of 0.03 ml/g of body weight on G8. Control dams were intraperitoneally injected with 0.03 ml/g of 0.9% saline solution on the same schedule as alcohol-treated dams to account for the effects of injection stress on behavior. For this procedure pregnant dams were weighed for calculation of their alcohol injection volume and given their first injection at 0900 hrs.

2.4.2 Postnatal Alcohol Treatment

Within each litter, pups were randomly assigned to receive twice daily injections of either alcohol (15% w/v in 0.9% w/v saline, 16.67ml/kg body weight) or physiological saline solution (0.9% w/v saline, 16.67ml/kg body weight) on P7. Injections were administered twice daily beginning between 0900-1100 hrs and again two hours after the first injection, as was reported in Wozniak et al. (2004). During the injection procedure all pups were removed from their dam, weighed and their injection volume calculated. Pups were then carefully injected subcutaneously at the nape of the neck. To ensure as little injection leakage as possible, the injection site was manipulated so that the needle could be pinched during it's withdrawal from the injection site. Pups were then held for 15 to 20 seconds to allow the injection volume to distribute and the injection site to better close. Pups were kept in a huddle on a 37ºC heating pad while other pups of that litter were manipulated. The injection procedure took approximately 10 minutes per litter, after which pups were returned to the home cage until time for the second injection.

2.5 Blood Alcohol Concentrations (BACs)

Separate groups of mice were used for blood alcohol determination. After decapitation,

$$\text{Alcohol} + \text{Oxygen} \xrightarrow{\text{Alcohol Oxidase}} \text{Alcetaldehyde} + \text{Water}$$

trunk blood samples were collected in heparinized centrifuge tubes one, four and eight hours after the final alcohol injection. Samples were then centrifuged at 13,000 revolutions per minute for 10 minutes, after which the plasma was extracted, sealed with parafilm to avoid evaporation and frozen at -20ºC for later analysis. BACs were assayed using an Analox® GL5 Alcohol Analyzer (Analox Instruments, Boston, MA), which uses alcohol oxidase to catalyze the oxidation of the alcohol in each sample to acetaldehyde and hydrogen peroxide. Because alcohol oxidase is specific to low molecular weight alcohols, the maximum rate of oxygen consumption is directly related to the alcohol concentration in a given sample.

2.6 Peptide Treatment

On P67, all offspring were treated with 40 μg D-NAP [Biosynthesis, > 95% purity] and 40 μg D-SAL [Abbiotec, >90% purity] (200μg of each peptide/ml) or vehicle (0.20 ml). Solutions were made from 10 mg D-SAL dissolved and diluted in 5 ml of filtered Dulbecco's phosphate-buffered saline solution. 10 mg D-NAP were diluted in 0.5 ml of dimethyl sulfoxide and further diluted with the 4.5 ml of the filtered Dulbecco's phosphate-buffered saline solution. This stock solution was diluted with filtered Dulbecco's phosphate-buffered saline to reach the appropriate concentrations and then

frozen in 1ml aliquots. A volume of 0.2 ml of this solution was administered via gavage between 0700 and 0900 hours for 11 days beginning on P67.

2.7 Morris Water Maze

2.7.1 Water Maze Procedure

The offspring began training in the water maze at 70 days of age, three hours after peptide co-treatment, by an experimenter blind to alcohol and peptide treatment conditions. Training consisted of four acquisition trials each day for seven consecutive days in the Morris water maze. During each training trial, mice were given 60 seconds to navigate a 125cm (diameter) pool filled to within 25cm of the rim of the tank with 26°C water made opaque by adding non-toxic white tempera paint (Dick Blick Art Materials, Galesburg IL) in which a 9cm (diameter) platform was placed. The top of the submerged platform was 0.5cm below the surface of the water rendering it invisible to the test subject. The pool rested on top of a 12" stand made of cinder blocks. The testing environment (280 X 390cm), illuminated by indirect incandescent lighting, contained fixed audio (radio kept at low volume) and visual reference cues (e.g. posters, mobile, sink, computer, and experimenter). The platform position remained constant throughout training, with each squad randomly assigned a platform position. Throughout trials, mice learn to use the distal room cues to locate the escape platform. As training progressed shorter escape path lengths and decreased latencies to find the hidden platform were used as indices of spatial learning.

A probe trial, administered after the last acquisition trial on day 7 of training, was used to examine each animal's search strategy. During probe trials, subjects were given 60 seconds to navigate the pool without the presence of the escape platform, while the escape path was monitored. Each trial, including the probe, was initiated by introducing the animal to the pool (facing the wall) at one of 8 starting positions (corresponding to the 8 cardinal directions) and lasted until the animal mounted the platform or 60 seconds had expired. Different, pseudo-randomly determined, starting points were used for each trial. Mice were not started within the same quadrant as the target, nor were start positions repeated on a given day.

If a mouse was unable to find the platform, it was guided to the platform by the experimenter. All subjects remained on the platform for 10 seconds before being returned to their respective incubators (30±1°C), used to limit hypothermia during the intertrial interval. Mice were tested in squads of 3 or 4, resulting in an intertrial interval of approximately three minutes. At the end of each day of testing, the mice were allowed to remain in the incubators until warm and mostly dry (approximately 10 minutes) before being returned to the vivarium.

2.7.2 Water Maze Data Collection

Escape paths were monitored by a video camera interfaced with computer-controlled tracking software (HVS Image, Hampton, UK) and recorded by a JVC VCR. The computer-controlled tracking software displayed the moment-to-moment position of the animal in the pool. Dependent variables assessed by this software included escape latency,

escape path length, swim speed, initial heading angle and time spent in thigmotaxis (swimming the perimeter of the pool).

During probe trials, the computerized video tracking system superimposed virtual counting discs (27cm, i.e., three times the size of the platform) over the pool in the location of the animal's designated platform position. The video tracking software obtained measures of time spent in, latency to enter, and numbers of passes through the target virtual counting disc; these measures were used to quantify the spatial distribution of the search strategy of each animal.

CHAPTER 3 POSTNATAL ALCOHOL RESULTS

3.1 Blood Alcohol Concentrations

The mean BACs on P7 are shown in Table 3.1. The mean (±SEM) BAC on P7, one hour after the second alcohol injection, was 472 (±16) mg/dl. The alcohol clearance rate, calculated using the values obtained at 4 and 7 hours after the second alcohol injection, was 27.7 mg/dl/hr (Table 1). Substantially higher than BACs Kelly and colleagues (1987a, peak 411 mg/dl) reported to disrupt typical sensory and motor development.

Table 3.1.1 Blood Alcohol Concentrations from trunk blood samples taken at 1, 4 and 7 hours after the final alcohol injection.

1 Hour	4 Hours	7 hours
472 ±16	385 ±8	302 ±13
n = 13	n = 12	n = 11

3.2 Body Weight Analysis

As shown in Figure 3.2.1, all mice gained weight during the early postnatal and pre-weaning period [main effect of day $F(4,344) = 4019.9$, $p<0.005$], with males weighing significantly more than females [day X sex interaction: $F(4,344) = 2.85$, $p<0.05$]. Alcohol

treatments on P7 resulted in a significant body weight deficit during the pre-weaning period [day X alcohol treatment interaction: $F(4,344) = 6.40$, $p<0.005$].

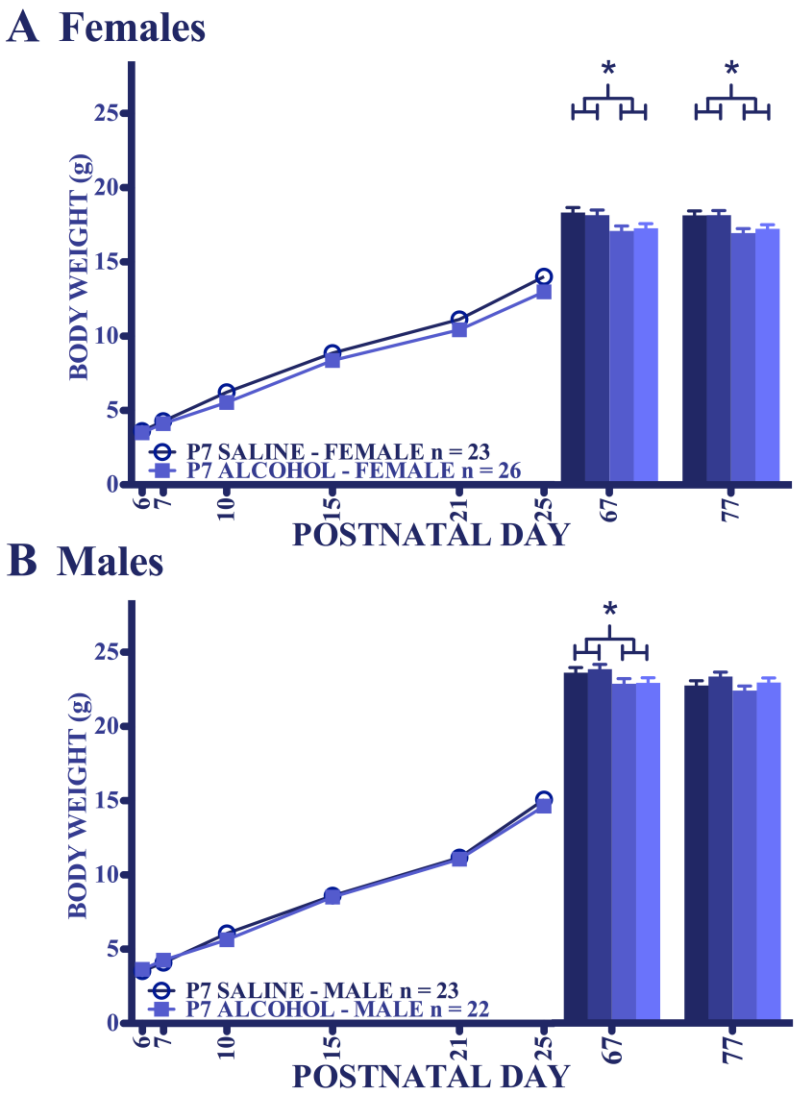

Figure 3.2.1 Offspring body weights. Open circles represent animals given saline on P7 closed squares represent mice given alcohol on P7. The bar graph separates mice further into their peptide treatment group. From left to right and dark to light the groups are P7 saline – vehicle; P7 saline – peptide; P7 alcohol – vehicle; P7 alcohol – peptide. ★indicates $p<0.05$

Looking at each sex separately, females gained weight over the pre-weaning period as expected [main effect of day: $F(4,180) = 2724.0$, $p<0.005$]; however, alcohol treatment on P7 caused a significant delay in body weight gain [main effect of alcohol treatment: $F(1,45) = 7.02$, $p<0.05$; day X alcohol treatment interaction: $F(4,180) = 5.65$, $p<0.005$]. This effect persisted through weaning on P25 until the end of behavioral testing on P77 [main effect of alcohol treatment on P25: $F(1,45) = 9.18$, $P<0.005$; main effect of alcohol treatment on P67: $F(1,45) = 15.07$, $p<0.005$; main effect of alcohol treatment on P77 $F(1,45) = 18.89$, $p<0.005$]. There were no peptide treatment effects on body weights at any time. In contrast, males only showed alcohol treatment effects on body weight on P67 [main effect of alcohol treatment: $F(1,41) = 4.48$, $p<0.05$], when alcohol-treated mice weighed 0.969g less than their saline-treated controls ($p<0.05$). As was the case with female mice, there were never any peptide-treatment effects in bodyweights.

3.3 Morris Water Maze Acquisition

All offspring improved performance in the water maze task over time by decreasing the average path length swum to escape over days [Figure 3.3.1; main effect of day: $F(6,516) = 67.10$, $p<0.005$]; however, this improvement was impaired by alcohol exposure on P7 [main effect of alcohol $F(1,86) = 33.33$, $p>0.005$; alcohol treatment X day interaction $F(6,516) = 2.49$, $p<0.05$]. Performance was also found to be sex dependent, as males decreased escape path lengths faster than females and ended with lower terminal performances [main effect of sex $F(1,86) = 8.74$, $p<0.005$]. To investigate well-established sex differences in water maze learning, males and females were subsequently analyzed separately.

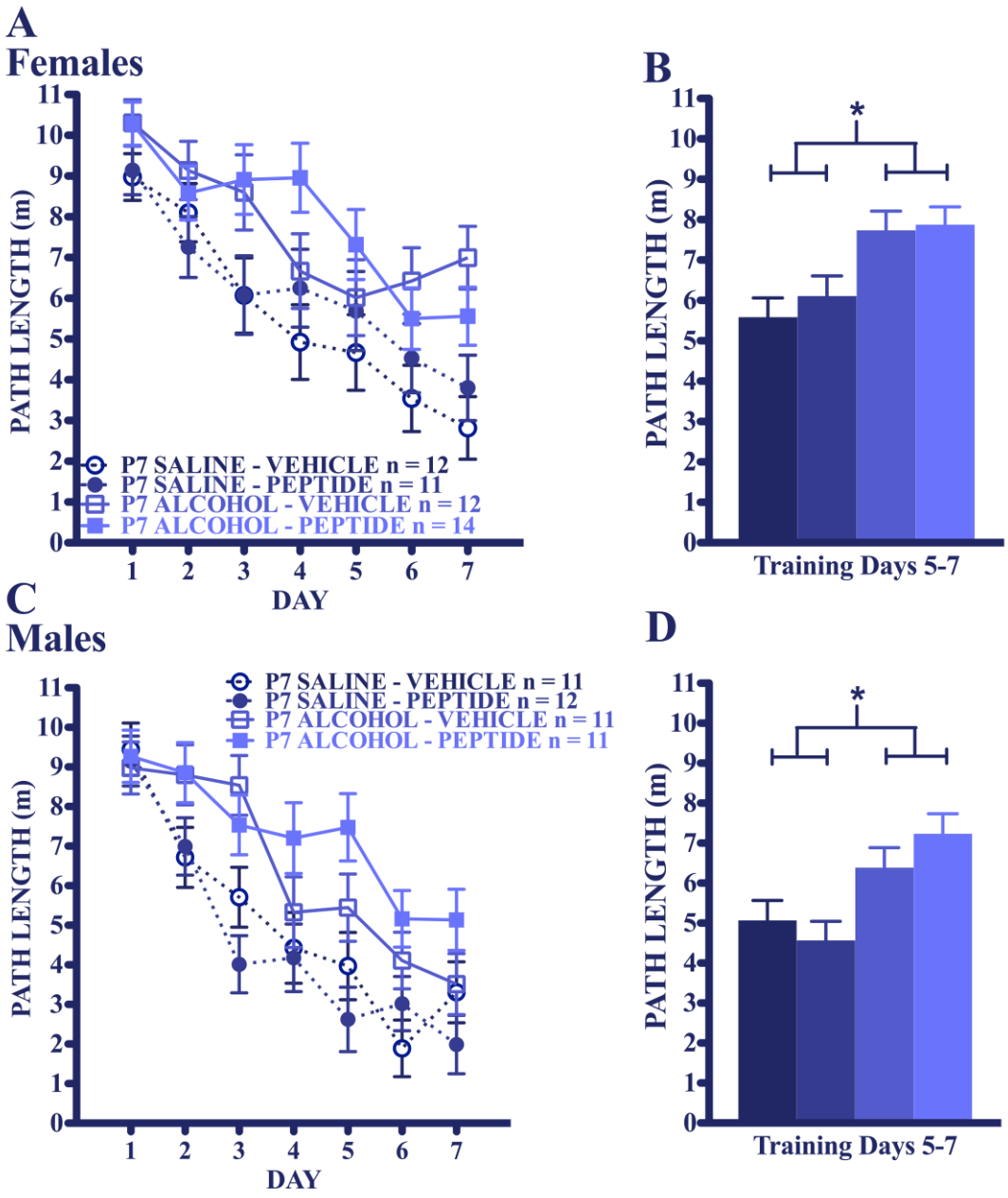

Figure 3.3.1 Morris Water Maze Acquisition. Panel A&C illustrate acquisition rates of each group over testing days, while B&D show performance collapsed over the last three days of training (separated by sex; females Panel A&C, males Panels B&D). Circles indicate mice given saline on P7. Squares represent mice given alcohol on P7. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide. In Panels B&D from left to right and dark to light the groups are P7 saline – vehicle; P7 saline – peptide; P7 alcohol – vehicle; P7 alcohol – peptide. ★indicates $p<0.05$

Females

Females improved performance over the course of training indicated by shorter path lengths to reach the escape platform [Figure 3.3.1 A&C; main effect of day: $F(6,270) = 27.23$, $p<0.005$]. Alcohol exposure on P7 impaired water maze acquisition [$F(1,45) = 14.78$, $p<0.005$]. Within females, there were no main or interactive effects of post-weaning peptide treatment.

Males

Males also improved performance over training days showing shorter escape path lengths across training [Figure 3.3.1 B&D; main effect of day $F(6,246) = 41.14$, $p<0.005$]. P7 alcohol exposure induced a deficit in acquisition [main effect of alcohol treatment $F(1,41) = 19.62$, $p<0.005$; alcohol treatment X day interaction $F(6,246) = 2.82$, $p<0.05$]. Within males, there were no main or interactive effects of post-weaning peptide treatment.

3.4 Water Maze Probe

3.4.1 Time Spent in the Target vs Non-Target Platform Regions

Seven days of water maze training significantly increased the amount of time spent searching virtual counting area three times the size of the platform superimposed over the region of the pool that previously contained the platform compared to the average time spent in three equally sized regions superimposed in three other parts of the pool [main effect of pool region: $F(1,59) = 117.76$, $p<0.005$]. The preference for the target region of the pool was significantly decreased by P7 alcohol exposure [main effect of P7 alcohol treatment: $F(1,59) = 15.03$, $p<0.005$, P7 alcohol treatment x pool region interaction: $F(1,59) = 15.15$, $p<0.005$]. Female mice spent significantly less time in the target region

vs the average non-target regions than males [sex X pool region interaction: $F(1,59) = 4.61$, $p<0.05$].

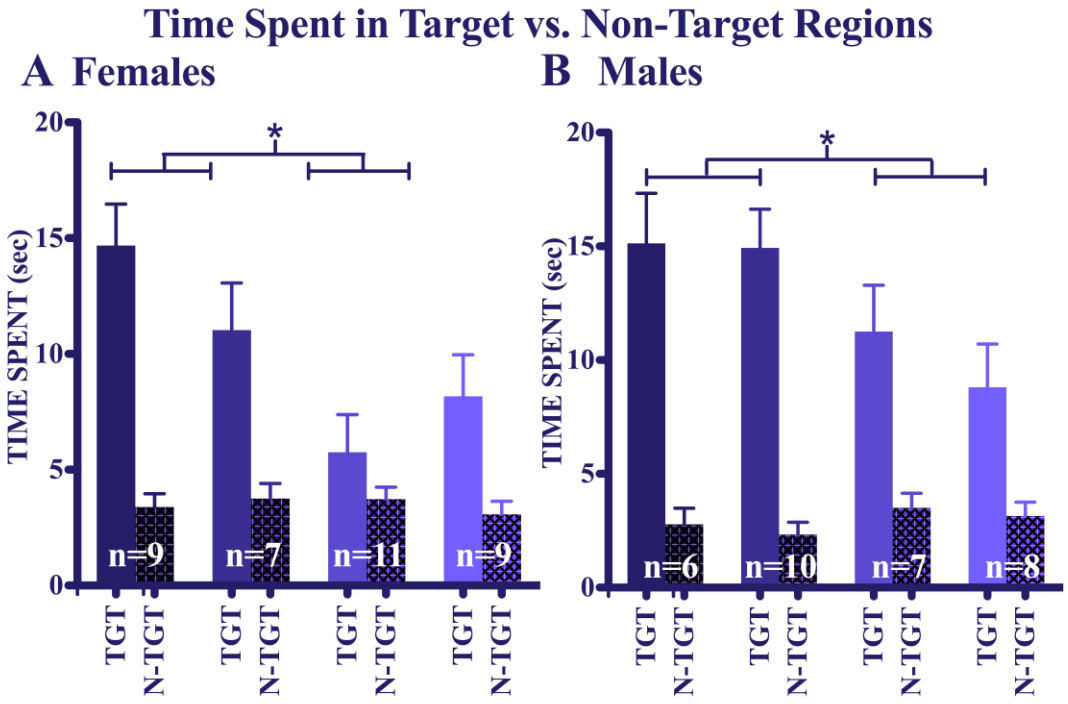

Figure 3.4.1 Time Spent in the Target vs. Non-Target Regions of the Pool. From left to right and dark to light the groups are P7 saline – vehicle; P7 saline – peptide; P7 alcohol – vehicle; P7 alcohol – peptide. Solid bars represent time spent in the target while hatched bars represent time spent in an average of the three non-target platform regions. ★indicates $p<0.05$

Females

Seven days of water maze training led to a significant preference for the target platform region [Figure 3.4.1A; main effect of pool region: $F(1,32) = 43.33$, $p<0.005$], spending more time in that region during probe trials than the average time spent in the other three non-target regions. Alcohol treatment on P7 did disrupt the preference for the target region [main effect of P7 alcohol treatment: $F(1,32) = 9.84$, $p<0.005$; P7 alcohol

treatment X pool region interaction: $F(1,32) = 8.59$, $p<0.01$]. Alcohol-treated animals given peptide treatment tended to prefer the target vs non-target platform more than alcohol-treated females given vehicle, but it failed to reach significance [$F(1,32) = 3.30$, $p=0.079$].

Males

Males also preferred the target platform region over the non-target region after 7 days of water maze training [Figure 3.4.1B; $F(1,27) = 73.87$, $p<0.005$]. Alcohol treatment on P7 disrupted this preference [main effect of P7 alcohol treatment: $F(1,27) = 5.73$, $p<0.05$; P7 alcohol treatment X pool region interaction $F(1,27) = 6.68$, $p<0.05$], yielding a less organized search strategy. There were no main or interactive effects of post-weaning peptide co-treatment.

3.4.2 Passes Through the Target vs Non-Target Platform Regions

After 7 days of water maze training, all mice showed a preference for the region of the pool that contained the escape platform, illustrated by an increase in the number of times the mice passed through the superimposed target region vs. an average of three identically sized regions in other portions of the pool [main effect of pool region: $F(1,59) = 107.8$, $p<0.005$]. Alcohol treatment on P7 significantly impaired preference for the target platform region, decreasing the number of passes through the target region compared to the three other non-target regions [main effect of P7 alcohol treatment: $F(1,59) = 8.81$, $p<0.005$; P7 alcohol treatment X pool region interaction: $F(1,59) = 8.22$, $p<0.01$]. Performance on this measure was also found to be dependent on sex and peptide treatment such that peptide treatment significantly increased the number of passes

through the target platform region of alcohol treated female mice [P7 alcohol treatment X peptide co-treatment X sex X pool region interaction: $F(1,59) = 4.12$, $p<0.05$; P7 alcohol treatment X peptide co-treatment X sex interaction: $F(1,59) = 6.45$, $p<0.05$].

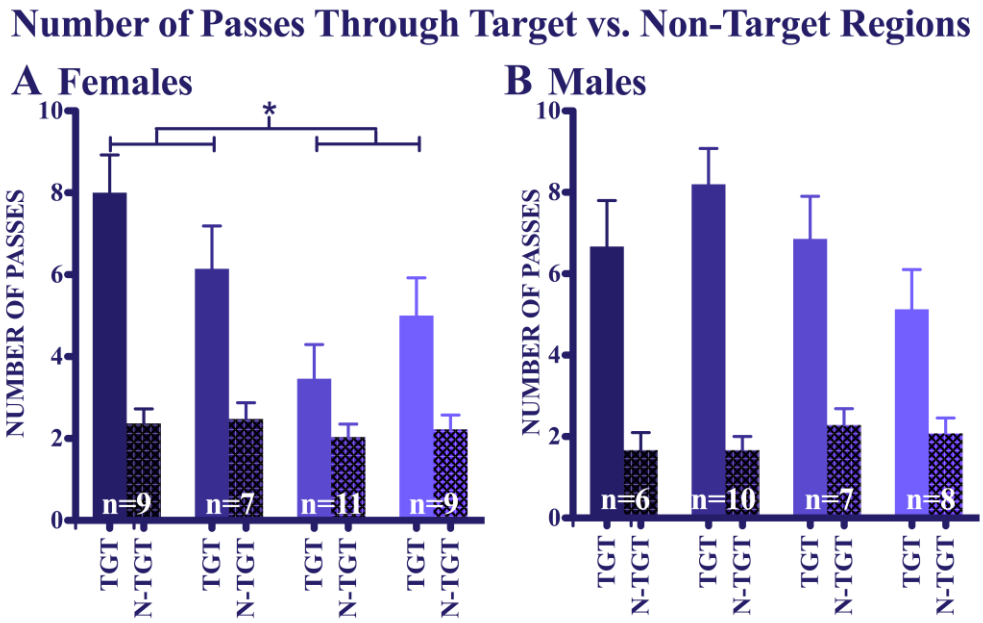

Figure 3.4.2 Number of Passes Through Target vs. Non-Target Regions of the Pool. From left to right and dark to light the groups are P7 saline – vehicle; P7 saline – peptide; P7 alcohol – vehicle; P7 alcohol – peptide. Solid bars represent time spent in the target while hatched bars represent time spent in an average of the three non-target platform regions. ★indicates $p<0.05$

Females

Female mice made more passes through the target vs an average of the other three non-target platform regions [Figure 3.4.2A; main effect of region type: $F(1,32) = 53.50$, $p<0.005$]. Alcohol treatment on P7 significantly decreased the number of passes through the target vs non-target platform regions [main effect of P7 alcohol treatment: $F(1,32) = 10.91$, $p<0.005$; P7 alcohol treatment X region type interaction: $F(1,32) = 7.62$, $p<0.01$]. There was a trend toward an interaction between P7 alcohol treatment, post-weaning

peptide treatment and region type due to the modest increases in passes through the target region in mice given P7 alcohol treatment, while those given saline on P7 did not improve [P7 alcohol treatment X peptide co-treatment X region type interaction $F(1,32) = 3.23$, $p=0.082$; P7 alcohol treatment X post-weaning peptide treatment interaction: $F(1,32) = 3.37$, $p=0.076$].

Males

Male mice also preferred the target to an average of the non-target regions of the pool [Figure 3.4.2B; $F(1,27) = 52.96$, $p<0.005$]. P7 alcohol treatment did not significantly reduce the number of passes through the target vs the non-target platform region. There was a trend toward an interaction between P7 alcohol treatment and post-weaning peptide treatment on the preference for the target region due to the slight peptide-induced impairment in P7 alcohol-treated mice, while mice given saline on P7 saw slight increases in the number of passes through the target vs non-target platform regions [P7 alcohol treatment x post-weaning peptide treatment interaction: $F(1,27) = 3.15$, $p=0.087$].

CHAPTER 4PRENATAL ALCOHOL SULIK MODEL RESULTS

4.1 Blood Alcohol Concentrations

The mean BACs on G8 are shown in Figure 4.1. The mean (±SEM) BAC on G8 after the first alcohol injection was 297 (±23) mg/dl at 30 minutes, and declined to 243 (±29) mg/dl at one hour, 134 (±22) mg/dl at 2.5 hours and 72 (±16) mg/dl at the time of the second injection. The next peak came 30 minutes after the second alcohol injection, was 481 (±30) mg/dl and then declined to 461 (±27) mg/dl at one hour, 417 (±27) mg/dl at 90 minutes and 370 (±28) at the last measurement two hours after the final alcohol injection. The alcohol clearance rate, calculated using the values obtained at 1 and 2 hours after the second alcohol injection to be 91.9 mg/dl/hr (Figure 4.1.1).

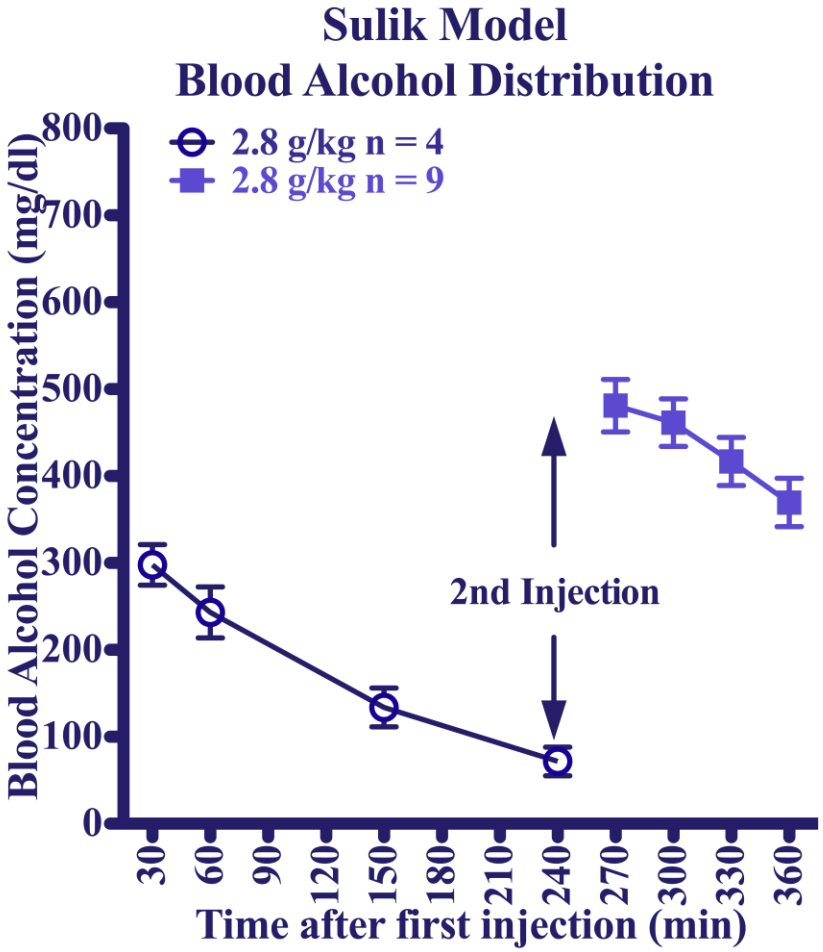

Figure 4.1.1 Blood Alcohol Concentrations. Tail bloods taken from 15 separate pregnant dams at 8 days gestation. The arrow indicates the time point of the second alcohol dose (2.8g/kg in Ringer's solution)

4.2Body Weight Analysis

Body weights of all pregnant dams were measured on the day of sperm plug G0, 6, 8, 10 and 15. All pregnant dams gained weight over the gestational period as expected [Figure 4.2.1; main effect of day: $F(4,104) = 1346.47$, $p<0.005$] although the pattern of weight gain over the gestational period depended on gestational treatment [day X treatment interaction: $F(4,104) = 2.95$, $p<0.05$]. Narrowing the analysis to the two gestational data

points after the alcohol treatment reveals that these differences in weight gain are not a function of the gestational alcohol treatment as only the main effect of day remains significant [$F(1,26) = 1452.07$, $p<0.005$].

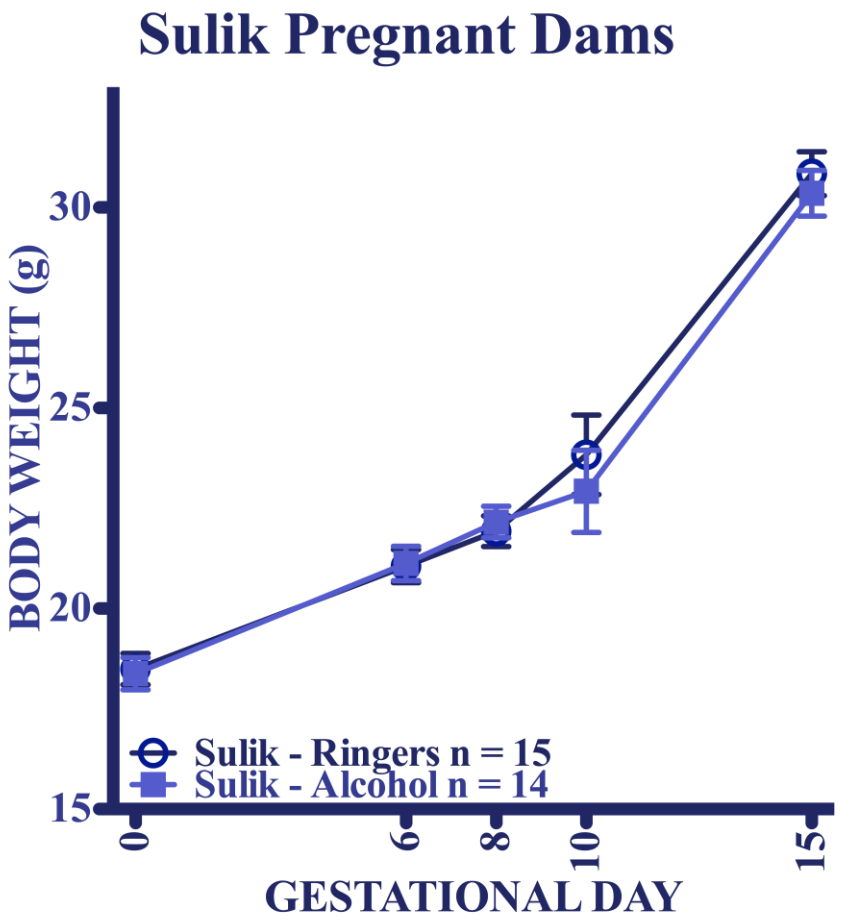

Figure 4.2.1 Pregnant Dam Body Weights. Open circles represent dams given saline on G8 closed squares represent dams given alcohol on G8.

Weights of offspring were measured on P6, 7, 10, 15, 21 and at weaning on P25. All offspring gained significant amounts of weight during the neonatal and early postnatal period [main effect of day: $F(5,260) = 481.71$, $p<0.005$] an effect that depended on sex as

males gained weight faster than females [day X sex interaction: $F(5,260) = 2.39$, $p<0.05$]. All female offspring gained weight throughout this period [Figure 4.2.2 A; main effect of day: $F(5,130) = 238.58$, $p<0.005$] an effect that gestational alcohol exposure did not alter. Male offspring showed the same pattern of weight gain increasing over the neonatal and early postnatal period [Figure 4.2.2 C; main effect of day: $F(5,130) = 244.90$, $p<0.005$]. Animal weights just before the initiation of the peptide co-treatment on P67 revealed only a main effect of sex [Figure 4.2.2 B&D; $F(1,50) = 28.57$, $p<0.005$], but no effect of gestational alcohol exposure on G8. Analysis of body weights after water maze training on P77 also showed only the expected main effect of sex [$F(1,137) = 512.63$, $p<0.005$] with no effects of either E8 alcohol exposure or the peptide co-treatment on P67 - 76.

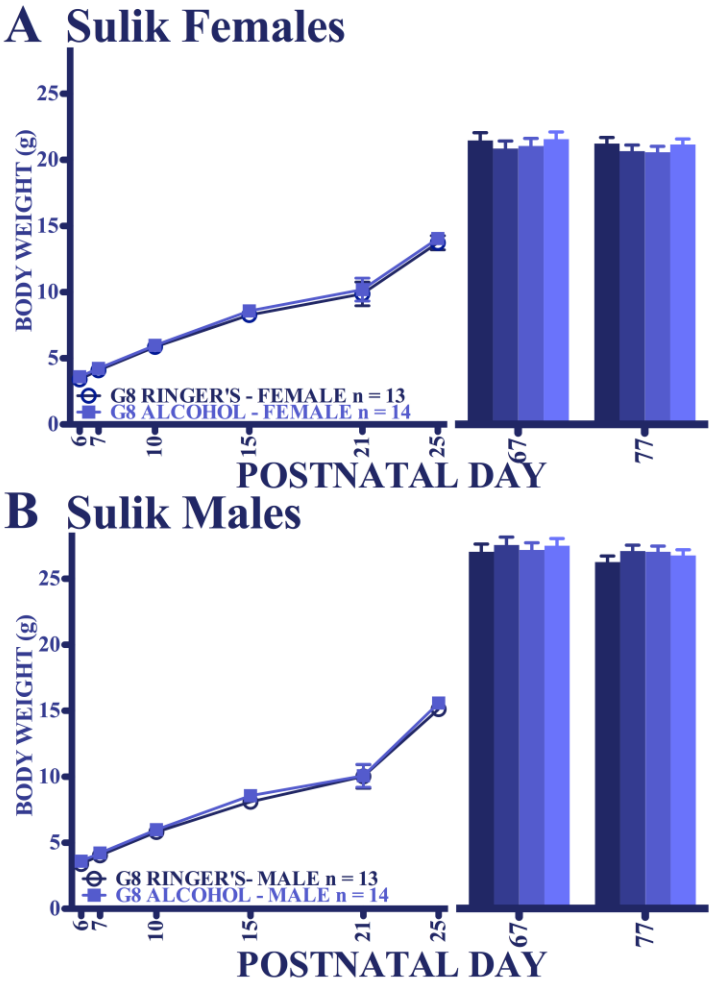

Figure 4.2.2 Offspring body weights. Open circles represent offspring of mice given saline on G8 closed squares represent offspring of mice given alcohol on G8. The bar graph separates mice further into their peptide treatment group. From left to right and dark to light the groups are G8 Ringer's – vehicle; G8 Ringer's – peptide; G8 alcohol – vehicle; G8 alcohol – peptide.

4.3 Morris Water Maze Acquisition

All offspring were able to increase water maze performance decreasing average path lengths swum to escape over training days [Figure 4.3.1; main effect of day $F(6,432) = 93.40$, $p<0.005$]. There were no main or interactive effects of G8 alcohol treatment, pre-

training co-peptide treatment or sex. As sex differences are often present in measures of spatial learning and memory, each sex was be evaluated separately to ensure differences aren't disguised by other performance measures.

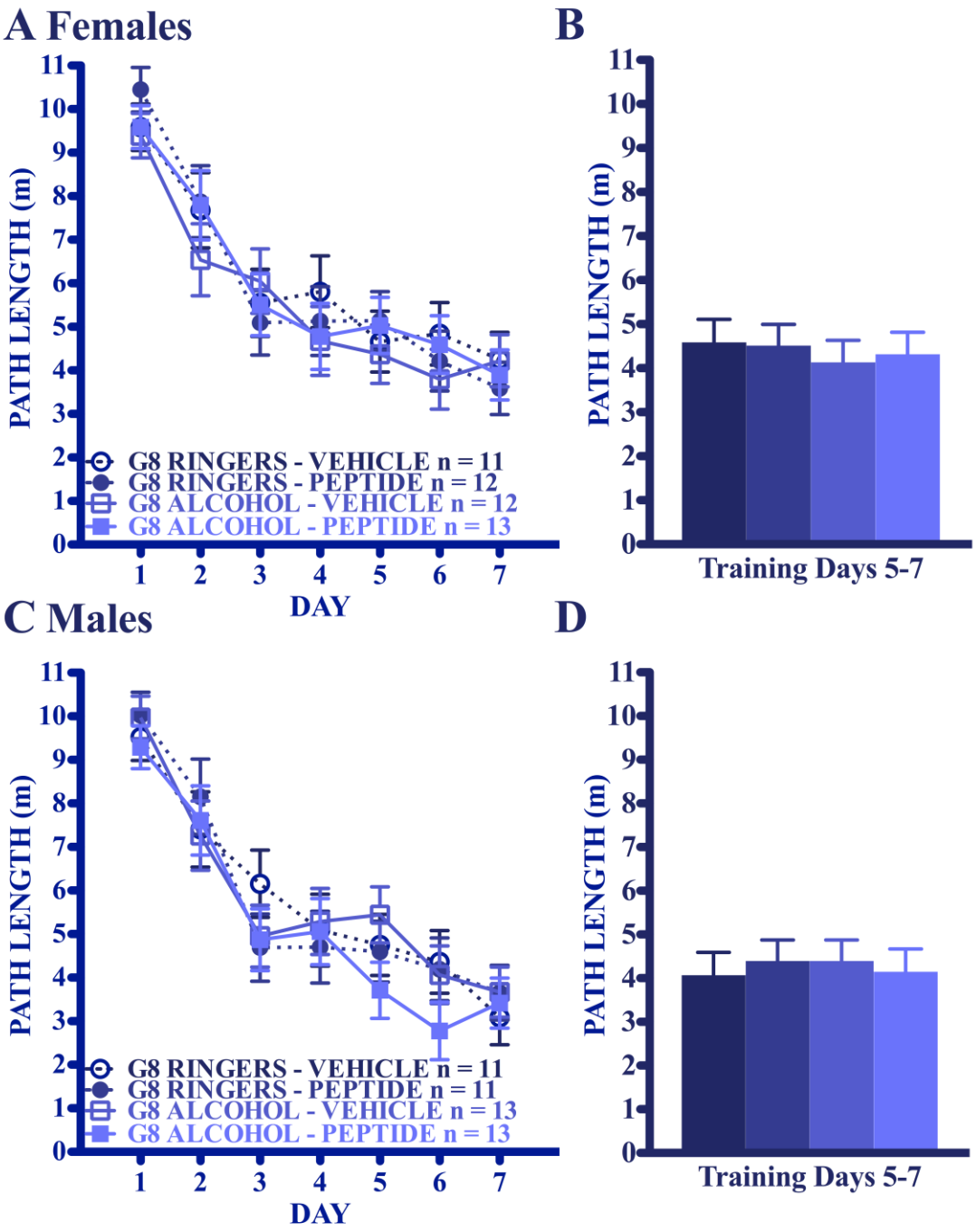

Figure 4.3.1 Morris Water Maze Acquisition. Panel A&C illustrate acquisition rates of each group over testing days, while B&D show performance collapsed over the last three days of training (separated by sex; females Panel A&C, males Panels B&D). Circles indicate offspring of mice given saline on G8. Squares represent offspring of mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide. In Panels B&D from left to right and dark to light the groups are G8 Ringer's – vehicle; G8 Ringer's – peptide; G8 alcohol – vehicle; G8 alcohol – peptide.

Females

Post hoc analyses revealed only the expected main effect of day [Figure 4.3.1A; $F(6,264) = 49.61$, $p<0.005$], illustrating that all female offspring were able to acquire the task regardless of pre-training co-peptide treatment.

Males

The same patterns were shown in male offspring with only the expected main effect of day [Figure 4.3.1C; $F(6,264) = 52.71$, $p<0.005$] reaching significance.

4.4 Water Maze Probe

There were no differences in performance measures during the probe trials such as path length swum, swimming speed or time spent in thigmotaxis.

4.4.1 Time Spent in the Target vs Non-Target Platform Regions

All offspring given 7 days of water maze training spent more time in the target platform region of the pool compared to the average time spent in the other three non-target platform regions of the pool [Figure 4.4.1; $F(1,88) = 100.49$, $p<0.005$], an effect that did not depend on gestational alcohol treatment on G8 or peptide co-treatment.

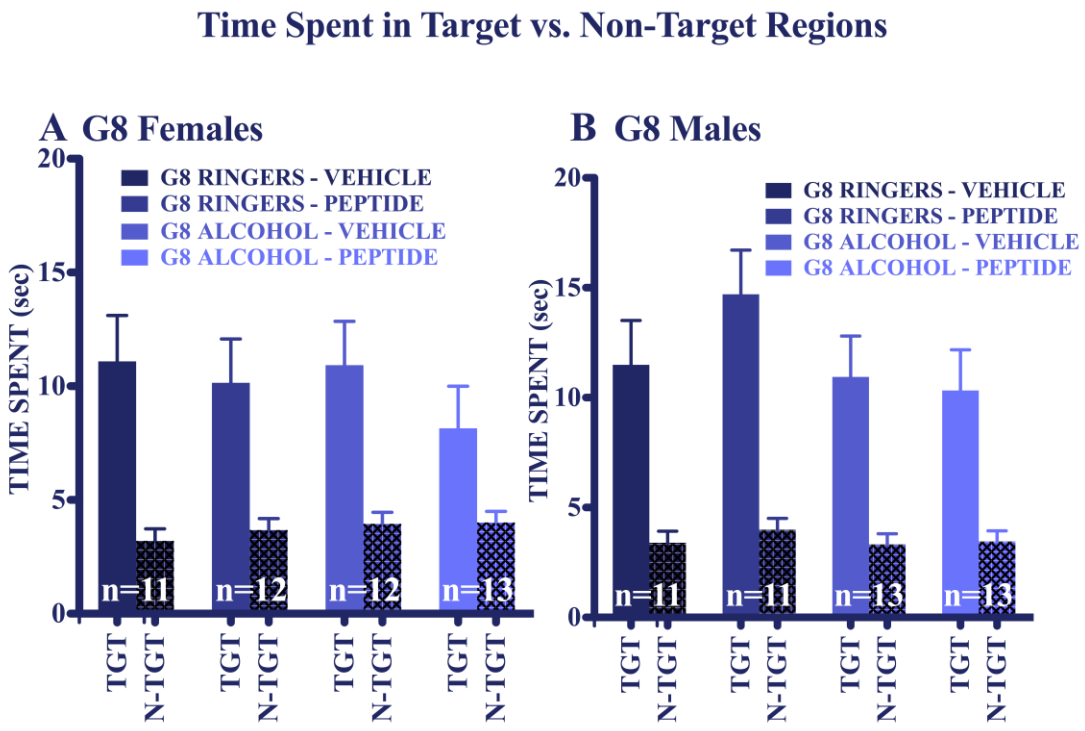

Figure 4.4.1 Time Spent in the Target vs. Non-Target Regions of the Pool. From left to right and dark to light the groups are G8 Ringer's – vehicle; G8 Ringer's – peptide; G8 alcohol – vehicle; G8 alcohol – peptide. Solid bars represent time spent in the target while hatched bars represent time spent in an average of the three non-target platform regions.

Females

Female offspring preferred the former platform-containing region to the average of the other three non-platform regions [Figure 4.4.1A; main effect of pool region: $F(1,44) = 43.46$, $p<0.005$]. This preference was not affected by gestational alcohol treatment on G8 or the peptide co-treatment.

Males

Male offspring also preferred the region of the pool that previously contained the platform over an average of the other three non-platform containing regions [Figure

4.4.1B; main effect of pool region: $F(1,44) = 57.00$, $p<0.005$], an effect that was not affected by alcohol exposure on G8 or peptide co-treatment group.

4.4.2 Passes Through the Target vs Non-Target Platform Regions

Similar patterns of performance were seen when evaluating the number of passes made through the region of the pool that had formerly contained the platform region compared to the average of the other three regions of the pool that had never contained the escape platform [Figure 4.4.2; main effect of pool region $F(1,65) = 212.74$, $p<0.005$]. This performance measure was also not affected by sex, gestational alcohol exposure on G8 or peptide co-treatment group.

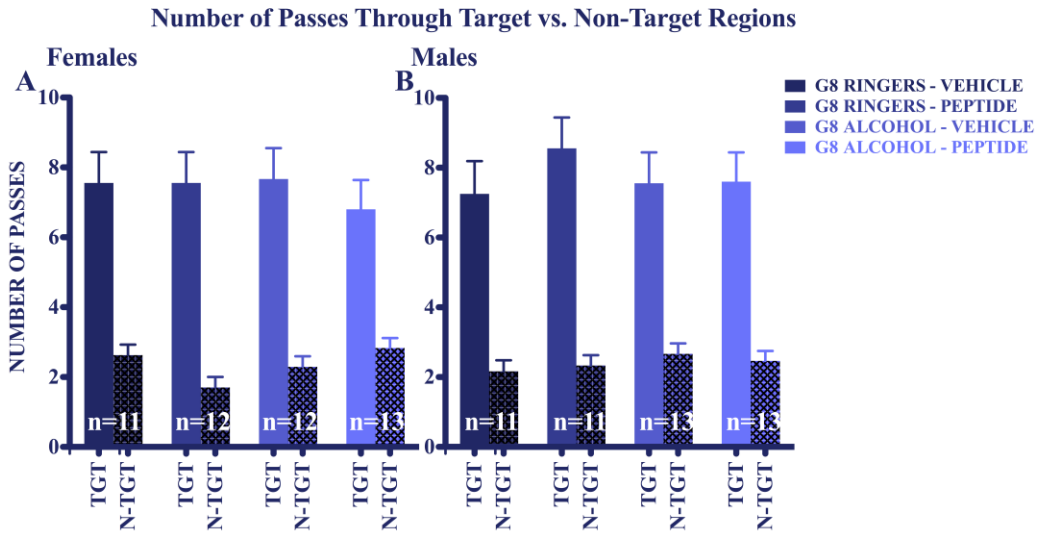

Figure 4.4.2 Number of Passes Through Target vs. Non-Target Regions of the Pool. From left to right and dark to light the groups are G8 Ringer's – vehicle; G8 Ringer's – peptide; G8 alcohol – vehicle; G8 alcohol – peptide. Solid bars represent time spent in the target while hatched bars represent time spent in an average of the three non-target platform regions.

Females

Female offspring showed similar performance, making more passes through the platform region that formerly contained the platform than the average number of passes through the other three regions of the pool [Figure 4.4.2A; main effect of pool region: $F(1,33) = 108.94$, $p<0.005$].

Males

Male offspring also made more passes through the region of the pool that had contained the platform compared to the average number of passes through the regions of the pool that had never contained the platform [Figure 4.4.2B; main effect of pool region: $F(1,32) = 103.95$, $p<0.005$].

CHAPTER 5 PRENATAL ALCOHOL WEBSTER MODEL RESULTS

5.1 Blood Alcohol Concentrations

The mean BACs on G8 are shown in Figure 5.1. The mean (±SEM) BAC on G8 after the alcohol injection was 731 (±22) mg/dl at 30 minutes, and declined to 656 (±12) mg/dl at one hour, 602 (±11) mg/dl at 1.5 hours and 557 (±14) mg/dl at two hours after the alcohol injection. The alcohol clearance rate, calculated using the values obtained at 1 and 2 hours after the second alcohol injection to be 99.1 mg/dl/hr (Figure 5.1.1).

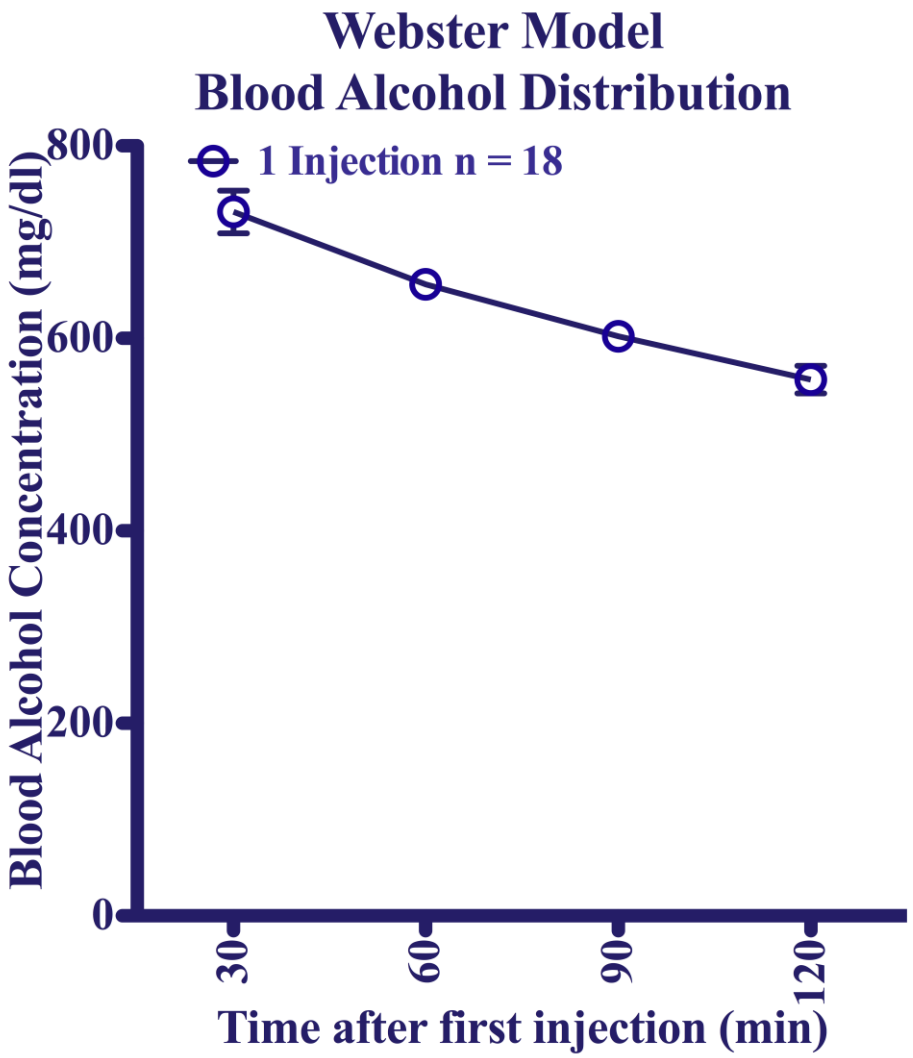

Figure 5.1.1 Blood Alcohol Concentrations. Tail bloods taken from 18 separate pregnant dams at 8 days gestation.

5.2Body Weight Analysis

Body weights of all pregnant dams were measured on the day of sperm plug G0, 6, 8, 10 and 15. All pregnant dams gained weight over the gestational period as expected [Figure 5.2.1; main effect of day: $F(4,72) = 49.74$, $p<0.005$]. In contrast to the Sulik alcohol exposure model there were no effects of alcohol exposure on G8 on maternal weight gain.

Narrowing the analysis to the two gestational data points after the alcohol treatment confirms gestational weight gain was not dependent on the gestational alcohol treatment as only the main effect of day remains significant [$F(1,18) = 35.85$, $p<0.005$].

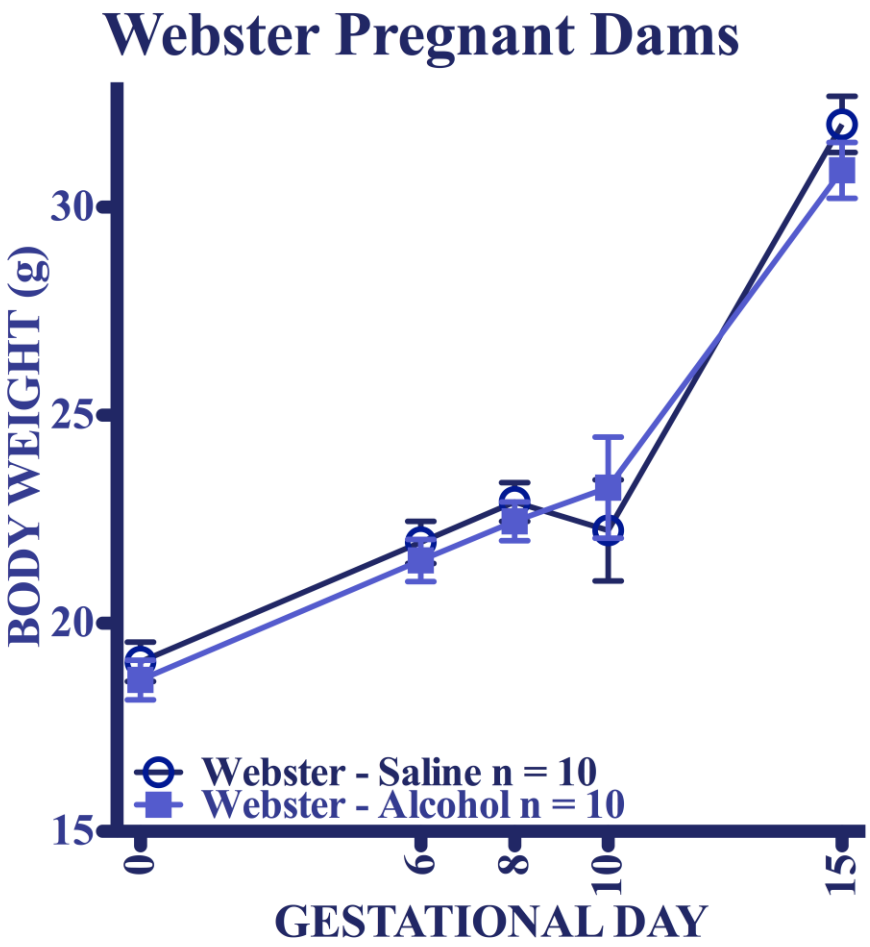

Figure 5.2.1 Pregnant Dam Body Weights. Open circles represent dams given saline on G8 closed squares represent dams given alcohol on G8.

Weights of offspring were measured on P6, 7, 10, 15, 21 and at weaning on P25. All offspring gained significant amounts of weight during the neonatal and early postnatal period [Figure 5.2.2; main effect of day: $F(5,175) = 1542.83$, $p<0.005$] Males gained

weight more rapidly than females [day X sex interaction: $F(5,175) = 11.15$, $p<0.005$] and G8 alcohol exposure delayed weight gain [day X alcohol treatment interaction: $F(5,175) = 2.41$, $p<0.05$] . All female offspring gained weight throughout this period [Figure 5.2.2A; main effect of day: $F(5,80) = 505.21$, $p<0.005$], an effect that gestational alcohol exposure did not alter. Male offspring showed the same pattern of weight gain increasing over the neonatal and early postnatal period [Figure 5.2.1B; main effect of day: $F(5,95) = 1189.46$, $p<0.005$]. Body weights just before the initiation of the peptide co-treatment on P67 were not affected by G8 alcohol exposure. Analysis of body weights after water maze training on P77 also showed no effects of either G8 alcohol exposure or the peptide co-treatment on P67 - 76.

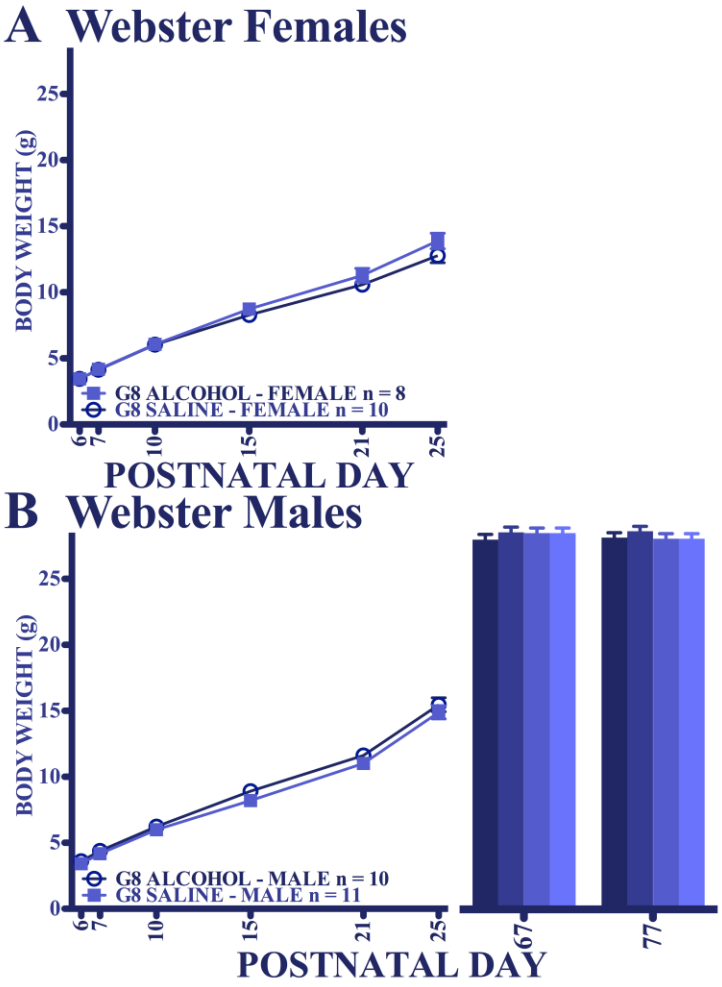

Figure 5.2.2 Offspring body weights. Open circles represent offspring of mice given saline on G8 closed squares represent offspring of mice given alcohol on G8. The bar graph separates mice further into their peptide treatment group. From left to right and dark to light the groups are G8 saline – vehicle; G8 saline – peptide; G8 alcohol – vehicle; G8 alcohol – peptide.

5.3 Morris Water Maze Acquisition

Males

All mice showed the typical performance improvement over the seven days of training illustrated by the expected main effect of day [Figure 5.3.1A; $F(6,234) = 33.78$, $p<0.005$].

While there were no main effects of G8 alcohol treatment or pre-training peptide co-treatment, analyses revealed a significant interaction of G8 alcohol exposure and peptide treatment over days [alcohol treatment X pre-training peptide co-treatment x day interaction $F(6,234) = 2.23$, $p<0.05$. Subsequent analyses yielded no further two way interactions, therefore, the three way interaction was driven by the change in groups over days not effects of either the alcohol treatment or peptide treatment on performance. A chi-square test of independence was performed to examine whether peptide treatment improved water maze acquisition in animals given alcohol treatment on G8. The criteria for acquisition was set as having an average path length of 5 meters or less on each day of training. There was no significant relationship between water maze acquisition in alcohol-treated animals as a function of peptide treatment on any testing day, or when collapsed over the first three days of training, as shown in figure 5.3.1b.

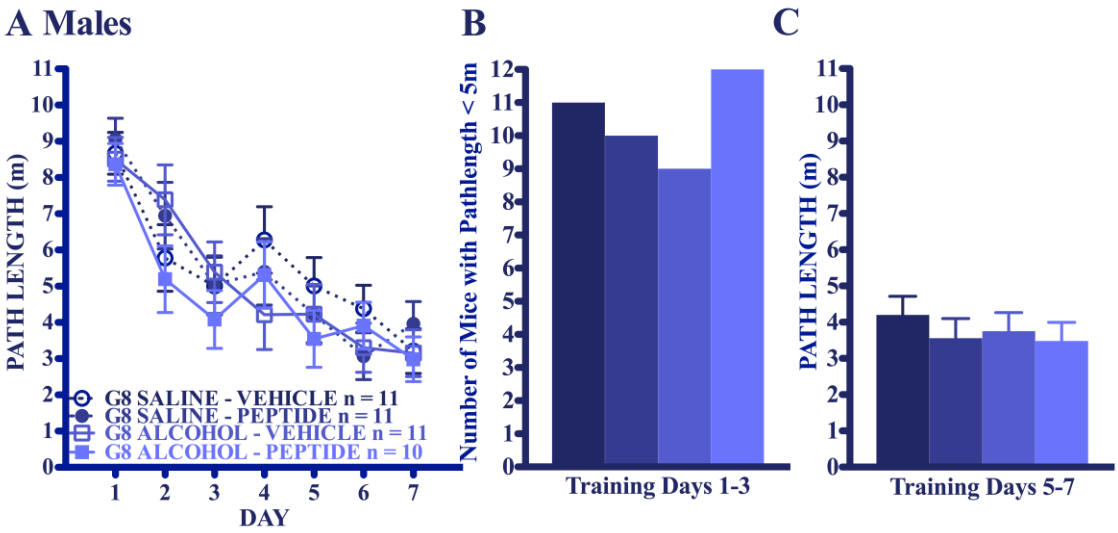

Figure 5.3.1 Morris Water Maze Acquisition. Panel A illustrates acquisition rates of each group over testing days, while B shows the number of mice meeting the 5m path length criteria over the first three days. C shows performance collapsed over the last three days of training. Circles indicate offspring of mice given saline on G8. Squares represent offspring of mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide. In Panel B and C from left to right and dark to light the groups are G8 saline – vehicle; G8 saline – peptide; G8 alcohol – vehicle; G8 alcohol – peptide.

5.4 Water Maze Probe

There were no differences in performance measures during the probe trials, such as path length swum, swimming speed or time spent in thigmotaxis.

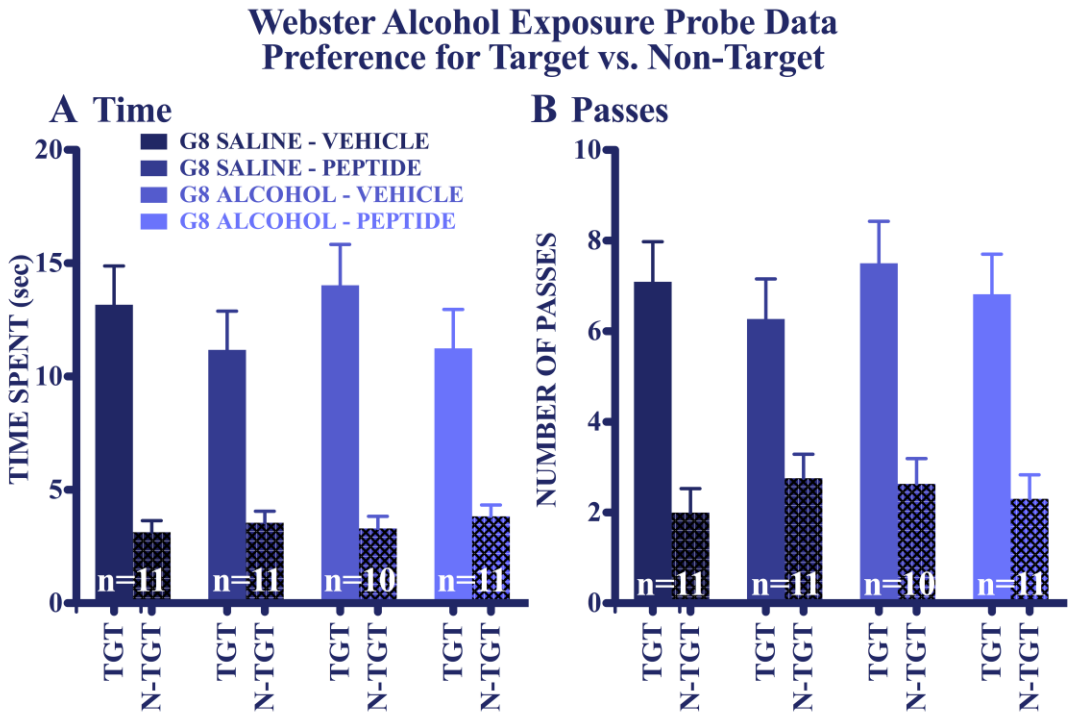

Figure 5.4.1 Probe Performance Panel A Time Spent in the Target vs. Non-Target Regions of the Pool. Panel B Number of Passes Through Target vs. Non-Target Regions of the Pool. Solid bars represent time spent in the target while hatched bars represent time spent in an average of the three non-target platform regions. From left to right and dark to light the groups are G8 saline – vehicle; G8 saline – peptide; G8 alcohol – vehicle; G8 alcohol – peptide.

5.4.1 Time Spent in the Target vs Non-Target Platform Regions

Males

After 7 days of water maze training all animals spent significantly more time in the region of the pool that had contained the escape platform [Figure 5.4.1A; main effect of

pool region: $F(1,39) = 86.40$, $p<0.005$]. This preference was affected by neither alcohol treatment on G8 nor the peptide co-treatment.

5.4.2 Passes Through the Target vs Non-Target Platform Regions

Males

Likewise all mice passed through the target platform region significantly more times than the average number of passes through the other three regions that never contained the platform [Figure 5.4.1B; main effect of pool region: $F(1,39) = 67.71$, $p<0.005$]. Passes through the platform region was also not affected by either G8 alcohol exposure or peptide co-treatment.

CHAPTER 6. DISCUSSION

6.1 Overall Summary

The primary objectives of the present experiments were to evaluate the therapeutic potential of the activity-dependent neuropeptides D-NAP and D-SAL in rescuing alcohol-induced deficits in the acquisition of spatial learning and memory. Herein it is reported that a single episode of a high-dose, binge-like alcohol exposure on P7 induces spatial learning deficits that persist into adulthood. These findings replicate previous reports that high-dose, binge-like alcohol exposure on P7 produces significant acquisition and retention deficits in Morris water maze learning in adult C57BL/6 mice (Wagner et al., submitted, Furumiya and Hashimoto, 2011). Peptide treatment with 40µg D-NAP and 40µg D-SAL did not have any effect on spatial learning in this model of FASD.

Surprisingly, neither model of a single episode of a high-dose, binge-like alcohol exposure on G8 produced significant learning deficits in the Morris water maze, even though the maternal peak BACs produced by the two models were very high (481 mg/dl for the Sulik model and 731mg/dl for the Webster model). The lack of effect in the latter case meant that this study failed to replicate the key previous study on which the current study was based, i.e., the study by Incerti et al. (2010c) that reported very large acquisition deficits with the same gestational treatment. Of course, the lack of a learning

deficit induced by the gestational alcohol treatment precluded the ability to determine peptide treatment effects. These findings underscore the need for confirmation of brain malformations due to the alcohol treatment, as there is thus far, no positive control to verify that the alcohol treatment had any effects. Tissues have been collected, but their analysis was outside the scope of this thesis.

6.2 Potential reasons for failure to replicate Incerti et al. (2010c)

Neither the Sulik nor the Webster model of alcohol exposure was able to replicate the spatial learning deficits found in the Incerti et al. (2010c) study. One major difference between the current studies and the Incerti et al. (2010c) study are the ages that Morris water maze testing occurred. Incerti et al. (2010c) tested their mice starting at P43, however, the current study sought to test animals that were through adolescence and well into adulthood (P70). Choosing to test animals in adulthood ensures animals are not experiencing a developmental delay in their spatial learning abilities, but, in fact, have enduring impairments, as a result of their developmental alcohol exposure.

Incerti and colleagues (2010c) do not report the diameter of the water maze used in their study. An earlier study from the same lab (Endres et al., 2005) reports using an 80cm diameter Morris water maze pool. The pool used in the current study was 126 cm in diameter. This size difference results in two and a half times the amount of searchable surface area, which would lead to a much more challenging task for the animals in the current study. In addition to having a smaller pool, mice in the Incerti et al. (2010) study were given two consecutive trials each day compared to four trials per day in this study.

Consecutive trials or ‘massed practice’ has been shown to produce slower acquisition than ‘distributed practice’ in the Morris water maze, suggesting a post trial rest period is necessary for acquisition of water maze place learning (Hasegawa et al., 1988, Commins et al., 2003). While the exact trial time was not reported, one can glean that the maximum trial time was 60 seconds based on the y-axis of the acquisition graph. The authors also fail to report the intertrial interval in which the animal is left on the platform. Previous reports from this lab have allowed mice 15 seconds on top of the platform before being placed in the pool for each trial, except for the first day when the mice are allowed 60 seconds before the trial begins (Endres et al., 2005). It is not clear from the methods of the Incerti et al. (2010c) paper if these are the methods used, but they are the most similar to those published by the same lab in other reports. Despite these methodological differences, control mice in the current study showed nearly identical terminal performance to control mice of the Incerti et al. (2010c) study; therefore, the differences in alcohol effects between the two studies can not be explained by differences in general performance or task parameters.

Another possibility for failure to replicate the peptide-treatment effect in our neonatal alcohol-treatment group could be the source of the peptides. The Incerti study does not report the source or purity of the peptides used in their study. The peptides D-NAP and D-SAL were obtained from Bio-Synthesis, Inc. and ABBIOTEC, respectively. Both peptide sources guaranteed products were >95% purity by high performance liquid chromatography and mass spectrometry analysis. There are no reports on the long-term stability of this peptide, thus great care was taken to ensure quality and viability. All

peptides were stored at -20ºC until prepared for gavage treatments. Fresh peptide solutions were prepared before each cohort began treatment. The peptide solutions were aliquotted and stored at -20ºC until the morning they were to be used. Once thawed, peptide solution was never refrozen, but was stored in a 4ºC refrigerator for use the next morning. A study by Brenneman and colleagues (1998), which discusses a steep decrease in bioavailability of SAL after even one freeaze-thaw cycle, had been overlooked until final revision of this thesis. Although, SAL is known to be the least potent and efficacious of the two peptides used, it is uncertain as to whether the lack of significant effects of the peptide treatments in this study and the differences between this study and that of Incerti and colleagues (2010c) can be fully explained by the decreased bioavailability of D-SAL. Further, this error could only account for differences in the peptide effect and cannot explain the lack of alcohol effects on water maze learning. Experiment 3 with the Webster model was intended to serve as a positive control, but since there were no significant alcohol- or peptide-treatment effects, the current studies have no positive control. However, given the guaranteed quality and careful handling of the peptides, it is unlikely that they were inactive.

The neuropeptides NAP and SAL are able to protect against alcohol-induced neurodegeneration when administered with or just following alcohol administration. Although not much is known about their upstream or downstream effects, recent work has determined that NAP and SAL exert their neuroprotective effects in part by interfering with proapoptotic and inducing antiapoptotic cytokines and chemokines (Roberson et al., 2012, Zamostiano et al., 1999), preventing mitochondrial accumulation

of reactive oxygen species (Glazner et al., 1999), and promoting axonal elongation (Smith-Swintosky et al., 2005) and synapse formation (Blondel et al., 2000). Other studies illustrating NAP- and SAL-induced increases in hippocampal cell density (Kumral et al., 2006) gave promise to the rehabilitative potential of these neuropeptides, but in studies showing these promising results, the treatment is either applied just after the inflammation event, or during a period in which the brain is already in a very plastic state.

Perhaps the lack of a peptide-treatment effect in the postnatal study could be indicative of a critical window for peptide treatment exposure, rather than an inability to recover spatial learning as measured by the Morris water maze. For example, alcohol exposure on G8, producing peak BACs of 381mg/dl (Parnell et al., 2009), has been shown to disrupt development of the neural plate (Sulik and Johnston, 1983, Sulik et al., 1981, Sulik et al., 1984), producing volumetric reductions in olfactory bulbs, hippocampus and septal regions, as well as the cerebellum and pituitary gland (Parnell et al., 2009). It is possible that the peptide treatment interacts with these damaged neural substrates in a particular way during adolescence that increases the functional integrity of systems necessary for spatial learning and memory. It is possible that adolescence is a critical period for influencing synaptic plasticity in brains that have undergone this particular type of damage that results in significant hippocampal cell loss and that peptide treatments given during this time period are able to integrate the surviving cells into a more functional network. While there is no evidence for NAP and SAL inducing hippocampal LTP, there

is evidence for NAP potentiating alcohol-induced inhibition synaptic plasticity through its interactions with the NMDA receptor (Zhang et al., 2005).

Given the lack of an alcohol treatment effect in the current study using the Sulik model, the final aim of this thesis was to replicate the alcohol treatment paradigm used by Incerti et al. (2010c) with the expectation that the most severe alcohol administration of a 5.8g/kg bolus on G8 could produce a spatial learning deficit that would allow the peptide co-treatment effects to be adequately evaluated. However, despite producing "heroic" (and near toxic) peak BACs of 730mg/dl on G8, this model surprisingly failed to produce a significant deficit in Morris water maze acquisition in the young adult male C57BL/6 mice. Therefore, the effects of the peptide co-treatment on G8 alcohol-induced learning deficits could not be evaluated in this study. Although the peak BACs reached in the current study are approximately 91% of that reported by the Webster and colleagues (1980), from whom Incerti modeled their exposure, it is implausible that a spatial learning deficit could have been elicited from a G8 treatment with peak BACs of 800mg/dl, but not a similar one with peak BACs of 730mg/dl. There have been decades of work illustrating a timing- and does-dependent relationship between developmental alcohol exposure and its detrimental effects, but those studies generally evaluate differences in effects with doses ranging between 50 and 400mg/dl. The BACs generated in all of the current experiments are well above any that have been evaluated as "threshold" doses for hippocampal neurotoxicity or behavioral dysfunction (Goodlett and Johnson, 1997, Hunt et al., 2009, West et al., 1990, West et al., 1987, Ikonomidou et al., 2000).

It should be noted that all of the aforementioned studies that evaluated hippocampal neurotoxic or behavioral thresholds were evaluated using one form or another of third-trimester equivalent exposure. Incerti et al. (2010c) appear to be the first to report deficits in Morris water maze acquisition after a G8 alcohol exposure, but they were not the first to investigate the effects of this exposure on spatial learning and memory. Minetti and colleagues (1996) failed to show water maze acquisition deficits in both 45- and 90-day old rats that had been exposed to a Sulik-style alcohol exposure on G8 that produced BACs of 457mg/dl 60 minutes after the second alcohol injection.

Our findings are consistent with many reports of spatial learning and memory deficits induced by high-dose alcohol insults during the third trimester equivalent (Furumiya and Hashimoto, 2011, Goodlett and Peterson, 1995, Johnson and Goodlett, 2002, Pauli et al., 1995, Tomlinson et al., 1998, Goodlett and Johnson, 1997). Our previous study of P7 binge-like exposure in C57BL6 mice – that formed the basis for the current test of the neuroprotective peptides in the neonatal alcohol exposure model – showed that the single-day, binge-like alcohol exposure on P7 induced significant spatial learning deficits in adulthood, supporting it as an excellent model by which to assess the therapeutic potential of the activity-dependent, neuroprotective peptide co-treatment. The current results failed to confirm (in P70 adults) the rescue effects of the peptide treatment reported for (P40 late adolescents) by Incerti et al., (2010c). In our hands, the 40µg D-NAP plus 40µg D-SAL administered three days prior to and throughout Morris water maze training failed to influence spatial learning and memory deficits induced by P7

alcohol exposure in our version of the Morris water maze task. Due to the laborious and costly nature of this protocol, a dose-response peptide treatment was determined to be prohibitively expensive. However, it is possible, especially given the freeze-thaw related decrease in bioavailability of SAL, that the doses of peptides were insufficient to rescue such severe spatial learning deficits.

The results of the Incerti et al. (2010c) study were interesting and exciting to say the least. No other pharmacological studies have shown such a successful recovery of functioning after such a devastating developmental, alcohol-induced learning deficit. At this point, pharmacotherapeutic interventions have been more successful in the attenuation or prevention of developmental alcohol-induced neurobiological and behavioral dysfunction when administered concurrently with the alcohol treatment, whether the insult occurs gestationally or in the early postnatal period. The antioxidant/vitamin work is a great example of this concept. The antioxidants exert their effects against alcohol-induced apoptosis by scavenging the excessive reactive oxygen species, which are agents that cause activation of the apoptosis cascades. The antioxidant mechanisms of neuroprotection are, therefore, most beneficial during an inflammation event, such as binge-like alcohol exposure, when there are reactive oxygen species being produced. As such, administration of antioxidants after the damage has already occurred would be expected to have little or no effect. While the work on choline supplementation seems to suggest that co-administration with the alcohol insult is helpful in the reversal of alcohol-induced behavioral and neurobiological disruptions, the fact that choline supplementation has a more global effect on learning, increasing performance of controls as well as

alcohol-exposed animals, suggests that it may not be necessary that the choline supplementation be administered at the time of the alcohol insult. However, the choline supplementation in these studies was all administered during a time when the brain is very plastic. Future studies are needed to examine the potential of choline supplementation when administered later in life in the reversal of developmental alcohol-induced learning deficits.

Other more classic pharmacotherapies, such as drugs that inhibit NMDAR hyperexcitability in response to alcohol withdrawal (Gibson et al., 2003, Lewis et al., 2012) and 5HT1A agonists– which in culture have been shown to stimulate astrocytic release of trophic support (Eriksen and Druse, 2001) thereby preventing alcohol-induced cell loss (Tajuddin and Druse, 1999) – have shown therapeutic potential, but could have potentially dangerous consequences if administered in vivo due to the important role both serotonin and NMDA play in the migration, synaptogenesis and organogenesis during gestational and perinatal development (Zhou et al., 2002, Xu et al., 2004, Zhou et al., 2005, Xiao et al., 2013).

Finally, behavioral therapies have shown promise in the rehabilitation of a variety of alcohol–induced learning deficits. Importantly, behavioral therapies investigated in the treatment of alcohol-induced behavioral and neurological dysfunction have been shown to be effective long after the alcohol-induced damage has occurred. Environmental enrichment and voluntary exercise are both able to stimulate neurogenesis (Hamilton et al., 2012), improve alcohol-induced motor coordination deficits (Klintsova et al., 1997,

Munn et al., 2011), normalize alcohol-induced hyperactivity (Thomas et al., 2008), and improve alcohol-induced spatial learning deficits (Berman and Hannigan, 2000, Thomas et al., 2008). This profile of benefits makes these interventions well suited for the treatment of damage induced by alcohol at any point in development. In contrast, acrobatic motor learning has been shown to increase cerebellar synaptogenesis (Klintsova et al., 2000) and improve alcohol-induced deficits in motor performance (Klintsova et al., 2000, Klintsova et al., 1998) and cerebellar-dependent eyeblink classical conditioning (Wagner et al., 2013), but have not yet been shown to improve deficits in hippocampal-dependent tasks induced by alcohol exposure during the third trimester equivalent. Therefore, acrobatic motor learning is well suited for treatment of a third trimester alcohol insult, but would be best used in combination with environmental enrichment, voluntary exercise or choline supplementation.

Although behavioral therapies and choline supplementation have shown promise, it's important to pursue the therapeutic potential of activity-dependent neuroprotective peptides. These peptides are able to limit damage induced by alcohol, and in at least one report (Incerti et al., 2010c), completely recover gestational alcohol-induced deficits in adult offspring. The lack of a positive control in the current studies means it is too early to close the book on the rehabilitative potential of the activity dependent neuropeptides D-NAP and D-SAL. First, NAP and SAL should be tested against a reliable spatial learning deficit in adolescent mice to determine whether the peptide treatment is more effective during adolescence and why. If adolescence is a critical period for synaptogenesis, which drove the success in the Incerti et al. (2010c) study, the peptide

treatment should be successful in correcting the spatial learning deficit resulting from a postnatal alcohol insult. Because the G8 alcohol insult was unable to produce a deficit by which to test the peptide treatment, the next logical step would be to increase task demands by adding reversal, which has purportedly been able to elucidate subtle deficits in spatial learning following a G8 alcohol exposure (Wieczorek et al., 2013). If both of these experiments fail to confirm the peptide effect of Incerti et al. (2010c), I think the route of administration of the peptide should be changed. Whereas oral administration does have its translational flare, administration via an intraperitoneal injection or intranasally could ensure enough of the peptide is getting to the brain to be efficacious. However, given the lack of bioavailability data from these routes of administration this idea is a generalization based on the routes of administration of drugs in general. If the peptide treatment can be shown to be successful in reversing spatial learning deficits, it will be important to determine if this effect generalizes to other tests of spatial learning and memory and then finally to other tests of hippocampal-dependent functioning that are independent of spatial processing.

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APPENDIX

APPENDIX

A.1 Postnatal Alcohol Acquisition

A 2 (alcohol treatment) x 2 (peptide treatment) x 2 (sex) x 7 (day) repeated measures ANOVA revealed that all offspring improved performance in the water maze task over time by decreasing the average latency to escape over days [Figure A.1.1; main effect of day: $F(6,516) = 56.95$, $p<0.005$]; however, this improvement was impaired by alcohol exposure on P7 [main effect of alcohol $F(1,86) = 26.88$, $p>0.005$]. Unlike path length performance only tended to be sex dependent, as males decreased escape path lengths faster than females and ended with lower terminal performances [main effect of sex $F(1,86) = 2.95$, $p=0.089$]. A 2 (alcohol treatment) x 2 (peptide treatment) x 2 (sex) x 7 (day) repeated measures ANOVA revealed that changes in swimming speed over training days depended on both alcohol treatment and sex [main effect of alcohol treatment: $F(1,86) = 9.52$, $p<0.005$; alcohol treatment x day interaction: $F(6,516) = 4.28$, $p<0.005$; alcohol treatment x sex x day interaction: $F(6,516) = 2.61$, $p<0.05$]. As shown in figure A.1.2, a 2 (alcohol treatment) x 2 (peptide treatment) x 7 (day) repeated measures ANOVA revealed that Male animals given saline during the neonatal period swam faster than those given alcohol in the neonatal period [main effect of alcohol: $F(1,41) = 5.00$, $p<0.005$; alcohol x day interaction $F(6,246) = 6.57$, $p<0.005$].

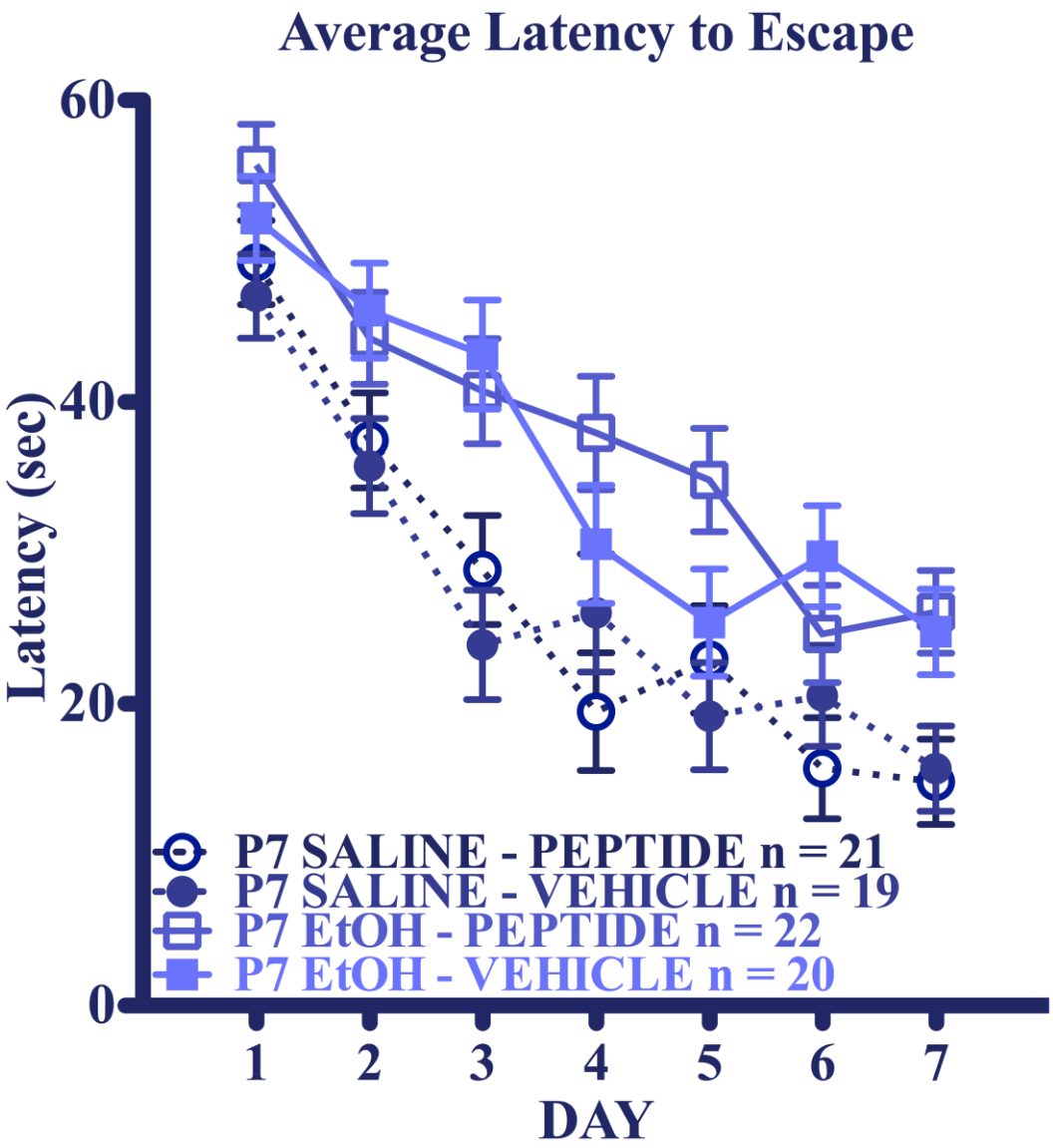

Figure A.1.1 Morris Water Maze Acquisition Mean Latency to Escape. Circles indicate mice given saline on P7. Squares represent mice given alcohol on P7. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

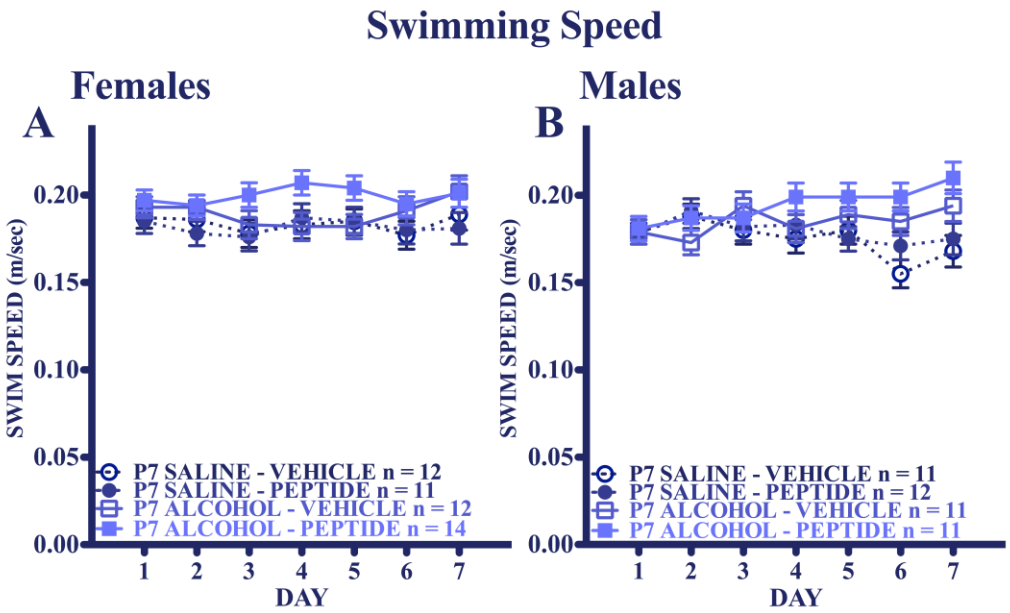

Figure A.1.2 Morris Water Maze Swimming Speed During Acquisition. Circles indicate mice given saline on P7. Squares represent mice given alcohol on P7. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

A.2 Sulik Model Acquisition

A 2 (alcohol treatment) x 2 (peptide treatment) x 2 (sex) x 7 (day) repeated measures ANOVA revealed that all offspring improved performance in the water maze task over time by decreasing the average latency to escape over days [Figure A.2.1; main effect of day: $F(6,528) = 84.62$, $p<0.005$]; an effect that was in no way influenced by alcohol exposure on G8 or peptide treatment during the training period. A 2 (alcohol treatment) x 2 (peptide treatment) x 2 (sex) x 7 (day) repeated measures ANOVA revealed that changes in swimming speed over training days depended on both alcohol treatment and sex [main effect of alcohol treatment: $F(1,86) = 9.52$, $p<0.005$; alcohol

treatment x day interaction: $F(6,516) = 4.28$, $p<0.005$; alcohol treatment x sex x day interaction: $F(6,516) = 2.61$, $p<0.05$]. As shown in figure A.2.2, swimming speed was influenced by a combination of factors. Specifically, throughout training, mice given alcohol treatment swam significantly slower than those given Ringer's solution on G8 [main effect of alcohol treatment: $F(1,88) = 4.18$, $p<0.05$] an effect that was also found to be dependent on peptide treatment and sex [alcohol treatment x peptide treatment x sex interaction: $F(1,88) = 14.23$, $p<0.005$]. A 2 (alcohol treatment) x 2 (peptide treatment) x 7 (day) repeated measures ANOVA confirmed that this effect was driven by the fact that female mice given alcohol on G8 and then given vehicle intubations during the training period swam significantly slower than all other groups [alcohol treatment x peptide treatment interaction: $F(1,44) = 5.37$, $p<0.05$].

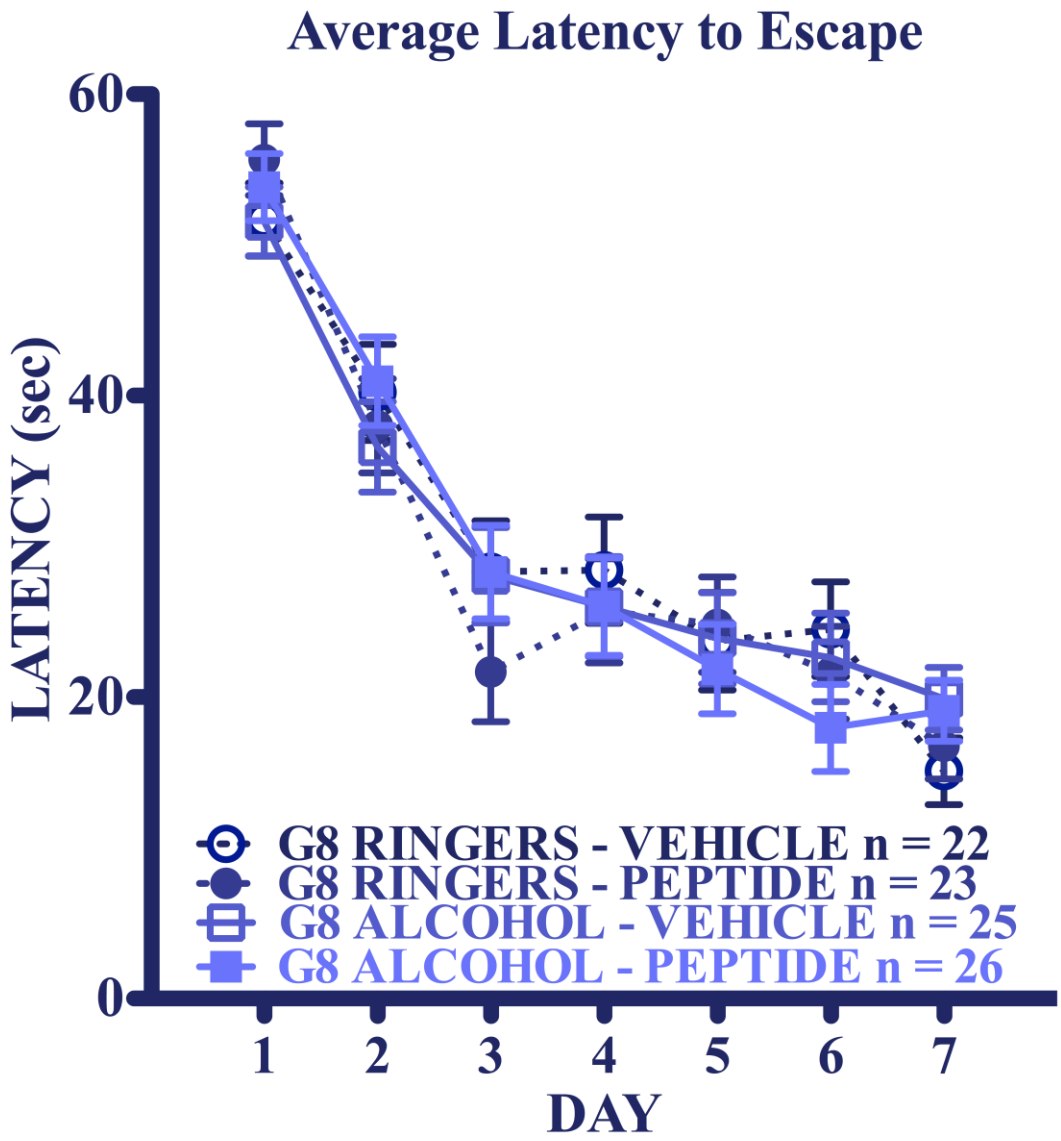

Figure A.2.1 Sulik Model Morris Water Maze Acquisition Mean Latency to Escape. Circles indicate mice given Ringer's Solution on G8. Squares represent mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

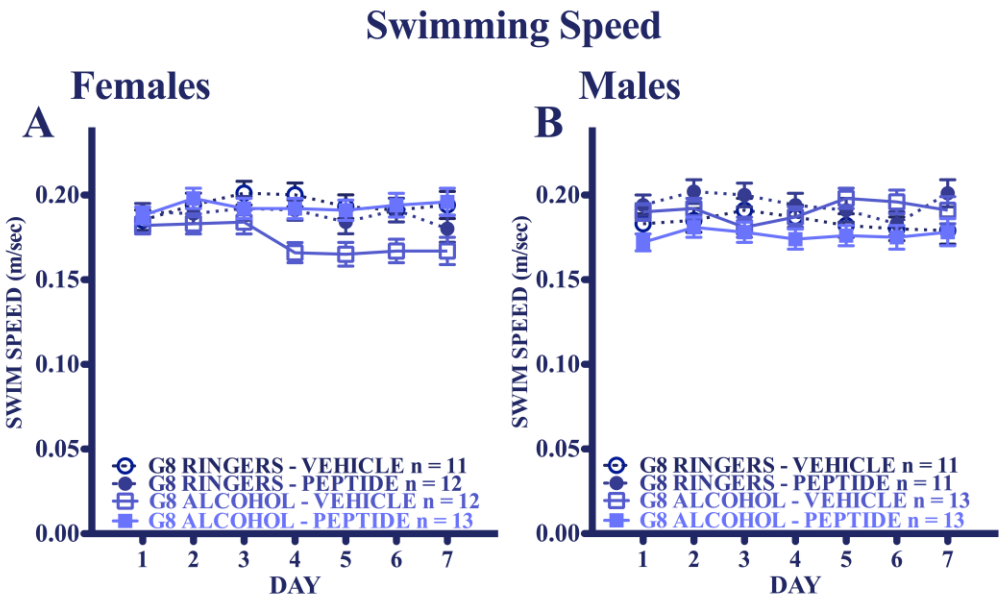

Figure A.2.2 Sulik Model Morris Water Maze Swimming Speed During Acquisition. Circles indicate mice given Ringer's solution on G8. Squares represent mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

A.3 Webster Model Acquisition

A 2 (alcohol treatment) x 2 (peptide treatment) x 7 (day) repeated measures ANOVA revealed that all offspring improved performance in the water maze task over time by decreasing the average latency to escape over days [Figure A.3.1; main effect of day: $F(6,234) = 1.67$, $p<0.005$]; an effect that was in no way influenced by alcohol exposure on G8 or peptide treatment during the training period. A 2 (alcohol treatment) x 2 (peptide treatment) x 7 (day) repeated measures ANOVA revealed that all animals increased swimming speed over the course of training [main effect of day: $F(6,234) = 5.16$, $p<0.005$]. However, alcohol animals swam slower over the course of training

compared to controls [alcohol treatment x day interaction: $F(6,234) = 2.86$, $p<0.05$; figure A.3.2].

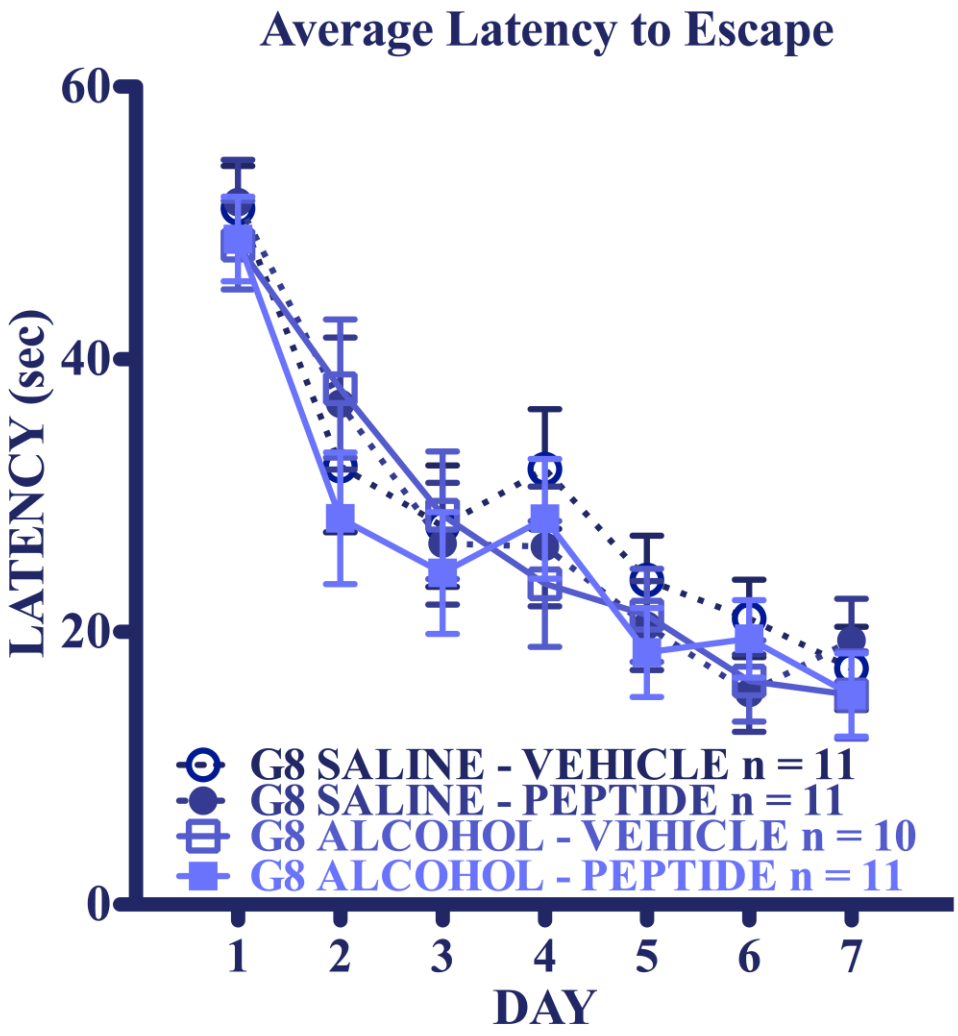

Figure A.3.1 Webster Model Morris Water Maze Acquisition Mean Latency to Escape. Circles indicate mice given saline on G8. Squares represent mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

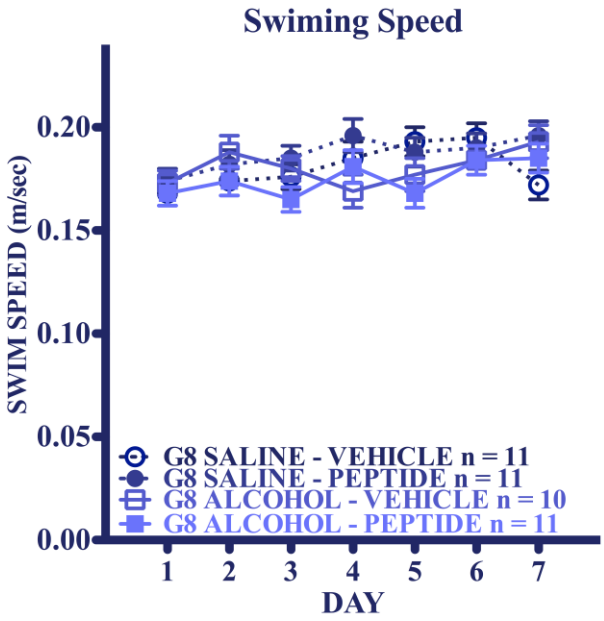

Figure A.3.2 Webster Model Morris Water Maze Swimming Speed During Acquisition. Circles indicate mice given saline on G8. Squares represent mice given alcohol on G8. Open symbols represent animals given vehicle in the post-weaning period. Closed symbols represent mice given peptide.

VITA

VITA

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Education

Ph.D. **Indiana University Purdue University Indianapolis, Indianapolis, IN**
2013 Area: Addiction Neuroscience
Dissertation: Effects of Coadministration of D-Napvsipq and D-Sallrsipa on spatial learning after developmental alcohol exposure.

M.S. **Indiana University Purdue University Indianapolis, Indianapolis, IN**
2008 Area: Psychobiology of Addictions
Thesis: Effects of One- and Three-Day Binge Ethanol Exposure in Neonatal C57BL/6 Mice on Spatial Learning in Adolescence and Adulthood.

B.S. **Indiana University Purdue University Indianapolis, Indianapolis, IN**
2005 Major: Psychology, Minor: Chemistry, Concentration(s): Behavioral Neuroscience, Psychobiology of Addictions

Refereed Journal Articles

Wagner, J.L., Klintsova, A.Y., Greenough, W.T., and Goodlett, C.R. (2013) Rehabilitation training using complex motor learning rescues deficits in eyeblink classical conditioning in female rats induced by binge-like neonatal alcohol exposure. Alcoholism: Clinical and Experimental Research, 37, 1561-1570. PMID:23647404
.

Papers Under Review or In Preparation

Wagner J.L., Zhou, F.C., and Goodlett C.R. Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning and memory in adolescence and adulthood. Alcohol, submitted.

Wagner J.L., Anthony B., Goodlett C.R., Zhou F.C. Effects of alcohol and NAPVSIPQ (NAP) on locomotor activity levels and spatial learning. In Preparation.

Wagner J.L. and Goodlett C.R. Effects of Coadministration of Oral D-NAPVSIPQ and D-SALLRSIPA on Spatial Learning and Memory Deficits in C57BL/6J Mice Induced by Developmental Alcohol Exposure. In Preparation.

Published Abstracts and Paper Presentations

Wagner J.L., Beck B.M., Neal-Beliveau B.S. (2005) Conditioned place preference for methamphetamine in adolescent and adult rats after chronic exposure to methylphenidate. International Society for Developmental Psychobiology annual meeting, Washington D.C., November 12, 2005

Wagner J.L., Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning in adolescence, Department of Psychological Sciences, Purdue University, April 16, 2007.

Wagner J.L. and Goodlett C.R. (2007) Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning in adolescence, Research Society on Alcoholism annual meeting, Chicago, IL. July 10, 2007

Wagner J.L. and Goodlett C.R. (2007) Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning in adolescence
NIAAA Training Workshop, Indianapolis, IN. September 29, 2007

Wagner J.L. and Goodlett C.R. (2007) Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning in adolescence
Local Society for Neuroscience annual meeting, Indianapolis, IN. October 26, 2007

Wagner J.L. Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning, Department of Psychological Sciences, Purdue University, April 23, 2008.

Wagner J.L. and Goodlett C.R. (2008) Effects of one- and three-day binge ethanol exposure in neonatal C57BL/6 mice on spatial learning
Research Society on Alcoholism annual meeting, Washington D.C. July 2, 2008

Wagner J.L., Garzon D., Klintsova A.Y., Greenough W.T., and Goodlett C.R.(2008) Complex motor learning ameliorates deficits in eyeblink classical conditioning in a rat model of binge alcohol exposure during the third trimester equivalent.
Fetal Alcohol Spectrum Disorder Study Group annual meeting, Washington D.C. June 28, 2008

Wagner J.L., Garzon D., McKay J., Klintsova A.Y., Greenough W.T., and Goodlett C.R. (2008) Complex motor learning ameliorates deficits in eyeblink classical conditioning in a rat model of binge alcohol exposure during the third trimester equivalent.
International Society for Developmental Psychobiology annual meeting, Washington D.C. November 13, 2008

Wagner J.L. and Goodlett C.R. (2009) Complex motor learning ameliorates neonatal alcohol-induced deficits in short-delay but not trace eyeblink conditioning. NIAAA Trainee Conference, New Orleans L.A. March 26, 2009

Wagner J.L., Loghmani J.S., and Goodlett C.R. (2009) Complex motor learning ameliorates neonatal alcohol-induced deficits in short-delay but not trace eyeblink conditioning. Research Society on Alcoholism annual meeting, San Diego, C.A, June 21, 2009

Wagner J.L. Acrobatic training ameliorates neonatal alcohol-induced deficits in short-delay but not trace eyeblink conditioning. Local Society for Neuroscience annual meeting, Indianapolis IN, October 30, 2009

Wagner J.L. Twenty days of complex motor learning rehabilitates alcohol-induced deficits in cerebellar-dependent tasks in an animal model of fetal alcohol spectrum disorders. First Annual Ann Daugherty Symposium for Basic Science and Addiction Recovery, Franklin IN, May 14, 2010

Wagner J.L., Loghmani J.S., Federici L.M., and Goodlett C.R. (2010) The benefits of complex motor learning are specific to cerebellar-dependent tasks that do not rely on forebrain structures. Research Society on Alcoholism annual meeting, San Antonio TX, June 28, 2010

Wagner J.L. Cognitive and behavioral deficits associated with fetal alcohol spectrum disorders. Second Annual Ann Daugherty Symposium for Basic Science and Addiction Recovery, Franklin IN, May 26, 2011

Wagner J.L., Loghmani J.S., Anthony B., Goodlett C.R., Zhou F.C. (2011) Effects of alcohol and NAPVSIPQ (NAP) on locomotor activity levels and spatial learning. Research Society on Alcoholism annual meeting, Atlanta GA, June 26, 2011

Wagner J.L. and Goodlett C.R. (2012) Oral D-NAPVSIPQ and D-SALLRSIPA fails to rescue spatial learning and memory deficits resulting from a single heavy binge-like alcohol exposure on postnatal day 7. Society for Neuroscience annual meeting, New Orleans LA, October 14, 2012

Wagner J.L. and Goodlett C.R. (2013) Acute alcohol on gestational day 8 fails to disrupt spatial learning and memory in adult mice. Research Society on Alcoholism annual meeting, Orlando FL, June 24, 2013

Professional Memberships

International Society for Developmental Psychobiology (2004)

Research Society on Alcoholism (2006)

Fetal Alcohol Spectrum Disorder Study Group (2006)

Indianapolis Society for Neuroscience (2004)
Society for Neuroscience (2012)

Psychology Graduate Student Organization
– Psychobiology of Addictions Representative (2010)

Awards

Undergraduate Research Opportunities Program Grant
Indiana University Purdue University Indianapolis, March 2003

NIH Student Travel Award
International Society for Developmental Psychobiology, November 2005

Guze Symposium Meeting Award
Washington University in St. Louis School of Medicine, March 2006

Student Merit Award
Research Society on Alcoholism, June 2008
Research Society on Alcoholism, June 2009
Research Society on Alcoholism, June 2011
Research Society on Alcoholism, June 2013

Travel Award
IUPUI School of Science Graduate Student Council, June 2008

NIAAA Institutional Training Grant #T32-AA07462
Dr. William McBride Indiana University School of Medicine, August 2008 – July 2013
Faculty Sponsor: Dr. Charles R. Goodlett

NIH (NICHD) Sackler Institute Student Travel Award
International Society for Developmental Psychobiology, November 2008

NIAAA Trainee Conference Travel Award
Louisiana State University Health Science Center, New Orleans, LA 2009

Research and Teaching Experience

8/06-Present	**Graduate Research Assistant to Dr. Charles R. Goodlett, Department of Psychology, Purdue School of Science, Indiana University-Purdue University Indianapolis**
8/07-12/07	**Instructor for Introductory to Psychology as a Social Science, Department of Psychology; Indiana University-Purdue University Indianapolis**
8/05-5/08	**Graduate Teaching Assistant for Introduction to Psychology as a Biological Science, Department of Psychology, Purdue School of Science, Indiana University-Purdue University Indianapolis**
8/05-6/06	**Graduate Teaching Assistant to Dr. Shenan Kroupa, Department of Psychology, Purdue School of Science, Indiana University-Purdue University Indianapolis**
6/04-7/06	**Undergraduate Research Assistant to Dr. Bethany Neal-Beliveau, Department of Psychology, Purdue School of Science, Indiana University-Purdue University Indianapolis**
6/02-8/04	**Assistant Course Coordinator to Dr. John Kremer, Department of Psychology, Purdue School of Science, Indiana University-Purdue University Indianapolis**