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BEHAVIORAL DEFICITS ACROSS DEVELOPMENT IN A NOVEL MOUSE MODEL OF FETAL ETHANOL EFFECTS

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DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

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2017

ABSTRACT OF DISSERTATION

BEHAVIORAL DEFICITS ACROSS DEVELOPMENT
IN A NOVEL MOUSE MODEL OF
FETAL ETHANOL EFFECTS

Fetal Alcohol Spectrum Disorders (FASD) are a spectrum of anatomical, developmental and neurobehavioral impairments resulting from ethanol (ETOH) exposure during fetal development. Efforts to develop and screen novel pharmacotherapies against fetal ETOH effects depend heavily upon rodent models to provide indicators of the safety and efficacy of such compounds, in addition to helping better understand the underlying mechanisms to develop and test these pharmacotherapies. The following experiments describe the development of a novel mouse model of FASD using behavioral batteries to assess behavioral or cognitive deficits in juvenile and adolescent offspring (Experiment 1, Experiment 2) and whether deficits with this model extend into adulthood (Experiment 3). In Exp 1, three ETOH exposure paradigms were generated to model ETOH exposure during periods of mouse development analogous to the three trimesters of human central nervous system (CNS) development. These included a prenatal (first-second trimester) model, a neonatal (third trimester) model, and a combined prenatal-neonatal (3-trimester) model. Exploratory behavior and learning performance were assessed in juvenile and adolescent development through a behavioral test battery that included open-field (OF), elevated plus maze (EPM) and Morris water maze (MWM). These tasks were selected to model deficits in behavior regulation that are reported in humans with FASD, including hyperactivity, impulse control deficits and learning impairments. The results of this study showed that the neonatal ETOH exposure paradigm produced hyperactivity, inhibitory failures and impaired learning. These effects were not altered by pre-exposure to the present prenatal exposure paradigm. Based on these findings, the neonatal ETOH exposure model was selected for a follow-up study (Experiment 2-3) to provide additional data on the behavioral profile of this model using two novel tasks and a wider testing window extending into adulthood. Testing was conducted in the juvenile, adolescent and early adult stages in the hole-board test (HB), open-field (OF), and attentional set shifting task (ASST) respectively. These tasks and ages were selected to provide additional data on various aspects of the behavioral profile in this model, including distinguishing between enhanced locomotion or investigation in hyperactivity, the potential for OF effects to persist into adolescence, and to determine whether these

mice show cognitive inflexibility in adulthood. Results showed that neonatal ETOH exposure led to increased investigation that may be related to both hyperactivity and inhibitory failures, persistent effects on OF activity and exploration in adolescence, and learning deficits in adulthood. It was concluded that the neonatal ETOH exposure model shows substantial face validity for behavioral symptoms in FASD and is a strong rodent model for future use in neurogenetic studies and drug development.

KEYWORDS: Fetal Alcohol, Ethanol, Rodent model, C57BL6/J, Neuroscience, Development

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April 19, 2017
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BEHAVIORAL DEFICITS ACROSS DEVELOPMENT
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Chapter 1

Introduction and Background

4.4 Epidemiology

Since the discovery of ETOH's teratogenic effects (Jones & Smith, 1973), efforts have been made to inform the public and reduce drinking during pregnancy. Even so, 10% of pregnant women in the US report drinking within the last 30 days, with 3% reporting binge drinking in the same time period (Tan et al., 2015). Epidemiological studies estimate that as many as 2-5% of live births in the United States may exhibit developmental effects as a result of drinking during pregnancy (May et al., 2009). Worldwide, these statistics vary considerably, with some regions and subpopulations showing higher risk (Roozen et al., 2016). Across the world, children displaced from their biological families into child care systems (e.g. orphanages, foster care, child welfare programs) show a particularly high prevalence. In a meta-analysis, Lange et al. (2013) assessed FASD in child care systems internationally and estimated FAS to occur in 6% of children, with FASD occurring in 13% of children. Much more research will be required to assess the full impact of fetal ETOH, though current estimations underline the need for accurate identification of individuals with FASD and for the development of efficacious interventions.

1.2 Diagnosis

FASD is not a diagnostic term, but rather an umbrella term for disorders resulting from fetal ETOH exposure. The initial discovery of fetal ETOH effects was heavily based on visible features of the fetal alcohol syndrome (FAS), including low birth weight, evidence of CNS damage and a set of craniofacial dysmorphologies (Smith & Graden,

1998). These symptoms are associated with extreme ETOH exposure. Over time though, it has become evident that ETOH exposure during fetal development often results in functional and behavioral deficits without apparent changes in facial structure (e.g. Stratton et al, 1996). Individuals without facial dysmorphology still display evidence of CNS damage, suggested by deficits in behavior and development (Hoyme et al., 2016). These less severe cases of FASD have been diagnosed using a number of classifications, including partial FAS (pFAS), alcohol-related neurobehavioral disorder (ARND), alcohol related birth defect (ARBD), and most recently, neurobehavioral disorder with prenatal alcohol exposure (ND-PAE). ND-PAE is included in a subsection of disorders requiring additional research in the DSM-V (APA, 2015) and focuses on cognitive and behavioral abnormalities which are more characteristic across the spectrum.

ND-PAE, as included in the DSM-V, outlines 3 functional areas which are commonly affected by fetal ETOH exposure: *neurocognitive functioning, self-regulation* and *adaptive functioning* (APA, 2015). *Neurocognitive functioning* refers to deficits in complex behavior that are associated with higher cognitive processes (Kabel et al., 2015), such as global intellect or IQ, executive function, learning, memory and visuospatial reasoning. *Self-regulation* refers to deficits in more basic behavioral or cognitive functions (Kabel et al., 2015) including weak or inconsistent control of attention, mood and impulsive behaviors. *Adaptive function* refers to deficits in the ability to complete a range of daily tasks and learn complex skills (Kabel et al., 2015) including language or communication, social skills, and the ability to complete daily tasks independently.

The addition of ND-PAE to the DSM-V is promising in that it provides functional domains for classifying behavioral symptoms and a diagnostic code that can be

used by non-medical health care providers (Hoyme & Coles, 2016). However, recent reports suggest that missed or misdiagnosis of FASD may be a major obstacle to treatment. Chasnoff, Wells & King (2015) reported that among a clinical sample of foster or adopted children, the rate of FASD was 28.5%. However, of those children, only 1 in 5 had a documented FASD. The remaining subjects with FASD were either referred without any previous diagnosis or with other diagnoses, including ADHD and a variety of mood, learning and developmental disorders. In many cases, ongoing treatment plans were discontinued or altered based on the new FASD diagnosis. These findings may reflect the limited ability or willingness of clinicians to make these diagnoses. It has been reported that pediatricians infrequently ask about drinking during pregnancy when assessing child behavioral health (Gahagan et al., 2006) and often feel unprepared or uncomfortable making these ETOH-related diagnoses in children (Elliot et al., 2006).

1.3 Co-morbidity and Conceptual Issues

An additional complicating factor for diagnosis is that young individuals with FASD are frequently also diagnosed with other psychiatric disorders including anxiety, depressive disorders, oppositional-defiant disorder, conduct disorder and ADHD (e.g. Chasnoff et al., 2015, Fryer et al., 2007). It remains controversial how to interpret the co-diagnosis of FASD with such disorders, as these disorders do not have identifiable causes. This is particularly a problem for ADHD, which is co-diagnosed in 40-75% of individuals with FASD (e.g. Burd et al., 2003, Chasnoff et al., 2007, Fryer et al., 2007). Co-diagnoses may be approached as either separate, co-morbid disorders (e.g. O'Malley & Nanson, 2002), or a situation where fetal ETOH exposure produces impulsive,

inattentive and hyperactive phenotypes which meet the classification for ADHD (e.g. Glass et al., 2013). This latter explanation is likely, as clinical reports and animal models have each shown that ETOH exposure on the developing brain can lead to patterns of hyperactivity, impulse control problems and inattention (Klingdon, Cardoso & McGrath, 2016, Patten, Fontaine & Christie, 2015). These patterns may mimic the primary symptoms of ADHD and encourage co-diagnosis rather than discrimination between these disorders.

The similarity in behavioral symptoms between ADHD and FASD has spurred a number of studies addressing and comparing these disorders. Pharmacological studies have been conducted to evaluate the efficacy of typical ADHD medications for individuals with FASD. Results have been mixed, although several studies suggest that typical ADHD treatments may be less effective in treating behavioral symptoms among subjects with FASD, particularly inattentive symptoms (e.g. Coles et al., 1997, Oesterheld et al., 1998, Doig et al., 2007). Although the behavioral disorders appear superficially similar, the underlying deficits in the brain may be quite different. Both ADHD and FASD are associated with deficiencies in prefrontal cortex function and related circuitry (e.g. Durston et al., 2003, Riley et al., 2004) and fetal ETOH exposure may contribute to these deficiencies by altering the development of numerous behaviorally relevant regions or pathways, including cortical structures, basal ganglia, and the corpus callosum, hippocampus and cerebellum (e.g. Riley, McGee & Sowell, 2004). Given these differences and the lower efficacy of psychostimulants for those with FASD, it is likely that more subtle features of these disorders are unique and may be used to discriminate between ADHD and FASD. To parse out these critical differences, some

researchers have sought to identify more subtle clinical or cognitive characteristics that may be more or less relevant to each diagnosis.

Comparisons of children with ADHD, prenatal ETOH exposure or both suggest that ETOH exposure is more closely associated with deficits in verbal comprehension and perceptual reasoning (Glass et al., 2013), evidence of lying, cheating and stealing (Nash et al., 2006), and difficulties in social cognition and emotion processing (Greenbaum et al., 2009) than ADHD. Neuropsychological testing further suggests that ETOH exposure is also more often associated with deficits in visuospatial reasoning, information processing and cognitive flexibility than sustained attention, which is more relevant in ADHD (e.g. Coles et al., 1997, Vaurio et al, 2008). While results have been mixed (e.g. Nanson and Hiscock, 1990), these data generally suggest that using performance based tests to assess cognitive deficits could improve the ability of clinicians to discriminate between disorders and intervene accordingly.

1.4 Consequences of Behavioral Deficits

As discussed above, FASD are characterized by diverse deficits which provide unique challenges to accurate estimation and diagnosis. Behavioral deficits lead to broad, negative consequences for these individuals as they develop. For children with FASD, among the most commonly reported issues relate to patterns of hyperactivity, attentional problems, poor adherence to rules, impaired learning and cognitive inflexibility (Chudley et al., 2007; Coles et al., 2002; Franklin et al., 2008, Mattson, Crocker & Nguyen, 2011, Rasmussen, 2005). These issues interfere with many aspects of early life, particularly in the classroom, where cognitive difficulties and conduct issues impede many social and academic activities (Green et al., 2007). As these students grow up, they are

disproportionately likely to experience a variety of secondary disabilities as well, including interruptions in education (e.g. suspension, expulsion or drop-out), trouble with the law, risky or inappropriate sexual behavior, substance abuse, unemployment and financial troubles (e.g. Moore & Riley, 2015, Streissguth et al., 1992, Streissguth et al., 2004). In adulthood, individuals with FASD are also likely to experience a range of mental health issues, such as major depression, bipolar disorders, anxiety, addiction-related issues and personality disorders, such as avoidant or antisocial personality disorders (Streissguth & O'Malley, 2000). These outcomes likely reflect interactions between adverse experiences over the lifetime and direct ETOH-effects on the developing brain. Given the profound disruptions that fetal ETOH effects can produce in essential tasks and outcomes, it is critical that treatments are developed that address the underlying mechanisms producing these effects.

1.5 Neural Mechanisms

As discussed above, fetal ETOH exposure produces a wide array of effects including primary symptoms and secondary consequences. At present, there are no approved pharmacotherapies for the prevention or treatment of fetal ETOH effects on behavior, although a number of brain regions and pharmacological systems have been identified which may represent key targets for intervention.

Brain regions of interest: The cognitive control of behavior results from complex interactions between cortical and subcortical structures which process, bias and integrate sensory inputs. Prefrontal structures and networks are particularly tied to cognitive regulation, as they serve to integrate signals arriving from a number of cortical and subcortical structures (Miller & Cohen, 2001) and regulate them through feedback

inhibition. The prefrontal cortex (PFC) communicates in this way with sensory processing areas (e.g. Carmichael & Price, 1995, Groenewegen & Uylings, 2000) as well as subcortical regions including the amygdala, basal ganglia, hippocampus and thalamus (e.g. Haber et al., 1995; Laroache, David & Jay, 2000; Quirk et al., 2003; Vertes, 2006). Limbic structures are believed to regulate the processing, integration and recall of information through their cortical interactions and through interactions with one another (e.g. Atallah, Frank & O'Reilly, 2004, Vertes, 2006, Vuilleumier, 2005). Each structure contributes to behavioral regulation and selection in relatively separable ways. For example, the amygdala is associated with anxiety, fear conditioning and attention to stressors (Pourtois, Schettino & Vuilleumier, 2013), the basal ganglia are associated with goal-oriented behaviors and motivated learning (Yin & Knowlton, 2006), and the hippocampus is associated with spatial processing and memory formation (Bannerman et al., 2004). Additionally, certain cortical zones appear to be more specialized for regulatory functions, such as the basal forebrain, implicated in the direction of attention (Baxter & Chiba, 1999), and the anterior cingulate gyrus, implicated in "error detection" and the inhibitory regulation of ongoing behavior (Botvinick, Cohen & Carter, 2004). The integrity of these structures and their interactions are critical to the ongoing regulation of behavior.

Imaging studies suggest structural and activational changes in several behaviorally relevant areas following prenatal ETOH exposure. ETOH exposure during fetal CNS development can result in reduced brain weight and gross alterations in the size and shape of various brain structures (Norman, Crocker, Mattson & Riley, 2009), although the sensitivity of each structure or subregion is variable. In the cortex, fetal

ETOH exposure can alter cortical organization and cell densities, either producing cortical thinning or thickening depending upon the region (Yang et al., 2011, Zhou et al., 2011). Cortical function may be further perturbed by regional losses in white matter and damage to the corpus callosum (Green et al., 2003), which may lead to reduced network efficiency and impaired cooperation between networks across the midline respectively. Functional imaging studies show that organizational changes in the cortex lead to differential activation patterns at rest and during cognitive testing (See Review, Moore, Migliorini, Infante & Riley, 2014). In these studies, activational changes correlate with behavioral performance deficits. Structural and functional changes have similarly been shown in the basal ganglia and hippocampus, although the amygdala appears less sensitive to these effects (See Review, Norman et al., 2009).

Pharmacological targets: In order to reduce the broad deficits in CNS development and function, pharmacological studies have sought to identify pharmacological targets and relevant compounds which can either prevent these effects or attenuate symptoms produced by them at a later age. As discussed previously, typical 8ehavior88ve medications (such as stimulants typically prescribed for ADHD), may have limited efficacy for FASD due to the unique etiology of FASD. Alternative mechanisms for cognitive enhancement, such as through nicotinic acetylcholine receptors (nAChR) (e.g. Wilens & Decker, 2007), may yield different results, although this remains to be investigated. Given current obstacles to identification and treatment of FASD later in development, prevention of ETOH-induced neural damage may offer a more promising avenue to treatment. Of particular interest, research suggests that ETOH withdrawal plays a major role in the teratology of ETOH. Using a rat model, Ikonomidou and

colleagues (2000) demonstrated that withdrawal after a binge-like ETOH treatment triggered widespread apoptotic cell-death which mimics patterns observed after administration of N-methyl-D-aspartate receptor (NMDAr) antagonists and/or gamma aminobutyric acid receptor (GABA-Ar) agonists. This affected a number of relevant brain structures including multiple layers and locations in the cortex, the hippocampus, the caudate (part of the basal ganglia) and multiple subregions of the thalamus. A number of studies have similarly shown damaging effects of ETOH withdrawal using both *in vitro and in vivo* models (e.g. Barron et al., 2008, Goodlett & Horn 2001, Prendergast et al., 2001). Further studies suggest that antagonism of the NMDAr during ETOH withdrawal can reduce cell death (e.g. Thomas, Fleming & Riley, 2001), although NMDAr-antagonists can produce toxicity in the developing brain as well, leading future drug development to search for alternative ways to modulate NMDA activity (See Review, Barron et al., 2016). Additional work aiming to reduce ETOH-related neurotoxicity has focused on other neuroprotective mechanisms, such as reducing ETOH-induced oxidative stress (e.g. Peng et al., 2005) and actions on cholinergic mechanisms, such as choline supplementation (e.g. Thomas et al., 2004) or modulation of nAChR receptors (e.g. de Fiebre and de Fiebre, 2003). These pharmacological targets provide exciting avenues for drug development, although much more work will need to be done to develop and screen viable compounds that address these mechanisms.

1.6 Need for Improved Methods and Animal Models

A number of key neural and pharmacological systems have been investigated for their roles in FASD although it is recognized that many neurochemical and genetic mechanisms need to be clarified and/or discovered. As new mechanisms of damage and

targets for intervention are investigated with an ever growing array of tools and technologies in neuroscience, the success of these investigations rests heavily on the availability and validity of laboratory models which form the basis of such work. It is pivotal then that laboratory models of FASD and relevant mechanisms are able to address the needs, limitations and potential for growth in these areas of research. To provide valid baselines for investigation, preclinical research often uses model systems, such as rodent species, to isolate and identify key processes and outcomes in experiments where genetic, pharmacological and experiential factors can be more precisely controlled.

Rodent models have provided valuable avenues to address research questions which are not practically or ethically feasible to test in human subjects. Rodents and humans share many important anatomical and physiological characteristics, and this similarity can be used to experimentally model many aspects of nervous system function and dysfunction across species (McGonigle, 2014). With respect to fetal ETOH effects, rodents have shown face validity in modeling a range of outcomes related to FASDs, including neurotoxicity, developmental deficits, psychomotor effects and neurocognitive impairments (Goodlett, Horn & Zhou, 2005, Patten, Fontaine & Christie, 2014). Presently, there are a number of exposure paradigms available for use in rodent models of fetal ETOH effects. These vary on a number of factors, including the timing, dosing and route of administration for ETOH and model species.

The mammalian central nervous system (CNS) develops not through a single process, but through a complex series of processes over the lifetime. These specialized processes create critical periods when development may be facilitated or interrupted in specific ways by an intervention. For instance, in the third trimester of human

pregnancy, the brain undergoes a "growth spurt", characterized by increases in brain mass and synaptogenesis, which is highly sensitive to the neurotoxic effects of ETOH exposure (Ikonomidou et al, 2000, Olney et al., 2000). Rodent models simulate ETOH effects on these processes by exposing subjects to ETOH for brief periods at an analogous time point in development. However, it is important to note that the timing of these critical periods differs across species (Dobbing and Sands, 1979). At birth, brain development in the rat and mouse has only reached a stage analogous to the second trimester in humans, meaning that a model of ETOH effects on the "third trimester growth spurt" must administer ETOH to neonatal pups, rather than pups *in utero* (e.g. Lewis et al., 2012). Models which mimic exposure at limited time points do not model all aspects of FASD or all processes that contribute to them, but rather replicate particular features which may be used to identify mechanisms of damage or evaluate potential interventions against them.

An alternative technique to providing ETOH exposure during limited windows is to develop full term (3-trimester) exposure models (e.g. Tran et al., 2000). These models simulate the effects of chronic ETOH exposure across wider windows of development, in this case across multiple trimester-equivalent periods. Although these techniques lack the specificity of shorter term exposure models, they capture an additional element of fetal ETOH effects; that multiple ETOH exposures over a wide developmental window may alter CNS development in ways that are distinct from their effects in the individual critical periods. For example, Cronise et al (2001) showed that binge-like exposure to ETOH (3g/kg) failed to produce persistent behavioral deficits in rats when the exposures were limited to a time window in the first, second or third trimester-equivalent alone.

However, behavioral deficits were apparent when ETOH exposure occurred during all 3 trimester-equivalent periods. Such interactions provide additional directions for mechanism identification and therapy development beyond critical period models.

In addition to the timing of exposure, the ETOH dosing paradigm is critical to modeling ETOH effects. More specifically, FASD models must take into account the peak blood ETOH content (BEC) achieved following exposure rather than the total dose administered (Maier & West, 2001). Compressing a total dose into shorter cyclic exposures, modeling "binge-like" ETOH exposure, produces higher peak BECs and more robust developmental deficits than a more continuous exposure method with lower peak BECs (e.g. West, Goodlett, Bonthius & Pierce, 1988), although moderate or low doses may also produce meaningful effects on certain tests (e.g. Choi, Allen & Cunningham, 2005, Wigal & Amsel, 1990). Importantly, peak BEC varies greatly based on the route of administration. Self-administration paradigms have strong face validity, although few rodent strains will voluntarily consume ETOH to BECs achieved by humans. Even in ETOH-preferring C57BL/6J mice, voluntary consumption during pregnancy produces only moderate BECs with few long term effects on behavior (Boehm et al., 2009). Involuntary exposure paradigms, including injection, intragastric exposure and inhalation produce higher BECs (>200 mg/dl), although these methods produce somewhat different pharmacokinetic profiles (e.g. Kelly & Lawrence, 2008). These dosing procedures are valuable in that they can be used to consistently produce high, clinically relevant BECs like those produced by alcoholics.

An additional consideration for rodent models is the species and strain selected. While rats and mice show comparable behavioral symptoms when used to model FASD

(Patten, Fontaine & Christie, 2014), the diversity and significance of models available differ between them. Given current advances in technology, mice are better suited for use in neurogenetic manipulation and testing, offering many exciting directions for behavioral teratology. However, mouse models have not matched the diversity of rat models of FASD. Existing mouse models of third trimester ETOH exposure have been largely limited to neonatal injection (e.g. Mantha, Kleiber & Singh, 2013), a practice which could lead to drug leakage due to the porous skin of neonatal pups (Zoetis & Walls, 2003). Rat models often administer ETOH through an intragastric gavage instead (e.g. Lewis et al., 2012). Recently, a few studies have extended this technique to mouse models of FASD in brief binge-like exposures (Drew et al., 2015, Bearer et al., 2015) and as part of a 3-trimester model (Louth et al., 2016). Such models are promising, although behavioral data remains very limited to date. Notably lacking is a third-trimester exposure model showing the behavioral effects of a binge-like exposure period covering several days in the neonatal period, as in many rat models (e.g. Lewis et al., 2012). Additionally, the single 3-trimester exposure model available in mice (Louth et al., 2016) provided very limited data on the validity of this model, either in terms of behavioral data in early development or the relevance of the component treatments (e.g. prenatal vs neonatal vs combined) to the effects observed in adulthood.

Chapter 2

Experiment 1: Initial Model Development

2.1 Study Design

Limitations in the procedural and theoretical diversity of mouse models negatively impact the utility of mice in addressing FASD. Based on these gaps in the literature, Experiment 1 aimed to develop additional models that utilize repeated neonatal intragastric intubation and/or a 3-trimester paradigm with appropriate comparison groups. These models utilized C57BL/6J mice, a common strain used in both neurogenetic and pharmacological studies. Three ETOH-treated groups were generated: prenatal-ETOH-exposure (2g/kg i.g. GD 7-16), neonatal-ETOH-exposure (4g/kg i.g. PD 4-10), and a combined prenatal-neonatal (perinatal) exposure. These groups were generated in order to analyze the effects of a neonatal ETOH exposure model and to determine whether these effects might be augmented by prenatal exposure to ETOH. Control groups consisted of a handled control and a control group intubated with a maltose-milk formula. Behavioral outcomes in juvenile and adolescent mice were selected to model deficits that are often reported in individuals with FASD, including hyperactivity, anxiety-like behavior, disinhibition and deficits in learning and memory. This work was completed as part of a collaboration between the laboratories of Dr. Susan Barron and Dr. Gang Chen. The manuscript below has recently been submitted for review to the journal *Public Library of Science – ONE.* First authorship of this manuscript is shared between Wenhua Xu, Andrew Hawkey, Hui Li and Lu Dai.

Early Neonatal Ethanol Exposure Causes Behavioral Deficits in Young Mice

Wenhua Xu; Andrew B. Hawkey; Hui Li; Lu Dai; Howard H. Brim; Jonathan W. Handshoe; Jacqueline A. Frank ; Jia Luo; Susan Barron and Gang Chen.

2.2 Abstract

Background: Fetal ethanol (ETOH) exposure can damage the developing central nervous system (CNS) and lead to cognitive and behavioral deficits, known as fetal alcohol spectrum disorders (FASD). A variety of rodent models have been developed for FASD research. However, as most of the rodent models use rats, there is a demand for mouse models that can show behavioral deficits induced by ETOH exposure during early CNS development.

Methods: C57BL/6J male and female mice were exposed to ETOH prenatally, perinatally or during early neonatal days by intubation. ETOH was administered to pregnant dams (2 g/kg/day) on gestational day (GD) 7-16 and/or to neonatal pups (4 g/kg/day) on neonatal days (PD) 4-10 by intubation. Testing of FASD-related behaviors of the offspring, including open-field, elevated plus maze and Morris water maze, were performed during PD 20 – 45.

Results: ETOH exposure during the early neonatal period, with or without prenatal treatment, resulted in hyperactivity, disinhibition and deficits in learning and memory in young offspring without any observed sex differences in sensitivity. Exposure to the lower dose of ETOH during pregnancy did not result in adverse behavioral effects.

Conclusion: Based on these data, the current neonatal intubation model may be useful for future mechanistic studies of FASD and for screening of novel therapeutic

approaches.

2.3 Introduction

Ethanol (ETOH) consumption during pregnancy may result in a range of developmental deficits collectively known as the fetal alcohol spectrum disorders (FASD). Fetal ETOH exposure may damage the developing central nervous system (CNS) and lead to cognitive and behavioral dysfunctions, such as hyperactivity, disinhibition and deficits in learning, attention and executive functions (Jacobson, 1998, Riley and McGee, 2005). The underlying mechanisms of these effects remain unclear.

Studying the underlying mechanisms relies on the development of appropriate animal models. A variety of animal models have been developed for FASD studies, among which mouse models have become increasingly useful due to extensive knowledge of mouse genetics for studies on the etiology of neurological disorders. Depending on the goal of a study, various rodent models have been used, including acute or chronic exposure paradigms, varying routes of administration and varying dosage or timing of ETOH exposure to mimic binge-like, moderate or low-dose drinking behavior as well as the first, second or third trimester alcohol consumption in humans (Patten et al., 2014). In addition, the early neonatal period of mouse brain development represents a critical period for ETOH neurotoxicity (Maier et al., 1999, Olney et al., 2002) as it parallels the rapid growth, gliogenesis and synaptogenesis observed in the "third trimester brain growth spurt" in humans (Dobbing and Sands, 1979).

Currently, the number of studies using mice to study fetal ETOH effects has been limited and the results are less consistent than in rat studies. In studies assessing prenatal ETOH exposure, voluntary drinking models have produced behavioral impairments

including hypoactivity, and deficits in tasks that tap into hippocampal function or sensorimotor integration although these data are mixed (Allan et al., 2003, Becker et al., 1993, Brady et al., 2012, Abbott et al., 2016). Prenatal ETOH exposure has also been administered by oral intubation to the dam with some studies reporting hyperactivity in ETOH-exposed adolescent mice (Sanchez Vega et al., 2013, Fish et al., 2016), while others report minimal effects on activity (Downing et al., 2009a).

Fewer studies have focused on ETOH exposure during the early neonatal period. Olney and colleagues have shown that a single neonatal ETOH injection induces neuronal apoptosis (Ikonomidou et al., 2000). Similar single-exposure models have not shown corresponding alterations in activity but caused learning and memory deficit (Wozniak et al., 2004), while multiple injections may alter performance on hippocampal and cerebellar tasks (Bearer et al., 2015, Wagner et al., 2014). At present, there is a limitation in the availability of mouse models that show behavioral deficits induced by ETOH exposure during early stage of CNS development (Wozniak et al., 2004, Vink et al., 2005, Kleiber et al., 2011, Wainwright et al., 1990).

The aim of the current study was to determine the effects of perinatal, prenatal or early neonatal ETOH exposure on FASD-related behaviors in C57BL/6 mice. ETOH was delivered to pregnant dams, their offspring or both. Offspring were tested on PD 20-21 for activity levels using an open-field, on PD 25-26 for exploration on an elevated plus maze and on PD 35-45 for spatial learning and memory in a Morris water maze.

2.4 Materials and Methods

Animals: Adult C57BL/6 mice were obtained from Harlan Labs (Indianapolis, IN). Offspring were generated in the University of Kentucky Medical Center's breeding

colony. Male and female pups, representing at least 8 litters for each treatment group were used and randomly assigned to treatment conditions to preclude potential litter effects (Abbey and Howard, 1973). Mice were maintained on a 14:10 light-dark cycle (lights on at 07:00 h, off at 21:00 h). All procedures were approved by the NIH and the Animal Care and Use Committee of the University of Kentucky.

Breeding: Adult female and male C57BL/6 mice were caged in a ratio of 2:1 and seminal plugs were examined in the morning as evidence that copulation occurred. If a seminal plug was detected, it was designated as GD 0 and the pregnant female was singly housed and randomly assigned to either an ethanol-intubation, maltose-intubation or non-intubation control group.

Ethanol administration: ETOH was administered at 09:00 h either during pregnancy on gestation day (GD) 7-16 (as a "first and second trimester exposure" model) (Kim et al., 2010), after birth to neonatal pups on neonatal day (PD) 4-10 (as a "third trimester exposure" model) (Kelly et al., 1988) or combined (as a "3-trimester" model) as outlined in Table 1. For prenatal treatment, pregnant dams were intubated daily on GD 7-16 with either 2 g/kg ETOH (20% w/v) or an isocaloric amount of maltose. ETOH or maltose was added to a milk solution developed to mimic rodent milk (Kelly and Lawrence, 2008). The 2 g/kg ETOH dose was chosen based on our preliminary study and published data (Kim et al., 2010) showing that this dose did not reduce litter size, while higher doses did. Non-intubated control litters were also included. Mice were weighed daily through the intubation period. As parturition approached, cages were checked daily for pups. The day of birth was designated PD 0. On PD 4, litters were weighed and pseudo randomly assigned to either a neonatal ETOH intubation, isocaloric intubated

control or a non-intubated control group (Table 1). Pups in the ETOH group received 4 g/kg/day of ETOH delivered via oral intubation in a 0.02 ml/g body weight of the milk solution (Kelly and Lawrence, 2008) on PD 4–10. This dose was chosen because it has been shown to produce significant neurotoxicity during the third trimester equivalent and may lead to neurobehavioral deficits (Dursun et al., 2013). The intubated control, whose dam was intubated with maltose prenatally, received isocaloric intubations of the maltose solution neonatally. The non-intubated controls received no treatment. The pups were returned to the dam and allowed to nurse immediately after intubations. Pup body weights were recorded daily. Mortality from intubation was low (approximately 5%).

Table 1.
Summary of the treatment groups.

	NTC	Maltose	Prenatal	Neonatal	Perinatal
Dams	Non-treated	Maltose i.g. GD 7-16	2g/kg/day i.g. GD 7-16	Non-treated	2g/kg/day i.g. GD 7-16
Pups	Non-treated	Maltose i.g. PD 4-10	Non-treated	4g/kg/day i.g. PD 4-10	4g/kg/day i.g. PD 4-10
Exposure Model	Non-treated control	Intubated control	1^{st} and 2^{nd} trimester model	3^{rd} trimester model	All 3-trimesters model

BEC measurement: For BEC of pregnant dams, blood samples were obtained from a 1-2 mm tail clip 30, 60, 90, 120, 180, 240, 360 and 480 min after ETOH administration. For BEC of the pups, separate groups of pups were treated and trunk blood of pups was collected following decapitation 30, 60, 90, 120, 180, 240, 360 and 480 min after ETOH intubation. BEC was determined using an assay kit from Sigma-Aldrich, St. Louis, MO (product number: MAK076). The data were collected from multiple samples (n=4).

Behavioral testing: Offspring were tested for activity in an open-field (OF) for 2

days in the morning between 08:00 – 12:00 h on PD 20-22 (Mei et al., 2016), and for activity, anxiety and exploration in an elevated plus maze (EPM) at similar times in the morning on PD 25–27 (Mei et al., 2016). Offspring were then weaned on PD 28, housed with 2–3 same sex littermates and allowed to acclimate for one week prior to Morris water maze (MWM). Spatial learning and memory in a MWM testing was then conducted between at 12:00– 16:00 h on PD 35-45 (Chen et al., 2013). These tasks and ages were selected to respectively assess the presence of deficits in behavior regulation in juvenile development and learning deficits in adolescence, as have been reported in children and adolescents with FASD (Mattson et al., 2011). All behavioral testing was conducted under low ambient light conditions with white noise to reduce extraneous auditory stimuli. Surfaces and holding cages were cleaned before and after testing with Nature's Miracle© enzymatic cleaning solution to remove animal odors. Animal movements were recorded using the AnyMaze tracking system (Stoelting Co.)

Open-field: OF testing is commonly used to measure levels of activity, habituation to novel environments and patterns of exploration which may indicate impulse control or anxiety (Bailey and Crawley, 2009). Each mouse was removed from its home cage and brought into the test room in a clean holding cage for a 10 min habituation period. The OF was a round chamber
(diameter 39.4 cm) with opaque white walls and floor. Subjects were tested 30 min daily for two consecutive days. The dependent measures included total distance traveled and distance traveled in the center. The center was defined as a circular zone in the center of the OF with a diameter half of the width of the OF. Measurements related to activity in the center are often used as a measure of anxiety and/or inhibitory control since rodents

typically display thigmotaxis and avoid the center of open arenas (Bailey and Crawley, 2009). Additional analyses were run on center exploration when controlling for total distance traveled to produce a preference score.

Elevated plus maze: EPM is used to measure exploration and alterations may be a measure of impulse control and/or anxiety issues (Bailey and Crawley, 2009). The EPM apparatus consisted of plus-shaped (+) Plexiglas maze with clear walls bordering two of the four arms (30 x 6 cm). The mouse was placed halfway down one open arm of the maze, facing away from the center, and was then allowed to explore the maze for 5 min. Subjects were tested once between PD 25-27. The dependent measures were distanced traveled overall, distance traveled in the open arms and number of entries into open arms. Additional analyses were run on open arm exploration when controlling for total distance traveled.

Morris water maze: MWM is used to measure spatial learning and retention (Vorhees and Williams, 2006). The MWM consisted of a round plastic tub (diameter 107.6 cm) filled with water (22-23^{o}C) made opaque using white non-toxic water-based paint. A platform (15.2 x 15.2cm) was submerged 0.75cm under the surface of the water in a fixed location. Four visible extra-maze cues were placed at various points around the maze. Each mouse was placed in one of four starting positions on the far side of the pool (120, 150, 210 and 240^{o} from the platform) and allowed to swim until either they found the submerged platform or they reached a ceiling of 60 sec. If they did not find the platform, they were gently guided to the platform. Subjects remained on the platform for 5 sec before being removed for a 5 min inter-trial interval (ITI).

During maze acquisition, four trials were completed per day for four days. On the

fifth day, the platform was removed for a single probe trial. The mouse was placed in the opposite quadrant and the swim pattern was recorded for 60 sec. Four hours after the probe trial, subjects completed a reversal learning phase, which consisted of four trials with the platform replaced one quadrant away from its original location. Following reversal learning (ITI 5 min), a single visible platform trial was conducted where the platform's location was indicated by a visible rod above the surface of the water. The visible platform component was included to ensure that there were no visual or motor deficits potentially contributing to performance on this task following developmental ETOH exposure. MWM testing was conducted over 5 days between PD 35-45.

The dependent measures during acquisition included latency to reach the platform and distance traveled for each trial. For the probe trial, performance was analyzed for crossings of the annulas zone, defined as the square zone where the platform was placed throughout acquisition, and the distance traveled within the quadrant containing the acquired platform location. For the reversal phase, the dependent measures included latency to reach and distance traveled to the new platform location. During the visible platform trial, the dependent measure was swim speed.

Statistical Analysis: Data were analyzed using SPSS software version 21 (IBM). To account for differences in variability within/between litters of mice, behavioral data from same sex siblings were averaged together to produce a single data point per sex, per treatment, per litter for each measure. A subset of mice failed to learn to swim to the platform during acquisition, often floating rather than searching for the platform. Failure to acquire the task was defined as the failure to reach the platform on all 4 trials on

acquisition day 4. Treatment did not significantly influence the number of mice meeting this criterion (total mice affected: NTC= 3, Maltose= 8, Dam ETOH= 8, Pup ETOH= 3, Dam + Pup ETOH=4). Adjusted sample sizes for MWM analyses reflect the removal of subjects which were unable to acquire the task, although all treatment groups still represent data from 8-9 separate litters of mice. For each analysis, univariate or mixed-factors analysis of variance (ANOVA) was performed with treatment and sex as grouping factors and repeated measures when warranted. Significant interactions were broken down by simple main effect and/or post hoc Tukey test. The Greenhouse-Geisser correction was used to correct for violations in homogeneity of variance when necessary. In some cases, this correction resulted in degrees of freedom that were not whole numbers. If there was no main effect or interaction with sex or across multiple time points (e.g. days, time bins, etc), the data were collapsed across this variable for ease of presentation.

2.5 Results

BEC data, body weight and litter size: BEC levels of pregnant dams were examined on GD 7 after exposure to 2 g/kg/day ETOH as shown in Figure 1 (upper panel). BEC levels peaked at 144.4 + 12.4 mg/dl at 1 hour post intubation and declined to 28.1 + 8.2 mg/dl by 8 hours. BEC levels of the pups was determined from the two groups that received neonatal ETOH exposure on PD 7 to ensure that in utero ETOH exposure had no impact on neonatal ETOH pharmacokinetics (Figure 1 lower panel). BEC curves did not differ in these two groups with a peak at 335.5 + 14.3 and 325.9 + 18.8 mg/dl for the pups with or without prenatal ETOH exposure, respectively.

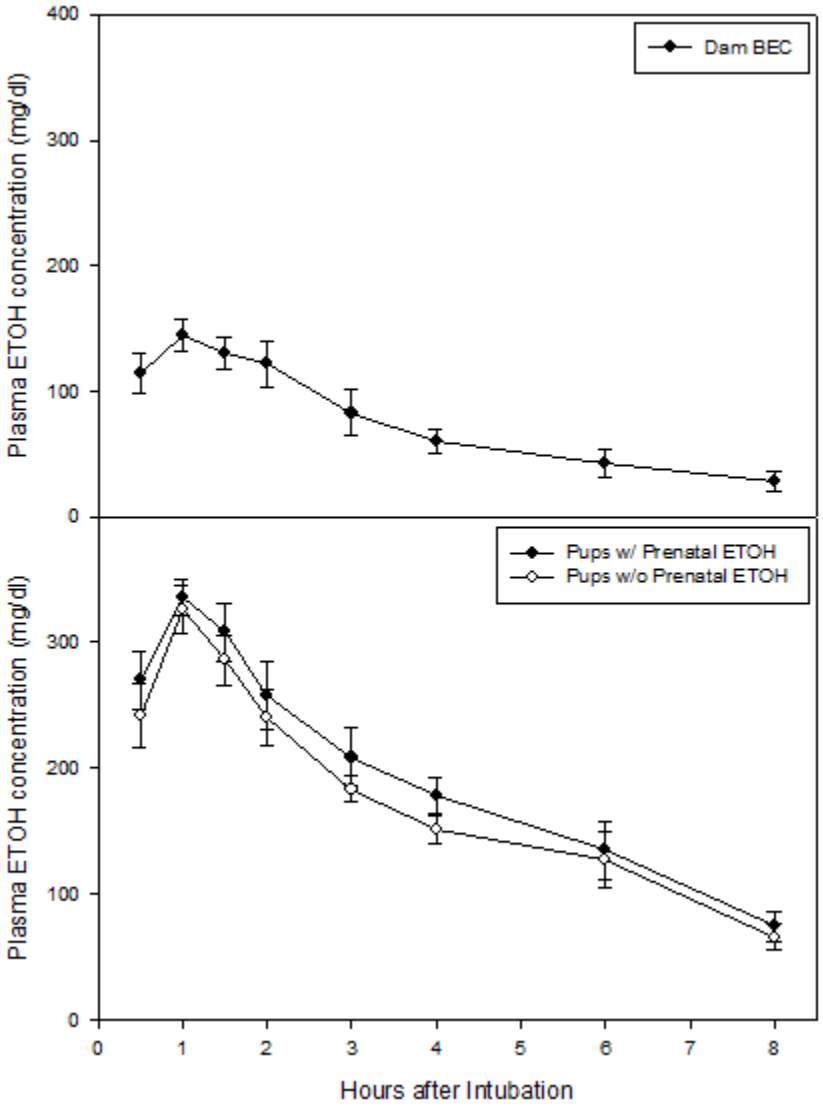

Figure 1. BEC profile of dams and pups. Upper panel: Ethanol (2g/kg) was administered to the dams via intragastric intubation. Lower panel: Ethanol (4g/kg) was administered to neonatal (PD 7) pups via intragastric intubation. BECs were measured at 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours post-treatment. Data are expressed as the mean +/- SEM (N=4).

Body weight gain of dams was recorded daily during GD 7-16. A one-way ANOVA indicated a significant main effect of dam treatment on maternal body weight gain, $F(2, 23) = 74.78$, $p < 0.05$. ETOH treated dams gained significantly less than than non-treated or maltose-treated dams (p's < 0.05) (34.2, 42.8 and 47.4% for ETOH,

maltose and non-treated dams, respectively). Dam treatment had no significant effect on litter size.

Offspring body weights on PD 4-10 are presented in Table 2. A mixed factors ANOVA demonstrated a significant interaction of treatment and age on weight, $F(28, 245) = 6.16$, $p < 0.05$. Post hoc tests indicated no significant difference in body weights across groups on PD 4. Pups with perinatal ETOH exposure weighed less than non-treated control on PD 5-10 ($p < 0.05$). Neonatal exposure alone decreased body weight when compared with non-treated controls only on PD 10. Prenatal ETOH exposure alone did not alter pup body weight significantly.

Table 2:
*Offspring weight profile***.**

Postnatal Day (PD)	NTC (g)+/- SEM		Maltose (g)+/- SEM		Prenatal (g)+/- SEM		Neonatal (g)+/- SEM		Perinatal (g)+/- SEM	
4	2.57±0.22		2.47±0.23		2.39±0.17		2.48±0.19		2.36±0.14	
5	3.04±0.25		2.93±0.28		2.84±0.21		2.86±0.20		2.69±0.21*	
6	3.49±0.23		3.35±0.30		3.25±0.18		3.20±0.25		3.06±0.19*	
7	3.95±0.21		3.79±0.29		3.67±0.21		3.65±0.22		3.48±0.25*	
8	4.39±0.22		4.20±0.35		4.08±0.27		4.02±0.28		3.80±0.26*	
9	4.85±0.22		4.64±0.42		4.49±0.30		4.46±0.32		4.26±0.31*	
10	5.30±0.22		5.08±0.45		4.96±0.31		4.76±0.32*		4.71±0.30*	
	M	F	M	F	M	F	M	F	M	F
20	10.27 ±0.44	9.33± 0.43	10.58 ±0.20	9.98± 0.19	9.83± 0.33	8.91± 0.21	8.42± 0.30*	7.98± 0.34*	8.20± 0.28*	8.33± 0.29
24	14.24 ±0.68	12.55 ±0.66	11.43 ±0.55 *	11.00 ±0.55	13.27 ±0.35	12.27 ±0.25	10.61 ±0.52 *	10.58 ±0.62 *	10.78 ±0.34 *	10.24 ±0.30 *
35	19.47 ±0.31	16.56 ±0.39	21.89 ±0.33 *	18.08 ±0.23	19.55 ±0.38	16.73 ±0.28	19.02 ±0.42	15.55 ±0.59	17.36 ±0.34 *	16.20 ±0.50

Table 2: Body weights during neonatal and testing phases (split by sex) were altered relative to NTC as indicated by asterisks *. * = $p < .05$. Data are expressed as the mean (g) +/- SEM (N = 8-9).

Neonatal or perinatal ETOH exposure also reduced body weight at the time of open-field and EPM testing (Table 2). There were significant interactions between treatment and age, $F(6.05, 205.62) = 3.61$, $p < 0.05$, and sex and age, $F(1.51, 205.62) =$

3.31, $p < 0.05$. Post hoc tests were conducted for males and females separately. For males, neonatal ETOH treatment resulted in lower body weights for both OF and EPM ($p < 0.05$). By PD 35, the age for MWM testing, only males with perinatal ETOH treatment showed lower body weight relative to non-treated controls. Additionally, maltose treatment appeared to have effects on body weight at the time of EPM (reduced) and MWM (elevated) with no effect on body weight relative to controls during OF testing. Among females, neonatal ETOH treatment resulted in a reduction in body weight at the time of OF and EPM testing with no body weight differences for MWM.

Behavioral testing

Open-field: OF exploration was significantly altered by ETOH exposure, with similar patterns of hyperactivity being observed for the offspring in both groups that received neonatal ETOH exposure. Prenatal ETOH exposure alone did not appear to affect activity levels. Figure 2 shows activity in the OF, as measured by distance traveled. These data are shown collapsed across the two OF sessions and across sex due to the lack of day or sex interactions. The repeated measures ANOVA of distance traveled revealed significant main effects of treatment, $F(4, 73) = 9.42$, $p < 0.05$, day, $F(1, 73) = 20.71$, $p < 0.05$, and time bin, $F(2.95, 280.60) = 189.65$, $p < 0.05$, with significant interactions of bin by treatment, $F(11.79, 280.60) = 2.94$, $p < 0.05$, and day by bin, $F(3.84, 280.60) = 37.07$, $p < 0.05$. Post hoc tests showed that offspring with neonatal ETOH exposure, with and without prenatal treatment, were hyperactive across multiple time blocks relative to maltose-treated ($p < 0.05$) and non-treated controls ($p < 0.05$). Prenatal ETOH treatment did not significantly alter the effects of neonatal ETOH treatment, although scores for the perinatal exposure failed to reach significance in the

latter 3 time bins.

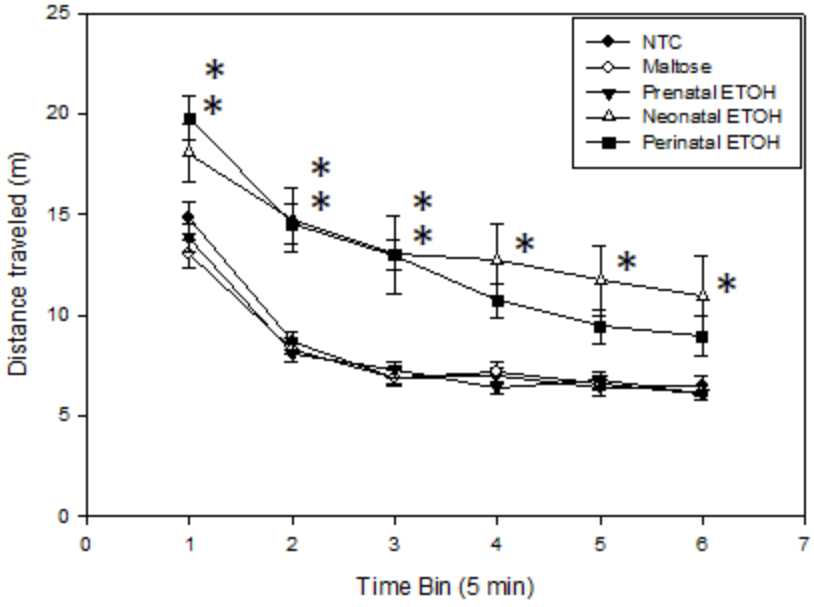

Figure 2. Open-field activity. Distance traveled across six 5-min time bins in the OF, shown collapsed across day. Offspring with neonatal or perinatal ETOH exposure were hyperactive in the OF. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Error bars indicate SEM (N = 8-9).

Additional analyses (Figure 3) assessed ETOH effects on center exploration and preference, respectively measured as the total distance traveled in the center zone alone and adjusted for the total amount of activity [(Distance traveled in the center zone/Total distance traveled) x 100]. As expected, control mice avoided the center zone, with only 19-20% of exploration typically occurring in this zone. Neonatal ETOH treatment was associated with increased distances traveled in the center zone, although this did not reflect a greater preference for open spaces than controls but more reflected the hyperactivity the ETOH exposed animals were displaying. For center exploration (Figure 3, upper panel), there were significant main effects of treatment, $F (4, 73) = 7.59$, $p < 0.05$, sex, $F (1, 73) = 8.57$, $p < 0.05$, day, $F (1, 73) = 35.13$, $p < 0.05$, and a day by

treatment interaction, $F(4, 73) = 3.11$, $p < 0.05$. Post hoc tests showed that offspring with perinatal ETOH exposure explored significantly more distance in the center than controls ($p < 0.05$) on day 1, but not day 2 ($p > 0.05$). No other treatment groups reached significance relative to controls. Offspring with neonatal or perinatal ETOH exposure did not significantly differ from one another. Male mice explored greater distances in the center than females. When adjusting these scores for the total level of activity to create a preference score, a different pattern was apparent (Figure 3, lower panel). There were no significant differences across groups in center preference on day 1 ($p > 0.05$). On day 2, there was a reduction in center preference among offspring with neonatal ETOH exposure alone ($p < 0.05$), with a similar trend in offspring with perinatal exposure failing to reach significance. This effect consisted of a weak shift in exploration (5-6%) away from the center, bringing center preference down to 14%.

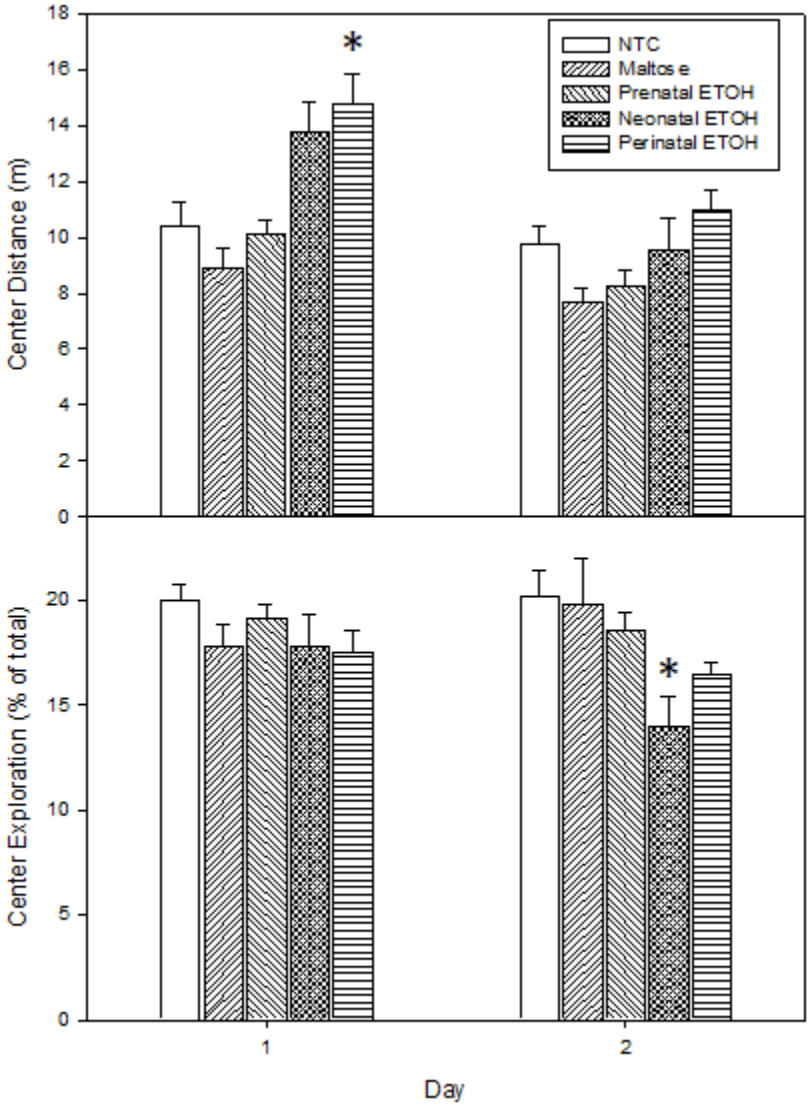

Figure 3: Open-field: Center exploration. Center exploration as distance traveled alone (upper panel) and as adjusted for total activity [(center distance/total distance)*100]. Offspring with perinatal ETOH exposure were more active in the center on session 1. Offspring with neonatal ETOH exposure alone showed greater center avoidance on session 2. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Error bars indicate SEM (N = 8-9).

Elevated plus maze: Offspring with neonatal ETOH exposure, alone or combined with prenatal exposure, were hyperactive and showed increased exploration of open arms in the EPM. Figure 4 shows performance in the EPM, as measured by total distance traveled (upper panel) and open arm exploration (lower panel). The offspring exposed to

neonatal ETOH exposure, independent of whether there was also prenatal exposure, were hyperactive relative to all other treatment groups. The univariate ANOVA of distance traveled revealed a significant main effect of treatment, $F(4, 73) = 15.52$, $p < 0.05$, with post hoc tests confirming that those groups with neonatal ETOH exposure were hyperactive ($p < 0.05$) relative to controls.

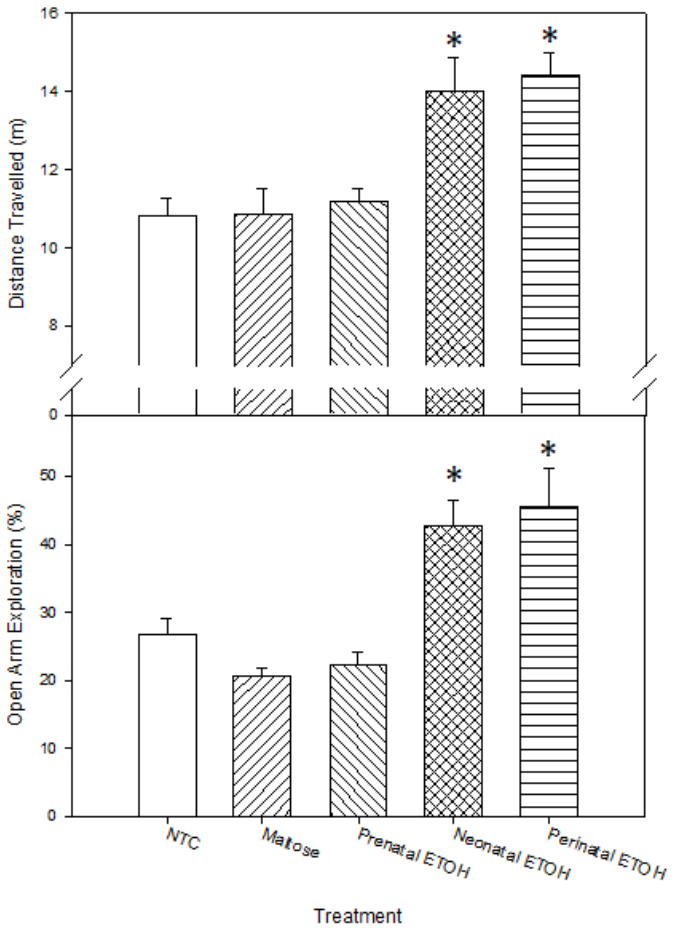

Figure 4. Elevated plus maze. Distance traveled in the EPM (upper panel) and distance travelled in the open arms adjusted for total activity [(open arm distance/total distance) * 100]. Offspring with neonatal or perinatal ETOH exposure were hyperactive and devoted a greater percentage of exploration to the open arms. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Error bars indicate SEM (N = 8-9).

Additional analyses assessed ETOH effects on distance traveled in (Figure 4, upper panel) and preference for the open arms of the maze (Figure 4, lower panel),

respectively measured as the total distance traveled in the open arms alone and adjusted for the total distance traveled in the maze [(Distance traveled in the open arms/Total distance traveled) x 100]. As expected, control mice avoided the open arms, with 27% (NTC) of exploration typically occurring in these areas of the maze. Neonatal exposure to ETOH increased exploration of the open arms, with and without controlling for total activity. Univariate ANOVA for distance traveled in the open arms showed a main effect of treatment, $F(4, 73) = 15.52$, $p < 0.05$, with post hoc tests showing that the neonatal ETOH treatment increased distance traveled with and without prenatal ETOH treatment relative to controls, ($p < 0.05$). The univariate ANOVA of preference scores yielded a significant main effect of treatment, $F(4, 73) = 12.90$, $p < 0.05$. Neonatal ETOH treatment increased open arm preference, with and without prenatal ETOH treatment, relative to controls ($p < 0.05$). This effect consisted of a ~16-18% increase in open arm preference, with ~43-45% of all exploration occurring in the open arms (See Figure 4, lower panel).

Morris water maze: Offspring exposed to neonatal ETOH exposure, independent of prenatal exposure, were impaired on acquisition in the MWM. Acquisition performance was measured by the latency (not shown) and distance traveled prior to escape (Figure 5) onto the platform. The mixed-factors ANOVA of escape latency revealed a significant main effect of treatment, $F(4, 67) = 7.71$, $p < 0.05$, and day, $F(3, 201) = 30.21$, $p < 0.05$. Post hoc tests confirmed that those groups with neonatal ETOH exposure took longer to find the platform (i.e. had longer escape latencies) than controls ($p < 0.05$). Similarly, distance traveled to the platform was greater during acquisition in offspring with neonatal ETOH treatment, with and without prenatal exposure. The

mixed-factors ANOVA of distance traveled to the platform revealed a significant main effect of day, $F(3, 201) = 67.78$, $p < 0.05$, and treatment, $F(4, 67) = 13.83$, $p < 0.05$. Post hoc tests confirmed that those groups with neonatal ETOH exposure traveled greater distances to find the platform ($p < 0.05$) relative to both non-treated and intubated control groups. An additional follow-up test was performed to determine whether group differences in the maze are generalized, perhaps impairing the ability to find the platform regardless of experience or consequence, or emerged as learning deficits during the acquisition period. To address this, additional univariate analysis tested whether ETOH treatment influenced latency or distance to escape on the first trial of day 1. No treatment effects were detected.

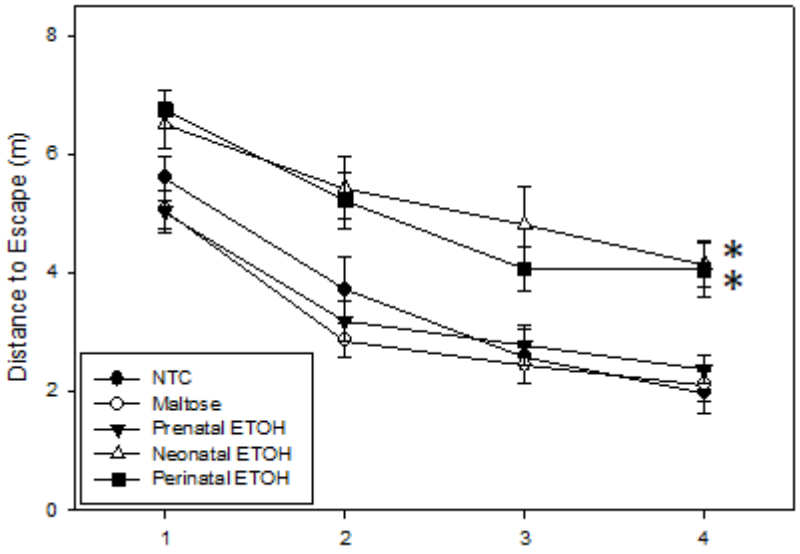

Figure 5. Morris water maze: Acquisition. Average distance travelled to the platform across four days of training. Offspring with neonatal ETOH exposure swam greater distances prior to reaching the platform. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Error bars indicate SEM (N = 8-9).

For the probe trial, there was a significant main effect of treatment, $F(4, 67) = 5.58$, $p < 0.05$. Post-hoc analysis revealed that that there was a reduction in the number

of annulus crossings, or crossings of the previous platform location, in offspring with prenatal ETOH treatment alone and neonatal ETOH treatment alone compared to maltose control ($p < 0.05$), but not non-treated controls ($p > 0.05$). There were no significant treatment effects on the distance explored in the target quadrant. Performance on the reversal component of the MWM is shown in Figure 6. Neonatal ETOH exposure alone increased escape latencies in the reversal phase relative controls. There was a main effect of treatment, $F(4, 67) = 3.75$, $p < 0.05$. The trend for a similar pattern among offspring with prenatal treatment alone and perinatal ETOH exposure failed to reach significance. There were no significant treatment effects on the distance traveled to the platform.

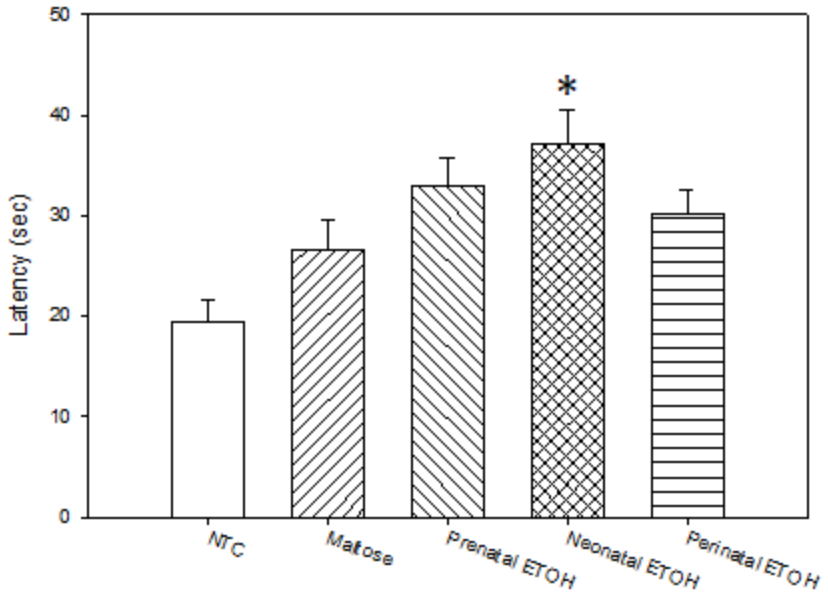

Figure 6. Morris water maze: Reversal. Latency to reach platform during reversal trials. Offspring with neonatal treatment alone showed greater latencies than NTC. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Error bars indicate SEM (N = 8-9).

To assess whether early ETOH exposure altered motor performance or visual ability, swim speed was measured in a visible platform probe (not shown). There were no significant effects of treatment or sex on swim speed to reach the visible platform.

2.6 Discussion

In the present study, we investigated the effects of prenatal, neonatal or perinatal ETOH exposure by intubation on activity in an open-field, exploration in the elevated plus maze and spatial learning and retention in a Morris water maze in male and female C57BL/6 mice. Hyperactivity, impulsivity and learning deficits are key diagnostic features of FASD and are particularly important for pharmacotherapy development. Analogous deficits have been examined in a variety of rat models for fetal ETOH effects, including those based on gestational exposure alone (Carneiro et al., 2005, Hofmann et al., 2005), neonatal exposure alone (Thomas et al., 2007, Lewis et al., 2012), and combined gestational and neonatal exposure (Cronise et al., 2001, Brocardo et al., 2012). A potential limitation of the current study is the two doses of ETOH used for dams and pups. Previous studies have emphasized the importance of peak BECs in ETOH's behavioral teratogenicity (Kelly et al., 1987) and that higher BECs, as the BECs observed in neonatal intubation but not those produced by maternal intubation in this study, are typically required for persistent behavioral effects (Allan et al., 2003). The prenatal dosing paradigm for this study was selected to avoid effects on litter size and neonatal body weight that are associated with higher doses (Downing et al., 2009b), but not this dose range (Kim et al., 2010). Although gestational treatments may augment the effects of later, neonatal doses in existing models (Cronise et al., 2001), these effects were limited in the current study, suggesting only minor differences in center exploration on the first session and a greater pattern of body weight reductions during and following neonatal treatment. Of note, the high BEC levels with neonatal treatment in this study are comparable to those reported in human alcoholics (Cartlidge and Redmond, 1990, Van

Zanten et al., 2013) and to those used in other rodent models (Goodlett et al., 1990). Advantages for the current paradigm also include the advantages of intubation over injection in neonatal rodents. Repeated injections can result in tissue damage and ETOH leakage due to their porous skin. Moreover, intubation allows for a consistent and well controlled BEC level in the animals.

Our data show that ETOH exposure during the third trimester equivalent, a critical period of CNS development, induced hyperactivity in the OF and EPM. This hyperactivity was observed at different ages and in two behavioral paradigms suggesting that this effect was long term and persistent at least in the young mouse. These patterns are consistent with previous mouse and rat models of FASD (Kim et al., 2013), including third trimester exposure models with rats (Smith et al., 2012, Thomas et al., 2007). Hyperactivity is an important aspect of the current model, as this symptom is highly prevalent in those with FASD and represents a prominent focus of drug development efforts for FASD (Koren, 2015). In addition to activity, both of these tasks also included a component typically used to assess anxiety, the open arms of the EPM and the center region of the OF. Mice generally avoid these open areas and control mice displayed this species-typical behavior (as measured by reduced distance traveled in and preference for the open arms and the center of the OF). Neonatal ETOH exposure increased exploration in these open areas, although interpretation was complicated by the fact that these offspring were hyperactive. To better understand this pattern, activity in the open areas was assessed relative to total activity for each subject and this led to different patterns on the two tasks. For the OF, the ETOH exposed mice were equally hyperactive in the periphery and in the center and so the increased exploration in the center was probably

best explained as just hyperactivity. In contrast, EPM exploration showed open arm preference approaching 50%, suggesting minimal preference between closed and open areas at all. These EPM findings are consistent with previous studies using young rodents with early ETOH exposure (Carneiro et al., 2005).

The disparity in ETOH effects on open area exploration in the OF and EPM is interesting. Although these open areas are both aversive, previous studies suggest that the EPM may be more sensitive to anxiolytic or disinhibitory effects than the OF (Schmitt and Hiemke, 1998, Acevedo et al., 2014), possibly due to the unique combination of open spaces and raised elevation in the EPM (Schmitt and Hiemke, 1998). From an ecological perspective, the failure of a rodent to avoid open spaces would lead to a higher "risk" of predation, suggesting that the observed ETOH effects would have real implications for natural consequences. These inhibitory failures are an important feature of the present mouse model, as those with FASD have difficulty avoiding risky behaviors or aversive consequences, leading to poorer outcomes through childhood and adolescence (Green et al., 2007) into adulthood (Moore and Riley, 2015). Although the present data do not implicate specific inhibitory control mechanisms which impact decision making (Bari and Robbins, 2013), future testing with this model may use more sophisticated tests of executive function to parse out these factors.

Neonatal ETOH exposure impaired acquisition of the MWM and this was observed in offspring treated perinatally or neonatally with ETOH. ETOH exposed offspring had longer swim paths and greater latencies to reach the platform during acquisition of the task. Swim speeds on the visible platform trial were equivalent across groups, so the differences in performance displayed by the ETOH exposed offspring

relative to controls could not be due to swimming deficits. Treatment effects were not evident on the first trial, but once evident, were consistent across acquisition. This did not translate to altered behavior during the probe trial relative to non-treated controls. Given this pattern, the observed performance impairments may be interpreted as impairments in spatial learning. These findings are consistent with previous third trimester ETOH exposure models reporting deficits in MWM acquisition (Goodlett and Peterson, 1995, Banuelos et al., 2012, Wagner et al., 2014). Deficits in memory and spatial abilities are also commonly reported among clinical populations with fetal ETOH exposure (Doyle and Mattson, 2015) and also represent key targets for intervention.

It is notable that male and female mice showed few meaningful differences in the current study. Male subjects appeared more sensitive to the weight reducing effects of early ETOH exposure, with weight reductions persisting into adolescence in male offspring with perinatal ETOH exposure, but not in females. In all other aspects, males and females showed equivalent behavioral deficits produced by early ETOH exposure. Previous mouse studies with relatively brief, 1 or 3 day, neonatal ETOH exposure paradigms have similarly shown only minor sex differences, if any. Consistent with the present data, Wozniak et al. reported that MWM deficits among young mice following neonatal ETOH on PD 7 did not differ by sex (Wozniak et al., 2004). Wagner et al. similarly found deficits in MWM performance following binge-like ETOH exposure on PD 7 or 7-9, with sex differences limited to performance on a probe trial (Wagner et al., 2014).

In conclusion, the current neonatal ETOH intubation paradigm induces hyperactivity, disinhibition and deficits in learning and memory in the young mice with

no sex difference. As the early neonatal period appears to be critical in ETOH's effects in the present study, it may be a useful tool for future mechanistic studies of FASD and for evaluating novel therapeutic approaches.

Chapter 3

Experiments 2-3: Neonatal ETOH Effects across Development

3.1. Study Design

Experiment 1 demonstrated the utility of the novel neonatal ETOH exposure paradigm through a test battery of 3 traditional behavioral assays. These data were consistent with the hypothesis that this ETOH treatment paradigm would produce diverse effects on behavior. Specifically, mice with neonatal ETOH exposure displayed increased locomotor activity, inhibitory failures, and poor response acquisition. Of particular interest, the effects of neonatal ETOH exposure on spontaneous exploration appeared to be only partially mediated by hyperactivity. Regulatory processes such as inhibitory control were implicated, but were unclear due to differences in the patterns across tasks and measures. These behavioral outcomes, while simple in practice, appeared to be sensitive to more complex features of behavior regulation and require additional study. The following experiments were designed to clarify the relevance of cognitive and regulatory deficits to behavioral performance in mice with neonatal ETOH exposure. To do this, a new behavioral battery was introduced that provided measures for activity levels, inhibitory control, anxiety-like behavior and deficits in higher cognitive processes, such as reversal learning and set shifting. These measures were selected for their relation to FASD symptoms with relevance to deficits in complex and essential behavioral functions across the lifespan. The following manuscript has been

prepared for the present dissertation and is currently being prepared for submission to the journal *Pharmacology, Biochemistry and Behavior*.

Choice Behavior and Learning across Development in a Mouse Model of Third-Trimester-like Ethanol Exposure

Andrew Hawkey, Logan Fields, Wenhua Xu, Hui Li, Gang Chen & Susan Barron

3.2 Abstract

Background: Consumption of ethanol (ETOH) during pregnancy can lead to diverse impairments in central nervous system (CNS) development and behavior, known as the Fetal Alcohol Spectrum Disorders (FASD). These symptoms may persist into adulthood and lead to secondary disabilities. Rodent models of third-trimester ETOH exposure have linked this critical period to cognitive and regulatory deficits in FASD which may be relevant to deficits in choice and other complex behaviors.

Methods: C57BL/6J male and female mice were exposed to ETOH on neonatal days (PD) 4-10 by intubation. In Experiment 2, behavioral testing was conducted on juveniles (PD 20-22) in the hole-board and adolescents (PD 35-37) in the open-field. In Experiment 3, male and female subjects were tested in adulthood (PD 60-75) in a nose-poke-based variant of the attentional set shifting task (ASST).

Results: In the hole-board, neonatal ETOH exposure increased exploration of holes as measured by the number of hole investigations. The ETOH-treated females also spent more time investigating the holes. In the open-field, neonatal ETOH exposure increased peak activity and reduced center exploration on the second session. In the ASST, ETOH-treated subjects showed impaired discrimination learning, requiring more

trials to reach criterion on the compound discrimination and three intradimensional shift phases. This poor performance was associated with greater numbers of committed errors and faster response times.

Conclusion: The current neonatal intubation model demonstrated multifaceted deficits in behavior regulation which persisted into adulthood. This model may be valuable for future mechanistic and drug development studies aiming to investigate or reduce deficits in choice and other complex outcomes.

3.3 Introduction

Ethanol (ETOH) consumption during pregnancy produces a range of deficits in central nervous system (CNS) development and behavior, collectively known as the fetal alcohol spectrum disorders (FASD). Fetal ETOH effects are estimated to impact as many as 2-5% of live births in the US (May et al., 2009), making fetal ETOH exposure the leading preventable cause of neurodevelopmental impairment. ETOH influences CNS development through a number of mechanisms, including cell death and alterations in cell signaling and gene expression (Muralidharan, Sarmah, Zhou & Marrs, 2013). Research with rodent models has worked to identify these mechanisms and the key factors which modulate them, including the dosing, timing, and schedule of ETOH exposure, as well as various pharmacological targets (e.g. Barron, Fields, Hawkey & Littleton, 2016, Goodlett, Horn & Zhou, 2005). Given this, rodent models are well suited to demonstrate fetal ETOH effects in controlled experiments and to assess the pharmacological or neurogenetic variables underlying them. However, for a rodent model to provide compelling support for a mechanism or compound with treatment potential, it is essential that appropriate behavioral outcomes are identified which map onto the

common symptoms of FASD.

The behavioral profile of FASD is multifaceted. Recent diagnostic guides indicate that fetal ETOH exposure is associated with impairment in 12 behavioral areas representing neurocognitive, self-regulatory and adaptive functions (Doyle & Mattson, 2015). Cognitive and regulatory deficits emerge in a variety of settings, often as hyperactivity, inattention, inappropriate or impulsive behaviors, and difficulties in learning (Green et al., 2007). These disruptions also lead to high comorbidity with other disorders, such as ADHD and conduct disorders (Popova et al., 2016). Beyond clinical outcomes, impairments often manifest as secondary disabilities. Those with FASD are at elevated risk for a number of negative or disruptive outcomes as they approach and enter adulthood, including interrupted schooling (suspension, drop out, etc), substance abuse, risky or inappropriate sexual behavior, difficulty maintaining employment, financial difficulties and trouble with the law (Moore & Riley, 2015, Streissguth et al., 1992, Streissguth et al., 2004). The precise mechanisms leading to these disabilities remain unclear, as early ETOH exposure can impair several critical processes for the control of choice and complex behavior. These include neurocognitive functions, such as executive function and learning and memory, as well as regulatory functions, such as control of mood or behavior, impulse control and attention (Doyle & Mattson., 2015, Kodituwakku & Kodituwakku, 2014). In light of secondary deficits, it may be beneficial for drug development efforts targeting FASD symptoms to also address potential benefits to the control of complex behaviors, such as choice, alongside specific symptoms.

Choice behavior, defined as the allotment of responses among multiple alternatives, is a common outcome in rodent behavior analysis and has been adapted to

detect deficits in a variety of behavioral and cognitive functions. For example, rodent models of FASD exhibit impaired learning and/or memory in spatial choice tasks, such as single or multiple T-mazes (e.g. Lewis et al., 2012, Zimmerberg, Sukel & Stekler, 1991) or radial arm mazes (e.g. Reyes, Wolfe & Savage, 1989, Thomas, La Fiette, Quinn & Riley, 2000, Wozniak et al., 2004), impaired attention, as in the 5 choice serial response time task (5CSRTT) (Louth, Bignell, Taylor & Bailey, 2016), and altered motivational functions, as in a delay discounting paradigm (Bañuelos et al., 2008). Similarly, spontaneous or unlearned choices are often biased in rodent models of FASD, with ETOH-exposed offspring showing altered preferences for investigating other rodents (e.g. Kelly, Goodlett & Hannigan, 2009), objects (e.g. Allan, Chynoweth, Tyler & Caldwell, 2003) and open spaces, as in the open-field or elevated plus maze (e.g. Thomas, Sather & Whinery, 2008). Choice data from these paradigms can be adapted to assess yet more complex phenomena by assessing choice over time across time or repeated trials, revealing patterns in how these deficits manifest as subjects interact with their environments.

In line with previous work with rodent FASD models in choice designs, we have recently reported deficits in choice behavior in a novel mouse model of third-trimester-like ETOH exposure (i.g. 4g/kg ETOH, PD 4-10) (Xu et al., In Review). Specifically, juvenile offspring (PD 20-27) showed increased locomotor activity, as well as enhanced center avoidance on the second session of testing in the open-field (OF) and reduced open arm avoidance in a single session on the elevated plus maze (EPM). As adolescents (PD 35-45), these subjects also showed general deficits in spatial learning in the Morris water maze (MWM). Taken together, these findings suggest that neonatal ETOH exposure

leads to multiple deficits that are relevant to either spontaneous preference or operant learning, including hyperactivity, anxiety-like responses, disinhibition, and learning deficits. Importantly, these findings also link key cognitive and regulatory deficits to a critical period in CNS development that overlaps the "third trimester human brain growth spurt"; a period of rapid brain growth and synaptogenesis coinciding with the human third trimester or the rodent neonatal period (Dobbing and Sands, 1979). During this time, a number of behaviorally-relevant brain areas are highly sensitive to ETOH teratology, including the cortex and hippocampus (e.g. Ikonomidou et al., 2000, Livy, Miller, Maier & West, 2003).

The current study aimed to further investigate cognitive and regulatory deficits following neonatal ETOH exposure using a novel behavioral battery spanning into young adulthood. The behavioral batteries consisted of measures of investigatory and/or locomotor exploration, measured in the hole-board (HB) and open-field (OF), and adaptive or flexible learning, through the attentional set shifting test (ASST). The HB was selected to discriminate between locomotor and investigatory biases related to exploration of preweanling mice (PD 20-22). The OF was selected to test activity and exploration in adolescent mice (PD 35-37), showing whether previously reported effects in OF exploration among juveniles (Xu et al., In Review) persist into adolescence. The ASST was selected to provide data on rapid learning and cognitive flexibility in adulthood (PD 60-75) through a series of shifting response-consequence contingencies. Together, these data may then suggest novel outcomes in the mouse model with future implications for the identification of neural mechanisms underlying complex behavioral deficits in FASD and for the development of efficacious treatments for them.

3.4 Materials and methods

Animals: Litters of C57BL/6J mice were bred in the Medical Center Animal Facility at the University of Kentucky. The animals were maintained on a 12-h light/dark cycle with *ad libitum* access to water and standard laboratory chow in a nursery that was temperature and humidity controlled. All experimental procedures were conducted between 8:00 and 17:00 h. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Neonatal ETOH treatment: As parturition approached, cages were checked daily for pups. Birth was considered PD 0. On PD 3, litters were weighed and culled to 8 pups, when necessary, while litters with fewer than 4 pups were not included. Pups were quasi-randomly assigned to treatment groups (See table 3), with equal representation of males and females when possible. This split litter design was conducted to ensure a minimum of 8-10 litters represented per sex per treatment group to preclude potential litter effects (Abbey and Howard, 1973). Mice were treated as previously described by Xu et al (In Review). From PD 4-10, offspring assigned to the ETOH group were intubated once daily (1200hr) with 4 g/kg ETOH in a maltose formula developed to mimic mouse milk (Kelly & Lawrence, 2008) or the maltose formula alone by gavage (0.02 ml/g bw). Mortality due to gavage error was low (6%). A non-intubated control group was also included.

Table 3
Treatment Conditions

	NTC	Maltose	ETOH
Dam	Non-treated	Non-treated	Non-treated
Pups	Non-treated	Maltose i.g. PD 4-10	4g/kg/day i.g. PD 4-10

Table 3: Subjects were divided into 3 treatment conditions: non-treated controls (NTC), an intubated control (Maltose), and a neonatal ETOH condition (ETOH).

Behavioral testing: In Exp 2, juvenile subjects (PD 20-22) were first tested in a modified hole-board (HB) procedure for one session. Offspring were then weaned on PD 28 and housed with 1–3 same sex littermates. Subjects were allowed to acclimate for one week prior to further testing. Subjects were then tested in adolescence (PD 35-37) using a circular open-field (OF) for 2 sessions. Upon reaching adulthood (PD 60), a single subject per sex, per treatment group, per litter was randomly selected for inclusion in Exp 3. Subjects from one litter were not included, as they were used to pilot an alternative behavioral procedure in adulthood. One additional subject was excluded due to an unrelated medical issue. In adulthood (PD 60-75), subjects were food restricted to ~80% of free feeding body weight and tested in the ASST. All behavioral testing was conducted under low ambient light conditions with white noise to reduce extraneous auditory stimuli. Surfaces and holding cages were cleaned before and after testing with Nature's Miracle© enzymatic cleaning solution to remove animal odors. Within the HB and OF testing, animal movements were recorded using the AnyMaze tracking system (Stoelting Co.).

Experiment 2

Hole-board: The hole-board test (HB) is a procedure designed to measure investigatory exploration (File & Wardill, 1975). The HB was a square chamber (30.5 x

30.5 cm) with white walls and floor. The floor contained 16 equally-spaced holes (1.5cm in diameter). For one session between PD 20-22, mice were individually brought into the test room in a clean holding cage for a 10 min habituation period prior to testing. Each mouse was allowed to explore the HB for 5 min. Dependent measures included total distance traveled, as well as the number and total duration of visits to nose-poke holes. This procedure was selected because unlike other exploration paradigms, it allows potential effects on exploration to be divided into locomotor and investigatory responses, measured below as distance traveled and time/frequency of hole investigations respectively.

Open-field: Open-field testing is commonly used to measure levels of activity and patterns of exploration (Bailey & Crawley, 2009). During adolescence, (PD 35-37), subjects were individually placed into a clean holding cage and allowed to habituate for 10 min prior to each of two daily 30 min OF sessions. The OF apparatus was a circular Plexiglas chamber (diameter 39.4 cm) with opaque white walls. The circular OF was selected to prevent subjects from retreating to the corners, as in the square OF apparatus. Dependent measures included total distance traveled and exploration of the center zone, measured as the distance traveled in the zone alone and adjusted for the total distance traveled. The center was defined as a circular zone with a diameter half the width of the OF. Exploration of the center zone was included to address levels of anxiety-like behavior in the OF, as mice tend to avoid open spaces where they would be vulnerable to predators in the wild.

Experiment 3

Attentional Set Shifting Task: The ASST is a cognitive battery requiring subjects

to complete a series of increasingly difficult discriminations, assessing the functions of varying attentional, inhibitory and learning functions (Birrell & Brown, 2000). Testing was performed in a modified rodent cage (20.32 x 45.72cm) with two alcoves on one end, each containing platforms (9 x 14cm) (See Figure 7). Each platform had a centered nose-poke hole (1.5cm) and was covered in distinctly-textured materials which varied across testing phases. Prior to the discrimination learning phases, animals were shaped to retrieve food reinforcers (⅙ of a Cocoa Pebble™, Post Foods) from nose-poke holes in uncovered wooden platforms which alternated (left-right) across shaping trials. After meeting a criterion of three consecutive trials with nose-poke latencies of <30 seconds, ASST testing began.

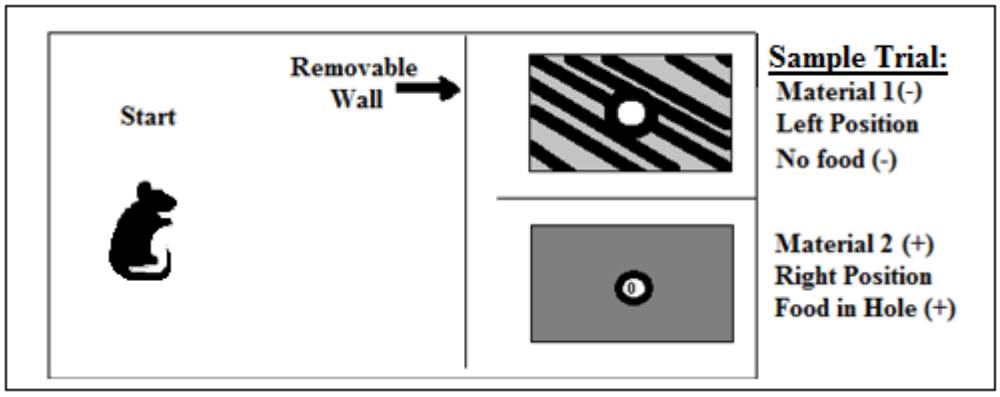

Figure 7. Two-Choice Apparatus for Attentional Set Shifting Task (ASST).

The training and testing sequence in the ASST was adapted from a procedure used by Bissonnette and colleagues (2005) (see Table 4 for phases and example stimuli). Subjects completed a series of discriminations whereby each pair of stimuli could be discriminated according to the material covering the platform (material) or the location of the platform (position). Subjects first completed compound discrimination training and 3 intradimensional shifts (ID) where subjects learn to find the hidden food by attending to

the material. Following the ID phases, subjects completed 3a reversal learning phase, where the reinforcer is suddenly paired with the alternative cue which previously predicted no reinforcer. This challenged subjects to inhibit the learned response and respond to the alternative. Following this, subjects completed an extradimensional shift (ED) in which the previously irrelevant stimulus dimension (platform position) predicted the location of the reinforcer. This shift challenged subjects to inhibit the previous attentional strategy and adapt to use the previously irrelevant cues.

Table 4.
Attentional set shifting task design

Phase	Platform Material		Position	
CD	Rubber (+)	Burlap (-)	Left	Right
ID1	Cardboard (+)	Cellophane (-)	Left	Right
ID2	Duct Tape (+)	Scour Pad (-)	Left	Right
ID3	Paper (+)	Foil (-)	Left	Right
Reversal	Paper (-)	Foil (+)	Left	Right
ED	Tule	Vinyl	Left (+)	Right(-)

Table 4. Example stimulus sets in the rodent ASST. Plus (+) indicates the location of the 48ehavior48. Minus (-) indicates no 48ehavior48. Abbreviations: CD= Compound Discrimination, IDS = Intradimensional Shift, ED= Extradimensional Shift.

To begin each phase, subjects were given 4 trials where they were allowed to sample both platforms while retrieving the reinforcer. This allowed subjects to investigate the new stimuli and learn the appropriate response for the new trials. For the remaining trials, subjects were only allowed to make one response (any part of the snout dipping below the lip of the hole) and it was scored as either a correct or incorrect trial. At this time, latency for the trial was also recorded. Animals proceeded to the next phase of the sequence by achieving a learning criterion of six consecutive correct

responses. If a subject failed to reach criterion within 50 trials, a failure to discriminate was recorded and the subject was automatically moved to the next phase. The platform materials and locations (left or right) of each on a given trial were pseudorandomly assigned, with the restriction that the reinforcer could not be located in the same location on more than two consecutive trials. A failure to respond within two min resulted in a time-out. Although rare, if a subject recorded time-outs on three consecutive trials (i.e. six min without a valid nose-poke), the session was terminated. Platforms of each material were exchanged in between trials to prevent scent marking by the mice. A trace amount of the food (powdered) was added to the bottom of each nose-poke hole to prevent detection of the through olfactory means.

Statistical Methods: Statistical analyses were conducted using SPSS v.22 (IBM) for analysis of variance (ANOVA) and follow-up testing. An initial analysis was conducted to determine whether the control groups (non-treated and maltose-intubated) differed on any measure included in the study. No differences were detected. Based on these findings, these treatment groups were collapsed into a single control group. Subsequently, univariate or mixed factors ANOVAs were performed, as appropriate, to detect main effects of treatment, sex and day/time, as well as interactions. If no sex*treatment interaction was found, data were collapsed across sex for follow-up analyses. Data is shown collapsed across all factors that did not interact with treatment for ease of presentation. In cases where homogeneity of variance was violated, the Greenhouse Geisser correction was used, resulting in degrees of freedom which were not whole numbers. For Exp 2, an adjustment was made to preclude litter effects whereby same sex siblings of a given treatment were averaged together, when applicable, to

produce a single data point, per sex, per treatment group, per litter. For Exp 3, a single subject per sex, per treatment group per litter was randomly selected for inclusion in ASST testing to provide appropriate representation of litters per cell.

3.5 Results

Body growth: Body weight data were collected throughout the treatment period and prior to each behavioral test in the sequence (see Table 3). A mixed factors ANOVA of body weight during the neonatal treatment period revealed a significant effect of age, F (7, 336) = 1392.73, $p < 0.05$, and an age*treatment interaction, F (14, 336) = 9.89, $p < 0.05$. Post hoc tukey tests showed that ETOH-treated offspring weighed less than non-treated controls on the final day of treatment ($p < .05$). A mixed factors ANOVA of body weight at the ages of testing showed significant effects of age , F (2, 94) = 2197.67, $p < 0.05$, and sex, F (2, 47) = 93.9, $p < 0.05$, whereby males weighed more than females and all subjects gained weight across development. This analysis also detected significant age*sex, F (2, 94) = 90.67, $p < 0.05$, and age*treatment, F (4, 94) = 2.60, $p < 0.05$, interactions. Follow-up testing performed pairwise comparisons of weights at each age and for each sex, with tukey correction for multiple tests. Maltose-intubated female mice were significantly larger than non-treated controls at 60 days of age ($p < .05$). Trends for reduced weights among ETOH treated males relative to non-treated controls failed to reach significance.

Table 5.
Offspring weights

Postnatal Day (PD)	NTC (g)+/- SEM		Maltose (g)+/- SEM		ETOH (g)+/- SEM	
4	2.54±0.11		2.49±0.12		2.51±0.86	
5	3.00±0.13		2.93±0.12		2.88±0.82	
6	3.57±0.15		3.57±0.15		3.27±0.10	
7	4.13±0.16		4.07±0.16		3.72±0.11	
8	4.59±0.17		4.63±0.16		4.17±0.12	
9	5.10±0.18		5.21±0.17		4.59±0.12	
10	5.65±0.18		5.76±0.18		5.06±0.12*	
	M	F	M	F	M	F
20	10.96±0.60	9.30±0.61	11.01±0.49	10.13±0.52	9.81±0.62	10.03±0.43
35	20.88±0.39	16.13±0.53	20.33±0.41	16.36±0.42	19.53±0.51	16.58±0.15
60	25.49±0.73	18.41±0.51	25.44±0.73	20.00*±0.32	23.49±0.38	18.81±0.28

Table 5. Body weights (in g) during the treatment period and behavioral testing (split by sex). ETOH treated subjects showed lower body weights on the final day of treatment, but not at testing ages. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. Data are expressed as the mean (g) +/- SEM (N = 8-11).

Experiment 2:

Hole-board: In the HB, ETOH treated subjects made a greater number of hole investigations than controls, with ETOH-treated females also showing increased total time spent investigating the holes. Statistical analyses assessed ETOH effects on hole investigation, measured as the number of hole investigations (Fig. 1) and total time spent investigating the holes (Fig. 2). The univariate ANOVA of number of hole investigations revealed a significant effect of treatment, F (1, 50) = 4.80, $p < 0.05$, which did not interact with sex. ETOH-treated subjects made a greater number of hole visits than controls ($p < .05$). The univariate analysis of investigation time revealed a significant treatment effect, F (1, 50) = 4.62, $p < 0.05$, and a significant sex*treatment interaction, F (1, 50) = 4.52, $p < 0.05$. Among females, there was a significant main effect of

treatment, F (1, 25) = 10.88, $p < 0.05$, whereby ETOH-treated offspring showed spent more time investigating the holes than controls ($p < .05$), while no treatment effects were found among males. Additionally, locomotor activity, measured as total distance traveled, was analyzed by univariate ANOVA, revealing no significant effects of treatment or sex, and no interactions.

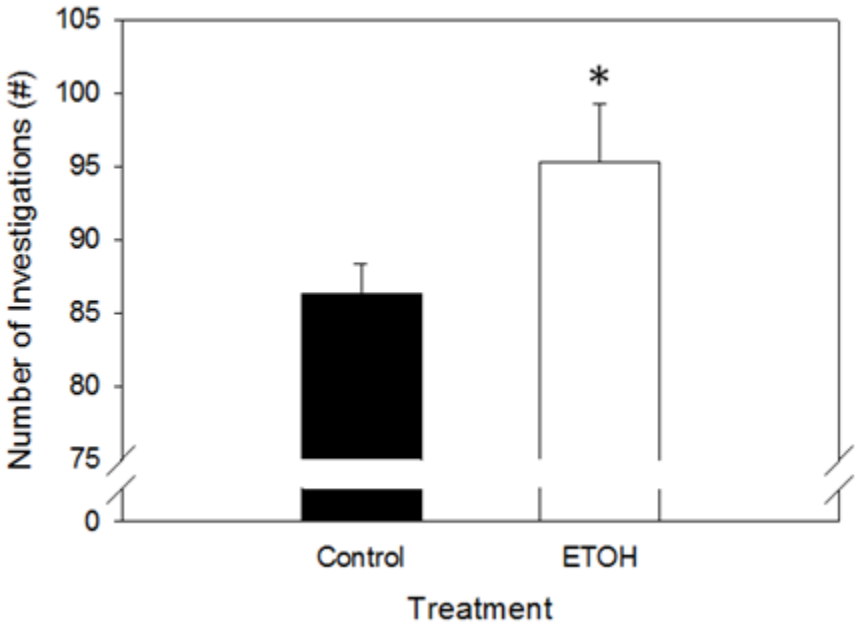

Figure 8. Holeboard: Number of hole investigations. Total number of visits to the holes. Asterisk * indicates a significant difference relative to controls. Error bars indicate SEM. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. (N = 10-11).

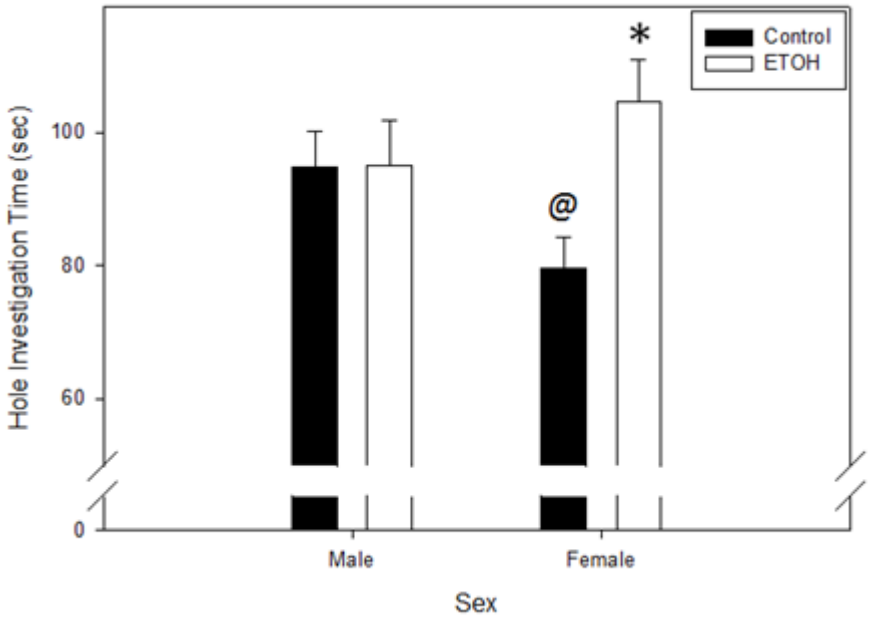

Figure 9. Holeboard: Hole investigation time. Total time spent investigating the holes. Asterisk shows significant difference relative to controls. "At"@ indicates a significant sex difference. Error bars indicate SEM. A = $p < .05$. (N = 10-11).

Open-field: In the OF, ETOH-treated adolescent mice showed greater initial activity after entering the apparatus, as well as reduced relative exploration of the center zone on the second testing session. Figure 10 shows activity in the OF, as measured by distance traveled. Data from the two OF sessions are collapsed into a single curve, as day did not interact with treatment effects on activity. Mixed factors ANOVA detected effects of day, F (1, 50) = 4.02, $p < 0.05$, and time, F (3.41, 170.38) = 70.69, $p < 0.05$, as well as interactions of day*time, F (4, 199.818) = 11.63, $p < 0.05$, and time*treatment, F (3.41, 170.38) = 3.81, $p < 0.05$. Post hoc testing determined that ETOH treated offspring were hyperactive in the first two time bins within the OF session ($p < .05$), but not later in the session.

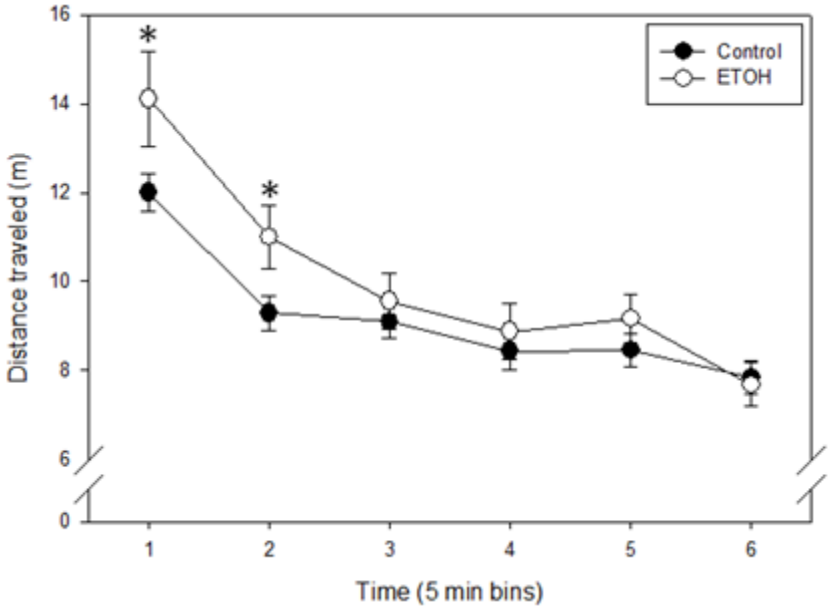

Figure 10. Open-field Activity. Distance traveled over time. ETOH-treated subjects were hyperactive relative to controls in the first two time bins. Error bars indicate SEM. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. (N = 10-11).

Additional analyses assessed center exploration, measured as the distance traveled in the center, alone (not shown) and adjusted for the total amount of exploration (Fig. 11). The mixed factors ANOVA of center distance detected a significant effect of day, F (1, 50) = 21.31, $p < 0.05$, but no other effects. When adjusted for the total level of activity, effects of day, F (1, 50) = 25.25, $p < 0.05$, and treatment, F (1, 50) = 4.40, $p < 0.05$, were found, as well as a day*treatment interaction, F (1, 50) = 7.42, $p < 0.05$. Post hoc tests showed ETOH-exposed adolescent offspring avoided the center zone to a greater degree than controls on day 2 ($p < .05$), devoting proportionately less of their exploration to this zone.

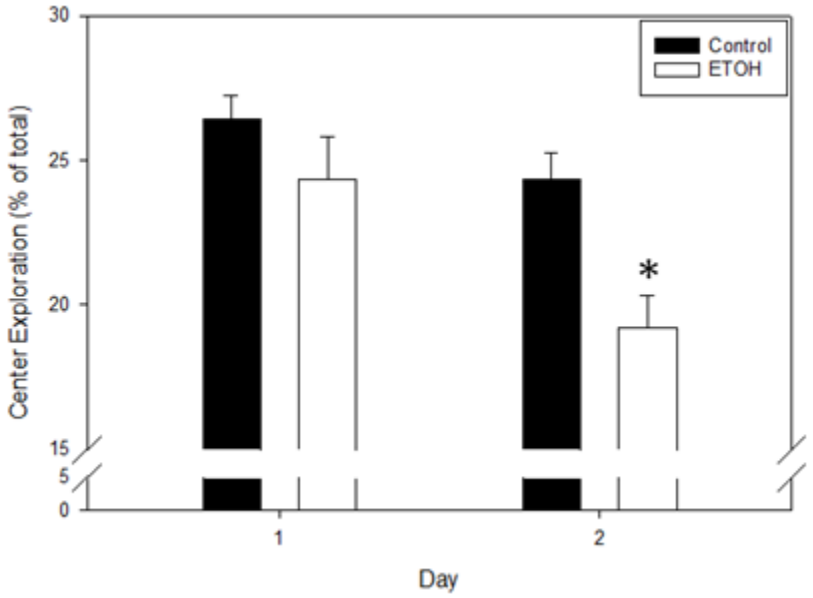

Figure 11. Open-field: Center exploration. Center activity adjusted for total distance traveled. [(center distance/total distance)*100]. ETOH-treated subjects were more center avoidant on session 2. Error bars indicate SEM. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. (N = 10-11).

Experiment 3:

ASST: ETOH treatment impaired ASST performance, reducing the latency to respond overall, and impairing learning across the initial discrimination phases. Figure 12 shows performance in the ASST, as measured by number of trials to criterion (upper panel). Univariate ANOVAs detected significant effects of treatment on the number of trials to criterion in the CD, F (1, 49) = 7.47, $p < 0.05$, ID1, F (1, 49) = 22.78, $p < 0.05$, ID2, F (1, 49) = 8.64, $p < 0.05$, and ID3, F (1, 48) = 5.35, $p < 0.05$ phases of testing. ETOH-treated offspring required more trials to reach criterion in each of these phases (p <.05). A follow-up analysis (not shown) was performed to determine whether poor learning performance following neonatal ETOH exposure reflected perseveration, or the repetition of responses. To assess this, errors were analyzed based on whether they occurred in the same location as the previous response or in the alternate location as the previous response. Mixed factors ANOVA of errors revealed a significant effect of

treatment (F (1, 46) = 21.70, $p < 0.05$) whereby ETOH-treated subjects committed greater numbers of errors regardless of error type. Finally, an analysis was performed to determine whether ETOH-treatment altered the latency to respond (See Figure 13). A univariate ANOVA of latency to respond observed an effect of treatment (F (1, 49) = 6.73, $p < 0.05$), whereby ETOH-exposed offspring responded more quickly than controls in the ASST ($p < .05$).

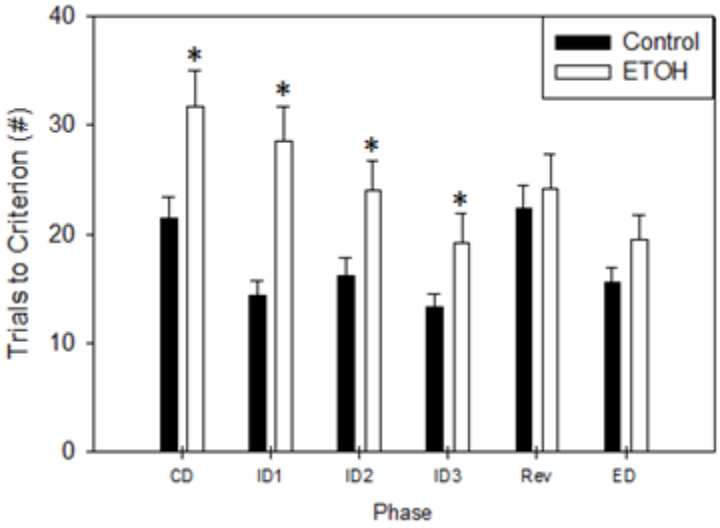

Figure 12. ASST: Trials to criterion. ASST performance measured as trials to criterion. ETOH-treated offspring required more trials to reach criterion on the CD and 3 ID phases of the ASST. Error bars indicate SEM. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. (N = 9-10).

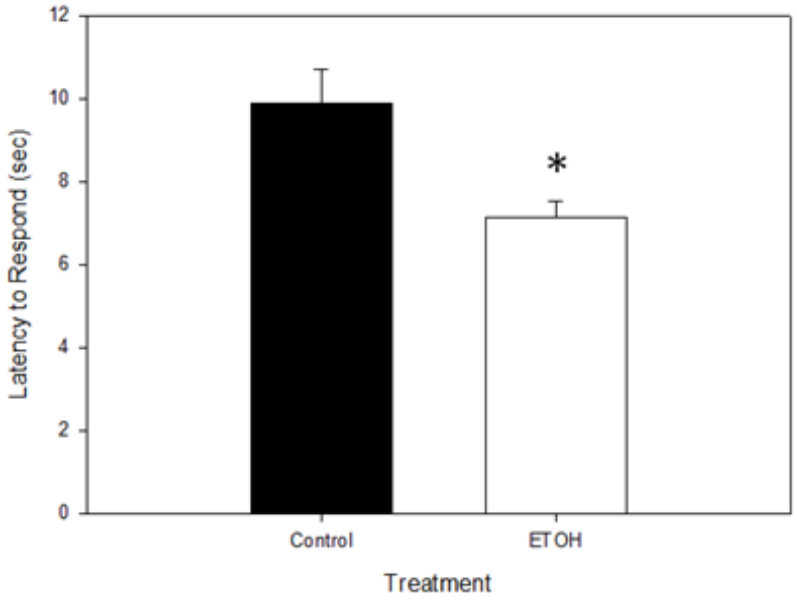

Figure 13. ASST: Response latencies. Latency to first response. ETOH-treated subjects responded more quickly than controls. Error bars indicate SEM. Significant differences from NTC are indicated by asterisks *. * = $p < .05$. (N = 9-10).

3.6 Discussion

In the current study, we investigated the nature and persistence of behavioral deficits following neonatal ETOH exposure in the C57BL/6J mouse. This exposure paradigm is a model of binge-like ETOH exposure during the third-trimester of CNS development. Exp 2 showed that neonatal ETOH exposure influences spontaneous exploration across the juvenile stage into adolescence, as evidenced by greater hole investigation in the holeboard (HB), as well as brief hyperactivity and enhanced center avoidance in the open-field (OF). These data further showed that these effects may be partially mediated by sex, at least among young animals. Both male and female offspring with neonatal ETOH exposure investigated holes more frequently than controls, although only females showed increases in investigation time as well. No sex differences were observed on OF exploration in adolescence.

Among juveniles in the HB, neonatal ETOH treatment was not shown to

influence locomotor activity. This finding lies in contrast with previous work reporting hyperactivity in the OF and EPM with this mouse model at comparable ages (Xu et al., In Review) and neonatal exposure models in rats (e.g. Smith et al., 2012, Thomas, Garrison & O'Neill, 2004). The lack of hyperactivity in the HB may be due to the interruption of locomotor activity by frequent, immobile investigations of the holes. One strength of the HB is that it separates investigatory responses from locomotion, which are confounded in the OF. Increases in hole investigation suggest that neonatal ETOH exposure produces a bias towards the investigation of new stimuli, in this case nose-poke holes, that is separate from any effects this treatment may have on locomotion. These effects may relate to patterns of greater exploration which appear in other spontaneous exploration tasks, such as the OF or EPM. Of note, ETOH-treated adolescent mice displayed hyperactivity in the present study, but only on the initial time blocks of the session. By contrast, previous work with the model has shown hyperactivity in juveniles that persisted across the session (Xu et al., In Review). This pattern suggests that in adolescents with neonatal ETOH exposure, hyperactivity may be observed as a brief hyperreactivity to entering the apparatus rather than generalized patterns of hyperactivity, as previously reported in younger offspring.

Neonatal ETOH-treatment was also shown to alter the pattern of exploration in Exp 2. In the HB, ETOH-treated subjects generally visited the holes more frequently than controls, with a corresponding increase in hole investigation time among ETOH-treated females. Previous studies have described hole investigations as ecologically "risky", in that the mouse will be less alert to detect the approach of a predator while nose-poking (e.g. Wardhill & File, 1975). Greater investigation of the holes in the present data may

then reflect deficits in the inhibitory processes that typically limit this behavior. Intriguingly, females appeared more sensitive to these effects than males. The greater sensitivity of females was related to a baseline sex difference in hole investigation time among controls, whereby females spend less time investigating the holes than males over the course of the session. ETOH treatment attenuated this sex difference. Consistent with this, previous studies indicate that early ETOH exposure may alter behavior by reducing or even reversing sexually dimorphic behaviors (e.g. Kelly & Dillingham, 1994, McGivern, Clancy, Hill & Noble, 1984, Ward et al., 2002). These effects are most often reported sexually mature animals, although ETOH-treated juvenile females may similarly appear "masculinized" on behaviors that are highly dimorphic at this age, such as social play (e.g. Meyer & Riley, 1986).

Within the OF, the pattern of exploration was measured in reference to the peripheral and the center zones in the arena. Mice tend to explore their surroundings thoroughly when placed in a new environment, although they naturally avoid open spaces to reduce their vulnerability to predators (e.g. Bailey & Crawley, 2009). Previous studies have shown that hyperactivity due to neonatal ETOH exposure can lead to greater exploration of the center zone (e.g. Thomas, Sathery & Whinery, 2008), although distance traveled in the center should also be assessed relative to total activity to measure center preference (as in Xu et al., resubmitted). When center exploration was converted to a percentage of total exploration, ETOH-treated subjects showed an enhanced aversion to the center zone on the second day of testing. Reduced center preference may reflect ETOH-induced deficits in the regulation of stress- or anxiety-related reactivity. These findings are in line with previous work showing that early ETOH exposure produces

hyperreactivity to stress and anxiety-like symptoms in humans and animal models (Hellemans et al., 2008). The present mouse model may then be valuable in addressing neural mechanisms which mediate these symptoms, such as alterations in the hypothalamus-pituitary-adrenal (HPA) axis (e.g. Hellemans et al., 2010) and the amygdala (e.g. Zhou, Wang, & Zhu, 2010).

An interesting aspect of the present model is that depending upon testing conditions, ETOH-treated subjects showed either enhanced or reduced aversive control of exploration. Comparison of the available data suggests that these effects may be experience-dependent. Presently, the HB was presented to the subjects briefly and only as a novel environment. Behavior was marked by inhibitory failures rather than anxiety-like behavior. This pattern mirrors previous data with this model from the elevated plus maze (EPM) (Xu et al., In Review), also presented briefly and only once. In the current study and previous work (Xu et al., In Review), ETOH-induced enhancements of center avoidance were only evident in the second OF session, when this environment was no longer novel. The striking differences in these patterns may be related to familiarization with novel environments. Previous analyses suggest that the sensitivity of exploration to anxiety-like symptoms can vary considerably based on familiarity with the relevant cues (File, 2001). For example, previous studies have shown that repeated exposure to an EPM results in a reduction in open arm exploration in mice (e.g. Espejo, 1997, Rodgers & Shepherd, 1993). The present data are in line with the conclusion that inhibitory and/or affective regulation of exploration depend upon experience and that the deficits associated with neonatal ETOH exposure are sensitive to those interactions.

Together, the findings of Exp 2 suggest that spontaneous exploration is controlled

by multiple processes that may be compromised due to neonatal ETOH exposure, including regulation of activity, inhibitory control and anxiety-like reactivity. Further, the present neonatal ETOH exposure model and behavioral battery provide a valuable set of baselines for investigating ETOH effects on the cortical, limbic and neuroendocrine systems underlying behavior regulation (Bari & Robbins, 2013, Chambers, Garavan & Bellgrove, 2009, Hellemans et al, 2010) and for the evaluation of novel treatments which may improve behavior regulation following early ETOH exposure.

Exp 3 addressed complex behavior in adulthood through the ASST, a rapidly acquired and multidimensional learning task which taps into learning, attention and cognitive flexibility (Birrell & Brown, 2000). In contrast to the paradigms in Exp 2, choice was not based on spontaneous or species-typical preferences. Instead, mice chose nose-poke holes to investigate based on recent experiences with hidden reinforcers and predictive cues. This required selective attention to the predictive cues and rapid learning to acquire and shift these choices over the course of each phase. ETOH-treated subjects required greater numbers of trials while learning to discriminate platforms of different materials across the initial CD and ID phases of the current ASST paradigm. Overall, neonatal ETOH exposure increased both perseveration and alternation errors, suggesting that increases in errors are not due to the use of an irrelevant spatial strategy (e.g. match-to-place, spatial alternation). The greater number of errors may be related in part to response times, as ETOH-treated subjects responded faster than controls. Altered latencies may indicate differences in motivation to respond and/or response inhibition.

Previous studies with the ASST have heavily focused on the reversal and ED components, in large part due to their close association with the orbital and medial

prefrontal cortex (e.g. Bissonette et al., 2007). As such, deficits in discrimination are largely considered confounds rather than deficits of interest. The current study, by contrast, found that the reversal and ED showed the least sensitivity to the treatment, rather than the most sensitivity. This may be due to the location of these tasks at the end of the current ASST design. Alternative designs for the ASST often include multiple reversals, usually three, over the course of the sequence (as in Birrell & Brown, 2000). When these studies report reversal deficits, those effects can be shown to weaken or disappear over successive repetitions (e.g. Cheng & Li, 2013, Gastambide et al., 2013, Laurent & Podhorna, 2004). This may account for the insensitivity of these final components to deficits which impaired the presumably less difficult discriminations and shifts beforehand.

Rapidly acquired discriminations are often categorized as working memory tasks, broadly defined as tasks where responding is based on experiences within a given session, rather than across sessions (Dudchenko, 2004). Current deficits in the CD and ID phases are consistent with deficits demonstrated in other working memory tasks using mice (Wozniak et al., 2004) or rats (e.g. Lewis et al., 2012) with binge-like neonatal ETOH exposure. In this case however, it is unclear whether working memory, as a cognitive process, is impaired. Working memory tasks are often sensitive to more basic disruptions, such as stereotypies and motivational deficits, a confound which has led to adaptations of common working memory tasks to include both within-session ("working memory") and across-session ("reference memory") components (e.g. Thompson & Moerschbacher, 1979). Treatments which impact both tasks are said to be nonspecific and attributed to more basic deficits. The current study did not include a similar control

condition, so it is perhaps more accurate to interpret the observed deficit as a general learning impairment than a deficit in working memory.

Of interest, recent data suggests that early ETOH exposure may impair learning in mice indirectly, through inattention. Louth and colleagues (2016) recently reported that male mice (PD 60+) with combined prenatal and neonatal ETOH exposure exhibited striking performance deficits in the 5CSRTT. These mice were slower to learn the task, showed poorer response accuracy and were more likely to fail to respond as the response windows grew shorter. These impairments were associated with electrophysiological deficits in the medial prefrontal cortex, mediated by nAChR and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) function. The relevance of these findings to the present deficits is unclear since the ASST does not specifically assess attention, beyond the use of attentional sets, and the mouse model used by Louth (2016) varied in dosing and timing of exposure. However, future testing with the current model may investigate the connection between the present learning deficits and the neuropharmacological targets identified by Louth et al (2016). Testing such a connection would be particularly beneficial given the current interest cholinergic and glutamatergic receptors as targets for pharmacological intervention against fetal ETOH effects (Barron et al., 2016).

In summary, the present neonatal exposure paradigm was used to model deficits in cognitive function and behavior regulation following binge-like ETOH exposure during the third trimester-equivalent of CNS development in mice. This exposure paradigm resulted in deficits that persisted into adulthood and affected the control of investigation and/or choice in multiple ways, depending upon the environment and

experience. Exp 2 described multiple ETOH effects on behavior in juvenile and adolescent mice, including hyperactivity, inhibitory failures and anxiety-like behavior. Exp 3 described additional deficits in adulthood in the rapid acquisition of discriminated choice. Taken together, alterations in investigatory and choice behavior in ETOH-treated offspring implicate multiple cognitive and regulatory functions that may independently or interactively contribute to deficits in complex behavior. Cognitive and regulatory symptoms in FASD are often cited as obstacles to social, academic and daily performance in juveniles, adolescents (e.g. Green, 2007) and adults (e.g. Moore & Riley, 2015), and have been key targets for the development and screening of traditional cognitive and behavior-based therapies (Paley & O'Connor, 2011). Analogous deficits shown in the present model may then provide valuable baselines for addressing the mechanisms of impairment, as well as in evaluating therapeutic compounds or strategies which may attenuate them.

Chapter 4

General Discussion

As indicated by the title, the experiments described in this dissertation aimed to identify key deficits of interest following neonatal ETOH exposure in the mouse and to assess the expression of these patterns across development. These aims were addressed using specialized behavioral batteries to assess spontaneous exploration and operant learning in juvenile- adolescent and adolescent-adult offspring respectively. The patterns of impairment shown in these studies show face validity for key symptoms and consequences of FASD and emphasize the importance of the "third trimester brain growth spurt" to the development of key cognitive and regulatory processes. Together,

these data provide support for the validity and continued use of the neonatal exposure paradigm for modeling behaviorally-relevant fetal ETOH effects using mice. This model appears well suited for future studies investigating the underlying mechanisms contributing to these effects and the efficacies of novel pharmacotherapies.

4.1 Validity and Utility of the Novel Treatment Paradigms

Initial testing (Exp 1) assessed the long term effects of binge-like neonatal ETOH exposure (4g/kg ETOH, PD 4-10) alone and when preceded by a lower prenatal dose (2g/kg ETOH, GD 7-16). The neonatal exposure paradigm was generated to model the effects of ETOH exposure during the third-trimester of CNS development. This model showed face validity for modeling multiple common FASD symptoms, including hyperactivity, disinhibition and impairments in learning (Kodituwakku & Kodituwakku, 2014). The perinatal (pre-/postnatal) treatment paradigm was generated to assess if ETOH pre-exposure would significantly alter the effects of neonatal treatment alone. Unlike with the single existing study with a 3-trimester mouse model (Louth et al., 2016), adequate control groups were included to assess whether the observed effects were due to the full span of the exposure paradigm, rather than a critical window within it. The primary influence of the present prenatal ETOH paradigm was that it led to more pronounced reductions in body weight during the neonatal ETOH treatment period, leading to more persistent reductions in weight, at least in males. The perinatal ETOH exposure model did not differ from the neonatal ETOH exposure model on the behavioral measures in this study, indicating that the behavioral outcomes seen in the perinatal model are due to the neonatal treatments. Minor differences in center exploration of the open-field (OF) were seen on the first session, where the trend toward greater center

distance only reached significance in the perinatal ETOH exposure group. The perinatal treatment paradigm was likely limited by the prenatal dose selected, which produced no measurable effects alone and was characterized by much lower peak BECs. Future development of 3-trimester ETOH exposure models with mice should consider higher prenatal doses with greater potential to produce unique or interactive effects. Although the 3-trimester model was not found to be a strong model of ETOH effects following multiple exposure periods, the neonatal exposure model showed considerable promise and was selected for further use.

4.2 Measurement and Interpretation of the Behavioral Effects

The initial findings and follow-up testing with the neonatal exposure paradigm suggest that the behavioral batteries were, by and large, well suited to evaluating the behavioral deficits exhibited by this model. Across the two studies, three tests of spontaneous exploration were used: the open-field (OF), elevated plus maze (EPM) and the hole-board (HB). These tasks provided unique measures that tapped into varying components of behavior regulation, collectively showing the multifaceted effects of neonatal ETOH exposure on mouse behavior across juvenile and adolescent development.

In the initial study, OF and EPM showed patterns of neonatal ETOH-induced hyperactivity in young mice, with the second study partially replicating this effect among adolescents in the OF. The OF consisted of two 30 min sessions, providing valuable data on the time course and persistence of activity changes across multiple sessions. Among the juvenile mice, neonatal ETOH exposure was associated with a general increase in

activity which was evident across time bins and testing sessions. By adolescence, the pattern of hyperactivity was much more limited in time, appearing at the beginning of the testing sessions and disappearing by the third time bin. A higher level of initial activity can be interpreted as reactivity to entering the apparatus, although the striking difference is that over the course of the session, adolescents return to control levels of activity, while juveniles do not. It is not clear whether this distinction indicates that these ETOH-treated juveniles have a higher baseline level of exploration, or whether they simply fail to habituate rapidly enough to reach control levels within the session. In either case, this does not appear to be an issue for adolescent animals. Previous research has suggested that some fetal ETOH effects may weaken or shift over the course of development (e.g. Wozniak et al., 2004), which may account for these differences. Even so, it is significant that hyperreactivity to being placed into the apparatus is an effect that can persist into adolescence, as future investigations of hyperactivity in this model may be able to examine a developmental window which includes both juvenile and adolescent development.

With respect to the pattern of exploration, the tasks selected demonstrated that neonatal ETOH exposure may bias exploration in one of two ways: enhancing either approach or avoidance of certain locations. Neonatal ETOH exposure enhanced approach of the open arms of the EPM and holes in the HB. These enhancements were interpreted as inhibitory failures, in that these responses may lead to greater risk in the

natural world. The consistency between these different responses and apparatus' can be viewed as a conceptual replication of inhibitory failures among juvenile mice with neonatal ETOH exposure. As discussed in each study, these inhibitory failures have face validity for deficits in impulse control that are often reported among those with FASD (Palozza et al., 2014). Lending additional significance to these findings, the HB and EPM data suggest overlapping mechanisms between hyperactivity, as is normally measured in OF or similar tests, and greater tendencies to investigate a new environment.

In the HB, juvenile offspring with neonatal ETOH exposure failed to show hyperactivity when nose-poke holes were available to investigate. In the EPM, juvenile mice with neonatal ETOH exposure were hyperactive and showed a proportionally greater amount of their exploration to the open arms than controls, suggesting increases in open arm investigation were more than expected given greater locomotion alone. These patterns suggest a bias in the processes that orient subjects to these areas and drive investigation of them. These patterns may be described as a motivational bias for novel environments, although further research is needed to directly assess this possibility. Some limited work in neuroeconomics has indicated potential for motivational biases in rodent models of FASD, including a recent pilot between Dr. Barron and Dr. Beckman (Dept. of Psychology, UK) suggesting that rats with neonatal ETOH exposure may show altered base motivation in an effort discounting task, and a handful of studies with delay-discounting or progressive ratio schedules (e.g. Bañuelos et al., 2012, Gentry, Merritt &

Middaugh, 1995). Previous research has implicated cortico-striatal and cortico-basal ganglia networks in motivation, most often implicating dopamine- and GABA-signalling in the regulation of these interactions (e.g. Yin et al., 2008). It may be beneficial to examine the status of these networks and neurotransmitter systems in the neonatal exposure model to determine whether these behavioral effects reflect organizational changes and/or cell death in the cortex and basal ganglia.

Interestingly, neonatal ETOH exposure also enhanced center avoidance, measured as the percent of all locomotion occurring in the center, among both juvenile and adolescent mice in the OF. In Exp 1, the relevance of the change in center avoidance on day 2 was unclear, in part due to the weaker and less consistent pattern observed relative to the inhibitory deficits observed in the EPM. For these reasons, the significance of these findings was not heavily emphasized. However, replication of these effects in somewhat older mice suggests this pattern may require further examination. As discussed in the context of Exp 2, reductions in center preference align with deficits in mood regulation and higher incidences of anxiety-like behavior among those with FASD (Hellemans et al., 2008, Pei et al., 2011). Based on available data, it is hypothesized that situational factors, such as the familiarity of an environment or certain cues may mediate the expression of these phenotypes. Future testing may be able to address this possibility by conducting additional exploration tests in extended and/or repeated exposures to a given apparatus, perhaps an EPM or HB, and assessing if and how these exposures alter

the expression of inhibitory phenotypes. Exploratory avoidance has been shown to be regulated by neuroendocrine functions mediated by the HPA axis (e.g. Aubry et al., 1995) and through interactions between the medial PFC, amygdala and hippocampus (e.g. Jacinto, Cerquiera & Sousa, 2016). Future studies with this model may benefit from examining these systems to determine whether these behavioral effects reflect organizational changes and/or cell death in the medial PFC or hippocampus, as well as dysregulation of HPA function.

The third task in each study used operant conditioning paradigms to assess cognitive functions relevant to learning and cognitive flexibility. In theory, these tests are designed to assess very different outcomes. The Morris water maze (MWM) uses escape-based conditioning to assess spatial learning and memory, as well as cognitive flexibility through a spatial reversal component. The attentional set shifting test (ASST) uses food-rewarded conditioning to assess non-spatial learning, reversal learning, attentional set formation and set shifting. Given these differences, it was not hypothesized that the patterns of impairment on these two tasks would mirror each other to the degree that they did. In each case, however, general learning impairments dramatically limited the interpretability of the components which fell after the initial acquisition phases. The striking pattern in each case was the persistence of the impaired performance, either across days in the MWM or across phases in the ASST. These data suggest that initial learning is a major obstacle to cognitive performance for mice with

fetal ETOH exposure. These paradigms were shown to be highly sensitive to the deficits produced by fetal ETOH exposure, although they provide less clarity as to the cognitive mechanisms responsible for these deficits than had been hypothesized.

Learning tasks involve somewhat varying circuitry depending upon the nature of the tests. MWM performance, particularly over multiple sessions, is associated with hippocampal functions (e.g. Redish & Touretzky, 1998), while nonspatial tests are less dependent upon the hippocampus (Aggleton, Hunt & Rawlins, 1986). In general, complex behavioral functions depend upon frontal lobe structures that perform tasks related to attention and working memory (Miyake et al., 2000, Prabhakaran, Narayanan, Zhao & Gabrieli, 2000). In the future, it may be beneficial to use the present neonatal mouse model to investigate organizational changes and/or cell death in both the hippocampus and frontal cortex and their relevance to the current learning impairments. Based on the general increase in errors in the ASST and implications for attentional function in mice with neonatal ETOH exposure (Louth et al., 2016), it may be particularly interesting to assess these changes in the anterior cingulate gyrus, associated with error detection (Botvinick, Cohen & Carter, 2004), and the medial PFC and/or basal forebrain, each implicated in attention (Baxter & Chiba, 1999, Louth et al., 2016).

The ASST was selected for its potential to provide unique measurements of cognitive flexibility that are otherwise difficult to assess in a single session or with clear distinctions between cognitive functions. Unfortunately, the pattern of impairment on

this task did not allow these typical elements to be evaluated. The basic discrimination required in the test was sensitive to the learning impairments produced by neonatal ETOH exposure. Given that, the present platform-based discrimination tests may be ideal for assessing deficits and treatment efficacies on non-spatial, short-term learning, although the full ASST paradigm may not be well suited to assessing executive-like deficits in mice with neonatal ETOH exposure.

4.3 Limitations

Although the present neonatal ETOH exposure model holds great promise, the initial projects developing and investigating this model were limited in some important areas. As noted above, a key aim of this research was to identify and clarify the deficit profile produced by novel ETOH treatment paradigms in prenatal and/or neonatal mice. With respect to the neonatal ETOH exposure model, a consistent pattern of hyperactivity was shown in the OF and EPM in juvenile mice. However, ETOH effects on the exploration of open areas of the mazes differed strikingly in these paradigms. Mice with neonatal ETOH exposure tended to show reduced exploration of open spaces in the OF, at least on day 2, but greater exploration of open spaces in the EPM. Given these seemingly opposing effects, it is unclear whether the observed biases in open area exploration are related to open area aversion, per se. The present discussion hypothesizes that the enhancement of open area avoidance may only be detectable given enough experience in the apparatus, although this possibility has not been directly tested. It also

remains to be tested whether other factors that differ between these paradigms, such as the shape of the apparatus and the elevation of the EPM, are relevant.

With respect to learning outcomes, both the MWM and ASST showed general deficits which were more similar than expected given the striking differences in the tests used. The ASST in particular was selected due to its unique design, utilizing rapidly acquired, non-spatial discrimination learning in an appetitive paradigm, in contrast to the multi-session, escape-based spatial paradigm of the MWM. It was hypothesized that these differences would allow unique impairments in executive-like cognitive functions to be described in the ASST without interruption by the effects observed in the MWM. However, the present ASST procedure was shown to be highly sensitive to general learning disruptions produced by neonatal ETOH exposure. Given these performance interruptions, it remains unknown whether executive-like deficits in cognitive flexibility are impaired in the present neonatal mouse model.

An additional limitation is related to the developmental trajectory of the deficits described in these studies. The current studies demonstrated alterations in several key outcomes from juvenile to young adult stages of development. One aim of the current project was to demonstrate the persistence of behavioral deficits into adolescence and adulthood. With respect to the open-field, it was demonstrated that enhancements in center avoidance can persist into adolescence, although hyperactivity may be less pronounced in adolescence than among juveniles. Other observations of similar effects at

multiple ages were accomplished using different tests which share key features but are not identical and therefore do not necessarily substitute for data showing outcomes from a single test at multiple ages. Therefore, it must be acknowledged that these tests offer unique information about the nature of ETOH-induced behavioral deficits, as well as complimentary information.

Finally, it is important to recognize the limitations of the 3rd trimester-like mouse model as a model for FASD and tool for drug development. Rodent models do not represent the full range of long term effects seen in FASD, nor do they represent all factors contributing to them. The current model of interest is best suited to representing effects on brain and behavior produced by binge-like ETOH exposure during the "third trimester equivalent" of CNS development. The present data show that this exposure is sufficient to produce lasting changes in behavioral function analogous to those seen in FASD. These observations suggest this model may be beneficial for identifying key mechanisms or mediators of ETOH teratology, specifically those relevant to neurodevelopment in the "third trimester equivalent" of CNS development. This is valuable for drug development, in that it adds greater control and provides baselines to indicate the relevance of key factors and the potential efficacy of compounds of interest. Given available data, the strength of this model is in its face validity. As the key outcomes and mechanisms remain to be identified and validated in relation to clinical outcomes and mechanisms, the predictive validity of this model for projecting the efficacy of potential pharmacological compounds is unknown. As such, this should be viewed as a useful tool for representing behavioral and pharmacological phenomena related to developmental ETOH effects in the lab, rather than as a "screen" for potential

therapeutic compounds.

4.4 Directions for Future Research

Given the breadth and persistence of behavioral deficits following the present neonatal ETOH treatment paradigm, it is generally concluded that this paradigm would provide a strong model for future investigations. Given the novelty of the procedure and the limited depth of the mouse literature on neonatal ETOH effects, there are a number of directions where this model may prove beneficial. Initially, it will be important to characterize changes in the CNS that correspond with the deficits observed in the present experiments. This could include the documentation of cell death following these procedures, as well as long term changes in the function and organization of surviving neurons and glia in key areas of the brain, such as the frontal cortex, basal ganglia and hippocampus. Using tissue collected from animals generated in the course of these experiments, some of this work is ongoing. Preliminary analyses from Dr. Chen's lab suggest that one area of particular interest will be in characterizing white matter losses due to neonatal ETOH exposure. At present, it is not clear whether such losses indicate cell damage or specific impairments in myelination, although ongoing analyses may suggest more specific mechanisms for further study. Additionally, the Butterfield lab (Dept. of Chemistry, UK) is currently assessing markers of oxidative stress in brain tissue collected from subjects used in Exp 2-3. When completed, these data may suggest pharmacological targets relevant to these mechanisms for further investigation.

Future work with this model will also likely lead to collaborative work investigating genetic and pharmacological factors that are relevant to the outcomes described in the studies above. As the C57BL/6J mouse is commonly used as a background for genetic models, the current neonatal exposure paradigm and behavioral paradigms will be useful for Dr. Chen's future investigations on the influence of gene expression patterns on the susceptibility of the developing brain the ETOH teratology. As this strain is also commonly used in behavioral pharmacology, the present model will also provide useful baselines for assessing the potential efficacy of novel therapeutic compounds. Previous and ongoing work in Dr. Barron's and collaborating labs has focused on a number of pharmacological targets which may also be investigated in this model. Several of these are targets for neuroprotective compounds, including antioxidant compounds, polyamine modulators that may reduce NMDA-mediated toxicity, and compounds with selective activity at nAChR such as the α7. The present model would also be appropriate for assessing the efficacy of compounds which may improve cognitive performance when given acutely, such as non-stimulant compounds that act upon nAChR. In conclusion, the neonatal ETOH exposure model is a valuable addition to the existing literature on fetal ETOH effects in mice and promises to serve as a beneficial baseline for future work in neurogenetics and drug development related to FASD.

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2014-2015	RA under Dr. Gang Chen	University of Kentucky
Fall 2013	TA Advanced Learning	University of Kentucky
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Publications

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