UNIVERSITY OF CALIFORNIA
RIVERSIDE

# The Effects of Parental Ethanol Exposure on Offspring Development

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Kathleen Elizabeth Conner

June 2024

Dissertation Committee:
Dr. Kelly Huffman, Chairperson
Dr. Edward Zagha
Dr. Glenn Stanley

The Dissertation of Kathleen Elizabeth Conner is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

Acknowledgements

First and foremost, I would like to thank my advisor Dr. Kelly Huffman for her support throughout graduate school. Her guidance has been important for my development as a scientist, and she has pushed me to become a better researcher throughout this process. I know taking on a student with no animal experience was a risk, but thank you for taking a chance on me and giving me the opportunity to do this type of research. Your expertise and constructive feedback have helped me navigate the complexities of my research with confidence.

I would like to thank my committee members Glenn Stanley and Edward Zagha for their support throughout this process. The interactions we've had (whether it be in the classroom or simply stopping to chat in the hallways or after seminar) have guided me to be a better thinker and teacher.

I would like to thank all the faculty and staff here at UCR that have influenced me along the way and made this degree possible. Thank you Micheal Erickson for your statistical expertise and assistance in learning Jamovi. Dr. Maggie Curras-Collazo and Dr. Hillary Jenks were particularly influential in helping me navigate the next steps in my career after graduate school. My previous research mentors Dr. Christopher Wilson, Dr. Jeremy Dodsworth, and Dr. Arturo Conception influenced my research skills and helped me realize that going to graduate was a possibility.

I engaged in several mentorship programs as a mentor while in graduate school (Graduate Student Mentorship Program, TRIO, women's resource center, and brain buddies). These programs, as well as the Student Mentoring Program at CSUSB, have influenced my interactions with students greatly. The University Teaching Certificate Program and Academy of Distinguished Teaching Faculty Learning Program have helped me improve my pedagogical skills and enhance my career prospects. The Region 9 Rising Faculty Fellowship Program and my faculty mentor Dr. Anjeanette Oberg also played a key role in my development as an educator of diverse populations.

Thank you to all the Huffman Lab members, past and present. Riley Bottom and David Rohac helped me throughout the early stages and trained me in new techniques. Roberto Perez, Mirembe Nabatanzi, and Angela Avitua were always there supporting me, whether it be through random conversations, sharing our successes and struggles, or troubleshooting protocols. To my many undergraduate research assistants, you were helpful for data collection, making solutions, and even doing the dreaded dishes; all important components to help streamline the scientific process. All of my lab members made the lab a fun environment to be in, thank you for putting up with my weirdness. I wish you the best of luck in your future endeavors. To all my friends at UCR and otherwise, thank you for your support throughout the years. I would like to specifically thank my cohort; we started this journey together and you became some of my first friends here at UCR.

I would like to thank my parents, Dave and Colleen Conner, for their support throughout my life. Your unwavering support, endless sacrifices, and unconditional love have shaped me into the person I am today. Thank you for teaching me that hard work and perseverance pays off and for supporting my goals. I would also like to thank my sister, Alison, for her support throughout the years. Thank you to all my family members who encouraged and supported me throughout the years.

Lastly, I would like to thank my wonderful fiancé David Serradell-Aldana. You have supported me throughout this entire process, through the ups and downs, and crazy schedules of academic life. Your encouragement, love, and support helped keep me sane and I'm excited to see what our future holds.

The chapters of this dissertation, in part or in whole, are reprints of materials as they appear in Alcoholism: Clinical and Experimental Research, January 2020 (V411, N1) (Conner et al., 2020). The co-author Kelly Huffman supervised this research while Riley Bottom contributed to data collection and analysis. Additional chapters will be published in the future.

ABSTRACT OF THE DISSERTATION

The Effects of Parental Ethanol Exposure on Offspring Development

by

Kathleen Elizabeth Conner

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, June 2024
Dr. Kelly J. Huffman, Chairperson

Fetal alcohol spectrum disorders (FASD) describe the wide array of long-lasting developmental abnormalities in offspring due to prenatal alcohol (ethanol [EtOH]) exposure via maternal gestational drinking. Our laboratory has identified many deleterious effects of prenatal ethanol exposure (PrEE) as well as paternal ethanol exposure (PatEE) prior to conception in a mouse model. Our results indicate that PrEE and PatEE can have substantial effects on the offspring including alterations in gross anatomy, neuroanatomy, gene expression, and behavior. In chapter 1, we show that PrEE can lead to craniofacial anomalies, decreased body weight and length, decreased brain weight and cortical length, as well as structural changes to the cortex and corpus callosum during embryonic development (E12.5, E14.5, E16.5, E18.5).

To further explore potential heritable effects of PrEE, we investigated brain and behavioral development in the F1 (directly exposed), F2 (indirectly exposed) and F3 (non-exposed) generations in Chapter 2. All generations of PrEE

newborns had decreased body weights, brain weights, and neocortical lengths compared to controls, although there were no differences in brain to body weight ratios. Hippocampal CA3 was significantly thinner in all generations of PrEE mice compared to controls. PrEE resulted in a significant rate of agenesis or partial development of the corpus callosum in the majority of F1 cases, with a less frequent, non-significant, occurrence in F2 and F3 mice. Disrupted sensorimotor integration, motor control, and anxiety-like behavior persisted to at least the F2 generation.

In Chapter 3, we investigated the transgenerational effects of paternal EtOH exposure (PatEE) on offspring brain and behavioral development. We observed no differences between control and PatEE (F1 and F2) mice in measures of neocortical length. Abnormal patterns of *Id2* and *RZRβ* gene expression were observed in the F1 generation but not the F2 at P0. Additionally, PatEE may generate sex-specific effects on offspring behavior that can last up to two generations after the sire's initial exposure.

We propose that the effects of drinking alcohol during pregnancy or prior to conception may be more serious than previously thought as transgenerational effects were observed in the offspring even though they never consumed alcohol themselves.

## Table of Contents

**List of Figures**

**Chapter 1:** Effects of ethanol exposure on embryonic gross anatomy and neuroanatomy in a mouse model



**Chapter 2:** Transgenerational effects of prenatal ethanol exposure on offspring neuroanatomy and behavior in a mouse model of FASD

**Abbreviations**

5-Methylcytosine (5-mC)

Accelerated rotarod (AR)

Adhesive Tape Removal (ATR)

Anterior cingulate cortex (ACC)

Anterior commissure (AC)

Auditory cortex (ACx)

Basolateral amygdala (BLA)

Basomedial amygdala (BMA)

Blood Ethanol Concentration (BEC)

Central nervous system (CNS)

Centers for Disease Control (CDC)

Cerebrospinal fluid (CSF)

Choroid plexus (ChP)

Corpus callosum (CC)

Corticosterone (CORT)

DNA methyltransferase (DNMT)

Dorsal lateral geniculate nucleus (dLGN)

Elevated Plus Maze (EPM)

Embryonic day (E)

Ethanol (EtOH)

Fetal Alcohol Spectrum Disorder (FASD)

Fetal Alcohol Syndrome (FAS)

First filial generation (F1)

Forced swim test (FST)

Histone acetyltransferase (HAT)

Hypothalamic-pituitary-adrenal axis (HPA)

Inhibitor of DNA binding (*Id2*)

Institutional Animal Care and Use Committee (IACUC)

Insulin growth factors (IGF)

Intraneocortical connections (INCs)

*In Situ* Hybridization (ISH)

Lateral ganglionic eminences (LGE)

Lateral geniculate nucleus (LGN)

Magnetic resonance imaging (MRI)

Medial ganglionic eminences (MGE)

Medial geniculate nucleus (MGN)

Medial nasal prominences (MNPs)

Messenger RNA (mRNA)

MicroRNA (miRNA)

Motor cortex (MCx)

Neuropilin 1 receptor (NRP1)

Nicotinic acetylcholine (nAChRs)

Paraformaldehyde (PFA)

Paternal Ethanol Exposure (PatEE)

Phosphate-Buffered Saline (PBS)

Piriform-amygdalar area (PAA)

Piriform cortex (PC)

Plasma osmolality (POSM)

Positron emission tomography (PET)

Postnatal day (P)

Prelimbic cortex (PrL)

Prenatal Ethanol Exposure (PrEE)

Primary Somatosensory Cortex (S1)

Primary Visual Cortex (V1)

Pro-opiomelanocortin (POMC)

Region of Interest (ROI)

Retinoid Z Receptor Beta (*RZRβ*)

Second filial generation (F2)

Scanning electron microscopy (SEM)

Small noncoding RNAs (sncRNAs)

Somatosensory cortex (SCx)

Third filial generation (F3)

Third ventricle (TV)

Ventral-posterior nucleus (VP)

Visual cortex (VCx)

## General Introduction

Fetal alcohol spectrum disorders (FASD) describe a wide array of developmental anomalies that occur as a result of ethanol (EtOH) exposure during pregnancy (Hoyme et al., 2016). Children diagnosed with FASD can potentially experience a range of developmental abnormalities including growth retardation, central nervous system issues, altered facial morphology, abnormal behavior, and cognitive deficits. However, absence of diagnosis or misdiagnosis of FASD has been shown to occur frequently (Chasnoff et al., 2015), leading to a severe underestimation of the true prevalence of FASD. Incidence rates for FASD are currently estimated to be as high as 5% in the United States (May et al., 2018) with the highest prevalence in South Africa at 11.1% (Lange et al., 2017).

The first health advisory issued by the National Institutes of Health (NIH) in June of 1977 (U.S. Food and Drug Administration, 1977) suggested a limit of two drinks per day for pregnant women and stipulated that consuming more than 6 drinks per day could pose a significant risk to the developing fetus (Warren, 2015). In July of 1981, the U.S. Surgeon General released a bulletin that advised women who are pregnant or considering becoming pregnant to not drink alcoholic beverages at all (FDA Drug Bulletin, 1981). However, warning labels on alcoholic beverages stating the dangers of consumption during pregnancy were not required until the passage of the Alcoholic Beverage Labeling Law in 1988, which mandated that these labels be placed on containers by 1989 (Warren,

2015). Similar recommendations were released by the U.S. Surgeon General in 2005 reiterated the 1981 bulletin and are continued to be made by professional societies around the world.

Despite warnings from the Centers for Disease Control (CDC) that no amount of alcohol is safe to consume during pregnancy (Green et al., 2016), the rates of alcohol consumption during pregnancy continue to remain high with about 14 percent of women reporting drinking alcohol at some point during pregnancy, most typically during the first trimester (Gosdin et al., 2022). Furthermore, about 5 percent of pregnant individuals reported binge drinking while pregnant (Gosdin et al., 2022). Additionally, the CDC reported that three out of four women who were actively trying to conceive did not stop drinking alcohol, posing a tremendous issue as a woman could get pregnant and not know for up to four to six weeks (Green et al., 2016).

*Background: PrEE, PatEE and the neocortex*:

The neocortex, the largest part of the human brain, facilitates emergent properties that mediate complex, higher order functions and behaviors. The neocortex relies on a tightly regulated temporal and spatial orchestration of genetic and environmental cues for proper development, a process that seems particularly susceptible to prenatal EtOH insult. Animal studies focusing on maternal EtOH exposure have found a plethora of atypical cortical phenotypes present in offspring including increased apoptosis (Ikonomidou  et al., 2000),

altered pyramidal cell morphology (Granato et al., 2003), modified development of anatomical regions or structures (Abbott et al., 2016), and atypical development of the intraneocortical circuitry (El Shawa et al., 2013). Human neuroimaging studies in children with FASD have also demonstrated abnormalities in neocortical development (Zhou et al., 2011), suggesting that irregular cortical phenotypes may underlie some PrEE-induced behavioral alterations.

One aspect of neocortical development affected by PrEE is arealization, or the patterning, of neurons into functionally and spatially distinct areas (Dye et al., 2011a, b). Specifically, PrEE results in aberrant intraneocortical connections (INCs), as well as altered expression of genes critical for proper patterning of the neocortex in mice (El Shawa et al., 2013).

Development of INCs has been shown to be governed by expression of patterning genes, such as *Id2* and *RZRβ* (Huffman et al. 2004), whose expression is highly regulated throughout development (Dye et al., 2011a; 2011b). Functionally, *Id2* is a helix–loop–helix transcription factor important for neural stem cell renewal and normal CNS development (Park et al., 2013), while *RZRβ*, a nuclear receptor, influences proper cortical structural patterning, including the development of the barrel cortex in primary somatosensory cortex (S1) (Jabaudon et al., 2012). Importantly, both genes are present within the murine cortex at embryonic and early postnatal ages and are expressed in distinct layer and area-specific patterns, suggesting their role in arealization

(Rubenstein et al., 1999; Dye et al., 2011a). In particular, *Id2* and *RZRβ* expression boundaries have been implicated in the guidance of regional development of early intraneocortical (INC) connectivity (Huffman et al., 2004). PrEE alters the expression of these patterning genes, and others, providing a potential mechanism on how EtOH disrupts INC development (El Shawa et al., 2013; Abbott et al., 2018). These changes in gene expression and connectivity could explain some of the behavioral effects observed in offspring with FASD. Recently, our laboratory has demonstrated that these phenotypes pass to second and third filial generations after an initial PrEE (Abbott et al., 2018), suggesting EtOH may have potent transgenerational effects. The second chapter will expand on these findings and discuss the transgenerational effects of PrEE on offspring neuroanatomy and behavior, while the first chapter will focus primarily on the development of embryonic neuroanatomy and gross anatomy.

As the CDC announced the lack of safety of any alcohol use during pregnancy and with most research focusing on the effects of women's drinking on their progeny, many individuals began to question why women were the only ones of concern when half of a baby's DNA comes from the father. This poses the important question of: does a father's alcohol consumption affect offspring brain and behavioral development? Although the teratogenic consequences of prenatal EtOH exposure (PrEE) are somewhat understood, much less is known about the impact of preconception paternal EtOH exposure (PatEE), despite a growing body of preclinical evidence indicating that offspring sired by males

exposed to EtOH prior to conception display altered brain and behavioral development similar to maternal-mediated prenatal EtOH exposure (Chang et al., 2017; Chang et al., 2019; Finegersh and Homanics 2014; Jamerson et al., 2004; Kim et al., 2014; Meek et al., 2007; Rompala et al., 2016; Rompala et al., 2017). It has been reported that up to 75% of children with FASD have biological fathers who are alcoholics (Abel, 2004). Lemoine and colleagues (1968) were the first to describe cases in which children with characteristics of fetal alcohol syndrome were born to mothers who did not drink, but fathers were known alcoholics. Approximately 58% of adult men report drinking alcohol in the last 30 days (CDC, 2020) with one study reporting that about 4.5% of men met the diagnostic criteria for alcohol dependence (Esser et al., 2014). Binge drinking also poses an issue as one in six US adults binge drinks around four times a month (~7 drinks per binge) resulting in approximately 17 billion total binge drinks consumed by adults annually (Kanny et al., 2018). Binge drinking behavior is more common in men (80% of all binge drinkers) and in younger adults aged 18-34 years, but more than half of the total binge drinks are consumed by those aged 35 and older (Kanny et al., 2018). This raises an important issue since many men consume alcohol and are unaware of the effects it may have on their sperm and future offspring as spermatogenesis occurs until very advanced ages (~95 years) (Dakouane et al., 2005).

Several studies (Oldereid et al., 1992; Marshburn et al., 1989; Little et al., 1986) have reported impotence rates ranging from 8% to 54% in males suffering

from chronic alcoholism compared to nondrinkers. Chronic alcohol exposure is associated with reduced seminiferous tubular diameter and germinal epithelium, fewer cells in ejaculate, increased morphologically abnormal sperm, and problems with sperm motility (Abel, 1983). It has been observed that male alcohol consumption one month before attempting in vitro fertilization (IVF) or gamete intrafallopian transfer (GIFT), independent of other covariates, increased the risk of miscarriage and failure of live birth (Klonoff-Cohen et al., 2003).

Additionally, clinical research in humans has found associations among heavy paternal EtOH consumption and adverse developmental outcomes in offspring (reviewed in Finegersh et al, 2015; Xia et al., 2018; Zuccolo et al., 2017), providing further support for the deleterious impact of paternal drinking. Specifically, children of alcoholic fathers have been shown to display lower birth weights (Abel, 2004; Little and Sing, 1987), increased risk of congenital defects (Zuccolo et al., 2017), cognitive impairments (Tarter et al., 1989), and altered reproductive development (Xia et al., 2018), suggesting PatEE may be more impactful on offspring development than previously thought.

Rodent studies, using a variety of species, strains, and exposure paradigms, have generally reinforced this idea with studies dating back to over 100 years ago (Stockard and Papanicolaou, 1918). Researchers have described various behavioral abnormalities (Abel, 1991; Abel and Lee, 1988; Hollander et al., 2019; Jamerson et al., 2004; Kim et al., 2014; Meek et al., 2007), as well as lower weights at birth (Bielawski et al., 2002), increased incidence of runts in

litters (Bielawski and Abel, 1997), smaller litter sizes (Liang et al., 2014; Meek et al., 2007), and congenital CNS anomalies (Lee et al., 2013). A decrease in pup size and weight were observed in both long term and acute alcohol exposure models (Abel, 1995; Abel, 2004; Adler, 1996; Bielawski and Abel 1997; Ceccanti et al., 2016, Cicero et al., 1994).

Preclinical studies focusing on PatEE's effects on the neocortex are sparse but have shown that affected offspring have increased cortical thickness (Jamerson et al., 2004) and altered expression and epigenetic regulation of the dopamine transporter in the frontal cortex (Kim et al., 2014). Importantly, to our knowledge, no study exists examining the effect of preconception paternal EtOH consumption on development of neocortical connections. We hypothesize that PatEE offspring could demonstrate abnormal neocortical development due to PatEE's ability to disrupt normal development in the neocortex, as well as EtOH's ability to modify INCs in the absence of direct exposure.

In previous rodent models, PatEE has been shown to disturb gene expression in the brain (Finegersh and Homanics, 2014; Kim et al., 2014; Liang et al., 2014; Przybycien-Szymanska et al., 2014; Rompala et al., 2017) and liver (Chang et al., 2017, 2019) of offspring. Chapter 3 investigates if similar alterations in neocortical patterning genes occur due to PatEE and what some potential mechanisms for these changes are. Since many prenatal EtOH exposure phenotypes have been shown to pass transgenerationally (Abbott et al., 2018; Gangisetty et al., 2020; see review by Chastain and Sarkar 2017), we

investigated whether the observed phenotypes from preconception PatEE also persist into future generations beyond the F1 generation. To our knowledge there has been no research on the transgenerational effects of PatEE on the neocortex. Although research investigating the impact of PatEE is on the rise, it remains greatly understudied compared to models of FASD generated from EtOH exposure via maternal drinking. It is not known whether PatEE, like PrEE, results in a heritable condition. Thus, this study is of critical importance to reveal risks for future generations of alcoholic fathers.

In summary, both prenatal exposure to alcohol and paternal exposure to alcohol prior to conception can cause a wide array of deleterious effects on offspring gross anatomy, neuroanatomy, and behavioral development. These effects can potentially be passed down to subsequent generations even if those offspring never drink themselves. Additionally, these changes may be mediated through damage to the neocortex. However, the spectrum of these effects remains largely unknown and the severity of them varies in each individual. Here, we will explore some of these changes more in depth as well as potential mechanisms for these changes.

## REFERENCES


Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K. M., & Huffman, K. J. (2016). The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice. *Alcohol Clin Exp Res, 40*,122–133.

Abbott, C. W., Rohac, D. J., Bottom, R. T., Patadia, S., & Huffman, K. J. (2018). Prenatal ethanol exposure and neocortical development: a transgenerational model of FASD. *Cereb Cortex, 28,* 2908–2921.

Abel, E. L.(1983). Pharmacology of alcohol. *Marijuana, tobacco, alcohol and reproduction*. Boca Raton, FL: CRC Press

Abel, E. L. (1991). Alcohol consumption does not affect fathers but does affect their offspring in the forced swimming test. *Pharmacol Toxicol, 68,* 68–69.

Abel, E. L. (1995). A surprising effect of paternal alcohol treatment on rat fetuses. *Alcohol, 12,* 1-6.

Abel, E. L. (2004). Paternal contribution to fetal alcohol syndrome. A*ddict Biol, 9,* 127-33.

Abel, E. L., & Lee, J. A. (1988). Paternal alcohol exposure affects offspring behavior but not body or organ weights in mice. *Alcohol Clin Exp Res, 12,* 49–355.

Adler, I. D. (1996). Comparison of the duration of spermatogenesis between male rodents and humans. *Mutat Res Mol Mech Mutagen, 352,*169–172.

Bielawski, D. M., & Abel, E.L. (1997). Acute treatment of paternal alcohol exposure produces malformations in offspring. *Alcohol, 14*, 397–401.

Bielawski, D. M., Zaher, F. M., Svinarich, D. M., & Abel, E. L. (2002). Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. *Alcohol Clin Exp Res, 26,* 347–351.

Chastain, L.G., &Sarkar, D.K. (2017). Alcohol effects on the epigenome in the germline: role of inheritance of alcohol-related pathology. *Alcohol, 60,* 53–66.

Church, M. W., & Kaltenbach, J. A. (1997). Hearing, speech, language, and vestibular disorders in the fetal alcohol syndrome: A literature review. *Alcohol Clin Exp Res., 21*(3), 495-512. doi: 10.1111/j.1530-0277.1997.tb03796.x.

Cicero, T. J, Nock, B., O'Connor, L., Adams, M.L., Sewing, B.N., & Meyer, E.R. (1994). Acute alcohol exposure markedly influences male fertility and fetal outcome in the male rat. *Life Sci, 55,* 901-910.

Centers for Disease Control and Prevention (CDC). (2020) *Behavioral Risk Factor Surveillance System data.* Atlanta, GA: CDC.

Ceccanti, M., Coccurello, R., Carito, V., Ciafre, S., Ferraguti, G., Giacovazzo, G., Mancinelli, R., Tirassa, P., Chaldakov, G.N., Pascale, E., Ceccanti, M., Codazzo, C., & Fiore, M. (2016). Paternal alcohol exposure in mice alters brain NGF and BDNF and increases ethanol-elicited preference in male offspring. *Addict Biol, 21,* 776–787.

Chang, R. C., Skiles, W. M., Chronister, S. S., Wang, H., Sutton, G.I., Bedi, Y.S., Snyder, M., Long, C. R., & Golding, M. C. (2017). DNA methylation-independent growth restriction and altered developmental programming in a mouse model of preconception male alcohol exposure. *Epigenetics, 12*, 841–853.

Chang, R. C., Wang, H., Bedi, Y., & Golding, M.C. (2019). Preconception paternal alcohol exposure exerts sex-specific effects on offspring growth and longterm metabolic programming. *Epigenet Chromatin 12*, 9. https://doi.org/10.1186/s13072-019-0254-0

Chasnoff, I. J., Wells, A. M., & King, L. (2015). Misdiagnosis and missed diagnoses in foster and adopted children with prenatal alcohol exposure. *Pediatrics, 135*(2), 264- 70.

Dakouane, M., Albert, M., Bergère, M., Sabbag, C., Brayotel, F., Vialard, F., Lombroso, R., Bicchieray, L., & Selva, J. (2005). Aging and spermatogenesis: A histologic, cytogenetic and apoptosis study. *Gynecol Obstet Fertil., 33*(9), 659-64. doi: 10.1016/j.gyobfe.2005.07.014.

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011a). A lifespan analysis of intraneocortical connections and gene expression in the mouse I. *Cereb Cortex, 21*, 1311–1330.

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011b). A lifespan analysis of intraneocortical connections and gene expression in the mouse II. *Cereb Cortex, 21*, 1331–1350.

El Shawa, H., Abbott, C.W., & Huffman, K.J. (2013). Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene expression, and behavior in a mouse model of FASD. *J Neurosci, 33,* 18893–18905.

Esser, M. B., Hedden, S. L., Kanny, D., Brewer, R. D., Gfroerer, J. C., & Naimi, T. S. (2014). Prevalence of alcohol dependence among US adult drinkers, 2009–2011. *Prev Chronic Dis, 11,* 140329.

FDA Drug Bulletin (1981). *Office of the Surgeon General (US) Surgeon General's advisory on alcohol and pregnancy.* 11, 9–10.

Finegersh, A., & Homanics, G. E. (2014). Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. *PLoS ONE, 9,* e99078.

Finegersh, A., Rompala, G. R., Martin, D. I. K., & Homanics, G. E. (2015). Drinking beyond a lifetime: New and emerging insights into paternal alcohol exposure on subsequent generations. *Alcohol, 49,* 461–470.

Gangisetty, O., Palagani, A., & Sarkar, D.K. (2020). Transgenerational inheritance of fetal alcohol exposure adverse effects on immune gene interferon-Ɣ. *Clin Epigenetics,12*(1), 70. doi: 10.1186/s13148-020-00859-9.

Gosdin, L. K., Deputy, N. P., Kim, S. Y., Dang, E. P., & Denny, C. H. (2022). Alcohol consumption and binge drinking during pregnancy among adults aged 18–49 years — United States, 2018–2020. *MMWR Morb Mortal Wkly Rep, 71,*10–13. DOI: http://dx.doi.org/10.15585/mmwr.mm7101a2external icon.

Granato, A., Di Rocco, F., Zumbo, A., Toesca, A., & Giannetti, S. (2003). Organization of cortico-cortical associative projections in rats exposed to ethanol during early postnatal life. *Brain Res Bull, 60,* 339–344.

Green, P. P., McKnight-Eily, L. R., Tan, C. H., Mejia, R., & Denny, C.H. (2016). Vital signs: Alcohol-exposed pregnancies — United States, 2011–2013. *MMWR Morb Mortal Wkly Rep, 65,* 91–97. doi: http://dx.doi.org/10.15585/mmwr.mm6504a6external icon.

Hollander, J., McNivens, M., Pautassi, R. M., & Nizhnikov, M. E. (2019). Offspring of male rats exposed to binge alcohol exhibit heightened ethanol intake at infancy and alterations in T-maze performance. A*lcohol, 76,* 65–71.

Hoyme, H. E., Kalberg, W. O., Elliott, A. J., Blankenship, J., Buckley, D., Marais, A. S., Manning, M. A., Robinson, L. K., Adam, M. P., Abdul-Rahman, O., Jewett, T., Coles, C. D., Chambers, C., Jones, K.L., Adnams, C.M., Shah, P. E., Riley, E. P., Charness, M. E., Warren, K. R., & May, P. A. (2016). Updated clinical guidelines for diagnosing Fetal Alcohol Spectrum Disorders. *Pediatrics ,138,* e20154256–e20154256.

Huffman, K. J., Garel, S., & Rubenstein, J. L. (2004). Fgf8 regulates the development of intra-neocortical connections. *J Neurosci., 24*(41), 8917-23.

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, F., Koch, C., Genz, K., Price, M. T., Stefovska, V., Tenkova, T., Dikranian, K., & Olney, J. W. (2000). Ethanol-induced apoptotic neurodegeneration and Fetal Alcohol Syndrome. *Science, 287*, 1056–1060.

Jabaudon, D. J., Shnider, S. J., Tischfield, D. J., Galazo, M., & Macklis, J.D. (2012). RORβ induces barrel-like neuronal clusters in the developing neocortex. *Cereb Cortex, 22,* 996–1006.

Jamerson, P. A., Wulser, M. J., & Kimler, B. F. (2004). Neurobehavioral effects in rat pups whose sires were exposed to alcohol. *Dev Brain Res, 149,* 103–111.

Kanny, D., Naimi, T. S., Liu, Y., Lu, H., & Brewer, R.D. (2018). Annual total binge drinks consumed by U.S. adults, 2015. *Am J Prev Med, 54,* 486–496.

Kim, P., Choi, C. S., Park, J. H., Joo, S. H., Kim, S. Y., Ko, H. M., Kim, K. C., Jeon, S. J., Park, S. H., Han, S-H., Ryu, J. H., Cheong, J. H., Han, J. Y., Ko, K. N., & Shin, C. Y. (2014). Chronic exposure to ethanol of male mice before mating produces attention deficit hyperactivity disorder-like phenotype along with epigenetic dysregulation of dopamine transporter expression in mouse offspring. *J Neurosci Res, 92*, 658–670.

Klonoff-Cohen, H., Lam-Kruglick, P., & Gonzalez, C. (2003). Effects of maternal and paternal alcohol consumption on the success rates of in vitro fertilization and gamete intrafallopian transfer. *Fertil Steril., 79*(2), 330-9.

Krubitzer, L., & Huffman, K.J. (2000). Arealization of the neocortex in mammals: genetic and epigenetic contributions to the phenotype. *Brain Behav Evol, 55,* 322–335.

Lange, S., Probst, C., Gmel, G., Rehm, J., Burd, L., &Popova, S. (2017). Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis. *JAMA Pediatr, 171,* 948–56.

Lee, H.J., Ryu, J-S., Choi, N.Y., Park, Y.S., Kim, Y.I., Han, D.W., Ko, K., Shin, C. Y., Hwang, H.S., Kang, K-S., & Ko, K. (2013). Transgenerational effects of paternal alcohol exposure in mouse offspring. *Anim Cells Syst, 17*, 429–434.

Lemoine, P., Harrousseau, H., Borteyru, J., & Menuet, J. C. (1968). Children of alcoholic parents: Observed anomalies, *Ouest Med, 25*, 476– 482.

Liang, F., Diao, L., Liu, J., Jiang, N., Zhang, J., Wang, H., Zhou, W., Huang, G., & Ma, D. (2014). Paternal ethanol exposure and behavioral abnormities in offspring: associated alterations in imprinted gene methylation. *Neuropharmacology, 81,*126–133.

Little, R., Asker, R., Sampson, P., & Renwick, J. (1986). Fetal growth and moderate drinking in early pregnancy. *Am J Epidemiol., 123,* 270–278

Little, R. E. & Sing, C. F. (1987). Father's drinking and infant birth weight: Report of an association. *Teratology, 36*, 59–65.

Marshburn, P., Sloan, C., & Hammond, M. (1989). Semen quality and association with coffee drinking, cigarette smoking and ethanol consumption. *Fertil Steril., 52,* 162–165

May, P. A., Chambers, C. D., Kalberg, W. O., Zellner, J., Feldman, H., Buckley, D., Kopald, D., Hasken, J. M., Xu, R., Honerkamp-Smith, G., Taras, H., Manning, M. A., Robinson, L. K., Adam, M. P., Abdul-Rahman, O., Vaux, K., Jewett, T., Elliott, A .J., Kable, J.A., Akshoomoff, N., Daniel, F., Arroyo, J.A., Hereld, D., Riley, E. P., Charness, M. E., Coles, C. D., Warren, K.R., Jones, K. L., Hoyme, H. E. (2018). Prevalence of fetal alcohol spectrum disorders in 4 US communities. *J AmMed Assoc, 319,* 474–482.

Meek, L. R., Myren, K., Sturm, J., & Burau, D. (2007). Acute paternal alcohol use affects offspring development and adult behavior. *Physiol Behav, 91,* 154–160.

Oldereid, N., Rui, H., & Purvis, K. (1992). Lifestyles of men in barren couples and their relationship to sperm quality. *Eur J Obstet Gynecol Reprod Biol., 43,* 51–55.

Park, H. J., Hong, M., Bronson, R. T., Israel, M. A., Frankel, W. N., & Yun, K. (2013). Elevated *Id2* expression results in precocious neural stem cell depletion and abnormal brain development. *Stem Cells, 31,* 1010–1021.

Przybycien-Szymanska, M. M., Rao, Y. S., Prins, S. A., & Pak, T. R. (2014). Parental binge alcohol abuse alters F1 generation hypothalamic gene expression in the absence of direct fetal alcohol exposure. *PLoS ONE, 9,* e89320.

Rompala, G. R., Finegersh, A., & Homanics, G. E. (2016). Paternal preconception ethanol exposure blunts hypothalamic-pituitary-adrenal axis responsivity and stress-induced excessive fluid intake in male mice. *Alcohol, 53,*19–25.

Rompala, G. R., Finegersh, A., Slater, M., & Homanics, G. E. (2017). Paternal preconception alcohol exposure imparts intergenerational alcohol-related behaviors to male offspring on a pure C57BL/6J background. *Alcohol, 60,* 169–177.

Rubenstein, J. L. R., Anderson, S., Shi, L., Miyashita-Lin, E., Bulfone, A., & Hevner, R. (1999). Genetic control of cortical regionalization and connectivity. *Cereb Cortex, 9,* 524–532.

Stockard, C. R., & Papanicolaou, G. N. (1918). Further studies on the modification of the germ-cells in mammals: the effect of alcohol on treated guinea pigs and their descendants. *J Exp Zool, 26,* 119–226.

Tarter, R. E., Jacob, R., & Bremer, D. L. (1989). Specific cognitive impairment in sons of early onset alcoholics. *Alcohol. Clin. Exp. Res., 13,* 786–789.

U.S. Food and Drug Administration. (1977). *Alcohol Use During Pregnancy. FDA Drug Bulletin*, 7(6), 2-3.

Warren, K. R. (2015). A review of the history of attitudes toward drinking in pregnancy. *Alcohol Clin Exp Res. 39,* 1110–7.

Xia, R., Jin, L., Li, D., Liang, H., Yang, F., Chen, J., Yuan, W., & Miao, M. (2018). Association between paternal alcohol consumption before conception and anogenital distance of offspring. *Alcohol Clin Exp Res, 42,* 735–742

Zhou, D., Lebel, C., Lepage, C., Rasmussen, C., Evans, A., Wyper, K., Pei, J., Andrew, G., Massey, A., Massey, D., & Beaulieu, C. (2011). Developmental cortical thinning in fetal alcohol spectrum disorders. *NeuroImage, 58,* 16–25.

Zuccolo, L., DeRoo, L. A., Wills, A. K., Smith, G. D., Suren, P., Roth, C., Stoltenberg, C., & Magnus, P. (2017). Erratum: pre-conception and prenatal alcohol exposure from mothers and fathers drinking and head circumference: Results from the Norwegian Mother-Child Study (MoBa). *Sci Rep, 7,* 5877. https://doi.org/10.1038/srep45877

## Chapter 1: Effects of ethanol exposure on embryonic gross anatomy and neuroanatomy in a mouse model


### ABSTRACT

Fetal alcohol spectrum disorders (FASD) describe a wide array of developmental anomalies that occur as a result of ethanol (EtOH) exposure during pregnancy. Children diagnosed with FASD can potentially experience a range of developmental abnormalities including stunted growth, central nervous system issues, altered facial morphology, abnormal behavior, and cognitive deficits. A mouse model was used to investigate the effects of prenatal ethanol exposure (PrEE) on gross anatomy and neuroanatomy at various stages during embryonic development (embryonic day (E)12.5, E14.5, E16.5, and E18.5). Experimental dams self-administered a 25% EtOH solution throughout the time of their gestation until designated experimental timepoints. Results from this experiment reveal altered gross and neuroanatomy, including decreased body weight, body length, brain weight, cortical length, and neocortical thinning due to in utero ethanol exposure at all stages investigated. Additionally, abnormal corpus callosum shape and callosal agenesis was observed in some PrEE cases at E16.5 and E18.5. This study will bring insight to the developmental trajectories of PrEE at various stages during gestation by providing information on the gross anatomical and neuroanatomical differences seen in children with FASD that may underlie atypical behavior exhibited in these individuals.

## INTRODUCTION

The Centers for Disease Control (CDC, 2024b) have issued warnings about the dangers of drinking during pregnancy. It is known that no amount of alcohol (regardless of type) is safe to consume during pregnancy and that it is dangerous to consume alcohol during any stage of pregnancy, including before a person knows they are pregnant. Despite these warnings, the rates of alcohol, or ethanol, consumption during pregnancy continue to remain high with 18.6% of pregnant women between the ages of 30 and 44 reporting that they drank during their pregnancy (Tan et al., 2015). Current research from the CDC indicates nearly 14% (or 1 in 7) of pregnant people reported drinking alcohol in the past 30 days and roughly 5% (or 1 in 20) reported binge drinking in the past 30 days (CDC, 2024c). Prenatal ethanol exposure (PrEE) can lead to pregnancy complications or even result in miscarriage or stillbirth as well as a variety of long-lasting developmental abnormalities known as fetal alcohol spectrum disorders (FASD) (Hoyme et al., 2016). Incidence rates for FASDs are currently estimated to be as high as 5% in the United States (May et al., 2018) with some sub-populations as high as 7% (May et al., 2021) and the highest prevalence in South Africa at 11.1% (Lange et al., 2017). However, many cases go undiagnosed and incidence rates may be on rise as the amount and frequency of alcohol consumption increased during the COVID-19 Pandemic (Boschuetz et al., 2020).

The most severe of the FASDs is fetal alcohol syndrome (FAS) in which the child has growth problems, abnormal facial features, and central nervous system issues that can range from neurological, functional, or cognitive deficits (CDC, 2024a; Astley and Clarren, 2000; Jones et al., 1973; U.S. Food and Drug Administration, 1977). In the United States, the prevalence of FAS reached 0.8% and partial FAS varied within four different regions from 0.8 to 5.9 percent (May et al., 2018). Instances of FAS may be higher if the baby is exposed to alcohol during the first trimester. This stage of development is critical as major organs systems such as the brain, spinal cord, heart and digestive systems are forming. Recent studies have reported 19.6% of pregnant women in the United States drank alcohol during the first trimester (England et al., 2020). Unfortunately, many women may not know that they are pregnant at this stage and may continue to drink alcohol.

Previous work in our laboratory has documented many deleterious effects of PrEE via multilevel experiments in a novel CD-1 mouse model of FASD. For example, abnormal neocortical gene expression, altered neuroanatomy, and development of ectopic intraneocortical connections in postnatal mice were induced by PrEE (Abbott et al., 2016; El Shawa et al., 2013). Many of these phenotypes were passed to subsequent generations, as demonstrated in our transgenerational model of FASD (Abbott et al., 2018; Bottom et al.,2022; Perez et al., 2024, in preparation).

In the current study, we extended our research by investigating the effects of PrEE in embryonic mice. Our goal is to identify gross neuroanatomical phenotypes in PrEE and control offspring at different embryonic stages prior to birth (embryonic day (E) 12.5, E14.5, E16.5, E18.5) in our mouse model of FASD. By assessing areas within the rostral, middle, and caudal portions of the brain, we were able to map changes in neuroanatomy that occur in response to in utero exposure of EtOH that progress throughout murine development.

## MATERIALS AND METHODS

### *Animal care.*

All studies were performed in accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside. CD-1 mice that were initially purchased from Charles River Laboratories (Wilmington, MA/USA) were used for all experiments. All mice were housed in animal facilities at the University of California, Riverside that are kept at approximately 22°C on a standard 12 h light/12 h dark cycle. All efforts were made to minimize animal discomfort and usage in this study.

### *Ethanol administration and breeding.*

P90 female mice were paired with P90 male breeders; the male was removed after detection of the vaginal plug and gestation day was set to embryonic day (E) 0.5. Female mice were then separated into control and

experimental groups and housed individually. Control mice (n =16) were provided with chow and water *ad libitum* throughout gestation until designated sacrifice day. Experimental mice (n = 10) were given chow and 25% ethanol (EtOH) *ad libitum* throughout their gestational period until the corresponding time period. See Figure 1.1 for an experimental timeline.

*Blood ethanol concentration.*

Blood ethanol concentration (BEC) of E18.5 embryos and dams resulting from treatment of 25% EtOH in water or water alone was determined using an alcohol dehydrogenase-based enzymatic assay. Immediately following embryo extraction and assessment of litter size, blood was extracted from the heart with a syringe. Blood samples were collected from both dams and embryos via cardiac puncture at the time of embryonic extraction.

Samples of whole blood from dams were allowed to sit at room temperature for 30 minutes to allow clotting. After clotting, samples were centrifuged for 15 minutes at 4,000 x g at 4°C to obtain serum. Then 1 mL of alcohol reagent (Pointe Scientific; Canton, MI/USA) that was pre-heated to 30°C was mixed with 5µL of serum, reheated to 30°C and immediately assayed using a spectrophotometer.

At E18.5, 50µL of the whole blood sample was deproteinized with 450µL trichloroacetic acid solution, mixed vigorously, then allowed to stand at room temperature for 5 minutes. The sample was then centrifuged at 4,000 x g for 5

minutes to obtain the supernatant. 1mL of alcohol reagent (Pointe Scientific; Canton, MI/USA) was heated to 30°C and 5µL of supernatant was added to the tube and incubated at 30°C for 5 minutes.

All samples were analyzed in duplicate using a Nanodrop 2000 spectrophotometer at 340 nm wavelength. An ethanol standard (100 mg/dL) was also reacted with alcohol reagent and heated to 30°C to create a BEC standard. For embryonic samples, a 1:10 dilution of this ethanol standard (100 mg/dL) was reacted with alcohol reagent and heated to 30°C to create the BEC standard. Test samples were then compared to the ethanol standard to quantitatively determine BEC levels. Differences between EtOH exposed and control dams were compared via *t*-test analyses with statistical significance set at $p < 0.05$.

Samples were not taken from embryos that were younger than E18.5 as there is not enough blood to conduct a proper reaction. On average, mice have around 58.5 ml of blood per kg of body weight. Therefore, a mouse weighing 1.5 g would have a total blood volume of approximately 58.5 ml/kg x 0.0015 kg = 87.75 µL.

*Dam blood plasma osmolality measurements.*

Blood plasma osmolality was measured using an osmometer. Samples of whole blood were taken from the dams via cardiac puncture and allowed to sit at room temperature for 30 minutes to allow for clotting. After clotting, samples were then centrifuged for 10 minutes at 4,000 x g at 4°C to obtain serum.

Approximately 150mL of serum was loaded into a disposable sample tube and measured after the machine was properly calibrated. Experimental and control samples were then compared via *t*-test analyses with significance set to $p < .05$.

*Cesarean section and tissue preparation.*

Pregnant female mice from both groups were weighed on a balance then sacrificed via cervical dislocation when their gestation reached a designated time point of either E12.5, E14.5, E16.5, or E18.5. Immediately after, a postmortem cesarean section was performed on the mice and embryos were extracted and weighed individually on a standard analytical balance. For embryo extraction, the gravid uterus was removed and placed in phosphate-buffered saline (PBS), pH 7.4, on ice. A small vertical incision was made in the uterine wall along the short axis of the uterine horn so that the implantation site where the fetus with its placenta was isolated. Then an incision of the anti mesometrial side of the uterine wall was made so that the fetal membrane was exposed. The fetal membrane was then removed with forceps, the umbilical cord was cut, and the placenta was removed so that the embryo was extracted. The process was repeated until all embryos were extracted. At E12.5, once the umbilical cord was cut and embryos were extracted, they were allowed to bleed out in PBS on ice. Embryos at time point E14.5 were placed in PBS on ice and immediately decapitated and allowed to bleed out. E16.5 and E18.5 embryos were anesthetized via hypothermia then transcardially perfused with 4%

paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. Litter sizes were recorded for all ages.

*Gross Anatomical Measurements.*

Images were taken of the embryo bodies for identifying differences in gross anatomical features. Body length was measured from crown to rump (see Fig. 1.4C for representative image of this technique) using an electronic micrometer in ImageJ (NIH). Images of facial features were taken at E18.5 to investigate changes in facial morphology. Brains were removed from the skull and weighed on an analytical balance. Then whole brains were imaged dorsally using a Zeiss (Oberkochen/Germany) Axio HRm camera attached to a dissecting microscope. Cortical lengths were then measured with an electronic micrometer using the dorsal whole brain images. Comparisons between EtOH and control brains were accomplished using *t*-test analyses and statistical significance was set at $p < 0.05$. After extraction, brains were postfixed in 4% PFA and designated for anatomical assays.

*Neuroanatomical measurements.*

To measure anatomical differences in cortical thickness, embryonic brains were cryoprotected in 30% sucrose solution for 3 days. Whole brains were then sectioned on a Leica cryostat at 40μm on the coronal plane, mounted on subbed slides, and stained for Nissl bodies. Brain slices were imaged using a Zeiss

microscope, then rostral, middle, and caudal regions were measured via electronic micrometer in ImageJ (NIH) by a trained researcher blind to treatment. In E18.5 Embryos measurements were taken from putative frontal, somatosensory, auditory, and visual regions. Regions were identified and confirmed using Kaufman's Atlas of Mouse Development Supplement (Baldock et al., 2016). Electronic lines were drawn perpendicular to the cortical sheet from the most superficial layer I to the deepest region of layer IV to measure cortical thickness. Differences in cortical length were then determined via *t*-test analyses with statistical significance set to $p < .05$.

*Statistical Analysis.*

Experimental data were compared to controls to identify the effects of PrEE on embryonic development. All statistical analyses were completed using GraphPad Prism 10 (La Jolla, CA/USA). All data were analyzed using unpaired two-tailed *t*-tests comparing control and experimental groups within each individual age group. Statistical significance was set to $p < 0.05$ for all measures and all data was expressed as mean ± S.E.M.

## RESULTS

*BEC and osmolality.*

BECs were measured to confirm EtOH exposure and intoxication level in both dams and embryos at E18.5. We found an elevation of BEC in EtOH

exposed dams ($M$ = 147.8 ± 3.605) compared to controls which had no detectable BEC level (Fig. 1.2B, $p$ = 0.0018). Additionally, E18.5 embryos that were exposed to EtOH through gestation also had elevated BECs ($M$ = 118.5 ± 16.15) compared to controls which had no detectable BEC level (Fig. 1.2A, $p$ < 0.0001). Blood plasma osmolality was measured to confirm that dams were properly hydrated and no significant difference in this measure was observed between control ($M$ = 312.5) and EtOH exposed ($M$ = 311.0) dams (Fig 1.2C, $p$ = .831).

*Gross embryo measurements.*

Differences in facial morphology between PrEE and control embryos were observed at all ages (Fig. 1.3). Microcephaly was observed in some cases of PrEE embryos at all ages. Furthermore, PrEE embryos exhibited smaller facial structures overall and in many cases appeared underdeveloped compared to their control counterparts. In PrEE mice the snout is shorter and there are deficits along the midline. Specifically, the nose appears smaller, and the philtrum is less pronounced in PrEE embryos compared to controls. Additionally, the eyes are consistently smaller in PrEE embryos especially at ages E14.5, E16.5 and E18.5 (Fig1.3 B2,3,4).

Although litter sizes were generally smaller in EtOH exposed mothers, no significant difference was observed in the number of pups per litter compared to controls (Fig 1.2D) and some implantation site failure was observed. Embryonic

body weights were significantly smaller at all ages (Fig. 1.4A). Specifically, on average E18.5 control embryos weighed 1.333g and PrEE embryos weighed 1.224g ($p < 0.0001$). E16.5 controls weighed 0.7156g on average compared to PrEE embryos that weighed 0.5572g ($p < 0.0001$). E14.5 control embryos weighed 0.2734g and EtOH exposed weighed 0.2310g on average ($p < 0.01$). At E12.5, control embryos weighed 0.0889g and EtOH exposed weighed 0.0614g on average ($p < 0.0001$). In correspondence to embryonic weight, embryonic body lengths were also significantly smaller on average at all ages in PrEE embryos (Fig 1.4B, E12.5 control: 16.03mm , EtOH: 15.47mm, $p = 0.0468$; E14.5 control: 18.63mm, EtOH: 15.62mm, $p <0.0001$; E16.5 control: 22.1mm , EtOH: 20.0mm, $p <0.0001$; E18.5 control: 25.6mm, EtOH: 24.2mm, $p = .0223$).

Brain weights were observed to be significantly smaller on average in PrEE embryos at all ages (Fig. 1.5, E12.5 control: 0.0088g, n = 6, PrEE: 0.0066g, n = 6, $p = 0.0277$; E14.5 control: 0.02621g, n = 16, PrEE: 0.01514g, n = 15, $p <.0001$; E16.5 control: 0.05447g, n = 84; PrEE: 0.04027g, $p <.0001$, n = 53; E18.5 control: 0.08383g, n = 48, PrEE: 0.07160g, n = 24, $p <.0001$).

Cortical length was measured at each timepoint to analyze gross neocortical developmental abnormalities associated with PrEE. A significant reduction in cortical length was observed in PrEE embryos at all ages (Fig1.5, E12.5 control: 2.347mm, n = 7, PrEE: 2.101mm, n = 6, $p = .0329$; E14.5 control: 3.068mm, n = 16, PrEE: 2.830mm, n = 15, $p <.0001$; E16.5 control: 4.150mm, n

= 38, PrEE: 3.632, n = 41, *p* <.0001; E18.5 control: 4.714mm, n = 48, PrEE: 4.296mm, n = 24, *p* <.0001).

*Neuroanatomical Phenotypes.*

Differences in neuroanatomical phenotypes in E12.5, E14.5, E16.5, and E18.5 embryos were observed in coronal sections of Nissl-stained brain tissue (Figs. 1.6, 1.7, 1.8, 1.9). Specifically, at E12.5 the lateral ventricles (LV) and the third ventricle (TV) are larger in PrEE mice compared to controls (Fig. 1.6). Additionally, the lateral ganglionic eminences (LGE), and medial ganglionic eminences (MGE) are smaller in PrEE mice at E12.5 (Fig. 1.6B). The choroid plexus (ChP) is also underdeveloped in PrEE embryos at E12.5 (Fig. 1.6B) and E14.5 (Fig1.7B). It was also observed that the ventricular zone is increased in PrEE embryos at E14.5 (Fig. 1.6). At E14.5, the lateral ventricles of PrEE mice continue to appear larger, extending further downward toward the ventral side of the brain compared to controls (Fig. 1.7C2). However, at E14.5 the LGE and MGE appear to recover in size and are comparable to controls (Fig. 1.7B). Additionally, a reduction in the piriform-amygdalar area (PAA) was observed at E14.5 and E16.5 in PrEE embryos. At 16.5, the lateral ventricles appear more collapsed than their control counterparts (Fig. 1.8B, C). However, the cerebral aqueduct (Aq) and the third ventricle (TV) are larger in PrEE embryos compared to controls at E16.5 (Fig 1.8C). Additionally, the hippocampus is reduced in volume in PrEE E16.5 embryos (Fig 1.8C). The corpus callosum extends further

down the midline in PrEE E16.5 embryos and in some cases, agenesis occurs (as shown in Fig 1.8B2). The lateral ventricles also appear smaller, while the cerebral aqueduct (Aq) is larger at E18.5 in PrEE embryos (Fig. 1.9A, B). The corpus callosum exhibited an unusual “U” shape at the midline in E18.5 PrEE embryos and agenesis occurred in some cases (Fig. 1.9B2). Additionally, agenesis was also observed in the anterior commissure (AC) at E18.5 (Fig 1.9B2).

*Cortical measurements.*

To assess the effects of PrEE on cortical thickness development at E12.5, E14.5, E16.5, and E18.5, we measured from rostral, middle, and caudal regions in Nissl-stained coronal sections in control and PrEE embryos (Figs. 1.10, 1.11, 1.12, 1.13). A significant decrease in thickness was observed in E12.5 PrEE mice compared to controls in rostral (Control: $M$ = 0.211mm; PrEE: $M$ = 0.157mm ; $p$ = 0.0134), middle (Control: $M$ = 0.132mm; PrEE: $M$ = 0.0885mm; $p < 0.0001$), and caudal (Control: $M$ = 0.113mm; PrEE: $M$ = 0.0874mm; $p < 0.0003$) cortical regions (Fig 1.10). Additionally, a significant reduction in cortical thickness was identified in E14.5 PrEE mice in comparison to controls in rostral (Control: $M$ = 0.281mm; PrEE: $M$ = 0.164mm ; $p < 0.0001$), middle (Control: $M$ = 0.190mm; PrEE: $M$ = 0.113mm ; $p < 0.0001$), and caudal (Control: $M$ = 0.187mm; PrEE: $M$ = 0.109mm ; $p < 0.0001$) regions (Fig. 1.11). Cortical thickness was also significantly reduced in E16.5 PrEE mice compared to controls in rostral (Control:

*M* = 0.190mm; PrEE: *M* = 0.105mm ; $p < 0.0001$), middle (Control: *M* = 0.186mm; PrEE: *M* = 0.089mm ; $p < 0.0001$), and caudal (Control: *M* = 0.145mm; PrEE: *M* = 0.0723mm ; $p < 0.0001$) regions (Fig 1.12). A significant reduction in cortical thickness was observed in E18.5 PrEE mice compared to controls in putative frontal (Control: *M* = 0.751mm; PrEE: *M* = 0.653mm ; $p = 0.0292$), somatosensory (Control: *M* = 0.286mm; PrEE: *M* = 0.233mm ; $p = 0.0188$), auditory (Control: *M* = 0.251mm; PrEE: *M* = 0.210mm ; $p = 0.0099$), and visual (Control: *M* = 0.210mm; PrEE: *M* = 0.161mm ; $p < 0.0061$) regions (Fig. 1.13).

Additionally, many PrEE cases also exhibited an unusual “U” shape of the corpus callosum (CC) near the midline at E18.5 (Fig. 1.14). Callosal agenesis was also observed in some PrEE cases at E18.5 and E16.5.

**DISCUSSION**

In this study, we demonstrate the effects of prenatal ethanol exposure (PrEE) on embryonic development. Our data highlight the effects of PrEE on fetal neuroanatomical development at ages E12.5, E14.5, E16.5 and E18.5. Facial morphology differences include microcephaly, a smaller snout and eyes, as well as changes in the midline including a smaller nose and less pronounced philtrum. Additionally, the body weights and lengths of PrEE embryos were significantly smaller at all ages. Additionally, reduced cortical lengths and thickness were found in PrEE embryos at all ages. Other studies from our lab investigate differences in newborn (P0) cortical thickness finding significant decreases in the

prelimbic, auditory, and visual cortices of F1 mice, compared to controls (Perez, 2024, in preparation). In the current study, we observed increased ventricle size at E12.5 and E14.5 but decreased ventricle size at E16.5 and E18.5. The choroid plexus was also reduced in size in PrEE E12.5 embryos. The lateral and medial ganglionic eminences were also less prominent in PrEE embryos compared to controls in the earlier E12.5 stage. Additionally, there was a reduction in volume of the hippocampus at E16.5 in PrEE embryos. We also noted some cases of agenesis within the anterior commissure at E18.5. The anterior commissure is a white matter tract that connects the cerebral hemispheres across the midline. Additionally, we observed significant changes in corpus callosum morphology including an abnormal “U shape” at E18.5 and some cases of agenesis at E16.5 and E18.5. Cases of callosal agenesis in P0 mice will be discussed further in chapter 2 showing that PrEE can disrupt callosal development in some cases for three generations suggesting transgenerational transfer of the phenotype.

*Facial dysmorphology*

Craniofacial dysmorphologies are more likely to occur if the mother drinks within the first 3 months of pregnancy (CDC, 2024b). In FASD, craniofacial dysmorphology is often characterized by a smooth philtrum, small upper lip vermilion, and short palpebral fissures (Del Campo and Jones, 2017). These facial features tend to become less prominent over time and can often disappear as the person ages (Astley & Clarren, 1995). In a study by Clarren and Smith

(1978) of 245 individuals with FAS, 80% showed these features as well as microcephaly and retrognathism.

Since the 1980s, it has been known that even a single day of exposure to alcohol during pregnancy in mice (specifically E7) can result in the characteristic facial features seen in those with FAS (Sulik et al., 1981). This time period is consistent with mid-week three in human development. One study used 3D analysis and reported that children with FAS had a shallower philtrum by an average of 0.4mm compared to controls (Blanck-Labarsch et al., 2018). Mice aged E7 also displayed a reduction in the depth of the upper lip midline groove, indicating a deficient philtrum (Lipinski et al., 2012). This corroborates our current study's findings as the philtrum was less pronounced, especially at E14.5 and E16.5. Additionally, upper lip length changed in a time point specific manner as it increased at E7 but decreased at E8.5 relative to controls (Lipinski et al., 2012). Occasionally, people with FAS also have a cleft lip or cleft palate. Majewski (1981) reported that 7% of FAS children have cleft palates, while 39% have highly arched palates.

Magnetic resonance imaging (MRI) studies have shown that shortened palpebral fissures are correlated with a reduction in cortical and corpus callosum thickness (Yang et al., 2011a; 2011b). Reduced palpebral fissure size is associated with ocular abnormalities in both humans and animals (Chan et al., 1991; Hammond et al., 2011; Ribeiro et al., 2007). In the current study, embryos displayed reduced eye size. Additionally, up to 80% of individuals with FASD

may have pronounced epicanthal folds (larger than normal fold of skin near the inner corner of the eye) (Manning & Hoyme, 2007) or ptosis (drooping of the upper eyelids).

Other facial anomalies correlated with FASD include railroad-track ears, anteriorly facing (upturned) nostrils, and midface hypoplasia (underdeveloped upper jaw, cheekbones, and eye sockets) (Del Campo and Jones, 2017). In a study of 415 Caucasian children who were exposed to alcohol during pregnancy, 3-dimensional craniofacial images were taken at 12 months revealing that many differences were concentrated around the midface, nose, lips, and eyes (Muggli et al., 2017). As the effects of alcohol on the embryo become more severe there is typically a greater loss of facial midline structures. For example, the nostrils become more closely approximated and are smaller in size (Sulik, 2018) which was also the case for our study. The characteristically small and upturned nose observed in humans with FAS can also be accounted for by deficiencies in the medial nasal prominences (MNPs) which fuse to form the philtrum, medial upper lip, nasal tip, and columella (bridge of tissue that separates the nostrils at the base of the nose) (Sulik & Johnston, 1983).

In mice, alcohol exposure at particular time points during pregnancy results in differing patterns of craniofacial features. Specifically, alcohol exposure on embryonic day 7 of pregnancy can result in exencephaly, cyclopia, mandibular hypoplasia, and cleft lip; whereas exposure on E8 resulted in maxillary hypoplasia associated with median cleft lip and cleft palate, and

sometimes mandibular hypoplasia (Lipinski et al., 2012; Webster et al., 1980). E7 in mice corresponds to early in the third week of pregnancy in humans, a time where many women might not know they are pregnant (O'Leary-Moore et al., 2011).

Our research as well as other research in rodents and primate studies (Astley et al., 1999) provide evidence for alcohol-induced craniofacial malformations being induced early on in embryogenesis, consistent with alterations that appear in virtually all individuals with full blown FAS (Sulik et al., 1981). It is important to note that the degree of severity for alcohol-induced craniofacial defects is widely variable among individuals with the most subtle defects being hard to distinguish from normal. Additionally, not all progeny exposed to alcohol in utero experience facial dysmorphology but still sustain other neuroanatomical, cognitive, or behavioral deficits.

*Gross anatomical differences due to PrEE*

Both human and animal studies have shown that PrEE can contribute to intrauterine growth retardation specifically in body length, weight, and head circumference (Carter et al., 2013). Children with FASDs often have lower birth weights or are shorter in stature than normal (at or below the 10th percentile) (CDC, 2024b). These deficits often persist through infancy and sometimes even into adulthood (Carter et al., 2013). Studies indicate that nutrients such as folic

acid (Xu et al., 2008), choline (Bottom et al., 2020; Thomas et al., 2009) and zinc (Keen et al., 2010) may have a protective effect over these differences.

Previous studies in our laboratory found that PrEE newborn pups are significantly lighter in weight compared to controls and that this difference persisted for three generations of newborns even though the first filial generation (F1) were the only pups receiving direct exposure to EtOH (Abbott et al., 2018; Perez et al., 2024, in preparation). This reduction in body weight is known to persist until adulthood in mice (Abbott et al., 2016). These studies are consistent with our current results which show a reduction of body size and weight in PrEE embryos. In our current study we investigate these differences at ages E12.5, E14.5, E16.5, and E18.5. The earliest time point (E12.5) is equivalent to about 40 days in human embryonic development, at which many individuals might not even realize that they are pregnant.

In addition to reduced body size and weight, prenatal alcohol exposure can cause skeletal malformations including vertebral segmentation defects, large joint contractures (abnormal bending of joints), and scoliosis (curvature of the spine). Humans with FASD have lower bone mineral density (Young et al., 2022) and alterations in bone structural parameters at the growth plate and diaphysis (mid-section) which can contribute to increased risk of bone fractures (Parviainen et al., 2020).

Sensory systems including the visual, auditory, and olfactory systems are also susceptible to the effects of prenatal alcohol exposure. Both conductive and

sensorineural hearing loss as well as developmental delays in auditory maturation are common in children with FAS (Church & Kaltenbach, 1997). Conductive hearing loss is the most common type of hearing loss which arises from problems in the middle ear. This can be caused by recurrent otitis media, an infection that causes fluid buildup behind the eardrum, which is commonly observed in children with FAS at rates ranging from 38% to 93% (Church & Kaltenbach, 1997). On the other hand, sensorineural hearing loss results from damage to hair cells within the cochlea, or damage to the vestibulocochlear nerve and is known to affect between 27% to 29% of the population of people diagnosed with FAS (Church & Kaltenbach, 1997). Hearing loss such as these may contribute to the speech and language difficulties seen in children with FAS. These deficits in the auditory system could also be due in part to ethanol insult on the medial geniculate nucleus (MGN). The MGN is a region of the thalamus that relays auditory information to the cerebral cortex serving as a filter to enhance the representation and perception of acoustic features within the auditory cortex (Bartlett, 2013). In the current study there was a reduction in the volume of the MGN in PrEE embryos at E16.5, which may contribute to auditory deficits later on.

The visual system is also susceptible to the effects of prenatal alcohol exposure. Up to 90% of people with FAS may also experience vision problems (Strömland, 1987). These problems may include strabismus (misalignment of the eyes), or optic nerve hypoplasia (an underdevelopment of the nerve that causes

visual impairments that cannot be corrected with glasses). Additionally, coloboma iridis, a distinctive cleft in the iris giving the appearance of an elongated pupil within a U-shaped iris, is one of the key extensive eye malformations that can be found in individuals with FAS (Abdelrahman & Conn, 2009). Mice in our experiment exhibited smaller eyes when exposed to ethanol in utero and may also experience visual problems later in life, but more research is needed to explore these specific deficits. Furthermore, there is a reduction in the number of retinal ganglion cells and alteration in morphology exhibited in rodents with FASD (Dursun et al., 2011). The retinal ganglion cells project to the superior colliculus and dorsal lateral geniculate nucleus (dLGN). In addition to malformation of the eye itself, changes to the dLGN, a region in the thalamus responsible for relaying information from the retina to the visual cortex, may contribute to poor vision seen in children with FASD (Kerschensteiner & Guido, 2017). In our study, the lateral geniculate nucleus was smaller in PrEE embryos at E16.5. Similar effects were observed in another study from our lab in PrEE mice at P20 (Abbott et al., 2016).

Prenatal alcohol exposure can lead to impaired odor identification in children (Bower et al., 2013). Odor identification not only requires the detection of a certain odor but also the ability to name it, indicating that memory systems are also involved for odors that were smelled previously. It is known that many systems required for successful odor identification are affected by PrEE, including the orbitofrontal cortex, anterior and medial temporal lobes,

hippocampus, and limbic system (Jones-Gotman et al., 1997; Mattson et al., 2001, 2010). The piriform-amygdalar area (PAA) was reduced in E14.5 embryos. The PAA lies adjacent to the cortical amygdalar nucleus and piriform cortex (PC) and receives olfactory bulb input while projecting to several regions of the amygdala (Swanson & Petrovich, 1998). The piriform cortex is also involved in memory and olfaction and is also known as the primary olfactory cortex as it is the first cortical area to receive olfactory information from the olfactory bulbs (Chee et al., 2022). Altered projections within the PAA and PC may contribute to the deficits in odor identification seen in those with FASD.

*Gross differences in neuroanatomy*

Cases of reduced head size and microcephaly have been described in many human studies. In one case of a baby born at 32 weeks gestation, the head circumference was 27cm at birth and the brain was microcephalic weighing only 140g (the average weight of an embryo at 25 weeks gestation) and did not grow for the following 6 weeks that the baby survived, which is far below the 380g average normally seen at 38 weeks (Clarren et al., 1978). In rodents, microcephaly has also been observed in embryos exposed to alcohol at E7 (Lipinski et al., 2012). Examination of embryos 24 hours after first ethanol exposure at E7 using scanning electron microscopy (SEM) showed a clear decrease in size in the neural plate, which was especially prominent in the forebrain area. Specifically, the forebrains of embryos exposed to alcohol at E7

appeared notably narrower and pointed towards the anterior end; these differences are often asymmetric (Sulik & Johnston, 1983). Deficits in neural plate formation during these initial stages can lead to irregularities in brain and eye development. In turn, the diminished size of the forebrain may also impact the induction of the olfactory placode (Jacobson, 1966). Olfactory placode placement greatly affects facial morphology. Reduced head size as well as narrower forebrains were observed in our current study. These differences are particularly noticeable in E12.5 and E14.5 brains (Figs. 1.3B1, 1.3B2, 1.5A2, 1.5B2). Facial morphology appears to be affected by the changes mentioned above as the current study also reports changes within the midline around the nose, philtrum and eyes which could be due to deficits in neural plate formation.

Although microcephaly is common in individuals with FAS, hydrocephalus is less common but can occasionally occur (Jarmasz et al., 2017). Hydrocephalus is a neurological disorder caused by the buildup of cerebrospinal fluid (CSF) in the ventricles of the brain. If left untreated hydrocephalus can be fatal. The choroid plexus (ChP) is a complex secretory tissue network responsible for producing CSF via ependymal cells that line the ventricles in the brain. Proper formation of the choroid plexus is essential for the formation and integrity of the central nervous system (CNS). Too little CSF impairs brain growth as CSF pressure is necessary for proper brain development (Desmond & Jacobson, 1977) while excess CSF can lead to hydrocephalus. The choroid plexus is first observed as a bilateral ridge at E11 with major morphological

development occurring between E11 and E14 (Sturrock, 1979). In the current study we observed underdevelopment of the choroid plexus at E12.5 and E14.5 which could contribute to abnormal brain growth.

The present study corroborates the results seen in humans as brain weight and size was reduced in PrEE embryos bringing further insight into how gross anatomy can be affected by PrEE through development. Reduced brain weight and gross cortical length of PrEE mice can persist into the F3 generation even though the F1 was the only generation receiving direct EtOH exposure through maternal intake (Abbott et al., 2018; Perez et al., 2024, in preparation).

*Neuroanatomical differences*

Anatomical irregularities in the human brain due to PrEE have been documented through various neuroimaging techniques as well as autopsy studies. MRI studies have shown reductions in white matter tracts (Archibald et al., 2001). Additionally, both white and gray matter in the parietal lobe were disproportionately reduced (hypoplasia) in FAS participants compared to controls (Archibald et al., 2001). MRI studies have also revealed structural changes in cortical areas such as reduced frontal, parietal, temporal and occipital lobe size (Archibald et al., 2001; Sowell et al., 2002) and alterations in subcortical areas including reduced basal ganglia (Mattson et al., 1996) and left-right hippocampal asymmetry (Riikonen et al., 1999). Furthermore, positron emission tomography

(PET) scans revealed decreases in metabolic rate in the thalamus and caudate nucleus compared to controls (Clark et al., 2000).

The corpus callosum begins developing approximately around E15 and continues until P14 in rodents (Plachez & Richards, 2005). Abnormalities in the corpus callosum, ranging from agenesis (Astley et al., 2009), thinning (Clark et al., 2000), or hypoplasia (Boronat et al., 2017) to structural variability that can persist into adulthood (Bookstein et al., 2002, 2007) have been documented through neuroimaging studies. One structural anomaly strongly associated with PrEE is a hook-like appearance between the splenium and the long diameter of the arch in the corpus callosum (Bookstein et al., 2007). Although these effects were observed in humans, similar phenotypes were noted in our current study as the corpus callosum had an abnormal “U” or hook shape near the midline. Additionally, cases of callosal agenesis were observed in the current study as well.

Autopsy studies also lend support to alcohol causing developmental changes which may ultimately be responsible for cognitive and behavioral changes observed in children later in life. Autopsies of embryos (stillborn or premature) revealed a severe reduction of brain weight with 10 cases falling in less than the 5th percentile (Jarmasz et al., 2017). Ectopic neurons in white matter were found in autopsied brains of children with FAS, with the most extreme case showing absence of the corpus callosum and anterior commissure (Clarren et al., 1978). Agenesis of the corpus callosum occurred in several

infants and partial agenesis to the posterior corpus callosum of a fetus was also observed (Jarmasz et al., 2017). Another study of a two-month-old PrEE baby revealed fusion of anterior structures (thalamus, septum, and caudate nucleus) as well as poorly developed optic tracts and absence of olfactory bulbs and tracts (Coulter et al. 1993).

This abnormal neuroanatomical development of the corpus callosum and anterior commissure seen in humans is consistent with what we observed in the mice in this study as agenesis of both regions occurred at E18.5. This suggests that these developmental anomalies occur early on and persist as the child develops.

Animal models provide more evidence for variation for neuroanatomy after PrEE. For instance, in rats the size of the corpus callosum is significantly reduced following prenatal alcohol exposure (Zimmerberg & Scalzi 1989). Reduction in the caudate putamen and ventricular enlargement were found in rats with PrEE (Mattson et al., 1994). O'Leary-Moore and colleagues (2011) reviewed changes in the mouse brain following a single day of PrEE during early fetal development using magnetic resonance imaging (MRI) revealing that timing of this teratogenic exposure is an important influence of brain malformation. Alcohol exposure on E7 mice is known to damage medial forebrain regions (Godin et al. 2010), basal ganglia, hippocampus, and anterior cingulate cortex, as well as cause hypoplasia or agenesis of the corpus callosum (Sulik and Johnston, 1982). Morphological changes induced by PrEE on E8 mice include

disproportionate volume reductions in the olfactory bulbs, hippocampus, and cerebellum while pituitary and septal regions are increased (Parnell et al. 2009). Acute PrEE at E9 resulted in cerebellar volume reduction, ventricle enlargement, and alterations in the shape of the cerebral cortex, hippocampus, and right striatum in mice (Parnell et al. 2013). PrEE E10 mice displayed enlarged ventricles and cortical volume reductions (O'Leary-Moore et al. 2010). This is consistent with our current findings in the current study as ventricles were enlarged at E12.5 and E14.5 and the hippocampal volume was decreased at E16.5 in PrEE mice.

Additionally, the lateral ganglionic eminences (LGE), and medial ganglionic eminences (MGE) are smaller in PrEE mice at E12.5. Ganglionic eminences are transient structures present during embryonic development that function to guide cell and axon migration. These structures protrude into the lateral ventricles and guide the tangential migration of neural cells (Chen et al., 2017). The MGE contributes to the production of GABAergic interneurons that migrate to the striatum and cerebral cortex as well as GABAergic projection neurons that migrate to the globus pallidus (Marín & Rubenstein, 2001). On the other hand, the LGE gives rise to interneurons and projection neurons in the striatum which migrate to populate the granule cell and glomerular layers of the olfactory bulb (Chen et al., 2017; Stenman et al., 2003). Improper migration of neurons from the eminences may contribute to sensory abnormalities in the

olfactory system in individuals with FASD, as well as differences in cortical thickness observed in PrEE mice at older embryonic ages as well as postnatally.

Previous findings from our laboratory show neuroanatomical changes due to PrEE in mice at various ages. Specifically, thickening of the frontal cortex in newborn (P0), juvenile (P20), young adult (P50), thinning of the prelimbic cortex at P0 and P50 and thickening at P20 (Abbott et al., 2016) was observed. Additionally, thinning of somatosensory cortex was examined at P0 and P20, visual cortex thickening was observed at P0, and variation in auditory cortex was detected (thinning at P0 and P50, and thickening at P20) (Abbott et al., 2016). Subcortical variations were also observed as there was volume reduction in the dorsal lateral geniculate nucleus (dLGN) (P20) and Basal ganglia (P0) (Abbott et al., 2016). The CA3 of the hippocampus was also thinned at P0 and P20 but thickened at P50, and the corpus callosum was thinned at newborn and juvenile ages but thickened in early adulthood (Abbott et al., 2016). These variations coincide with the current findings of reduced hippocampal volume at E16.5, reduced cortical thickness in rostral, middle, and caudal brain sections at E12.5, E14.5, and E16.5 as well as reduced thickness in the putative frontal, somatosensory, auditory, and visual cortices at E18.5.

*Mechanisms contributing to anatomical changes due to PrEE*

*Neurodevelopmental disruptions*

Oxidative stress damages cellular components, including DNA, proteins, and lipids, leading to inflammation and cell death (apoptosis). Prenatal ethanol exposure is clearly damaging to the overall development of the central nervous system, but some groups have identified specific areas that are particularly sensitive to this teratogen. It has long been known that prenatal ethanol exposure results in apoptotic cell loss that leads to a decrease in volume in the following structures: cerebral cortex, amygdala, basal ganglia, corpus callosum, cerebellum, and the hippocampus (Archibald et al,. 2001, Autti-Rämö, 2002, Ikonomidou et al., 2000, Klintsova et al., 2007, Roebuck et al., 1998). This, in turn, can result in changes in observable behaviors associated with these areas. These effects can be potentially driven by deficits to neuronal progenitor cell proliferation due to alcohol exposure (Nixon & Crews, 2002).

Reductions in white matter and other white matter abnormalities have been observed in offspring exposed to ethanol during gestation (Archibald et al., 2001; Bookstein et al., 2002, 2007; Clarren et al., 1978) particularly at the midline in regions such as the cortex, cerebellum, and hippocampus (Matthews et al., 2021). Three guidance cue families are responsible for guiding these neural pathways: Slit-Robo, Netrin-DCC, and Semaphorin-Neuropilin. These guidance cue systems are a crucial component of neurodevelopment as they attract and repulse axons to ensure that developing axons are directed to their appropriate

targets and form proper connections in the developing nervous system (Matthews et al., 2021).

In PrEE exposed rats, the area of the cortex that receives thalamocortical axons is thinner and the termination of these thalamocortical connections is abnormal (Minciacchi et al., 1993). Additionally, PrEE alters GABAergic and glutamatergic cell populations and glial cells that serve as guidepost cells for axons in the corpus callosum (Matthews et al., 2021). Guidepost cells are crucial for neural development, located strategically along the routes that axons travel, offering intermediate targets and directional signals that steer growing axons to their ultimate destinations. Guidepost cells can secrete or present guidance cues on their surfaces providing both a physical and chemical landmark for axonal growth. Lack of GABAergic guidepost cells can lead to corpus callosum agenesis as these provide a necessary structural mechanism for guidance (Matthews et al., 2021). Physical and behavioral deficits observed in those with FASD often coincide with changes in guideposts and guidance cue/receptor signaling (Matthews et al., 2021).

One guidance receptor termed neuropilin 1 (NRP1), a receptor for the class III semaphorin guidance cue, plays an integral role in the development of the corpus callosum (Piper et al., 2009). This semaphorin signaling along with regulation by ephrin type-B receptor 1 (EphB1) and NRP1 mediates the midline formation of the corpus callosum (Mire et al., 2018). Additionally, Netrin-DCC is expressed at the cortical midline and directly attracts cingulate axons while

indirectly modulating the Slit-Robo repulsion of callosal axons (Fothergill et al., 2014). DCC is expressed highly before axons cross the midline and is later downregulated post crossing as Robo1 takes over guidance (Fothergill et al., 2014). Exposure to alcohol disrupts the functioning of these guidance molecules, contributing to changes in neuroanatomical structures and connectivity.

*Interference with Neurotrophic Factors*

Neurotrophic factors promote neuronal survival, growth, differentiation, and synaptic function. They also support axonal growth and guidance. Some common neurotrophic factors include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin-like growth factor (IGF-1), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF). PrEE can reduce expression of IGF-1 receptors (de la Monte & Wands, 2002) and downregulate BDNF mRNA (Light et al., 2001). Depletion of BDNF can lead to reduced dendritic spine complexity and apoptosis contributing to structural changes including underdevelopment of the corpus callosum and cerebellum (Kaufmann & Moser, 2000). Furthermore, reduced neurotrophic factors may increase neuronal apoptosis, further depreciating neuronal populations that contribute to callosal pathways (Gavrish et al., 2024). Deficiencies in neurotrophic factors can result in the congenital anomalies of the corpus callosum, such as hypoplasia or agenesis, observed in individuals with FASD (Gavrish et al., 2024).

*Genetic and Epigenetic Changes*

Exposure to alcohol prenatally via maternal consumption could result in heritable epigenetic changes lasting generations (Abbott et al., 2018). Epigenetics refers to modifications within the genome that change gene expression without changing the sequence of DNA. These modifications include DNA methylation, acetylation, phosphorylation, ubiquitination, as well as histone modifications and changes to small noncoding RNAs (sncRNAs) (Kugel & Goodrich, 2012). These changes have been suggested to be the potential biomarker and mediator for the effects observed in prenatal ethanol exposures, in human and animal models.

DNA methylation contributes to the regulation of embryonic development and cell programming early in life (Planques et al., 2021). Higher levels of 5-methylcytosine (5mC) promote long-term silencing of genes, and more than 80% of CpG sites, in humans, exist in a silenced hypermethylated state (Morris & Monteggia, 2014). Clusters of CpG sites are typically referred to as CpG islands. These areas are usually methylated and highly conserved and about 70% of promoter regions contain CpG islands (Jang et al., 2017). CpG island methylation in promoter regions leads to the regulation of gene transcription through multiple mechanisms (Saxonov et al., 2006). For example, the CpG regions of two genes crucial for cortical patterning, *Id2* and *RZRβ*, experience decreases in methylation due to prenatal ethanol exposure (PrEE) in a rodent model (Abbott et al., 2018). This effect can be observed well into the third filial

generation (F3). Moreover, decreased 5mC levels were observed in the CA1 of the hippocampus in humans at the early fetal stages (Jarmasz et al., 2019).

Histones are highly basic proteins containing arginine and lysine residues that help package DNA into nucleosomes, the building blocks of chromatin. The N-terminal tails of histones are essential modulators of nucleosome structure and function (Lussier, Weinberg & Kobor, 2017) and are the main target of epigenetic modifications which include acetylation, methylation, phosphorylation, sumoylation, ubiquitination, proline isomerization, and ADP ribosylation (Bowman & Poirier, 2015; Kouzarides, 2007). These epigenetic processes can be affected by alcohol use potentially leading to changes in gene expression and neuroanatomy in individuals and their offspring. For example, PrEE may lead to epigenetic changes within histones including global decreases in histone methylation, and global increases in histone acetylation (Jarmasz et al., 2019). A study in rats showed that PrEE from embryonic day (E)7 to E21 led to decreased histone methylation in H3K4me2 and H3K4me3 and increased H3K9me2 within the hypothalamus of adult offspring (Govorko et al., 2012). This resulted in decreased Pro-opiomelanocortin (POMC) expression which could contribute to the disruption of the HPA axis and subsequent increase of stress hormone secretion (Govorko et al., 2012). Hypermethylation of POMC genes and its effects on the HPA axis have been observed in three generations passed on through the male germline (Gangisetty et al., 2022). This dysregulation of the HPA axis seen with PrEE contributes to hyper-stress response, mental

dysfunction, and impaired immune system response. Additionally, injections of ethanol in E7 mice resulted in increased H3K9me2 and decreased H3K27me3, and at E17 ethanol injections were followed by modest changes to H3K4me3. These effects on histone methylation are exhibited in mice with craniofacial dysmorphology and midline brain defects (Veazey et al., 2015) To combat the modifications made by alcohol exposure, the co-administration of choline may normalize these changes (Bekdash et al., 2013).

MicroRNAs (miRNAs) are a type of non-coding RNA that silence genes via one of two distinct mechanisms: translational repression and mRNA degradation. These miRNAs can bind to messenger RNA (mRNA) and repress translation in the cytoplasm, thereby preventing production of proteins. Gene silencing can also occur via target mRNA cleavage and degradation. MiRNA acts as a guide by base pairing with a target mRNA, with the level of complementarity between miRNA and mRNA determining whether the translation inhibition mechanism (limited miRNA base pairing) or mRNA cleavage and subsequent degradation mechanism (extensive miRNA base pairing) will be used (MacFarlane & Murphy, 2010). Typically, these interactions between miRNA and the target mRNA occur at the 3’ untranslated regions of mRNA (O’Brien et al., 2018).

DNA methylation and histone modifications can occur through miRNAs and the expression of miRNAs can be modified through PrEE (Miranda, 2012). Some miRNAs are sensitive to ethanol exposure throughout development. For

example, miR-9 expression is increased by ethanol early in development (Balaraman et al., 2012) and decreased in later developmental stages (Wang et al., 2009) as well as adulthood (Pietrzykowski et al., 2008). MiR-9 is important for neural tube patterning (Leucht et al., 2008), neurogenesis in the telencephalon (Shibata et al., 2011), and early differentiation (Shibata et al., 2008). Ligand-gated ion channel receptors such as $GABA_A$ and nicotinic acetylcholine (nAChRs) could potentially mediate some of ethanol's effects on fetal miRNAs (Miranda, 2012). Ethanol may also regulate miRNA expression by epigenetic mechanisms since ethanol has been shown to alter methylation patterns in neural stem cells (Zhou et al., 2011). The first and second trimesters of gestation are a critical point during development because during this time neural stem cells produce the vast majority of adult neural stem cells. Even minor disruptions during this stage can be particularly dangerous as the rate of proliferation and maturation can amplify these effects causing changes in brain structure and function (Miranda, 2012). Additionally, many miRNAs that are sensitive to ethanol are located near CpG islands making them potential targets for epigenetic programming and DNA methylation (Miranda, 2012).

These epigenetic changes can result in significant structural and functional abnormalities in the brain of offspring exposed to alcohol during pregnancy, contributing to the cognitive and behavioral deficits observed in individuals with fetal alcohol spectrum disorders.

*Conclusion*

Alcohol can affect the development of the baby's brain, leading to structural and functional abnormalities which can result in intellectual and learning difficulties, as well as behavioral problems. These behavioral problems include but are not limited to attention deficits (Burd, 2016), hyperactivity, impulsivity, and difficulties with social interactions (Thomas et al., 1998; Streissguth & O'Malley, 2000). Specifically, children with FASD may have difficulty interpreting social cues (Kelly et al., 2000), forming relationships, or regulating their emotions (Temple et al., 2019). In fact, children with FASD often experience low frustration tolerance which contributes to difficulties in ignoring teasing and bullying directed at them as well as making them less likely to avoid conflict overall (Streissguth & O'Malley, 2000). Additionally, children with FASD may experience developmental delays in speech and language (Hendrick et al, 2019), cognition (Kodituwakku, 2009), and motor skills (Kalberg et al., 2006).

In conclusion, this study illustrates the important finding that significant anatomical alterations can occur due to prenatal alcohol exposure. These effects can still occur even later on in the pregnancy (equivalent to the second and third trimester). Babies that are exposed to alcohol *in utero* are more likely to be born prematurely which contributes to additional health challenges for the child. Unfortunately, many of the effects of alcohol exposure are lifelong with no cure, and many effects spread to further generations even if the offspring never drink alcohol (Abbott et al., 2018; Perez et al., 2024, in preparation). Therefore, it is

important to abstain from alcohol throughout the duration of the pregnancy to ensure proper embryonic development.

## FIGURES

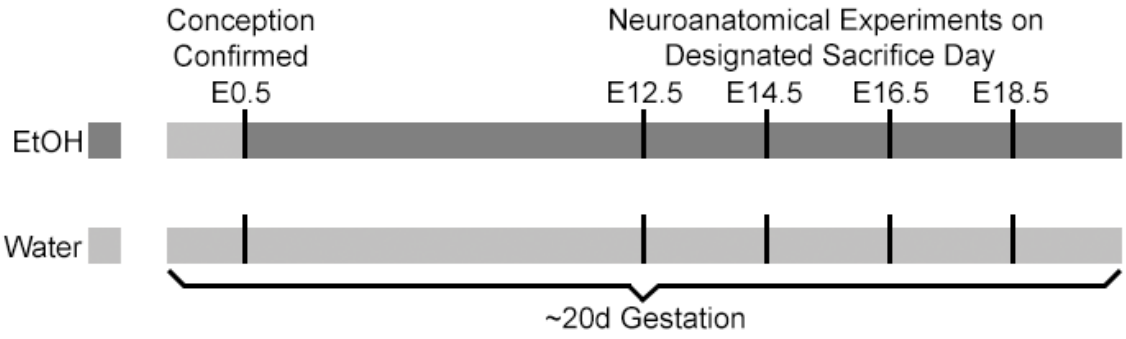


**Figure 1.1. Experimental Timeline.** After confirmation of conception, designated Embryonic day (E)0.5 females were randomly assigned to experimental or control groups. Control dams received water and experimental dams received 25% EtOH from conception until the designated sacrifice day (either E12.5, E14.5, E16.5, or E18.5) where neuroanatomical experiments were performed.

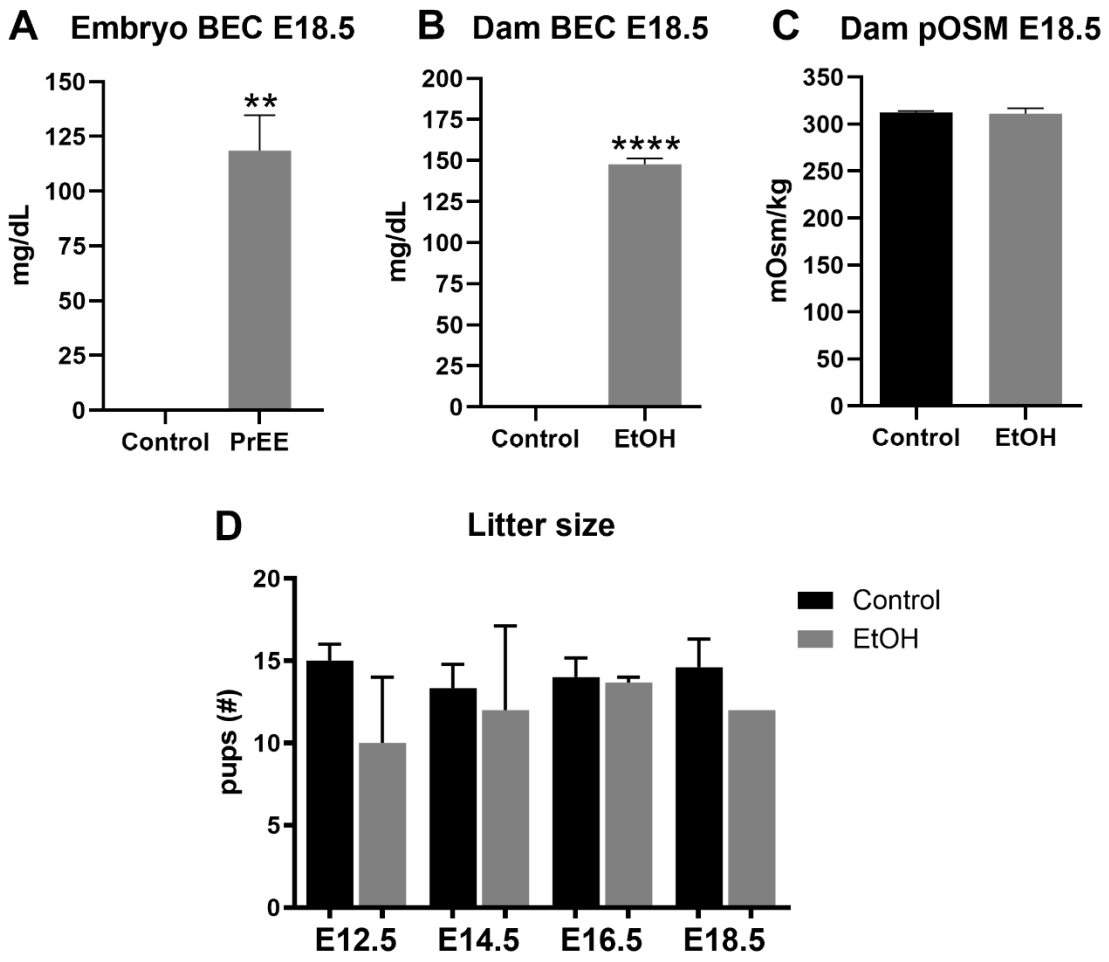


**Figure 1.2. Embryo BEC at E18.5, Dam BEC, Dam pOSM, Litter size.** **A.** Embryos exposed to 25% EtOH (n = 3) had an average BEC of 118.5 ± 16.15 mg/dL compared to controls (n = 3) which had a BEC of 0.0 ± 0.0 mg/dL at E18.5. **B.** Dams exposed to 25% EtOH (n = 4) had an average BEC of 147.8 ± 3.605 mg/dL compared to controls (n = 4) which had a BEC of 0.0 ± 0.0 mg/dL at E18.5. **C.** No significant difference in average dam plasma osmolality (mOsm/kg) between EtOH-treated (n = 2) and control (n = 2) dams at E18.5. **D.** No significant difference in average litter size between control and PrEE. Statistical significance was set to $p < 0.05$ and all data was expressed as mean ± S.E.M. (**$p < .01$, ****$p < .0001$).

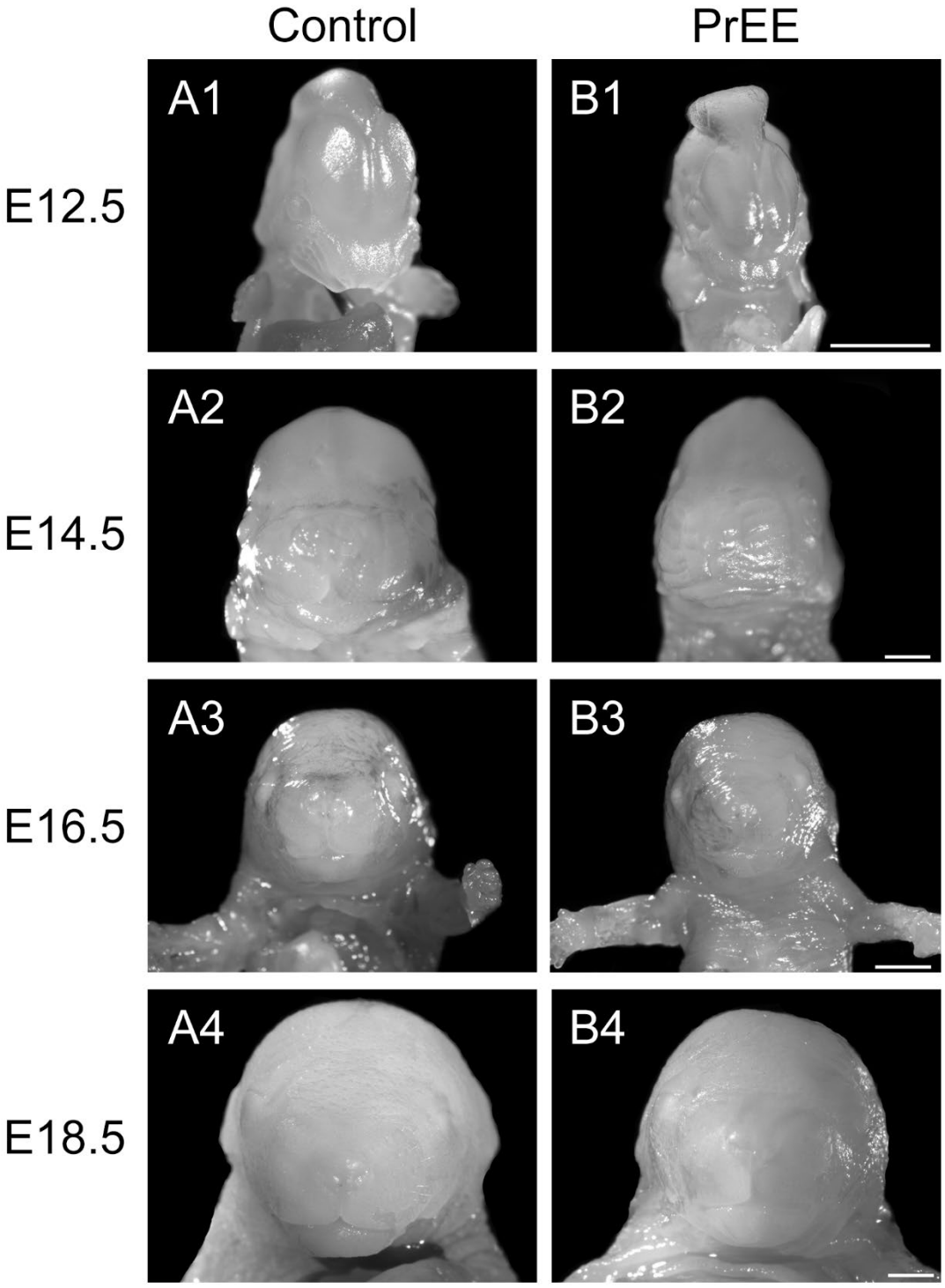


**Figure 1.3. Facial morphology.** Representative cases of facial morphology in control (**A1-A4**) and PrEE (**B1-B4**) mice. Scale bar, 2000μm.

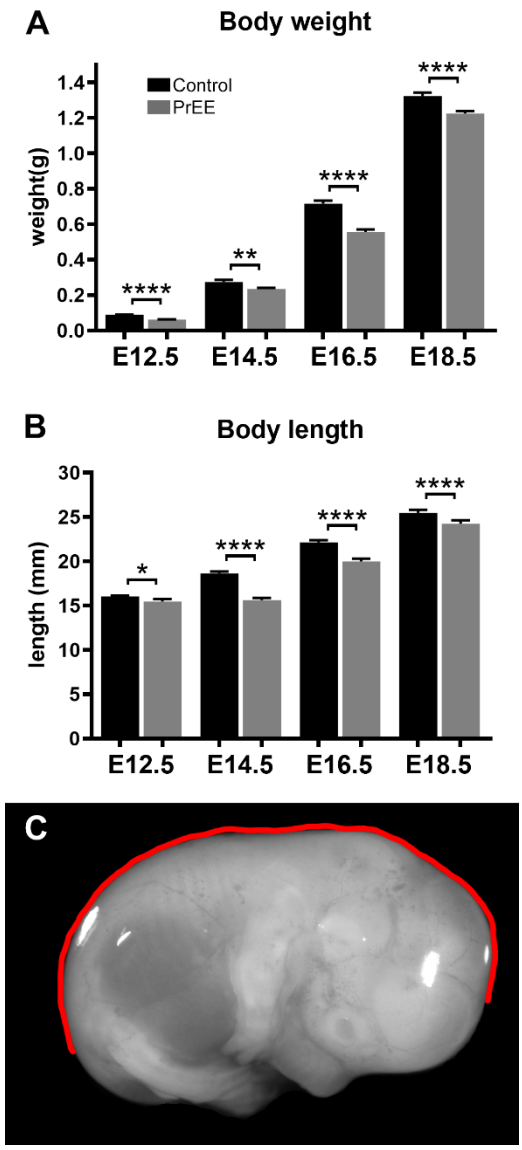


**Figure 1.4. Embryo Body weight, Embryo Body length.**
**A.** Embryonic body weights were significantly less in PrEE on average at all time points (E12.5: control: n = 45, PrEE: n = 20; E14.5: control: n = 23; PrEE: n = 20; E.16.5 control: n = 37, PrEE: n = 14; E.18.5 control: n = 34, PrEE: n = 24). **B.** Embryo body length was significantly greater in controls (E12.5: control: n = 41, PrEE: n = 8; E14.5: control: n = 24; PrEE: n = 21; E.16.5 control: n = 54, PrEE: n = 14; E.18.5 control: n = 26, PrEE: n = 12). **C.** A representative image (at E14.5) of how body length was measured using an electronic micrometer. Statistical significance was set to $p < 0.05$ and all data was expressed as mean ± S.E.M. (*$p$ < .05, **$p$ < .01, ****$p$ < .0001).

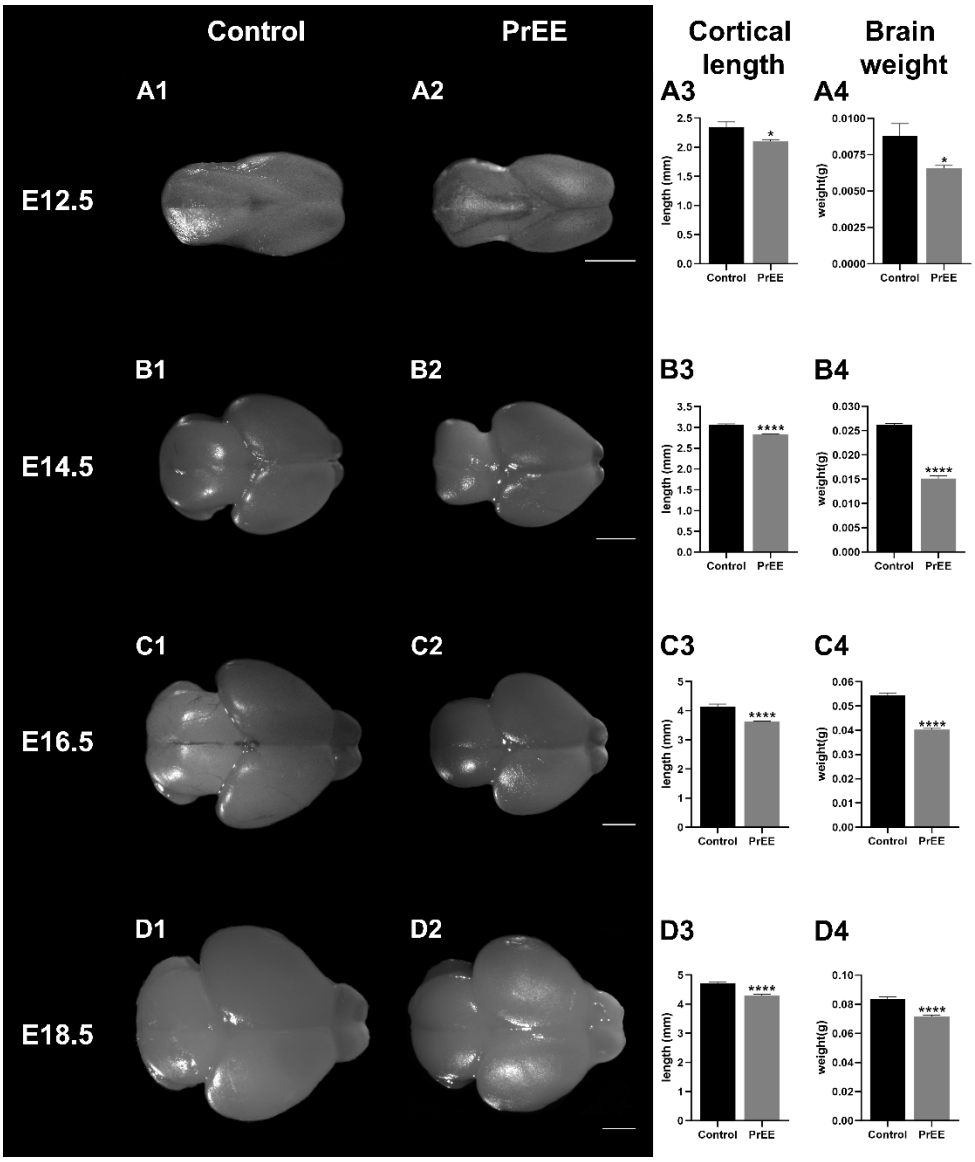


**Figure 1.5. Dorsal views, Cortical length, Brain weight.** Dorsal brain views and measurements. Dorsal brain views for control E12.5 (**A1**), E14.5 (**B1**), E16.5 (**C1**), E18.5 (**D1**) and PrEE E12.5 (**A2**), E14.5 (**B2**), E16.5 (**C2**), E18.5 (**D2**) embryos. (**A3-D3**) Significant reductions in cortical lengths of PrEE embryos at all ages (E12.5 control: n = 7, PrEE: n = 6; E14.5 control: n = 16, PrEE: n = 15; E16.5 control: n = 38, PrEE: n = 41; E18.5 control: n = 48, PrEE: n = 24).(**A4-D4**) Significant reduction in brain weights in EtOH-exposed embryos were found at all ages (E12.5 control: n = 6, PrEE: n = 6; E14.5 control: n = 16, PrEE: n = 15; E16.5 control: n = 84, PrEE: n = 53; E18.5 control: n = 48, PrEE: n = 24). Statistical significance was set to $p < 0.05$ and all data was expressed as mean ± S.E.M. (*$p$ < .05, ****$p$ < .0001). Scale bars, 2000μm.

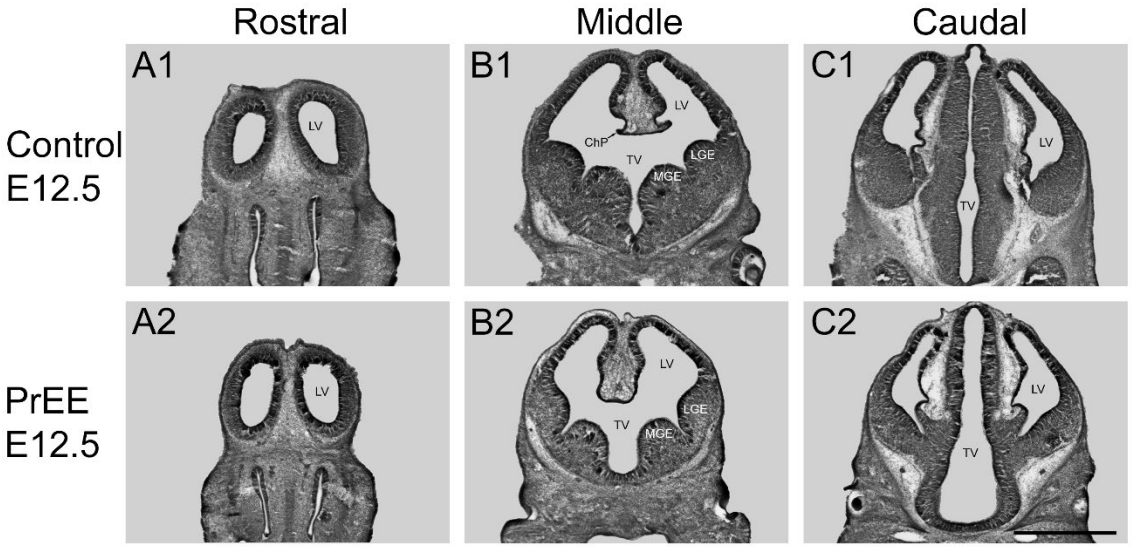


**Figure 1.6. Neuroanatomical phenotypes at E12.5.** Representative images of rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions in Nissl-stained coronal sections in control (n = 8) (**A1**, **B1**, **C1**) and PrEE (n = 8) (**A2**, **B2**, **C2**) embryos at E12.5. Lateral ventricles (LV) and the third ventricle (TV) are larger in PrEE embryos compared to controls. **B.** Lateral ganglionic eminences (LGE) and medial ganglionic eminences (MGE) are smaller in PrEE embryos. The choroid plexus is also underdeveloped in PrEE embryos compared to controls (**B1**). Scale bar 1000μm.

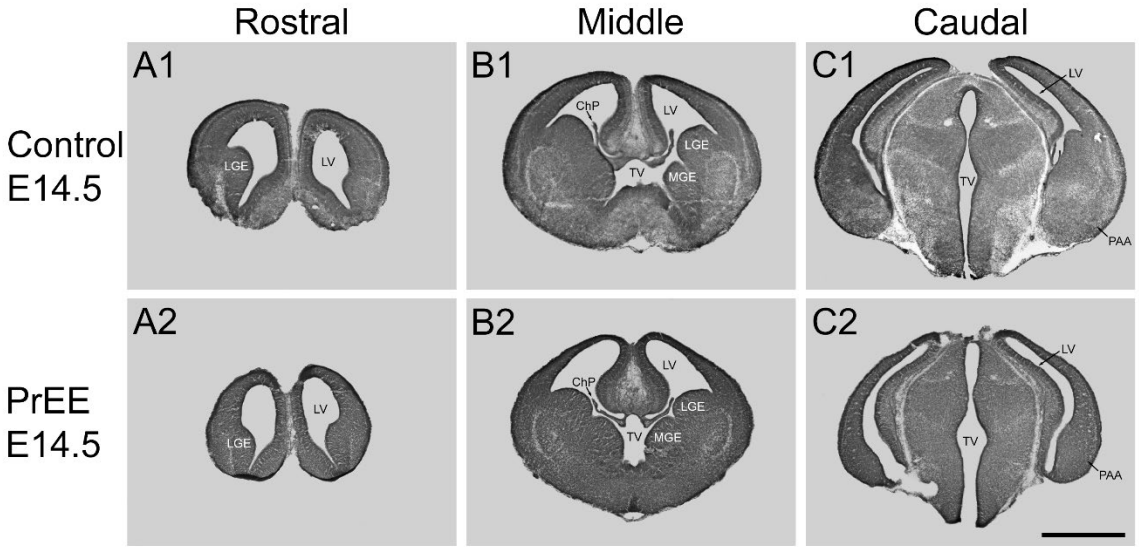


**Figure 1.7. Neuroanatomical phenotypes at E14.5.** Representative images of rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions in Nissl-stained coronal sections in control (n = 8) (**A1**, **B1**, **C1**) and PrEE (n = 9) (**A2**, **B2**, **C2**) embryos at E14.5. Lateral ventricles (LV) and the third ventricle (TV) are larger in PrEE embryos compared to controls. Lateral ganglionic eminences (LGE) (**A**, **B**) and medial ganglionic eminences (MGE) (**B**) are similar in size in PrEE and control embryos. The choroid plexus is smaller in PrEE embryos (**B2**) compared to controls (**B1**). **C1**, **C2**. The piriform-amygdalar area (PAA) is reduced in PrEE embryos compared to controls. Scale bar 1000μm.

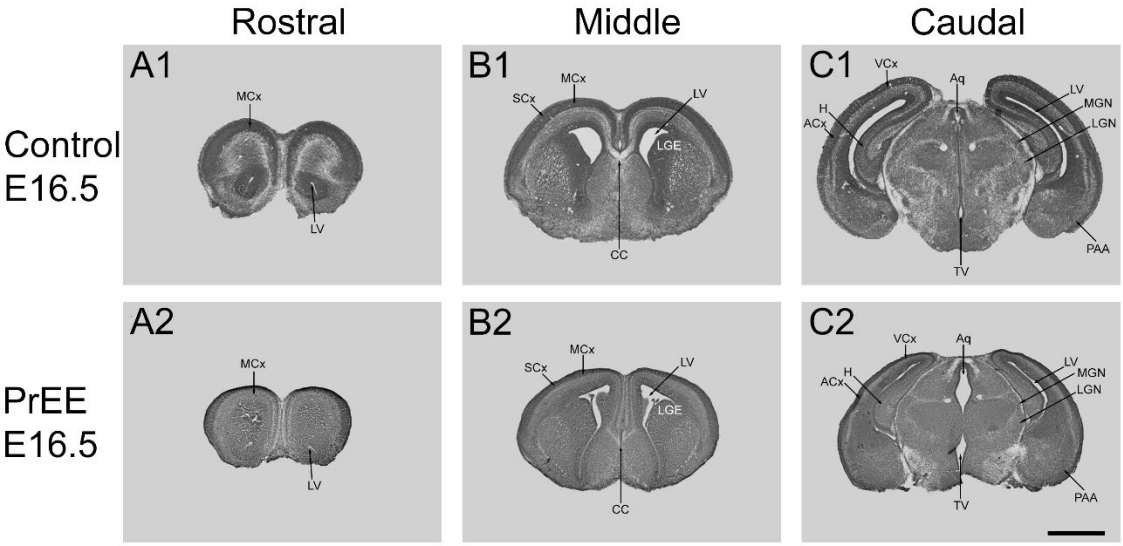


**Figure 1.8. Neuroanatomical phenotypes at E16.5.** Representative images of rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions in Nissl-stained coronal sections in control (n = 9) (**A1**, **B1**, **C1**) and PrEE (n = 8) (**A2**, **B2**, **C2**) embryos at E16.5. Motor cortex (MCx) is thinner in PrEE embryos (**A2**, **B2**) compared to controls (**A1**, **A2**). In PrEE embryos the somatosensory cortex (SCx) (**B2**), visual cortex (VCx) (**C2**) and auditory cortex (ACx) (**C2**) are smaller compared to controls. Lateral ventricles (LV) appear smaller in PrEE (**B2**, **C2**) compared to controls (**B1**, **C1**). **C**. The cerebral aqueduct (Aq) and the third ventricle (TV) are larger in PrEE embryos compared to controls. Lateral ganglionic eminences (LGE) (**A**, **B**) are similar in size in PrEE and control embryos. **C1**, **C2.** Volume of the hippocampus (H), medial geniculate nucleus (MGN), lateral geniculate nucleus (LGN), and piriform-amygdalar area (PAA) is reduced in PrEE embryos compared to controls. The corpus callosum exhibited agenesis in PrEE embryos (**B2**). Scale bar 1000μm.

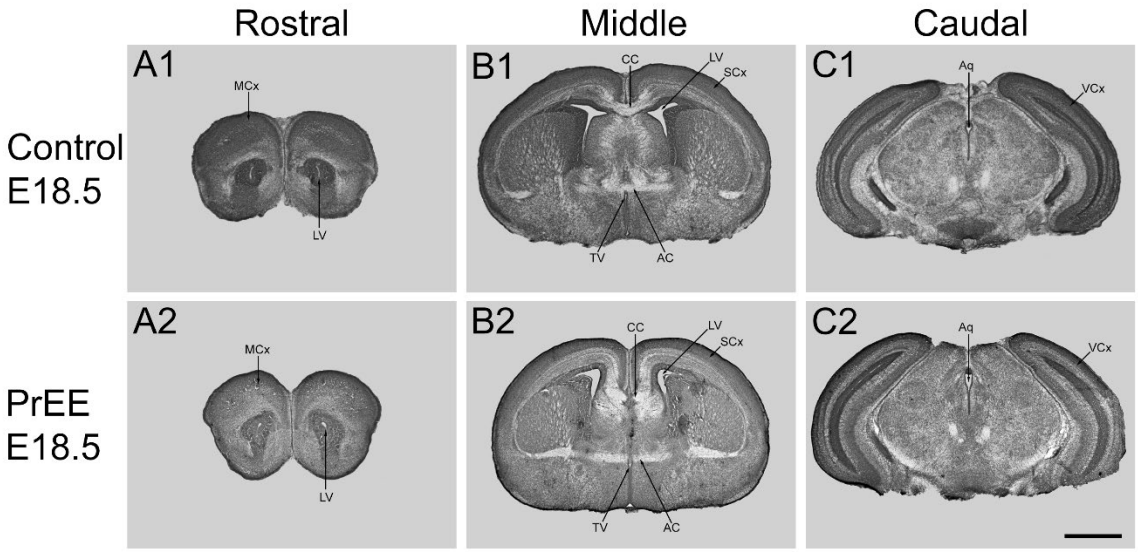


**Figure 1.9. Neuroanatomical phenotypes at E18.5.** Representative images of rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions in Nissl-stained coronal sections in control (n = 9) (**A1**, **B1**, **C1**) and PrEE (n = 7) (**A2**, **B2**, **C2**) embryos at E18.5. In PrEE embryos the motor cortex (MCx) (**A**), somatosensory cortex (SCx) (**B2**), and visual cortex (VCx) (**C2**) are smaller compared to controls. **B**. Agenesis was observed in the anterior commissure (AC) and corpus callosum at E18.5 in PrEE embryos (**B2**). The cerebral aqueduct (Aq) (**C**) and the third ventricle (TV) (**B**) are larger in PrEE embryos compared to controls. Scale bar 1000μm.

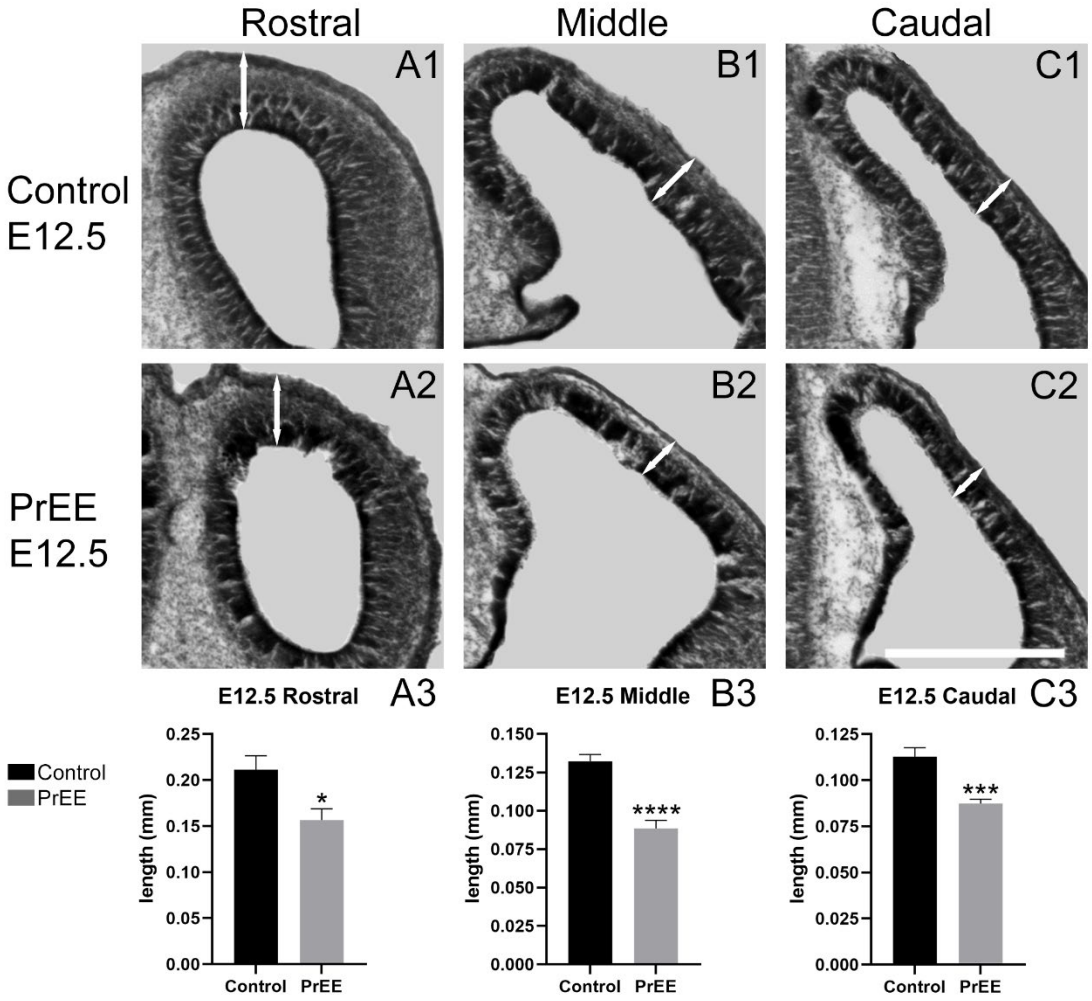


**Figure 1.10. Cortical thickness at E12.5.** A significant decrease in thickness was observed in E12.5 PrEE mice (n = 8) compared to controls (n = 8) in rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) cortical regions. (**p* < .05, ****p* < .001, *****p* < .0001). Scale bar 1000μm.

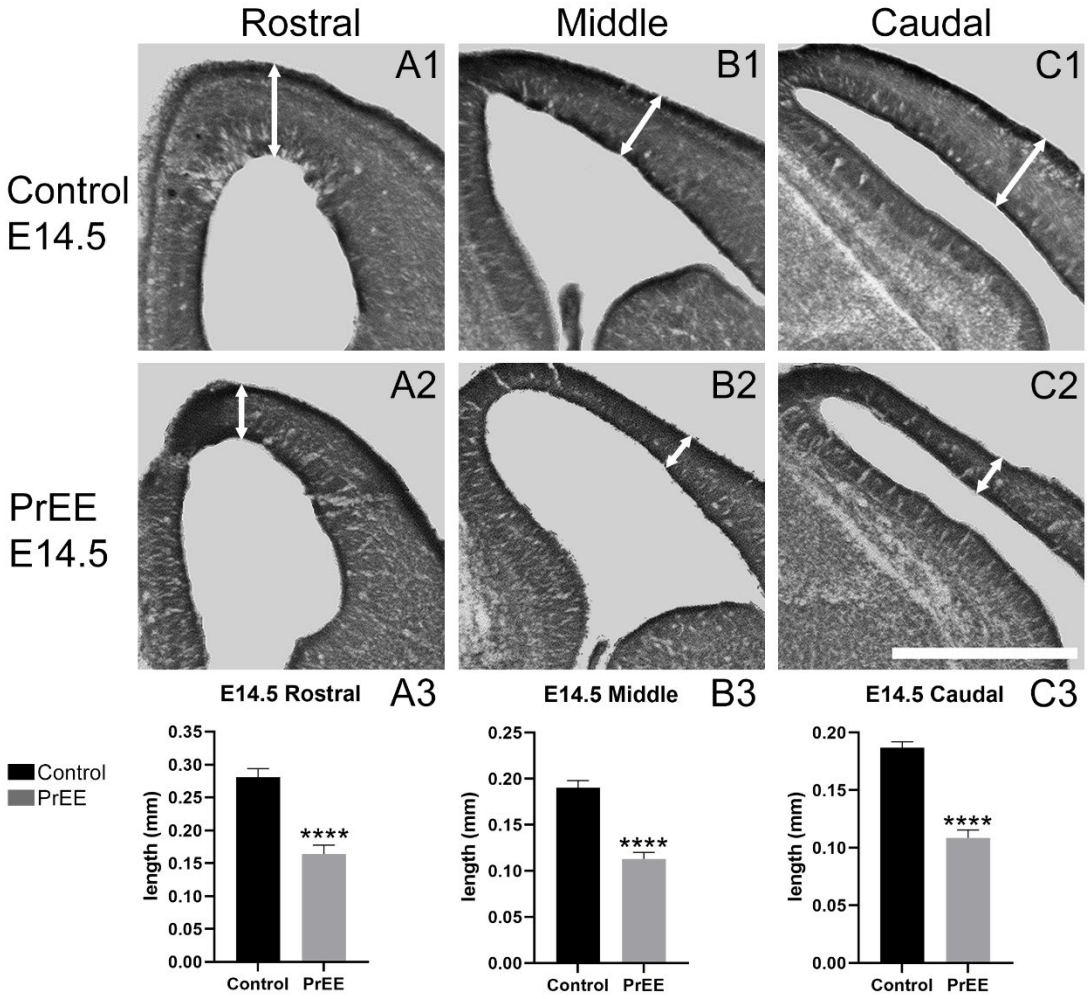


**Figure 1.11. Cortical thickness at E14.5.** A significant reduction in cortical thickness was identified in E14.5 PrEE mice (n = 9) in comparison to controls (n = 8) in rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions. (****$p$ < .0001). Scale bar 1000μm.

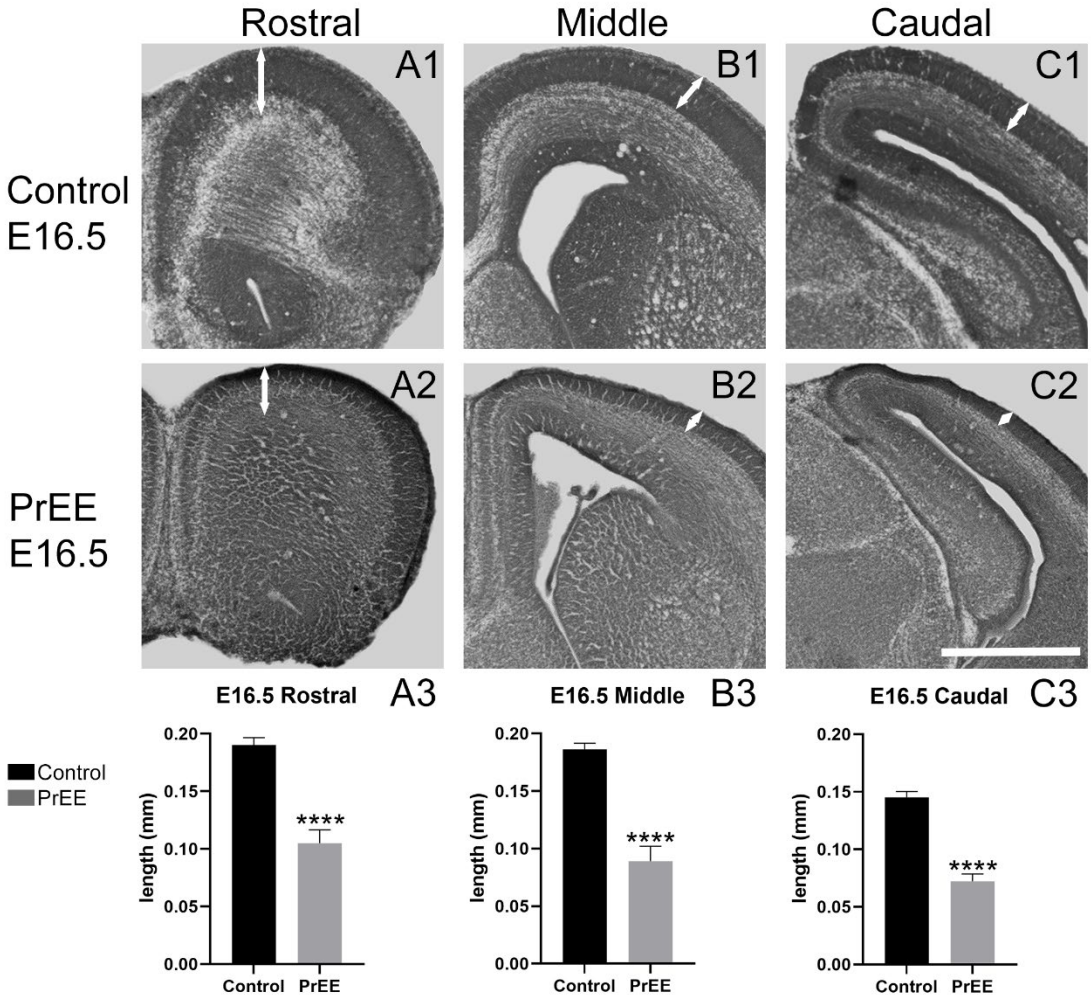


**Figure 1.12. Cortical thickness at E16.5.** Cortical thickness was also significantly reduced in E16.5 PrEE (n = 8) mice compared to controls (n = 9) in rostral (**A1**, **A2**), middle (**B1**, **B2**), and caudal (**C1**, **C2**) regions. (****$p$ < .0001). Scale bar 1000μm.

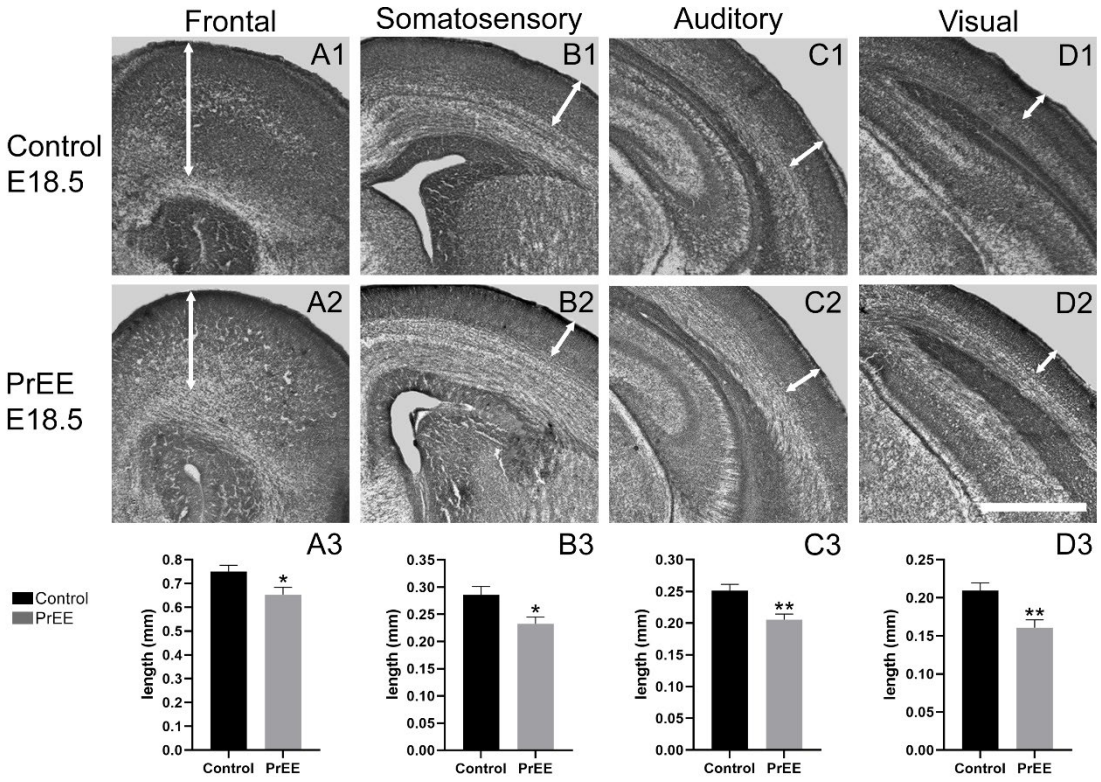


**Figure 1.13. Cortical thickness at E18.5.** A significant reduction in cortical thickness was observed in E18.5 PrEE (n = 7) mice compared to controls (n = 9) in frontal (**A1**, **A2**), somatosensory (**B1**, **B2**), auditory (**C1**, **C2**), and visual (**D1**, **D2**) regions of the cortex. (**p* < .05, ***p* < .01). Scale bar 500μm.

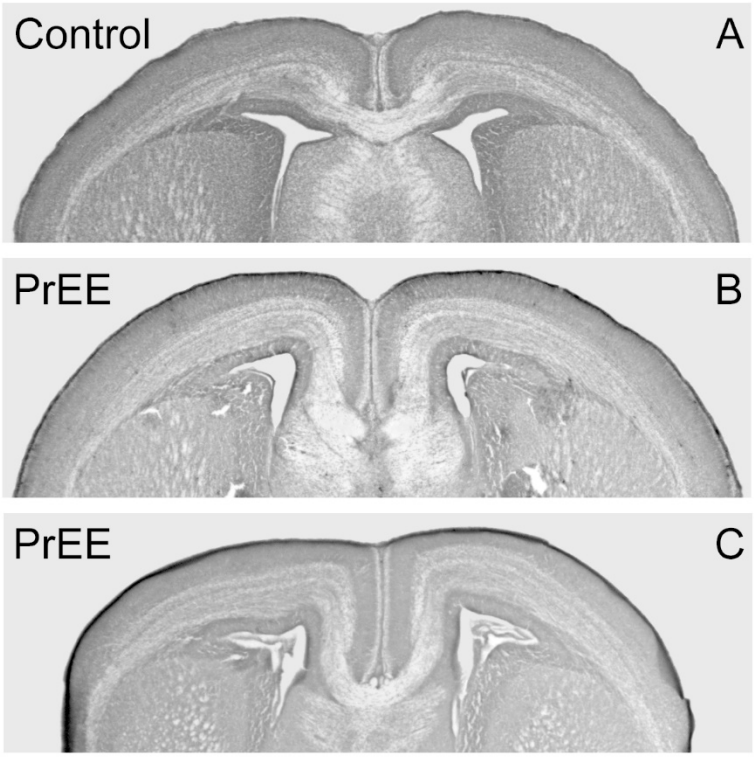


**Figure 1.14. Corpus Callosum at E18.5.** Representative cases showing differences in corpus callosum shape and thickness in control (n = 9) (**A**) and PrEE (n = 7) (**B, C**) Embryos at E18.5. Specifically, an abnormal “U” shape was observed at the midline in PrEE mice (**B**, **C**). Additionally, some cases of CC agenesis were observed in PrEE mice (**B**).

## REFERENCES


Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K.M., Huffman, K. J. (2016). The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice. *Alcohol Clin Exp Res., 40*(1),122–133.

Abbott, C. W., Rohac, D. J., Bottom, R. T., Patadia, S., Huffman, K. J. (2018). Prenatal ethanol exposure and neocortical development: a transgenerational model of FASD. *Cereb Cortex, 28*, 2908–2921.

Abdelrahman, A., & Conn, R. (2009). Eye abnormalities in fetal alcohol syndrome. *Ulster Med J., 78*(3), 164-5. PMID: 19907681; PMCID: PMC2773598.

Archibald, S. L., Fennema-Notestine, C., Gamst, A., Riley, E. P., Mattson, S. N., & Jernigan, T. L. (2001). Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev Med Child Neurol. 43(3),* 148-54. *Erratum in: Dev Med Child Neurol, 43(7),* 504. PMID: 11263683.

Astley, S. J., & Clarren, S. K. (2000). Diagnosing the full spectrum of fetal alcohol exposed individuals: introducing the 4-digit diagnostic code. *Alcohol Alcohol, 35*, 400–10.

Astley, S.J., Carmichael Olson, H., Kerns, K., Brooks, A., Alyward, E. H., Coggins, T. E., Davies, J., Dorn, S., Gendler, B., Jirikowic, T., Kraegel, P., Maravilla, K., & Richards, T. (2009). Neuropsychological and behavioral outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *Can J Clin Pharmacol. 16*(1), e178–e201.

Astley, S. J., & Clarren, S. K. (1995). A fetal alcohol syndrome screening tool. *Alcohol. Clin. Exp. Res., 19,* 1565–1571. doi: 10.1111/j.1530-0277.1995.tb01025.x.

Astley, S. J., Magnuson, S. I., Omnell, L. M., & Clarren, S. K. (1999). Fetal alcohol syndrome: Changes in craniofacial form with age, cognition, and timing of ethanol exposure in the macaque. *Teratology, 59,*163–172.

Autti-Rämö I. (2002). Foetal alcohol syndrome--a multifaceted condition. *Dev Med Child Neurol., 44*(2),141-4. doi: 10.1017/s0012162201001839.

Balaraman, S., Tingling, J. D., & Miranda, R. C. (2013). Dysregulation of microRNA expression and function contributes to the etiology of fetal alcohol spectrum disorders. *Alcohol Res 31*(1), 18-24.

Baldock, R., Bard, J., Davidson, D. R., Morriss-Kay, G. (2016). Kaufman's atlas of mouse development supplement.

Bartlett, E. L. (2013). The organization and physiology of the auditory thalamus and its role in processing acoustic features important for speech perception. *Brain Lang, 126*(1), 29-48. doi: 10.1016/j.bandl.2013.03.003

Bekdash, R. A., Zhang, C., & Sarkar, D.K. (2013). Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in β-endorphin-producing POMC neurons of the hypothalamus. *Alcohol Clin Exp Res. 37*(7),1133-42.

Blanck-Lubarsch, M., Dirksen, D., Feldmann, R., Sauerland, C., & Hohoff, A. (2019). Children with Fetal Alcohol Syndrome (FAS): 3D-analysis of palatal depth and 3D-metric facial length. *Int J Environ Res Public Health. 17*(1), 95. doi: 10.3390/ijerph17010095

Burd, L. (2016). FASD and ADHD: Are they related and how? *BMC Psychiatry*, *16*(1), 325.

Boschuetz, N., Cheng, S., Mei, L., & Loy, V. M. (2020). Changes in alcohol use patterns in the United States during COVID-19 pandemic. *WMJ, 119,* 171–176.

Bookstein, F. L., Connor, P. D., Huggins, J. E., Barr, H. M., Pimentel, K. D., Streissguth, A. P. (2007). Many infants prenatally exposed to high levels of alcohol show one particular anomaly of the corpus callosum. *Alcohol Clin Exp Res, 31,* 868–879.

Bookstein, F. L., Sampson, P. D., Connor, P.D., & Streissguth, A.P. (2002) Midline corpus callosum is a neuroanatomical focus of fetal alcohol damage. *Anat Rec B New Anat, 269,* 162–174.

Boronat, S., Sánchez-Montañez, A., Gómez-Barros, N., Jacas, C., Martínez-Ribot, L., Vázquez, E., & Del Campo, M. (2017). Correlation between morphological MRI findings and specific diagnostic categories in fetal alcohol spectrum disorders. *Eur J Med Genet., 60*(1), 65-71. doi: 10.1016/j.ejmg.2016.09.003.

Bottom, R. T., Abbott, C. W., 3rd, & Huffman, K. J. (2020). Rescue of ethanol-induced FASD-like phenotypes via prenatal co-administration of choline. *Neuropharmacology, 168,* 107990.doi:10.1016/j.neuropharm.2020.107990

Bottom, R. T., Kozanian, O. O., Rohac, D. J., Erickson, M. A., & Huffman, K. J. (2022). Transgenerational effects of prenatal ethanol exposure in prepubescent mice. *Front. Cell Dev. Biol., 10,* 812429. doi: 10.3389/fcell.2022.812429

Bower, E., Szajer, J., Mattson, S. N., Riley, E. P. & Murphy, C. (2013). Impaired odor identification in children with histories of heavy prenatal alcohol exposure. *Alcohol, 47*(4). doi: 10.1016/j.alcohol.2013.03.002

Bowman, G. D., & Poirier, M. G. (2015). Post-translational modifications of histones that influence nucleosome dynamics. *Chemical Reviews, 115*(6), 2274-2295. https://doi.org/10.1021/cr500350x

Carter, R. C., Jacobson, J. L., Sokol, R. J., Avison, M. J., & Jacobson, S. W. (2013). Fetal alcohol-related growth restriction from birth through young adulthood and moderating effects of maternal prepregnancy weight. *Alcoholism, clinical and experimental research, 37*(3), 452–462. https://doi.org/10.1111/j.1530-0277.2012.01940.x

Centers for Disease Control and Prevention. (2024a, May 15). *About alcohol use during pregnancy.* Centers for Disease Control and Prevention. https://www.cdc.gov/alcohol-pregnancy/about/index.html

Centers for Disease Control and Prevention. (2024b, May 15). *About fetal alcohol spectrum disorders (fasds).* Centers for Disease Control and Prevention. https://www.cdc.gov/fasd/about/index.html

Centers for Disease Control and Prevention. (2024c, May 15). *Data and statistics on alcohol and pregnancy*. Centers for Disease Control and Prevention. https://www.cdc.gov/alcohol-pregnancy/data-research/facts-stats/?CDC_AAref_Val=https%3A%2F%2Fwww.cdc.gov%2Fncbddd%2Ffasd%2Fdata.html

Chan, T., Bowell, R., O'Keefe, M., & Lanigan, B. (1991). Ocular manifestations in fetal alcohol syndrome. *The British journal of ophthalmology, 75,* 524–526.

Chee, K., Razmara, A., Geller, A. S., Harris, W. B., Restrepo, D., Thompson, J. A., & Kramer, D. R. (2022). The role of the piriform cortex in temporal lobe epilepsy: A current literature review. *Fron Neurol., 13*, 1042887 doi:10.3389/fneur.2022.1042887

Chen, V. S., Morrison, J. P., Southwell, M. F., Foley, J. F., Bolon, B., & Elmore, S. A. (2017). Histology atlas of the developing prenatal and postnatal mouse central nervous system, with emphasis on prenatal days E7.5 to E18.5. *Pathol., 45*(6), 705–744. doi:10.1177/0192623317728134.

Church, M.W., & Kaltenbach, J.A. (1997). Hearing, speech, language,and vestibular disorders in the fetal alcohol syndrome: A literature review. *Alcoholism Clinical and Experimental Research, 21*(3), 495–512.

Clark, C.M., Li, D., Conroy, J., Conroy, R. & Loock, C. (2000). Structural and functional brain integrity of fetal alcohol syndrome in nonretarded cases. *Pediatrics, 105,* 1069– 1099.

Clarren, S. K., Alvord, E. C., Sumi, S. M., Streissguth, A. P., & Smith, D. W. (1978). Brain malformations related to prenatal exposure to ethanol. *The Journal of Pediatrics, 92*(1), 64-67.

Clarren, S. K., & Smith, D.W. (1978). The fetal alcohol syndrome. *N Engl J Med, 92,* 878–883.

Coulter, C. L., Leech, R. W., Schaefer, G.B., Scheithauer, B. W., & Brumback, R. A. (1993). Midline cerebral dysgenesis, dysfunction of the hypothalamic–pituitary axis and fetal alcohol effects. Archives of *Neurology, 50*, 771–775.

de la Monte, S.M., & Wands, J.R. (2002). Chronic gestational exposure to ethanol impairs insulin-stimulated survival and mitochondrial function in cerebellar neurons. *Cell Mol. Life Sci., 59,* 882–893.

Del Campo, M., & Jones, K. L. (2017). A review of the physical features of the fetal alcohol spectrum disorders. *Eur J Med Genet, 60,* 55–64.

Desmond, M. E., & Jacobson, A.G. (1977). Embryonic brain enlargement requires cerebrospinal fluid pressure. *Dev Biol. 57,* 188–198.

Dursun, I., Jakubowska-Dogru, E., van der List, D., Liets, L. C., Coombs, J. L., & Berman, R. F. Effects of early postnatal exposure to ethanol on retinal ganglion cell morphology and numbers of neurons in the dorsolateral geniculate in mice. *Alcohol Clin Exp Res., 35*, 2063–2074.

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011a). A lifespan analysis of intraneocortical connections and gene expression in the mouse I. *Cereb Cortex, 21,* 1311–1330.

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011b). A lifespan analysis of intraneocortical connections and gene expression in the mouse II. *Cereb Cortex, 21,* 1331–1350.

El Shawa, H., Abbott, C. W., & Huffman, K. J. (2013). Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene Expression, and behavior in a mouse model of FASD. *J Neurosci, 33*, 18893–18905.

England, L. J., Bennett, C., Denny, C. H., Honein, M. A., Gilboa, S. M., Kim, S. Y., Gery, G. P., Tran, E. L., Rose, C. E. Bohm, M. K., & Boyle C. A. (2020). Alcohol use and co-use of other substances among pregnant females aged 12-44 Years - United States, 2015-2018. *MMWR Morb. Mortal. Wkly. Rep. 69,* 1009–1014. doi:10.15585/mmwr.mm6931a1

Fothergill, T., Donahoo, A. L., Douglass, A., Zalucki, O., Yuan, J., Shu, T., Goodhill, G. J., & Richards, L. J. (2014). Netrin-DCC signaling regulates corpus callosum formation through attraction of pioneering axons and by modulating Slit2-mediated repulsion. *Cereb Cortex, 24,*1138–51

Gangisetty, O., Chaudhary, S., Palagani, A., & Sarkar, D. K. (2022). Transgenerational inheritance of fetal alcohol effects on proopiomelanocortin gene expression and methylation, cortisol response to stress, and anxiety-like behaviors in offspring for three generations in rats: Evidence for male germline transmission. *PLoS ONE, 17*(2). https://doi.org/10.1371/journal.pone.0263340.

Gavrish, M., Kustova, A., Celis Suescún, J.C., Bessa, P., Mitina, N., & Tarabykin, V. (2024). Molecular mechanisms of corpus callosum development: a four-step journey. *Front Neuroanat., 17,* 1276325. doi: 10.3389/fnana.2023.1276325.

Godin, E. A., O'Leary-Moore, S. K., Khan, A. A., Parnell, S. E., Ament, J. J., Dehart, D. B., Johnson, B. W., Johnson, G. A., Styner, M. A., & Sulik K. K. (2010). Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: effects of acute insult on gestational day 7. *Alcoholism, Clinical and Experimental Research, 34*(1), 98–111.

Hammond, P., Suttie, M., Wetheril, L., Foroud, T., & Jacobson, S. (2011). Analysing facial dysmorphism in 3D. *Alcoholism: Clinical and Experimental Research 35,* 266A.

Hendricks, G., Malcolm-Smith, S., Adnams, C., Stein, D. J., & Donald, K. A. M. (2019). Effects of prenatal alcohol exposure on language, speech and communication outcomes: a review longitudinal studies. *Acta Neuropsychiatr. 31*(2), 74-83. doi: 10.1017/neu.2018.28.

Hoyme, H. E., Kalberg, W. O., Elliott, A. J., Blankenship, J., Buckley, D., Marais, A. S., Manning, M. A., Robinson, L. K., Adam, M. P., Abdul-Rahman, O., Jewett, T., Coles, C. D., Chambers, C., Jones, K.L., Adnams, C.M., Shah, P. E., Riley, E. P., Charness, M. E., Warren, K. R., & May, P. A. (2016). Updated clinical guidelines for diagnosing Fetal Alcohol Spectrum Disorders. *Pediatrics 138*, e20154256–e20154256.

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D.F., Koch, C., Genz, K., Price, M.T., Stefovska, V., Hörster, F., Tenkova, T., Dikranian, K., & Olney, J. W. (). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science, 287*(5455),1056-60. doi: 10.1126/science.287.5455.1056.

Jacobson, A. G. (1966). Inductive processes in embryonic development. *Science 152,* 25–34. 10.1126/science.152.3718.25.

Jang, H. S., Shin, W. J., Lee, J. E., & Do, J. T. (2017). CpG and non-CpG methylation in epigenetic gene regulation and brain function. *Genes, 8*(6), 148

Jarmasz, J. S., Basalah, D. A., Chudley, A. E., & Del Bigio, M. R. (2017). Human brain abnormalities associated with prenatal alcohol exposure and fetal alcohol spectrum disorder. *J Neuropathol Exp Neurol 76*(9) 813-833.

Jones, K. L., Smith, D. W., Ulleland, C. N., & Streissguth, P. (1973). Pattern of malformation in offspring of chronic alcoholic mothers. *The Lancet, 301*(7815), 1267-1271.

Jones-Gotman, M., Zatorre, R. J., Cendes, F., Olivier, A., Andermann, E., McMackin, D., Stauton, H., Siegel, A. M., & Wieser, H.G. (1997). Contribution of medial versus lateral temporal-lobe structures to human odour identification. *Brain, 120*(10,1845–1856.

Kalberg, W. O., Provost, B., Tollison, S. J., Tabachnick, B. G., Robinson, L. K., Hoyme, H. E., Trujillo, P. M. Buckley, D., Aragon, A. S., & May, P. A. (2006). Comparison of motor delays in young children with Fetal Alcohol Syndrome to those with prenatal alcohol exposure and with no prenatal alcohol exposure. *Alcohol. Clin. Exp. Res., 30*(12), 2037-2045. https://doi.org/10.1111/j.1530-0277.2006.00250.x

Kaufmann, W.E., & Moser, H.W. Dendritic anomalies in disorders associated with mental retardation. Cereb. Cortex 2000, 10, 981–991.

Keen, C. L., Uriu-Adams, J. Y., Skalny, A., Grabeklis, A., Grabeklis, S., Green, K., Yevtushok, L., Wertelecki, W. W., & Chambers, C.D. (2010). The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example. *Biofactors, 36*(2), 125-35. doi: 10.1002/biof.89. PMID: 20333752; PMCID: PMC2927848.

Kelly, S. J., Day, N., & Streissguth, A. P. (2000). Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicol Teratol., 22*(2):143-9. doi: 10.1016/s0892-0362(99)00073-2.

Kerschensteiner, D., & Guido, W. (2017). Organization of the dorsal lateral geniculate nucleus in the mouse. *Vis Neurosci., 34*, E008. doi: 10.1017/S0952523817000062.

Klintsova, A. Y., Helfer, J. L., Calizo, L. H., Dong, W. K., Goodlett, C. R., & Greenough, W. T. (2007). Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. *Alcohol Clin Exp Res., 31*(12), 2073-82. doi: 10.1111/j.1530-0277.2007.00528.x.

Kodituwakku, P. W. (2009). Neurocognitive profile in children with fetal alcohol spectrum disorders. *Dev Disabil Res Rev. 15*(3):218-24. doi: 10.1002/ddrr.73.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell, 128*(4), 693-705. doi: 10.1016/j.cell.2007.02.005.

Kugel, J. F., & Goodrich, J. A. (2012). Non-coding RNAs: key regulators of mammalian transcription. *Trends Biochem Sci, 37*(4),144-51. doi: 10.1016/j.tibs.2011.12.003.

Lange, S., Probst, C., Gmel, G., Rehm, J., Burd, L., & Popova, S. (2017). Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis. *JAMA Pediatr, 171,* 948–56.

Leucht, P., Kim, J. B., Amasha, R., James, A. W., Girod, S., & Helms, J.A. (2008). Embryonic origin and Hox status determine progenitor cell fate during adult bone regeneration. *Development, 135*(17):2845-54. doi: 10.1242/dev.023788.

Light, K. E., Ge, Y., & Belcher, S.M. (2001). Early postnatal ethanol exposure selectively decreases BDNF and truncated TrkB-T2 receptor mRNA expression in the rat cerebellum. *Brain Res. Mol. Brain Res, 93,* 46–55.

Lipinski, R. J., Hammond, P., O'Leary-Moore, S. K., Ament, J. J., Pecevich, S. J., Jiang, Y., Budin, F., Parnell, S. E., Suttie, M., Godin, E. A., Everson, J. L., Debart, D. B., Oguz, I., Holloway, H. T., Styner, M. A., Johnson, G. A., & Sulik, K. K. (2012). Ethanol-induced face-brain dysmorphology patterns are correlative and exposure-stage dependent. *PLOS One. 7*(8)

Lussier, A. A., Weinberg, J., & Kobor, M. S. (2017). Epigenetics studies of fetal alcohol spectrum disorder: where are we now? *Epigenomics, 9*(3), 291-311. doi: 10.2217/epi-2016-0163

MacFarlane, L., & Murphy, P. R. (2010). MicroRNA: Biogenesis, function and role in cancer. *Curr Genomics, 11*(7), 537-561. doi: 10.2174/138920210793175895

Majewski, F. 1981 Alcohol embryopathy: Some facts and speculations about pathogenesis. *Neurobehav. Toxicol. Teratol., 3*, 129-144.

Manning, M. A., & Hoyme, H. H. (2007). Fetal alcohol spectrum disorders: A practical clinical approach to diagnosis. *Neurosci Biobehav Rev, 31*(2), 230–8.

Marín O., & Rubenstein J. L. R. (2001). A long, remarkable journey: Tangential migration in the telencephalon. *Nat. Rev. Neurosci., 2,* 780–790. doi: 10.1038/35097509

Matthews, E., Dewees, K. Diaz, D. & Favero, C. (2021). White matter abnormalities in fetal alcohol spectrum disorders: Focus on axon growth and guidance. *Experimental Biology and Medicine*, *246*, 812–821. DOI: 10.1177/1535370220980398

Mattson, S. N., Riley, E. P., Jernigan, T. L., Garcia, A., Kaneko, W. M., Ehlers, C. L., & Jones, K. L. (1994). A decrease in the size of the basal ganglia following prenatal alcohol exposure: A preliminary report. *Neurotoxicol Teratol.16*(3), 283-9.

Mattson, S. N., Riley, E. P., Sowell, E. R., Jernigan, T. L., Sobel, D.F., & Jones, K. L. (1996) A decrease in the size of the basal ganglia in children with fetal alcohol syndrome. *Alcohol Clin Exp Res, 20,* 1088–1093.

Mattson, S. N., Schoenfeld, A. M., & Riley, E.P. (2001). Teratogenic effects of alcohol on brain and behavior. *Alcohol Research and Health, 25*(3), 185–191.

Mattson, S. N., Roesch, S. C., Fagerlund, A., Autti-Ramo, I., Jones, K. L., May, P. A., Adnams, C. M., Konovalova, V., & Riley, E. P. Toward a neurobehavioral profile of fetal alcohol spectrum disorders. Alcoholism: Clinical and Experimental Research. 2010;34(9):1640–1650.

May, P. A., Chambers, C. D., Kalberg, W. O., Zellner, J., Feldman, H., Buckley, D., Kopald, D., Hasken, J. M., Xu, R., Honerkamp-Smith, G., Taras, H., Manning MA, Robinson LK, Adam MP, Abdul-Rahman O, Vaux K, Jewett T., Elliott, A. J., Kable, J. A., Akshoomoff, N., Daniel, F., Arroyo, J. A., Hereld, D., Riley, E. P., Charness, M. E., Coles, C. D., Warren, K. R., Jones, K. L., & Hoyme, H. E. (2018). Prevalence of fetal alcohol spectrum disorders in 4 US communities. *J AmMed Assoc, 319*, 474–482.

May, P. A., Hasken, J. M., Hooper, S. R., Hedrick, D. M., Jackson-Newsom, J., Mullis, C. E., Dobyns, E., Kalberg, W., Buckley, D., Robinson. L., Abdul-Rahman, O., Adam, M.P., Manning. M.A., Jewett. T., & Hoyme, H. E. (2021). Estimating the community prevalence, child traits, and maternal risk factors of fetal alcohol spectrum disorders (FASD) from a random sample of school children. *Drug and Alcohol Dependence, 227,* https://doi.org/10.1016/j.drugalcdep.2021.108918

Miniaccchi, D., Granato, A., Santarelli, M., & Sbriccoli, A. (1993). Modifications of thalamo-cortical circuitry in rats prenatally exposed to ethanol. *Neuroreport, 4*(4), 415-418.

Miranda, R. C. (2012) MicroRNAs and fetal brain development: Implications for ethanol teratology during the second trimester period of neurogenesis. *Front Genet. 3,* 77. doi: 10.3389/fgene.2012.00077.

Mire, E., Hocine, M., Bazellieres, E., Jungas, T., Davy, A., Chauvet, S., & Mann, F. (2018). Developmental upregulation of ephrin-B1 silences Sema3C/ neuropilin-1 signaling during post-crossing navigation of corpus callosum axons. *Curr Biol, 28,*1768–82 e4.

Morris, M. J., & Monteggia, L.M. (2014). Role of DNA methylation and the DNA methyltransferases in learning and memory. *Dialogues Clin Neurosci., 16*(3), 359-71. doi: 10.31887/DCNS.2014.16.3/mmorris.

Muggli, E., Matthews, H., Penington, A., Claes, P., O'Leary, C., Forster, D., Donath, S., Anderson, P. J., Lewis, S., Nagle, C., Craig, J. M., White, S. M., Elliott, E. J., & Halliday, J. (2017). Association between prenatal alcohol exposure and craniofacial shape of children at 12 months of age. *JAMA Pediatr, 171*(8), 771-780. doi: 10.1001/jamapediatrics.2017.0778.

Nixon, K., &Crews, F.T. (2002). Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J Neurochem, 83*(5), 1087-93. doi: 10.1046/j.1471-4159.2002.01214.x.

O'Brien, J., Hayder, H., Zayed, Y., Peng, C. (2018). Overview of microRNA biogenesis, mechanisms of actions and circulation. *Front. Endocrinol, 9.* https://doi.org/10.3389/fendo.2018.00402

O'Leary-Moore, S. K., Parnell, S. E., Godin, E. A., Dehart, D. B., Ament, J. J., Khan, A. A., Johnson, G. A., Styner, M. A., & Sulik, K.K. (2010). Magnetic resonance microscopy-based analyses of the brains of normal and ethanol-exposed fetal mice. *Birth Defects Research Part A, Clinical and Molecular Teratology, 88*(11), 953–964.

O'Leary-Moore, S, K., Parnell, S. E., Lipinsky, R. J., & Sulik, K. K. (2011). Magnetic resonance-based imaging in animal models of Fetal Alcohol Spectrum Disorder. *Neuropsychol Rev, 21*,167–185.

Parnell, S. E., O'Leary-Moore, S. K., Godin, E. A., Dehart, D. B., Johnson, B. W., Johnson, G. A., Styner, M. A., & Sulik, K. K. (2009). Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: effects of acute insult on gestational day 8. *Alcoholism, Clinical and Experimental Research, 33*(6), 1001–1011.

Parnell, S. E., Holloway, H. T., O'Leary-Moore, S. K., Dehart, D. B., Paniaqua, B., Oguz, I., Budin, F., Styner, M. A., Johnson, G. A., & Sulik, K. K. (2013). Magnetic resonance microscopy-based analyses of the neuroanatomical effects of gestational day 9 ethanol exposure in mice. *Neurotoxicol Teratol, 39*, 77-83. doi: 10.1016/j.ntt.2013.07.009

Parviainen, R., Auvinen, J., Serlo, W., Järvelin, M. R., & Sinikumpu, J.J. (2020). Maternal alcohol consumption during pregnancy associates with bone fractures in early childhood. A birth-cohort study of 6718 participants. *Bone*, *137*, 115462, 10.1016/j.bone.2020.115462

Plachez, C., & Richards, L. J. (2005). Mechanisms of axon guidance in the developing nervous system. *Curr Top Dev Biol.* 69, 267–346.

Planques, A., Kerner, P., Ferry, L., Grunau, C., Gazave, E., & Vervoort, M. (2021). DNA methylation atlas and machinery in the developing and regenerating annelid Platynereis dumerilii. *BMC Biol, 19,* 148 https://doi.org/10.1186/s12915-021-01074-5.

Perez, R. F. Jr., Conner, K. E., Erickson, M. A., & Huffman, K. J. (2024). Transgenerational effects of prenatal ethanol exposure: Neuroanatomical and behavioral phenotypes in a mouse model of FASD. Manuscript in preparation.

Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C.L., Wynne, P. M., Siegelmann, H. T., & Treistman, S. N. (2008). Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron, 59*(2), 274-87. doi: 10.1016/j.neuron.2008.05.032.

Piper, M., Plachez, C., Zalucki, O., Fothergill, T., Goudreau, G., Erzurumlu, R., Gu, C., & Richards, L. J. (2009). Neuropilin 1-Sema signaling regulates crossing of cingulate pioneering axons during development of the corpus callosum. *Cereb Cortex, 19,* i11–21

Popova, S., Lange, S., Shield, K., Mihic, A., Chudley, A. E., Mukherjee, R. A. S., Bekmuradov, D., & Rehm, J. (2016). Comorbidity of fetal alcohol spectrum disorder: a systematic review and meta-analysis. *Lancet, 387*(10022). doi: 10.1016/S0140-6736(15)01345-8.

Ribeiro, I. M., Vale, P. J., Tenedorio, P. A., Rodrigues, P. A., Bilhoto, M.A., & Periera, H. C. (2007). Ocular manifestations in fetal alcohol syndrome. *European journal of ophthalmology, 17*, 104–109.

Riikonen, R. S., Salonen, I., Partanen, K., & Verho, S. (1999). Brain perfusion SPECT and MRI in fetal alcohol syndrome. *DevMed Child Neurol, 41*, 652–659.

Roebuck, T. M., Mattson, S. N., & Riley, E. P. (1998). A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcoholism: Clinical and Experimental Research, 22*(2), 339–344.

Saxonov, S., Berg, P., & Brutlag, D. L. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *PNAS, 103*(5), 1412-1417. https://doi.org/10.1073/pnas.0510310103

Shibata, M., Kurokawa, D., Nakao, H., Ohmura, T., & Aizawa, S. (2008). MicroRNA-9 modulates Cajal-Retzius cell differentiation by suppressing Foxg1 expression in mouse medial pallium. *J Neurosci, 28*, 10415–10421.

Shibata, M., Nakao, H., Kiyonari, H., Abe, T., & Aizawa, S. (2011). MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. *J Neurosci, 31*(9), 3407-22. doi: 10.1523/JNEUROSCI.5085-10.2011.

Sowell, E. R., Trauner, D. A., Gamst, A., & Jernigan, T. L. (2002). Development of cortical and subcortical brain structures in childhood and adolescence: A structural MRI study. D*ev Med Child Neurol, 44*(1), 4-16. doi: 10.1017/s0012162201001591. PMID: 11811649.

Stenman, J., Toresson, H. & Campbell, K. (2003). Identification of two distinct progenitor populations in the lateral ganglionic eminence: Implications for striatal and olfactory bulb neurogenesis. *Journal of Neuroscience, 23*(1) 167-174, DOI: https://doi.org/10.1523/JNEUROSCI.23-01-00167.2003

Streissguth, A.P, & O'Malley, K. (2000). Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. *Seminars in Clinical Neuropsychiatry, 5*(3), 177–190.

Strömland, K. (1987) Ocular involvement in the fetal alcohol syndrome. Surv *Ophthalmol, 31*(4), 277.

Sturrock, R. R. (1979). A morphological study of the development of the mouse choroid plexus. *J Anat, 129*, 777–793.

Sulik, K.K. (2018). *Prenatal alcohol exposure and abnormal brain development – findings from basic research.* In: Jonsson, E., Clarren, S., Binnie, I. (eds) Ethical and Legal Perspectives in Fetal Alcohol Spectrum Disorders (FASD). International Library of Ethics, Law, and the New Medicine, 75. Springer, Cham. https://doi.org/10.1007/978-3-319-71755-5_3

Sulik, K. K., Johnston, M. C., & Webb, M. A. (1981). Fetal alcohol syndrome: Embryogenesis in a mouse model. *Science, 214,* 936–938.

Sulik, K. K., & Johnston, M. C. (1982). Embryonic origin of holoprosencephaly: interrelationship of the developing brain and face. *Scan Electron Microsc, 1,* 309–322.

Sulik, K.K. & Johnston, M.C. (1983), Sequence of developmental alterations following acute ethanol exposure in mice: Craniofacial features of the fetal alcohol syndrome. *Am. J. Anat., 166*, 257-269. https://doi.org/10.1002/aja.1001660303

Swanson, L. W., & Petrovich, G. D. (1998). What is the amygdala? *Trends in Neuroscience, 21*(8), 323-331. https://doi.org/10.1016/S0166-2236(98)01265-X

Tan, C. H., Denny, C. H., Cheal, N. E., Sniezek, J. E., & Kanny, D. (2015). Alcohol use and binge drinking among women of childbearing age–United States 2011-2013. *MMWRMorb Mortal Wkly Rep, 64,* 1042–1046.

Temple, V.K., Cook, J.L., Unsworth, K., Rajani, H., & Mela, M. (2019). Mental health and affect regulation impairment in fetal alcohol spectrum disorder (FASD): Results from the Canadian National FASD Database. *Alcohol Alcohol, 54*(5), 545-550. doi: 10.1093/alcalc/agz049.

Thomas, J. D., Abou, E. J., & Domingues, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicol Teratol, 31*(5), 303-311.

Thomas, S. E., Kelly, S. J., Mattson, S. N., & Riley, E. P. (1998). Comparison of social abilities of children with fetal alcohol syndrome to those of children with similar IQ scores and normal controls. *Alcoholism: Clinical and Experimental Research, 22*, 528–533.

Veazey, K.J., Parnell, S.E., Miranda, R.C. & Golding, M. C. (2015). Dose-dependent alcohol-induced alterations in chromatin structure persist beyond the window of exposure and correlate with fetal alcohol syndrome birth defects. *Epigenetics & Chromatin, 8*, 39. https://doi.org/10.1186/s13072-015-0031-7

Webster, W. S., Walsh, D. A., Lipson, A. H., & McEwen, S. E. (1980). Teratogenesis after acute alcohol exposure in inbred and outbred mice. *Neurobehav Terato, 2*, 227–234,

Xu, Y., Tang, Y., & Li, Y. (2008). Effect of folic acid on prenatal alcohol-induced modification of brain proteome in mice. *Br J Nutr, 99*(3), 455-61. doi: 10.1017/S0007114507812074.

Yang, Y., Phillips, O. R., Kan, E., Sulik, K. K., Mattson, S. N., Riley, E. P., JOnes, K. L., Adnams, C. M. May, P. A. O'Connor, M. J., Narr, K. L. & Sowell, E. R. (2011a). Callosal thickness reductions relate to facial dysmorphology in fetal alcohol spectrum disorders. *Alcoholism, Clinical and Experimental Research, 36*(5). 798-806.

Yang, Y., Roussotte, F., Kan, E., Sulik, K. K., Mattson, S. N., Rile, E. P., Jones, K. L. Adnams, C. M., May, P. A. O'Connor, M. J., Narr, K. L., & Sowell, E. R. (2011b). Abnormal cortical thickness alterations in fetal alcohol spectrum disorders and their relationships with facial dysmorphology. *Cerebral cortex, 22*(5), 1170-1179.

Young, S. L., Gallo, L. A., Brookes, D. S. K., Hayes, N., Maloney, M. Liddle, K., James, A., Moritz, K. M., & Reid, N. (2022). Altered bone and body composition in children and adolescents with confirmed prenatal alcohol exposure. *Bone, 164*. https://doi.org/10.1016/j.bone.2022.116510

Zimmerberg, B., & Scalzi, L. V. (1989). Commissural size in neonatal rats: Effects of sex and prenatal alcohol exposure. *International Journal of Developmental Neuroscience, 7*, 81–86. doi: 10.1016/0736-5748(89)90046-4.

Zhou, V. W., Goren, A., & Bernstein, B. E. (2011). Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet, 12*(1), 7-18. doi: 10.1038/nrg2905.

## Chapter 2: Transgenerational effects of prenatal ethanol exposure on offspring neuroanatomy and behavior in a mouse model of FASD


### ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) encompass a range of biological and behavioral phenotypes in offspring exposed to ethanol via maternal consumption during pregnancy. In a series of studies, our laboratory has identified many deleterious effects of prenatal ethanol exposure (PrEE) in our FASD mouse model. In the first filial generation (F1) of exposed offspring, PrEE resulted in abnormal neocortical gene expression, ectopic intraneocortical connectivity, altered neuroanatomy, and disrupted behavior (El Shawa et al., 2013; Abbott et al., 2016; Kozanian et al., 2018). Additionally, our results suggest that PrEE can induce phenotypic change in brain and behavior that passes transgenerationally (Abbott et al., 2018; Bottom et al., 2022) most likely from epigenetic modifications. In the current study, to further explore potential heritable effects of PrEE, we investigate brain and behavioral development in the F1 (directly exposed), F2 (indirectly exposed) and F3 (non-exposed) generations. Comparative analyses of body weight, brain weight, cortical length, and measures from selected thalamic nuclei, and subcortical structures were evaluated in control, F1, F2, and F3 PrEE newborn mice as well as behavior at wean age. All generations of PrEE newborns had decreased body weights, brain weights and neocortical lengths compared to controls, although there were no

differences in brain to body weight ratios. Control litters were bred alongside the F1-3 PrEE generations and, due to low variability, were collapsed into a single control group for analyses. Quantitative measures in F1, F2, and F3 newborn PrEE mice demonstrated altered thickness and subcortical volumes in some areas. Specifically, hippocampal CA3 was significantly thinner in all generations of PrEE mice when compared to controls. Additionally, PrEE resulted in a significant rate of agenesis or partial development of the corpus callosum in the majority of F1 cases, with a less frequent, non-significant, occurrence in F2 and F3 mice. Finally, we found that disrupted sensorimotor integration, motor control, and anxiety-like behavior persisted to at least the F2 generation. Our data suggest that PrEE can result in abnormal brain and behavioral development with heritable effects that persist transgenerationally to subsequent generations of offspring.

## INTRODUCTION

Prenatal exposure to alcohol, or ethanol, can disrupt typical brain and behavioral development, and in humans, this can lead to the development of Fetal Alcohol Spectrum Disorders (FASD). The effects of prenatal ethanol exposure, or PrEE, on offspring are quite variable, with factors such as dosing, period of gestational exposure, and maternal tolerance playing key roles in the variability. Multiple studies have identified hallmarks of PrEE and FASD, including deficits in sensory-processing, behavior, motor learning, spatial

functioning, anxiety, and depression (Lemoine et al., 1968; Jones et al., 1973a; 1973b; Kalberg et al., 2006; Hellemans et al., 2010a; 2010b). Previous studies from our laboratory, in a PrEE mouse model of FASD, have demonstrated a host of phenotypes in the directly exposed offspring (first filial generation, F1). Specifically, we have shown developmental abnormalities in neuroanatomy, neocortical gene expression, neocortical connections, and behavior due to in utero ethanol exposure (El Shawa et al., 2013; Abbott et al., 2016; 2018, Kozanian et al., 2018; Bottom et al., 2020; 2022). PrEE-induced neural phenotypes observed in young PrEE animals may be substrates for sensorimotor, perceptual, cognitive, and behavioral deficits observed in humans with FASD.

It has been understood for some time that the consumption of alcohol during pregnancy increases the risk of complications or pregnancy loss (Aliyu et al., 2008; Strandberg-Larsen et al., 2008; Windham et al., 2015). Despite the CDC warning, which states that there is “no safe amount” of maternal alcohol consumption during pregnancy, incidence rates for FASD have been estimated to be around 5% in the United States (May et al., 2018), with some sub-populations as high as 7% (May et al., 2021). Actual incidence rates may be higher in the US and globally due to low maternal reporting. The CDC’s most recent statistics on alcohol use during pregnancy showed that 1 in 7 mothers or 14% drank at some point during their pregnancies, whereas in 2019, rates were lower with 1 in 9 mothers or about 11% drinking during pregnancy (CDC, 2022).

These data suggest an overall increase in alcohol consumption during pregnancy in recent years. This apparent rise in gestational drinking, coupled with the increase in alcohol use among American females during the COVID-19 pandemic (Kerr et al., 2022) makes understanding the biological and behavioral effects of PrEE critical for health and well-being.

Since Fetal Alcohol Syndrome, or FAS, was first described in the literature (Jones et al., 1973a), research efforts have focused on the F1 generation, the offspring directly exposed to alcohol while in utero, due to maternal consumption. Recently, however, we found that some of the phenotypes that characterize FASD in our mouse model were present in subsequent generations and that epigenetic modifications present in the brains of F1 offspring are likely playing a mechanistic role in the transgenerational inheritance of FASD-like phenotypes (Abbott et al., 2018; Bottom et al., 2022). To explore true transgenerational epigenetic change, filial generations must be extended beyond the directly exposed PrEE offspring. Any effects seen in the second generation (F2) would be considered intergenerational transmission, due to ethanol exposure of germ cells in the F1 animal (Gapp & Bohacek, 2018). Effects that persist to the third generation (F3) would represent true transgenerational transfer (Sarkar, 2015). Evidence is mounting for ethanol's ability to modify epigenetic pathways, subsequently resulting in a heritable pathology. Work published in our laboratory has demonstrated that PrEE induces epigenetic modifications in mice (Abbott et al., 2018). Specifically, an upregulation of neocortical gene expression was

observed along with promoter specific hypomethylation of specific genes (*RZRβ* & *Id2*) in conjunction with an overall decrease in global DNA methylation (Abbott et al., 2018). DNA methyltransferase (DNMT) expression was also lower in the P0 cortex. In conjunction, we have begun to unpack the slew of behavioral impacts observed in F2 and F3 generations (Abbott et al., 2018; Bottom et al., 2022). Notably, we reported the following alterations to behavioral phenotypes in peripubescent mice: abnormal sensorimotor processing, increased risk-taking behavior, and increased depressive-like behaviors that extend to the F3 generation.

In our current study, our goal was to extend upon the results reported in our previously published transgenerational PrEE research (Abbott et al., 2018; Bottom et al., 2022). To do this, we bred three generations of PrEE mice stemming from a single maternal ethanol exposure during pregnancy (the filial generation 0 dam). We bred control mice alongside each generation, to reduce the risk of confounds of breeding season or timing in our control data, and to produce alcohol naïve dams that recently gave birth for our cross fostering. We investigated whether PrEE could impact development of specific subcortical structures in offspring, beyond the directly exposed first generation. Additionally, using behavioral assays, we examined sensorimotor integration, motor control, and anxiety-like behavior as evaluated through the Suok test.

With rising alcohol consumption rates and relaxed views on drinking during pregnancy, we need to continue to deepen our understanding of the deleterious effects and heritability of FASD.

## MATERIALS AND METHODS

### *Animal Care*

All breeding and experimental studies were administered after careful consideration of the protocol guidelines approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. CD-1 mice used for breeding were purchased from Charles River Laboratories (Wilmington, MA/USA). We chose to perform our experiments with an outbred CD-1 mouse strain because these mice show superior resilience compared to inbred strains. Additionally, we have validated them as a model for prenatal ethanol exposure in our prior work (El Shawa et al., 2013) and as humans are outbred, they are a better model for human conditions such as FASD. Mice are housed in animal facilities located at the University of California, Riverside that are kept at approximately 22°C and are on a 12-hour light/dark cycle.

### *Ethanol administration and breeding paradigm*

The goal of the breeding was to produce 4 groups of pups: 1) control offspring born from ethanol-naïve dams and sires 2) F1 offspring born from ethanol-exposed dams (F0) and ethanol-naïve sires, 3) F2 offspring born from

and ethanol-naïve dams and F1 sires, 4) F3 offspring born from and ethanol-naïve dams and F2 sires.

To generate control and F1 offspring: Ethanol-naïve P90 female mice were paired with ethanol-naïve P90 male just before the dark cycle. Conception, embryonic day (E) 0.5, was determined by the presence of a vaginal plug and the pregnant female was moved to a separate cage. Water and mouse chow were provided to control dams *ad libitum*. Beginning on E 0.5, female dams in the ethanol-exposed group (F0) were given 25% ethanol solution in water and chow *ad libitum* until birth, pregnant control females were given *ad libitum* food and water that was calorie matched with maltodextrin. After a selection of control and PrEE F1 newborn mice were removed and euthanized for neuroanatomical studies, all remaining pups were cross-fostered with ethanol-naïve dams on the day of birth P0 to control for potential ethanol in breastmilk in experimental dams.

To generate F2/F3 offspring: F1 PrEE male offspring generated from F0 ethanol-treated dams were paired with alcohol-naïve females to breed the F2 generation. The subsequent generation (F3) was bred using F2 males paired with ethanol-naïve females. For the four conditions (Control, F1, F2, F3), 8-10 litters were bred per condition for neuroanatomical and behavioral measures. Dam data and pup brain and body weights across generations were taken from additional litters bred in the laboratory for other experiments, so the number of cases are higher. A summary of the breeding paradigm can be seen in Figure 2.1.

*Dam Data*

Food intake, weight gain and litter size: We measured food consumption each embryonic day to assess potential confounding differences in caloric intake between F1, F2, F3, and control dams. Mouse chow was weighed using a standard Fisher Scientific scale at the beginning of the active cycle and first thing in the morning, and the difference was calculated. Dam body weights were measured at conception, after detention of the plug which is during the light cycle and the day before birth, also during the light cycle, using a standard Fisher Scientific scale. The final weight gain was determined by subtraction.

Blood serum processing and ethanol concentration measurement

After control and ethanol-treated dams gave birth, pups were cross-fostered, and dams were euthanized by cervical dislocation and whole blood samples were collected via cardiac puncture. This was done early in the daytime, during the light cycle. Whole blood was incubated at room temperature (RT) for 15-20 minutes, then centrifuged for 15 minutes at 4,000G at 4°C in 1.5mL Eppendorf tubes to obtain serum. Serum was stored at 4°C and later used to quantify average blood ethanol concentration (BEC) using an alcohol reagent kit (Pointe Scientific; Canton, MI/USA). Briefly, 5µL of serum was combined with 1mL proprietary reagents from Pointe Scientific. Following a short incubation period, absorbance was read at 340 nm on a nanodrop 2000 spectrophotometer

for each sample. Each sample was analyzed in duplicate and compared to an alcohol standard.

This exposure paradigm is not designed to mimic any specific drinking habits observed in humans; however, it does produce an average BEC of around 100-140 mg/dL at peak times of consumption (El Shawa et al., 2013) which is similar to 0.08% blood alcohol concentration, or BAC, in humans. Although this is a high sustained alcohol level in human standards, murine models demonstrate a greater ability to break down alcohol based on a much higher metabolic rate (Cederbaum, 2012).

Dam data demonstrating the reliability of our PrEE model (El Shawa et al., 2013; Abbott et al., 2016; Bottom et al., 2020) and our transgenerational PrEE model (Abbott et al., 2018; Bottom et al., 2022) have been published repeatedly. Dam data collected for the current project (Figs. 2. 2, 2.3) did not differ significantly from data presented previously in our transgenerational model. For instance, in Abbott and colleagues (2018), we reported no significant differences in food intake or hydration (blood plasma osmolality) in the control and F1-3 dams. As expected, we found moderate BEC levels in ethanol-treated dams only, and zero BEC in ethanol-naïve dams (Fig. 2.2C). We did see consistent reductions in weight gains in F1-3 females when compared to control dams, however, this was correlated with a reduction in litter size for all three generations of PrEE offspring (see Fig. 2.3, Abbott et al., 2018).

*Pup weights and brain tissue preparation*

On the day of birth (Postnatal day (P) 0), control, F1, F2, and F3 pups were weighed. P0 pups to be used for neuroanatomical studies were euthanized via hypothermia and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were dissected from the skull, weighed, imaged, hemisected, and postfixed in 4% PFA.

*Anatomical measures*

Hemisected, post-fixed brains were cryoprotected in a 30% sucrose in phosphate-buffered saline (PBS) solution overnight and then cut in the coronal plane into 40 µm thick sections on a Leica cryostat. Sections were then stained with cresyl-violet following the standard protocol for Nissl, cover-slipped with Permount and imaged using a Zeiss Axio high-resolution (HRm) camera connected to a ZeissDiscovery.V12 stereomicroscope (Oberkochen, Germany). The areas measured were selected using recognizable landmarks based on a Developing Mouse Brain Atlas (Paxinos et al., 2007). Sections were measured across all cases using an electronic micrometer in ImageJ (NIH, Bethesda, MD, USA). Thickness measurements included the corpus callosum and the CA3 subregion of the hippocampus. Areal measurements included the dorsal lateral geniculate nucleus (dLGN), medial geniculate nucleus (MGN), ventral-posterior nucleus (VP) of the thalamus.

*Behavior analyses*

To evaluate behavioral phenotypes associated with transgenerational PrEE, we examined motor coordination, sensorimotor integration, and anxiety-like behavior in P20 control, F1, F2, and F3 mice through the use of a behavioral assay called the Suok test. Prior to commencement of the behavioral test, all mice were acclimated to the dimly lit behavioral room for a minimum of one hour. Behavioral sessions were recorded using an HD web camera and saved on a local desktop computer for documentation and further analyses. The apparatus was cleaned with Virkon between trials to eliminate olfactory cues.

The Suok test measures an animal's ability to integrate sensory input and control motor output while also reporting on anxiety-like behaviors (Kalueff et al., 2008; Glajch et al., 2012, El Shawa et al., 2013). The Suok apparatus was constructed in accordance with specifications published previously (Kalueff et al., 2008). The apparatus consists of a smooth 2-meter-long aluminum rod, 3-centimeters in diameter, elevated to a height of 20 centimeters. The rod is divided into 10-centimeter segments by colored markings and held in place between two clear acrylic walls. A 20-centimeter zone is marked at the center most point of the rod and serves as the placement location when starting the assay. At the start of the five-minute testing period, animals were placed on the center of the bar with the midline of the body parallel to the rod. Mice that fall off the apparatus are quickly placed back onto the rod in the position they fell from.

Several measures of behavior were observed and scored by trained observers: latency to leave the central zone, segments crossed, horizontal + vertical directed exploration, missteps, and falls. Reduced scores, with respect to control cases, signify a change in sensorimotor integration and motor coordination, while an increase in latency to leave center, decreases in exploration and grooming behaviors, and more instances of freezing behavior indicate anxiety-like behaviors. All time measures were documented with hand-held stopwatches and stereotyped behaviors were recorded during the test and verified using video recordings.

*Statistical Analyses*

All statistical analyses were completed using R (v4.1.2; R Core team, 2021). Between-subjects tests were carried out using analysis of variance (ANOVA). Repeated measures tests were performed using multilevel models via the lme4 R package (v1.1.27.1; Bates et al., 2015). Planned comparisons and simple effect tests were conducted using the emmeans R package (v1.7.2) using Dunnett's method to control for multiple comparisons between the control condition and the three treatment conditions and Bonferroni adjustments elsewhere (Dunnett, 1955; Lenth, 2022).

## RESULTS

### *Dam Measures*

In this study, we utilized our maternal ethanol self-administration paradigm to generate offspring prenatally exposed to ethanol, or F1 PrEE mice. In previous experiments, to ensure our exposure paradigm did not result in malnutrition or dehydration, we measured food and liquid intake, as well as blood ethanol concentration, blood plasma osmolality and dam weight changes throughout the pregnancy (El Shawa et al., 2013; Abbott et al., 2018; Bottom et al., 2020; 2022). No significant variation was detected in daily food or liquid intake consumption in our previous measures and those taken for the current study (Fig. 2.2A, B). Specifically, a one-way ANOVA failed to identify differences in the amount of food consumed per day by pregnant dams across the four conditions, $F(3,32)=0.24$, $p=0.8656$. Likewise, planned comparisons between the dams carrying the control mice and those carrying each filial generation failed to yield significant differences between the daily food consumption of dams in the control condition ($M=7.75$ g/day, $SD=1.34$ g/day, 95% CI [6.63, 8.87]) and in the F1 condition ($M=7.29$ g/day, $SD=1.77$ g/day, 95% CI [5.81, 8.77]), $t(32)=0.62$, $p=0.8417$, the F2 condition ($M=7.29$ g/day, $SD=1.28$ g/day, 95% CI [6.38, 8.21]), $t(32)=0.64$, $p=0.8292$, or the F3 condition ($M=7.18$ g/day, $SD=1.59$ g/day, 95% CI [6.04, 8.32]), $t(32)=0.81$, $p=0.7390$. Also, a one-way ANOVA failed to identify differences in the amount of liquid consumed per day by pregnant dams across the four conditions, $F(3,32)=2.03$, $p=0.1297$. Furthermore, planned comparisons

between the dams carrying the control mice and those carrying each filial generation failed to yield significant differences between the daily liquid consumption of dams in the control condition ($M$=13.69 mL/day, $SD$=0.70 mL/day, 95% CI [13.10, 14.28]) and in the F1 condition ($M$=12.34 mL/day, $SD$=0.99 mL/day, 95% CI [11.52, 13.17]), $t$(32)=2.29, $p$=0.0760, the F2 condition ($M$=12.58 mL/day, $SD$=1.21 mL/day, 95% CI [11.71, 13.45]), $t$(32)=1.99, $p$=0.1401, or the F3 condition ($M$=12.85 mL/day, $SD$=1.51 mL/day, 95% CI [11.77, 13.93]), $t$(32)=1.50, $p$=0.3251.

When measured at wean, EtOH exposed dams had greater blood ethanol concentrations (BEC; $M$=138.7 mg/dL, $SD$=8.0 mg/dL, 95% CI [130.3, 147.1]) than control dams ($M$=0.0 mg/dL, $SD$=0.0 mg/dL, 95% CI [0.0, 0.0]), $t$(5)=42.48, $p$<.001 (Fig. 2.2C). To ensure that the exposure paradigm did not result in dehydration, blood plasma osmolality (pOsm) was measured at wean (Fig. 2.2D). There was no evidence of a difference in blood plasma osmolality at wean between the EtOH exposed ($M$=311.81 mOsm/kg, $SD$=8.44 mOsm/kg, 95% CI [307.32, 316.31]) and the control dams ($M$=313.07 mOsm/kg, $SD$=6.82 mOsm/kg, 95% CI [309.13, 317.01]), $t$(27.85)=0.4515, $p$=.6551. This suggests that our exposure paradigm did not induce dehydration in experimental dams.

As previously reported (El Shawa et al., 2013; Abbott et al., 2018), this breeding paradigm results in a significant reduction in litter size for ethanol-treated dams with litter sizes differing across the four conditions, $F$(3,35)=5.10, $p$=0.0049 (Fig. 2.3A). Planned comparisons between the size of the control litters

and those of each filial generation showed greater litter sizes for the control mice ($M$=11.70 pups, $SD$=2.87 pups, 95% CI [9.65, 13.75]) than the F1 litters ($M$=8.33 pups, $SD$=1.58 pups, 95% CI [7.12, 9.55]), $t$(35)=3.39, $p$=0.005, the F2 litters ($M$=8.80 pups, $SD$=1.87 pups, 95% CI [7.46, 10.14]), $t$(35)=3.00, $p$=0.0139, and the F3 litters ($M$=8.70 pups, $SD$=2.06 pups, 95% CI [7.23, 10.17]), $t$(35)=3.10, $p$=0.0107. This is consistent with previous results obtained using this treatment paradigm (El Shawa et al., 2013; Abbott et al., 2016; Abbott et al., 2018; Bottom et al., 2022). Ethanol exposed dams gained less weight over the course of the pregnancy when compared to control dams (Fig. 2.3B). A one-way ANOVA identified differences in the dams' gestational weight change across the four conditions, $F$(3,32)=4, $p$=0.0158. Additionally, planned comparisons showed differences between the gestational weight change of control dams ($M$=24.39 g, $SD$=2.75 g, 95% CI [22.09, 26.69]) and the F1 dams ($M$=18.15 g, $SD$=5.21 g, 95% CI [13.79, 22.51]), $t$(32)=2.72, $p$=0.0288, the F2 dams ($M$=17.77 g, $SD$=4.52 g, 95% CI [14.54, 21.00]), $t$(32)=3.04, $p$=0.0131, and the F3 dams ($M$=18.18 g, $SD$=5.24 g, 95% CI [14.43, 21.93]), $t$(32)=2.85, $p$=0.0209. This effect was related to litter size, as a dam with fewer pups would show a reduced weight gain. We demonstrate this by analyzing weight gain while controlling for litter size (Fig. 2.3C). A one-way ANOVA failed to identify differences in the gestational weight change per pup by pregnant dams across the four conditions, $F$(3,32)=0.08, $p$=0.9716. Additionally, planned comparisons between the dams carrying the control mice and those carrying each filial generation failed to yield significant

differences between the gestational weight change per pup in the control condition ($M$=2.09 g, $SD$=0.60 g, 95% CI [1.58, 2.59]) and in the F1 condition ($M$=2.28 g, $SD$=0.93 g, 95% CI [1.50, 3.06]), $t$(32)=0.48, $p$=0.9036, the F2 condition ($M$=2.18 g, $SD$=0.90 g, 95% CI [1.54, 2.82]), $t$(32)=0.24, $p$=0.9761, or the F3 condition ($M$=2.19 g, $SD$=0.71 g, 95% CI [1.68, 2.69]), $t$(32)=0.27, $p$=0.9707.

*Pup Measures*

Body weight, brain weight, and brain-body weight ratio were measured in newborn (P0) control, F1, F2, and F3 mice (Fig. 2.4). A one-way ANOVA identified differences in body weight at P0 among the four conditions, $F$(3, 271) = 32.87, $p$<.001 (Fig. 2.4A). Additionally, planned comparisons provided evidence that the P0 control pups ($M$=1.74 g, $SD$=0.18 g, 95% CI [1.69, 1.79]) weighed more than the F1 mice ($M$=1.49 g, $SD$=0.18 g, 95% CI [1.46, 1.52]), $t$(271)=8.57, $p$<.001, the F2 mice ($M$=1.44 g, $SD$=0.18 g, 95% CI [1.39, 1.49]), $t$(271)=8.94, $p$<.001, and the F3 mice ($M$=1.54 g, $SD$=0.19 g, 95% CI [1.48, 1.60]), $t$(271)=5.56, $p$<.001. These effects persist to P20, as reported previously (Bottom et al., 2022). Thus, the prenatal exposure of the F1 pups to EtOH affected the body weight of all three filial generations.

Similarly, we identified differences in the weight of pups' brains across the four conditions, $F$(3,99)=12.86, $p$<.001 (Fig. 2.4B). Planned comparisons between the control pups and each filial generation showed the brain weight of

the control pups ($M$=0.103 g, $SD$=0.0140 g, 95% CI [0.098, 0.108]) was greater than the PrEE pups: F1 pups ($M$=0.088 g, $SD$=0.0055 g, 95% CI [0.085, 0.090]), $t$(99)=5.48, $p$<.001, F2 pups ($M$=0.094 g, $SD$=0.0114 g, 95% CI [0.090, 0.099]), $t$(99)=2.93, $p$=0.012, and F3 pups ($M$=0.087 g, $SD$=0.0094 g, 95% CI [0.082, 0.092]), $t$(99)=4.79, $p$<.001.These effects persist to P20, as reported previously (Bottom et al., 2022). These findings provide evidence that prenatal exposure of the F1 pups to EtOH affected brain weights across all three filial generations.

In addition to examining the raw brain and body weights, we computed the brain-body weight ratio for the pups in each condition (Fig. 2.4C). A one-way ANOVA identified differences in brain-body weight ratio at P0 between the four conditions, $F$(3,95)=3.51, $p$=0.0182. Planned comparisons between the control mice and each filial generation, however, failed to show significant differences between the brain-body weight ratio of the control mice ($M$=0.059 , $SD$=0.0080 , 95% CI [0.056, 0.062]) and the F1 mice ($M$=0.059 , $SD$=0.0037 , 95% CI [0.057, 0.060]), $t$(95)=0.19, $p$=0.9858, the F2 mice ($M$=0.063 , $SD$=0.0047 , 95% CI [0.061, 0.065]), $t$(95)=2.19, $p$=0.0818, or the F3 mice ($M$=0.056 , $SD$=0.0061 , 95% CI [0.053, 0.060]), $t$(95)=1.41, $p$=0.3643. Taken together, these results suggest that although brain and body weights are reduced in the three generations of PREE mice, the brain and body weights are scaling together across the conditions.

*Anatomical measures*

Analysis of anatomical measures identified additional changes in F1, F2, and F3 mice compared to controls on the day of birth. In addition to measurements in the neocortex, we assessed several nuclei of the thalamus, as well as CA3 in the hippocampus, to assess for anatomical phenotypes related to the ethanol exposure or transgenerational FASD (Fig. 2.5). A one-way ANOVA failed to find significant differences in the area of pups' dorsal lateral geniculate nucleus across the four conditions, $F(3,27)=0.40$, $p=0.7529$ (Fig. 2.5A1-5). Planned comparisons between control mice and each filial generation likewise failed to show significant differences between the section areas of the control mice ($M$=0.037 mm$^2$, $SD$=0.0102 mm$^2$, 95% CI [0.028, 0.045]) and the F1 mice ($M$=0.032 mm$^2$, $SD$=0.0073 mm$^2$, 95% CI [0.026, 0.039]), $t(27)=0.97$, $p=0.6374$, the F2 mice ($M$=0.036 mm$^2$, $SD$=0.0073 mm$^2$, 95% CI [0.030, 0.043]), $t(27)=0.13$, $p=0.9938$, or the F3 mice ($M$=0.036 mm$^2$, $SD$=0.0105 mm$^2$, 95% CI [0.028, 0.045]), $t(27)=0.11$, $p=0.9953$.

We similarly failed to find significant differences in the section areas of pups' medial geniculate nucleus across the four conditions, $F(3,24)=1.79$, $p=0.176$ (Fig. 2.5B1-5). Planned comparisons between the control mice and each filial generation likewise failed to show significant differences between the section areas of the control mice ($M$=0.103 mm$^2$, $SD$=0.048 mm$^2$, 95% CI [0.058, 0.15]) and the F1 mice ($M$=0.098 mm$^2$, $SD$=0.022 mm$^2$, 95% CI [0.077, 0.12]), $t(24)=0.29$, $p=0.9666$, the F2 mice ($M$=0.129 mm$^2$, $SD$=0.023 mm$^2$, 95% CI

[0.110, 0.15]), $t$(24)=1.69, $p$=0.2471, or the F3 mice ($M$=0.122 mm$^2$, $SD$=0.022 mm$^2$, 95% CI [0.100, 0.15]), $t$(24)=1.17, $p$=0.5172.

We also failed to find significant differences in the section areas of pups' ventral-posterior nucleus (VP) across the four conditions, $F$(3,27)=1.35, $p$=0.2792 (Fig. 2.5C1-5). Planned comparisons between the control mice and each filial generation also failed to show significant differences between the section areas of the control mice ($M$=0.25 mm, $SD$=0.035 mm, 95% CI [0.22, 0.28]) and the F1 mice ($M$=0.28 mm, $SD$=0.041 mm, 95% CI [0.25, 0.32]), $t$(27)=1.49, $p$=0.3345, the F2 mice ($M$=0.28 mm, $SD$=0.035 mm, 95% CI [0.25, 0.32]), $t$(27)=1.53, $p$=0.3165, or the F3 mice ($M$=0.25 mm, $SD$=0.059 mm, 95% CI [0.20, 0.30]), $t$(27)=0.19, $p$=0.9861.

The CA3 region of the pups' hippocampi was thinner in the three filial generations of the PrEE mice compared to the controls, $F$(3,25)=5.57, $p$=0.0046 (Fig. 2.5D1-5). Planned comparisons between the control mice and each filial generation showed this reduction in CA3 thickness between the control mice ($M$=0.107 mm, $SD$=0.0187 mm, 95% CI [0.090, 0.124]) and the F1 mice ($M$=0.088 mm, $SD$=0.0099 mm, 95% CI [0.079, 0.097]), $t$(25)=2.68, $p$=0.0352, the F2 mice ($M$=0.084 mm, $SD$=0.0091 mm, 95% CI [0.076, 0.093]), $t$(25)=3.27, $p$=0.0088, and the F3 mice ($M$=0.081 mm, $SD$=0.0125 mm, 95% CI [0.071, 0.092]), $t$(25)=3.76, $p$=0.0026. Thus, CA3 appears to be thinner in all three filial generations relative to controls.

Control and all three generations of PrEE pups' corpus callosa were examined for abnormalities (exemplar images from three cases per group—Control, F1, F2, F3—are shown in Fig. 2.6). A $\chi^2$ test found that the number of pups with typical corpus callosa varied among the four groups of mice, $\chi^2(3)=10.513$, $p=0.0087$ (Fig. 2.7). Because the number of pups in many of the cells was less than five, the $p$ value was computed via simulation. Three follow up tests were planned to compare each filial generation to the control pups. Only the F1 pups yielded a difference from the control pups, $\chi^2(1)=8.082$, $p=0.0345$ (Bonferroni adjusted). Prenatal exposure to ethanol via maternal consumption during pregnancy disrupts callosal development, and although we show significance only for the F1 generation, there were cases of agenesis in F2 and F3, and none in control, suggesting transgenerational transfer of the phenotype.

*Behavior*

Our behavioral analyses of results on the Suok test provided evidence of increased anxiety and reduced motor coordination starting in the first generation and continuing into the third generation (Fig. 2.8). Initial analyses included sex as a factor, but no main effects nor interactions with sex were significant. For the ease of exposition, the results in this section collapse over levels of sex. Using the Suok test, we evaluated correlates of both anxiety and motor development (sensorimotor integration and motor coordination) in a single assay. Behavioral analysis is examined 20 days after birth (P20). Significant differences were

observed between filial generations compared to controls. Increased latency to leave the center of the Suok bar suggests greater anxiety, and we identified overall differences between the four groups of mice in their latency to leave center, $F$(3,96)=10.45, $p$<.001 (Fig. 2.8A). Planned comparisons between the control mice and each filial generation provided evidence that the latency of the control mice ($M$=3.93s, $SD$=3.07s, 95% CI [2.64, 5.23]) was less than the F1 mice ($M$=22.45s, $SD$=12.72s, 95% CI [16.32, 28.58]), $t$(96)=2.85, $p$=0.015, and the F2 mice ($M$=31.06 s, $SD$=33.94s, 95% CI [19.41, 42.72]), $t$(96)=4.84, $p$<.001, but the difference between the latency of the control mice and the F3 mice ($M$=6.44s, $SD$=5.33s, 95% CI [4.08, 8.81]), did not reach significance, $t$(96)=0.40, $p$=0.933.

There were also differences in the number of exploratory behaviors between the four conditions, $F$(3,88)=6.91, $p$<.001 (Fig. 2.8B). Planned comparisons between the control mice and each filial generation identified fewer exploratory behaviors made by the F1 mice ($M$=51.88 behaviors, $SD$=11.89 behaviors, 95% CI [45.77, 58.00]), $t$(88)=4.35, $p$<.001, the F2 mice ($M$=59.97 behaviors, $SD$=11.60 behaviors, 95% CI [55.99, 63.96]), $t$(88)=3.15, $p$=0.0065, and the F3 mice ($M$=59.06 behaviors, $SD$=21.32 behaviors, 95% CI [47.70, 70.42]), $t$(88)=2.77, $p$=0.019, relative to the control mice ($M$=72.38 behaviors, $SD$=15.90 behaviors, 95% CI [65.66, 79.09]).

There were also differences in the number of stereotyped rearing/grooming events among the four groups, $F$(3,88)=4.95, $p$=0.0032 (Fig. 2.8C). Decreases in these behaviors are suggestive of increased anxiety.

Planned comparisons identified fewer rearing/grooming behaviors displayed by the F1 mice ($M$=1.75 behaviors, $SD$=0.94 behaviors, 95% CI [1.35, 2.15]), $t$(88)=2.97, $p$=0.0109, and the F3 mice ($M$=0.94 behaviors, $SD$=0.77 behaviors, 95% CI [0.53, 1.35]), $t$(88)=2.93, $p$=0.0122, relative to the control mice ($M$=1.75 behaviors, $SD$=0.94 behaviors, 95% CI [1.35, 2.15]). This reduction did not hold, however, for the F2 mice ($M$=1.57 behaviors, $SD$=0.85 behaviors, 95% CI [1.28, 1.86]), $t$(88)=0.78, $p$=0.7490.

We also identified differences in the number of missteps between the four conditions, $F$(3,83)=11.86, $p$<.001 (Fig. 2.8D). Planned comparisons between the control mice and each filial generation identified increases in the number of missteps made by the F1 mice ($M$=22.29 missteps, $SD$=7.46 missteps, 95% CI [18.46, 26.13]), $t$(83)=4.69, $p$<.001, the F2 mice ($M$=23.09 missteps, $SD$=10.17 missteps, 95% CI [19.54, 26.64]), $t$(83)=5.72, $p$<.001, and the F3 mice ($M$=18.80 missteps, $SD$=3.94 missteps, 95% CI [16.96, 20.64]), $t$(83)=3.52, $p$=0.0021, relative to the control mice ($M$=9.69 missteps, $SD$=4.84 missteps, 95% CI [7.11, 12.27]).

The number of falls from the bar in the Suok test differed across the four conditions, $F$(3,88)=7.54, $p$<.001 (Fig. 2.8E). Planned comparisons between the control mice and each filial generation found that the F1 mice ($M$=4.65 falls, $SD$=3.33 falls, 95% CI [2.93, 6.36]), $t$(88)=4.48, $p$<.001, the F2 mice ($M$=3.43 falls, $SD$=2.78 falls, 95% CI [2.47, 4.38]), $t$(88)=3.55, $p$=0.0018, and the F3 mice

($M$=3.25 falls, $SD$=2.32 falls, 95% CI [2.01, 4.49]), $t$(88)=2.7, $p$=0.0234 all fell more than the control mice ($M$=1.04 falls, $SD$=1.40 falls, 95% CI [0.45, 1.63]).

Segments crossed, a measure of the total distance traveled, differed among the four conditions, $F$(3,88)=2.73, $p$=0.0485 (Fig. 2.8F). Planned comparisons between the control mice and each filial generation, however, failed to show evidence of differences between the number of segments crossed by the control mice ($M$=87.46 segments, $SD$=24.57 segments, 95% CI [77.08, 97.83]) and the F1 mice ($M$ = 98.53 segments, $SD$=41.29 segments, 95% CI [77.30, 119.76]), $t$(88)=0.98, $p$=0.629, the F2 mice ($M$=80.00 segments, $SD$=43.98 segments, 95% CI [64.89, 95.11]), $t$(88)=0.79, $p$=0.7471, or the F3 mice ($M$=108.69 segments, $SD$=19.09 segments, 95% CI [98.51, 118.86]), $t$(88)=1.84, $p$=0.1727. Data from this behavioral test suggests a heritability of behavioral phenotypes that persist beyond the directly exposed generation.

*Summary of findings*

All generations of mice stemming from the first, directly exposed generation, F1, demonstrated lower body weights, and brain weights compared to controls. Thickness and volumes of some subcortical structures in F1, F2, and F3 newborn PrEE mice were impacted by the initial exposure. Hippocampal CA3 was significantly thinner in all generations of PrEE mice when compared to controls. Additionally, PrEE resulted in a significant rate of agenesis or partial development of the corpus callosum in the majority of F1 cases, with a less

frequent occurrence in F2 and F3 mice. Behavioral deficits were present in both the F1 and F2 PrEE generations but seemed to be rescued by F3. Our data suggest that PrEE can result in abnormal brain and behavioral development with heritable effects that persist transgenerationally to subsequent generations of offspring.

## DISCUSSION

For 50 years, scientists have tried to understand how and why developmental trajectories are changed by maternal consumption of alcohol during pregnancy. In those 50 years, how we study FAS and FASD has evolved tremendously. Research began in the early 1970s with simple recognition of facial dysmorphology in babies born to alcoholic mothers (Jones & Smith, 1973a) and fetal alcohol science marched forward to include complex cognitive and behavioral assessments to elucidate systemic dysfunction in children with FASD (Kerns et al., 1997; Nestler et al., 1981). More recently, the use of molecular biological methods has helped us begin to uncover the underlying mechanisms in the brain that are disturbed by ethanol exposure during early development (Abbott et al., 2018). Over 5 decades, scientists unveiled the dangers of drinking during pregnancy, detailed the ways in which the developing brain can be damaged by ethanol and investigated ways to help prevent FASD through abstinence and supplements such as choline (Bottom et al., 2020). The recent discovery that FASD is a heritable condition that can pass transgenerationally

without additional, subsequent ethanol exposures (Abbott et al, 2018), and the likely role of epigenetics in this process, has presented new challenges to scientists in the field. Notably, studies in rats show significant deficits in Pro-opiomelanocortin (POMC) neuronal functioning with altered levels of histone-modifying proteins and DNA methyltransferase levels in POMC neurons which persist into the F2 and F3 generations through the male germline (Govorko et al., 2012). Additionally, reduced Ifn-ɣ expression and increased promoter methylation of the Ifn-ɣ gene persisted in F2 and F3 male rats derived from the male germline (Gangisetty et al., 2020). However, both of these studies used an EtOH exposure window of embryonic day (E) 7 through 21. As these findings are relatively new, we do not know the full gamut of phenotypic variation that can stem from the initial F1 exposure, and how future generations are impacted in terms of brain and behavioral development. The current report continues our investigation of transgenerational phenotypes, expanding our knowledge of heritable changes that continue through familial lineages. To create ways to treat and improve the conditions of those with transgenerational FASD, we need to understand what neurological changes persist and what deficits carry over to non-exposed generations, and we need to better understand the epigenetic mechanisms that underlie the heritability of the disorder.

*FASD phenotypes in humans and rodent models*

FASD in humans is truly a spectrum disorder. Depending on the severity and timing of the exposure, people can have very mild to severe phenotypes. Despite this variability, there are a few hallmark features of FASD that connect the most disparate cases. Typically, people with FASD display alterations in sensory processing (Jirikowic et al., 2020), fine motor skills (Jones et al., 2010) and risk-taking behavior (Furtado & Roriz, 2016). Additionally, *in utero* exposure to ethanol can cause delays in cognitive development that may include deficits in general intelligence, attention, motor function and coordination, as well as higher executive functions (Harms et al., 2014; Mattson et al., 2019). Research on humans with FASD is limited, so implementing the use of non-human models has been critical to the field of research. Murine models of FASD provide a tool to evaluate the biological and behavioral effects of PrEE, in a more controlled environment. Our laboratory has reported changes in neuroanatomical development, intracortical connectivity, gene expression, epigenetics, and behavior in our mouse model of FASD (El Shawa et al., 2013; Abbott et al., 2016; 2018; Kozanain et al., 2018; Bottom et al., 2020; 2022). Many of the phenotypes observed in PrEE relate to what has been described in humans with FASD. Establishing comprehensive neurobehavioral, neuroanatomical, and molecular profiles in animal models of FASD is critical to the development of treatment and prevention strategies in humans.

*PrEE's impact on fetal growth and neuroanatomical development*

Several studies have demonstrated reduced body weights, brain weights, cortical lengths, cortical thinning, and other subcortical changes in humans with FASD, as well as rodent models (Zhou et al., 2011; Gautam et al., 2015; Abbott et al., 2018). Our findings in PrEE F1 mice here support previous reports and extend work into subsequent generations. Below we describe potential mechanisms underlying our findings PrEE mice and how these might be related to transgenerational FASD.

Growth restriction*:*

Although F1-3 dams tend to show smaller weight changes throughout pregnancy when compared to controls, this effect is likely to be linked to reduction in litter size as no differences were found when weight gain was assessed per pup, or by litter size. The F1-3 pups were also typically born smaller with smaller brains, however, the pup brain:body weight ratios across the conditions were not significantly different. Notably, F2 and F3 generation mice exhibit similar morphology to the F1 generation despite no significant difference in maternal nutrition as measured by food intake. This suggests a possible heritability of alcohol-related growth restriction that is not related to maternal food consumption. Epigenome alterations in the methylation of transcription factor promoter regions are heritable (Abbott et al., 2018; Almedia et al., 2020) and can potentially interfere with metabolism and IGF-2 levels during gestation (Almeida

et al., 2020). These metabolic effects may be responsible for the delays in growth metrics observed to those exposed to high levels of alcohol during pregnancy (Carter et al., 2016) and potentially a contributor to the transgenerational deficits in newborn brain and body weights we observed here. In summary, as the reductions in body weight, brain weight and cortical length are found in F2 and F3 generations, this suggests inter-and transgenerational transfer.

Our data from our mouse model of FASD are consistent with alcohol-related reductions in weight, height, weight-for-height/BMI, and head circumference in offspring born to women who consumed high doses of alcohol during pregnancy (Carter et al., 2013; 2016). It is thought that maternal ethanol consumption during pregnancy leads to alcohol-induced altered nutrient metabolism and malabsorption which, in turn, restricts fetal development (Naik et al., 2022). Also, insulin growth factors (IGF), especially IGF-1 and IGF-2 are critical to embryonic and fetal growth (Hellström et al., 2016; Kadakia & Josefson, 2016). Because serum concentrations of IGF-1 and IGF-2 are reduced in children with FASD, it is possible that by interfering with IGF function, alcohol can slow fetal growth (Andreu-Fernandez et al., 2019).

Smaller brain weights:

The central nervous system anomalies documented in FASD range from cellular and molecular aberrations to gross structural brain abnormalities (Norman et al., 2009; Riley et al., 2004). Among the most consistent findings in

brain imaging studies of FASD is the reduction in overall brain volume (Mattson et al., 1998; Archibald et al., 2001; Willoughby et al., 2008; Norman et al., 2009). The reductions in volume are detected throughout the brain, with frontal, temporal, and parietal lobes showing the most significant effects in individuals with FASD as compared with controls (Archibald et al. 2001; Yang et al., 2011). In our study, F1-3 pups demonstrated reduced brain weights compared to controls. Taken together, results of decreased brain weights and shortened cortical lengths are consistent with these as well as our previous findings (Abbott et al., 2016; Abbott et al., 2018).

There are a few potential mechanisms to explain microcephaly in FASD. One hypothesis is that neural crest cells suffer apoptosis early on due to ethanol exposure, which results in smaller brains. This can occur when ethanol generates a caspase cascade which can induce cell death in neural crest cells often by disrupting folic acid (folate) function (Muralidharan et al., 2013). Another possibility is that functional brain metabolism is disrupted by ethanol exposure (Fagerlund et al., 2006). Specifically, *N*-acetylaspartate/choline (NAA/Cho) and NAA/creatine (NAA/Cr) ratios appear reduced in the frontal and parietal lobes, corpus callosum and other subcortical structures in humans with FASD. This is suggestive of a reduction in glial cell proliferation, which could account for some of the observed reduction in brain size from PrEE.

Hippocampal changes: CA3

The hippocampus is a structure embedded deep within the temporal lobe that is important for learning and memory. Both human (Coles et al.,1991; Uecker and Nadel, 1996) and animal studies (Riley et al.,1984; Driscoll et al., 1990) have documented poor performance on learning and memory tasks due to prenatal alcohol exposure. A human longitudinal study of young adults used MRI coupled with the Verbal Selective Reminding Memory Test and its nonverbal counterpart, the Nonverbal Selective Reminding Memory Test, found significant deficits in memory performance along with decreased hippocampal volume in individual exposed to alcohol *in utero* (Coles et al., 2011). Another MRI study of children with FAS showed hippocampal asymmetry; left hemisphere hippocampal volume was smaller than the right (Riikonen et al., 1999).

Data from PrEE animal models show disruption in hippocampal development. For example, a study showed a decrease in dendritic arbor length and simplified dendritic branching within the hippocampal neurons of P14 PrEE mice (Davies & Smith, 1981). Additionally, decreases in hippocampal spine density and CA1/CA3 volumes have been documented in PrEE rodent models (Abel et al., 1983, Livy et al., 2003). Interestingly, pyramidal cell density was also reduced in the CA1 at all gestational ages, however CA3 pyramidal cell density was only reduced in animals treated with ethanol from P4-P9, the equivalent to the human third trimester (Livy et al., 2003). Therefore, the third trimester period in development may be a vulnerable time for alcohol exposure leading to altered

hippocampal development and memory deficits. Consistent with past research, our study showed a decrease in CA3 thickness in F1, replicating data from other laboratories. We discovered that this phenotype persists transgenerationally as well, with reduction in CA3 present in both the F2, and F3 generations. This suggests that there is a heritable component to hippocampal alteration due to PrEE in mice. In terms of potential molecular mechanisms, or epigenetic modifications that could lead to the transgenerational change, increases in gene expression of DNA methyltransferases, *Dnmt1* and *Dnmt3a*, in the hippocampus of PrEE rats have been reported. (Lucia et al., 2019; Gangisetty et al., 2015). These data suggest that epigenetic modifications generated from the exposure may play a role in the presumed heritability of hippocampal damage in PrEE. More research is needed to investigate the molecular mechanisms by which PrEE may induce heritable change in the structure and potentially function of the hippocampus.

Agenesis in corpus callosum:

The corpus callosum is a large white matter tract that connects the two hemispheres of the brain and allows for interhemispheric communication. This region connects the primary motor cortices and is also responsible for various high level cognitive functions such as verbal learning, memory, attention, and visuospatial abilities (O'Neil, 2010; Huang et al., 2015). The total number of callosal fibers is fixed at birth but the structure will continue developing

throughout life in terms of redirecting fibers, myelination, and pruning (Vannucci, 2017). The human corpus callosum begins developing during week five of gestation, which is after the time point neural crest cells are most vulnerable for developing into facial anomalies associated with FAS (O'Neil, 2010). This explains how central nervous system deficits, particularly those of the corpus callosum, can occur in children exposed to ethanol prenatally, even if facial phenotypes associated with FAS are not present.

Some of the most prominent changes in the brain due to PrEE are alterations to the corpus callosum. Imaging studies include cases of complete corpus callosum agenesis (Riley et al., 1995; Johnson et al.,1996; Swayze et al., 1997; Astley et al., 2009), partial agenesis and/or callosal thinning (Autti-Rämö, et al., 2002). In cases of partial or complete agenesis, the axons of the white matter tracts do not cross over but instead rest parallel to the hemispheres (Riley et al., 1995, O'Neil, 2010). These individuals experience severe deficits in motor control as well as executive functioning tasks such as decision making, and abstract thinking.

In our study, F1 PrEE mice showed significantly altered corpus callosal development. Similar to human studies, our data show corpus callosum thinning, full or parietal agenesis, in the most anterior portions of the region as well as a variation in crossover location. These abnormal callosal phenotypes could potentially explain some of the sensorimotor coordination difficulties observed in PrEE mice. Although the malformed callosa was prominent and frequent in F1

PrEE mice brains, the occurrence was rare but present in the subsequent generations. This suggests that the phenotypes can persist to F3, but with less frequency.

*PrEE and behavioral development: transgenerational effects.*

Sensory and motor deficits:

In humans with FASD, sensory processing difficulties such as visual, touch, body awareness, and planning are present during the peripubescent period (Hen-Herbst et al., 2020). Murine models of FASD often exhibit deficits in sensory and motor processing, social behaviors, motor learning, fear learning, spatial functioning and often show behavior associated with depression and anxiety (Kalberg et al., 2006; Hellemans et al., 2010ab; El Shawa et al., 2013; Kozanian et al., 2018). Errors in sensorimotor integration are common phenotypes in individuals with FASD and they are often used as diagnostic indicators using the Movement Assessment Battery for Children, second edition (MABC-2; Johnston et al., 2019). In our mouse model of FASD, PrEE mice slip (misstep) and fall significantly more than controls. This occurs in the absence of variable running rates (total segments crossed during the test did not differ across groups). Thus, these slips and falls are likely to be from sensorimotor integration errors rather than from increased speed of travel. Interestingly, these phenotypes persist to F2 and F3 generations, demonstrating transgenerational transfer. This is possibly related to the abnormal development of the corpus

callosum observed significantly in F1 PrEE mice and across generations in some cases in our results, as the corpus callosum is critical for bilateral coordination (Richmond & Fling, 2019).

In addition to deficits in Suok performance, our past reports reported deficits in adhesive tape removal and accelerated rotarod performance, which evaluates motor skills and motor learning, respectively, with effects persisting to F3 (Bottom et al., 2022). From our previous work, we found alterations in both DNA methylation and neocortical generational and we posit that these epigenetic mechanisms may form the basis for the transgenerational effects (Abbott et al., 2018).

Anxiety:

A hallmark of FASD in humans is increased incidence of anxiety and depression (Hellemans et al., 2010ab). These mental health problems can often be some of the most severe manifestations of FAS during adulthood (Lemoine et al., 2003). Socio-emotional anxious disturbances are common in humans with FASD. As with problems with sociality, this could be due to alterations in the amygdala, specifically reductions in volume of the basolateral complex (BLA) and basomedial nucleus (BMA) in adult F1 PrEE mice, which have been previously reported by our laboratory (Kozanian et al., 2018). The same study also showed an increase in cell packing density in the BLA, BMA, and central nucleus of the amygdala in the adult F1 PrEE mice. BMA neurons are involved in fear circuitry

as they are known to suppress fear-related freezing and help ameliorate elevated anxiety states (Adhikari et al., 2015). Another study found that PrEE during the rodent equivalent of the third trimester of human pregnancy can increase excitatory inputs to the BLA, leading to an increase in anxiety-like behaviors (Baculis et at., 2015). PrEE can also cause increased anxiety due to a dysregulation in functional connectivity between the BLA and anterior cingulate cortex (ACC) (Hwang & Hashimoto-Tori, 2022). Reduced excitation within the ACC could be a potential cause of anxiety in PrEE mice (Hwang & Hashimoto-Tori, 2022). These are all potential mediators for the increased anxiety-like behaviors observed in our F1 PrEE mice.

Our results show three behavioral markers for increased anxiety in our transgenerational PrEE model. F1 and F3 PrEE mice show decreased rearing and grooming, F1, F2 and F3 PrEE show decreased directed exploration, and F1 and F2 mice show an increased latency to leave center when compared to controls. These findings demonstrate an increase in anxiety-like behaviors across generations. Likewise, we discovered a transgenerational thinning of CA3 in the hippocampus that persists across all three generations. It is possible that this neuroanatomical phenotype is related to the behavioral phenotype of increased anxiety-like behaviors observed in our F1-3 generations, as the a2-containing GABAA receptors *(a2GABAARs)* within the CA3 have been found to be involved in the suppression of anxiety (Engin et al., 2016).

PrEE in both preclinical and clinical models has also been known to cause dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which plays a role in vulnerability to stress-related disorders such as anxiety (Haley et al., 2006; Wieczorik et al., 2015; Lam et al., 2019). The HPA axis is activated in response to stress and anxiety which causes the release of stress hormones such as corticosterone (CORT). In fact, one study showed increased CORT levels in PrEE mice after performing in the elevated plus maze, another test that can be used to detect anxiety-like behaviors in mice (Hwang and Hashimoto-Tori, 2022). Our model utilized the Suok test which revealed a decrease in exploratory behaviors and rearing/grooming events as well as an increase in latency to leave the center of the rod both of which are indicative of anxiety-like behaviors in rodents.

Sociality:

Characterization of FASD in humans has found that early, heavily exposed infants display higher levels of affective withdrawal, often observable in early infancy (Molteno et al., 2014). Additionally, emotional social withdrawal predicted poorer IQ ages 5 and 9 (Molteno et al., 2014). In extreme cases, adults with FASD exhibit social function at a level similar to a typically developing six-year-old (Streissguth et al., 1996). Our F1 PrEE mice also show social dysfunction, as measured in our sociability test, and this effect persists to the F2 generation. Specifically, F1 and F2 mice spent less time with a novel, social

mouse and spent more time in the empty chamber of the apparatus, possibly implicating that asocial behaviors from PrEE are heritable through the male germline to F2 progeny. There seems to be a step like improvement in sociality from F1 to F2 and F3. Notably, we did not observe any differences in F3 progeny compared to controls, implicating a recovery of asocial behaviors in F3. A key area that may modulate asocial behaviors could be the amygdala as it modulates social interactions in similar ways as it impacts anxiety (Amaral, 2003). As stated previously, PrEE has been shown to alter amygdalar volume and fear learning (Kozanian et al., 2018) and our data here shows that PrEE results in reduced thickness of the prelimbic area within neocortex in PrEE mice; thus, it is possible that amygdalar and prelimbic dysfunction might be mediating asocial behaviors in F1 and F2 mice.

*Heritability of FASD: potential mechanisms.*

In our current study, we found evidence for intergenerational and/or transgenerational transfer of several PrEE-related anatomical and behavioral phenotypes. One possible mechanism by which heritable phenotypes are being passed on is an alteration in epigenetic pathways. DNA methylation is a known mechanism that is involved in gene transcription silencing (Moore et al., 2013). Evidence suggests that alcohol exposure alters methylation profiles of mice when exposed *in utero* during neurulation (Liu et al., 2009), which may lead to some of the deficits observed in people with FASD. Additional work published by our

laboratory has shown that PrEE results in alterations in intraneocortical connectivity, upregulation of neocortical *RZRβ* and *Id2* expression accompanied by promoter hypomethylation and decreased global DNA methylation levels across generations with suppressed DNMT expression (Abbott et al., 2018). Our data in our transgenerational FASD model suggest that changes in DNA methylation may alter transcription of select developmental genes pertinent to cortical development, leading to altered expression, ectopic neural connections, and neuroanatomical restructuring that together may lead to atypical and problematic behavior. As neocortical patterning is governed by gene expression in early development, PrEE-induced shifts in gene expression lead to overall distortion of the cortical map (Fukuchi-Shimogori et al., 2001; Huffman et al., 2004; Dye et al., 2011, El Shawa et al., 2013; Abbott et al., 2018). If epigenetic modifications are generating shifts in gene expression, as we have hypothesized previously, then it follows that the origin of transgenerational transfer to F3 of neurobiological phenotypes is epigenetic in nature (Jirtle et al., 2007; Gapp et al., 2017; Abbott et al., 2018; Bottom et al., 2022). Our results support the hypothesis that maternal consumption of alcohol during pregnancy has the potential to induce stable epigenetic alterations; thus, leading to the persistence of the F1 PrEE phenotypes observed across three generations.

*Study Limitations*

A possible limitation is that our study uses a transgenerational model where transmission occurs via the male germline. Other studies investigated the epigenetic effects of PrEE transmitted through the female germline but found that effects did not persist into the F2 or F3 generations (Gangisetty et al., 2020; Govorko et al., 2012). Further research is needed to investigate if the effects of PrEE can be transmitted through the female germline. Additionally, we did not investigate potential mechanisms for why changes in anatomy and behavior occur, which is the focus of additional ongoing research in our laboratory. Therefore, future studies will be used to explore potential molecular and epigenetic mechanisms of PrEE.

*Conclusions*

Recent reports from our laboratory have demonstrated both intergenerational and transgenerational transfer of phenotypes related to PrEE, which suggests a heritability of FASD (Bottom et al., 2022, Abbott et al., 2018). Here we extend our prior work to show how some neuroanatomical and behavioral phenotypes associated with PrEE and FASD are passed on transgenerationally from the directly exposed generation. Understanding crucial features of FASD and transgenerational FASD, as well as uncovering the molecular mechanisms that underlie the phenomena are critical to the development of prevention strategies and therapeutics for FASD in both preclinical and clinical settings. Finally, the collective research on FASD from our

laboratory and others provides further support for abstaining from alcohol consumption during pregnancy to protect offspring and subsequent generations.

## FIGURES

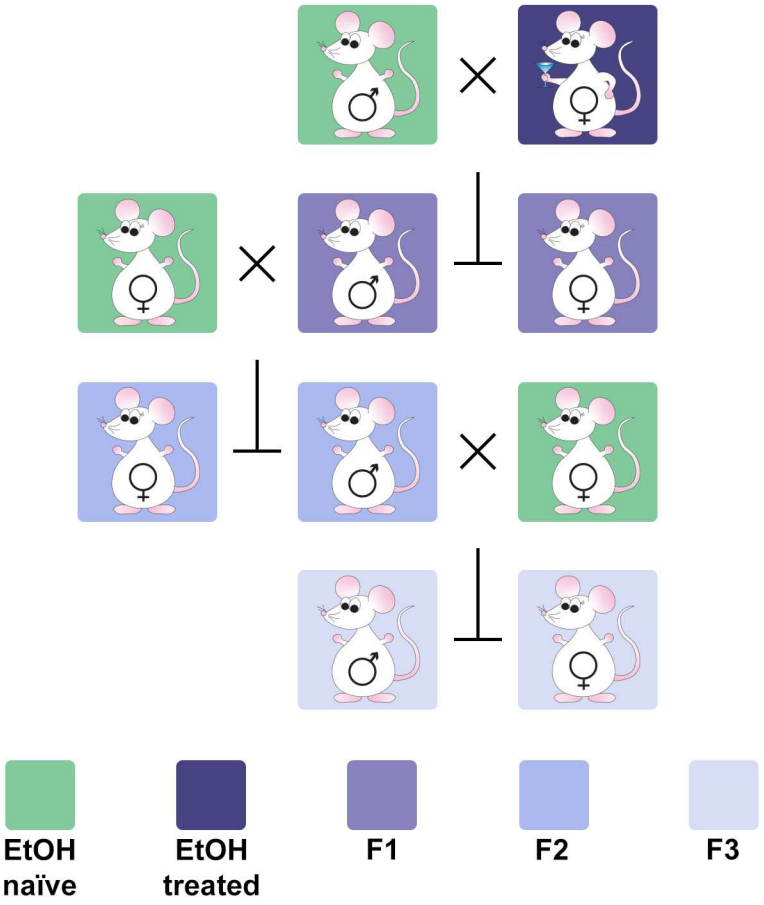


**Figure 2.1. Breeding paradigm.** Summarization of the breeding paradigm used to generate first, second and third generation FASD mice. Alcohol exposure occurs during pregnancy of the first filial (F) generation mice with 25% (v/v) ethanol consumed *ad libitum*. Breeding of second and third generation mice is done by pairing up first and second generation male mice with control, ethanol naive females, respectively.

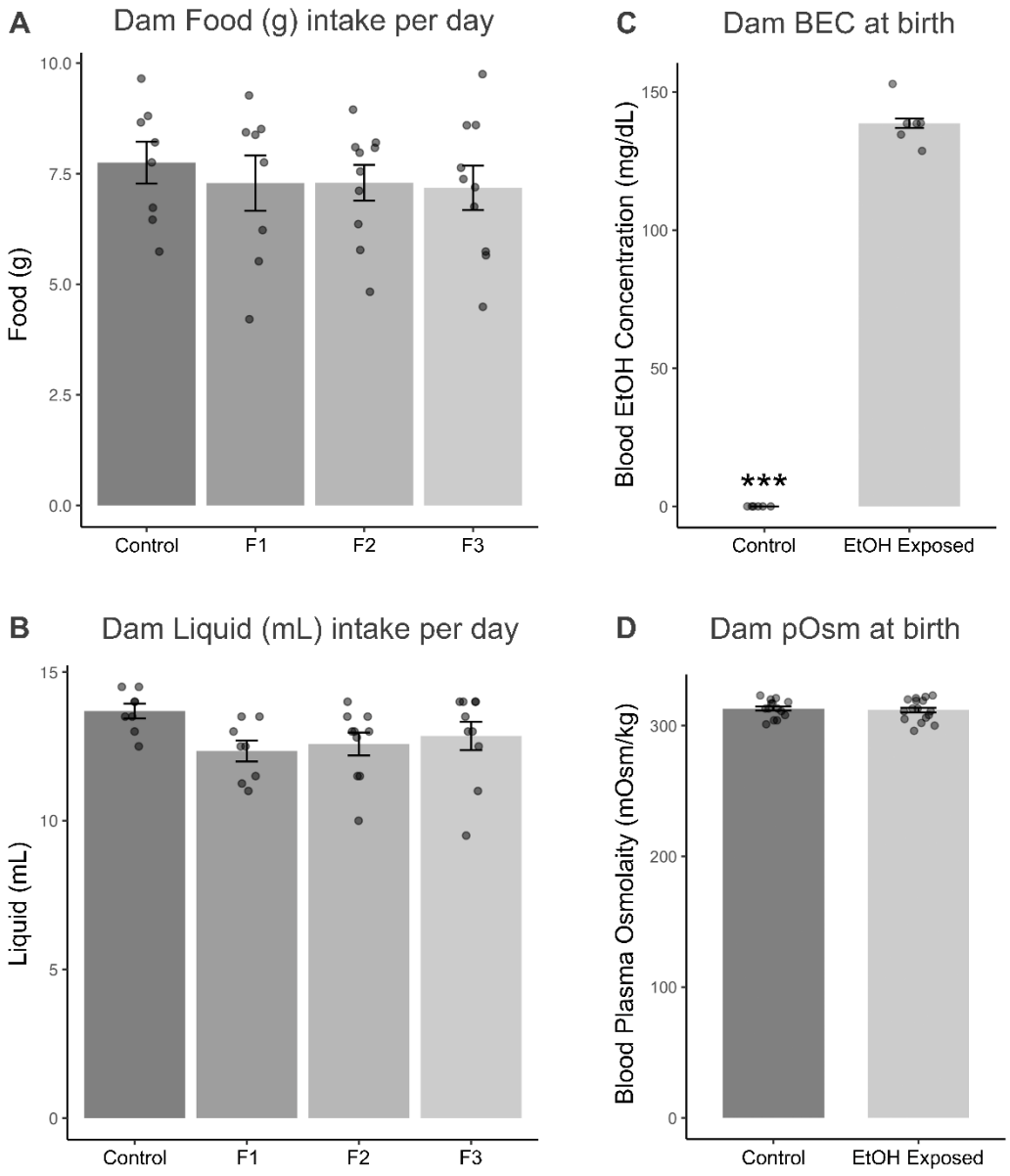


**Figure 2.2. Dam metrics at birth.** Evaluation of maternal metrics at birth of pups. **A.** No significant difference in food consumption between control (n = 8), F1 (n = 8), F2 (n = 10), and F3 (n = 10) dams. **B.** No significant differences in liquid intake were observed between control (n = 8), F1 (n = 8), F2 (n = 10), and F3 (n = 10) dams. **C.** Dam blood ethanol concentration (BEC) in mg/dL at birth. Elevated BEC levels were detected for EtOH exposed dams (n = 12). **D.** Dam plasma osmolality (pOsm) in millimole per kilogram (mOsm/kg). No significant differences were observed between control (n = 14) and EtOH exposed dams (n = 16). Variations in dot opacity are due to overlap of individual dots.

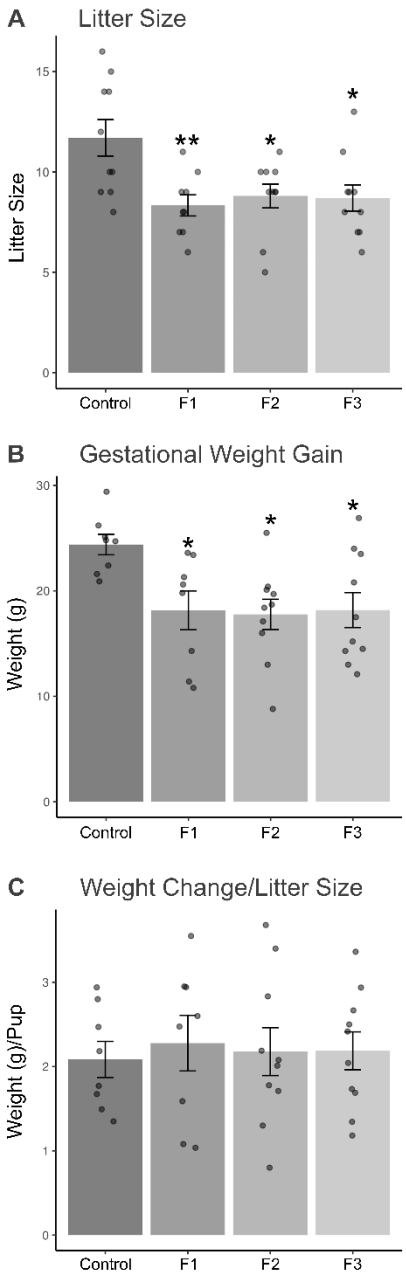


**Figure 2.3. Gestational metrics. A.** Litter sizes at birth for F1 (n = 9), F2 (n = 10), and F3 (n = 10) were significantly reduced compared to controls (n = 10). **B.** Gestational weight gain was significantly reduced in F1 (n = 8), F2 (n = 10), and F3 (n = 10) dams compared to controls (n = 8). **C.** No significant difference in weight change with respect to litter size for control (n = 8), F1 (n = 8), F2 (n = 10), and F3 (n = 10) dams. Data expressed as mean ± S.E.M. *$p < 0.05$. Variations in dot opacity are due to overlap of individual dots.

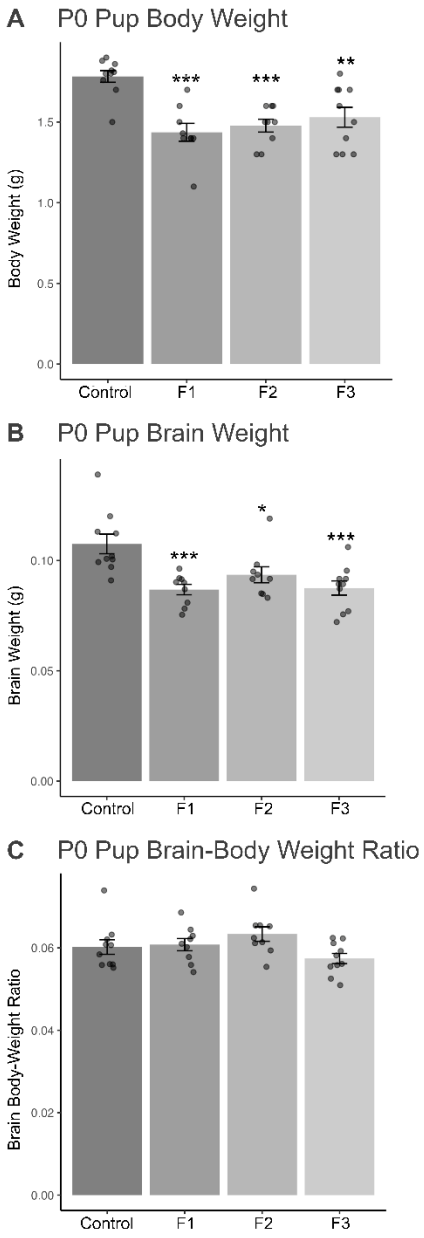


**Figure 2.4. Pup metrics at birth. A.** Significant decrease in F1 (n=110), F2 (n = 58), and F3 (n = 45) body weights of offsprings at P0, compared to controls (n = 62) at P0. **B.** Significant reductions in F1 (n = 28), F2 (n = 25), and F3 (n = 16) brain weights compared to controls (n = 34) were observed at P0. **C.** P0 Brain Weight/Body Weight (BrW/BdW) ratios were calculated for each experimental condition. No significant differences were observed between F1 (n = 28), F2 (n = 25), and F3 (n = 16) ratios compared to controls (n = 34). Data expressed as mean ± S.E.M. *$p$ < 0.05, **$p$<0.01, and ***$p$<0.001. Variations in dot opacity are due to overlap of individual dots.

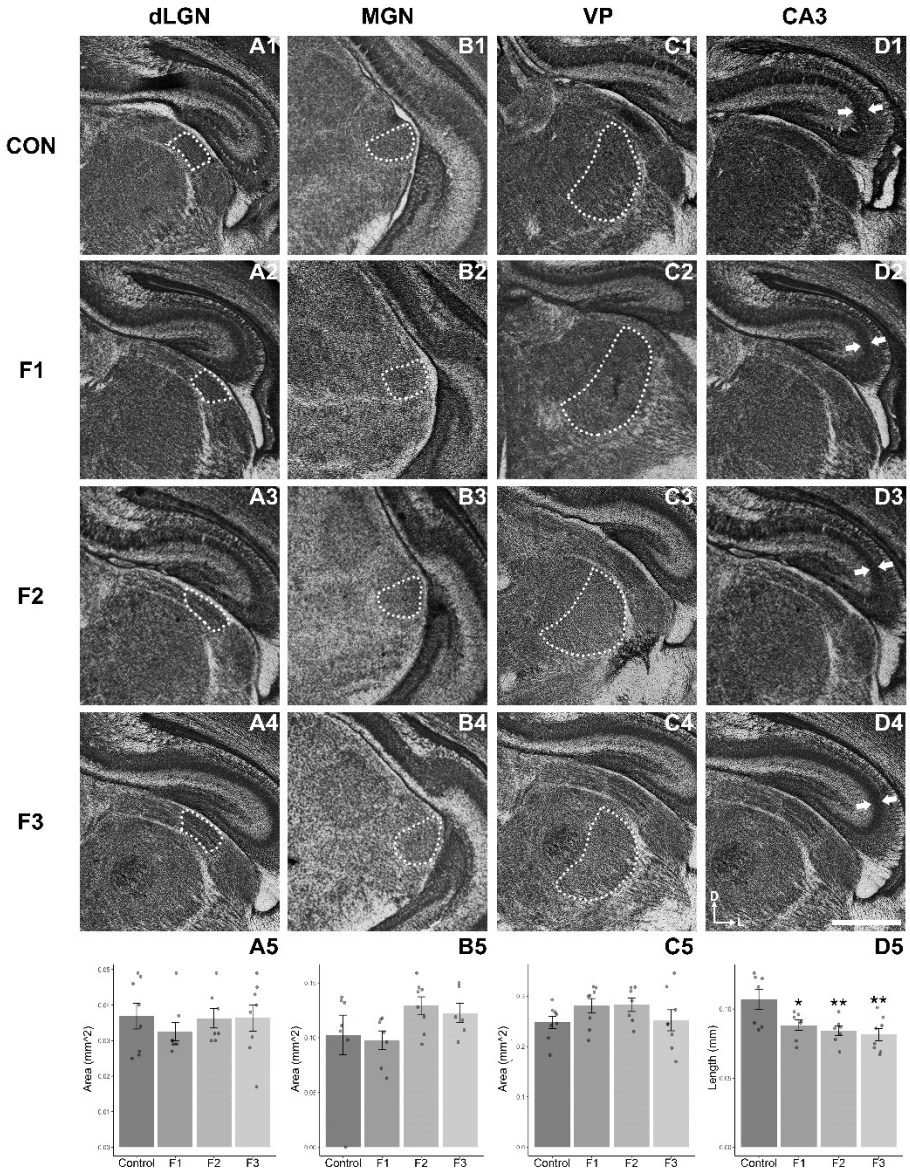


**Figure 2.5. Subcortical area and thickness measurements at P0.** Representative Nissl-stained coronal sections of P0 mouse brain tissues for all experimental groups (Control, F1, F2, and F3). Cortical areas evaluated include the dorsal lateral geniculate nucleus (dLGN; **A1–A5**; n = 31), medial geniculate nucleus (MGN; **B1–B5**; n = 28), ventral pallidum (VP; **C1–C5**; n = 31), and CA3 of the thalamus (**D1–D5**; n = 29). Significant decreases in thickness of CA3 were observed for F1, F2, and F3 mice, compared to controls. Data expressed as mean ± SEM. Images oriented dorsal (D) up and lateral (L) to the right. *$p < 0.05$ and **$p < 0.01$. Scale bar, 1 mm. Variations in dot opacity are due to overlap of individual dots.

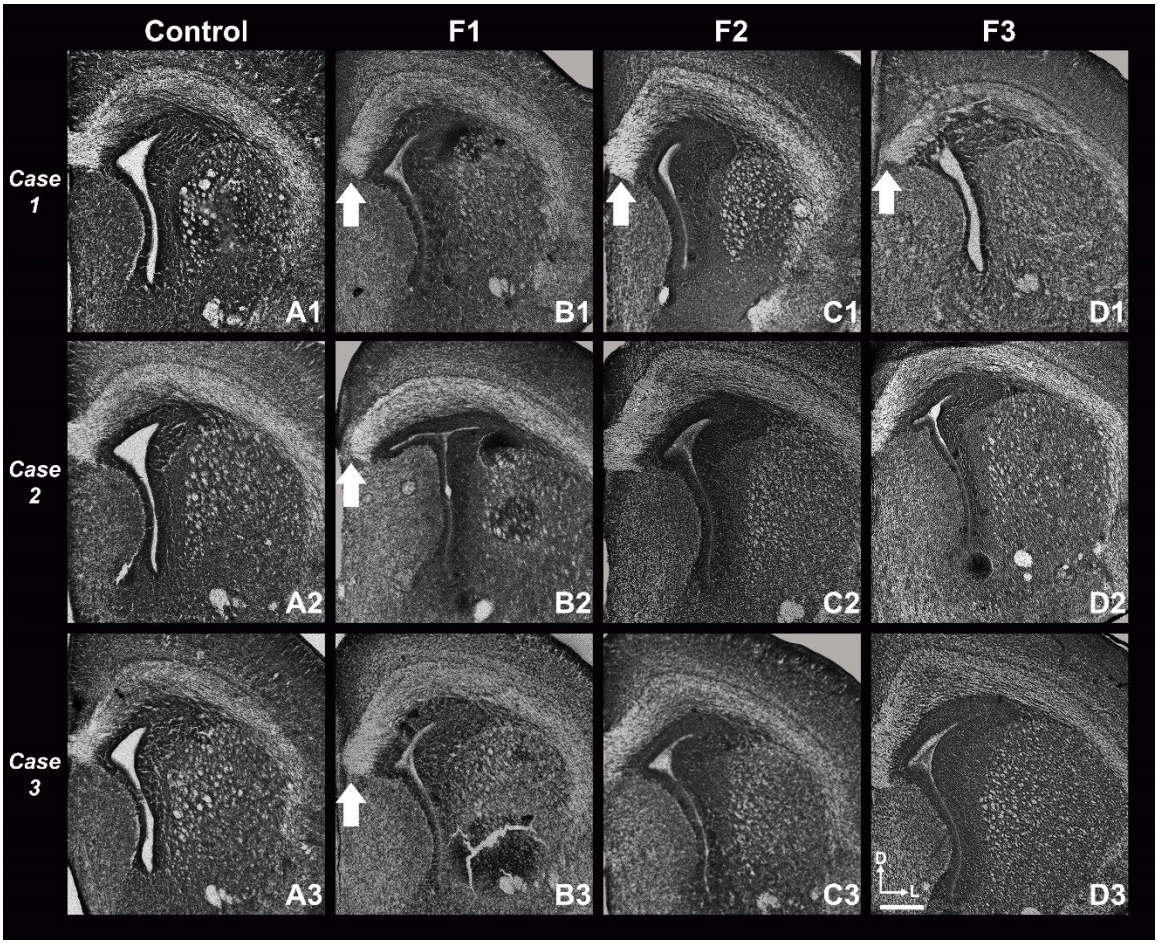


**Figure 2.6. Corpus callosum development evaluation at P0.** Three representative Nissl-stained coronal sections for each experimental group. Control (**A1-3**; n = 11), F1 (**B1-B3**; n = 7), F2 (**C1-C3**; n = 8), and F3 (**D1-D3**; n = 9). We observed abnormalities in the development of the corpus callosum in F1, F2, and F3 progeny. Percent typical development was calculated for each condition and dramatic alterations were statistically significant only for F1 progeny. White arrows indicate aberrant corpus callosum development. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar, 1 mm.

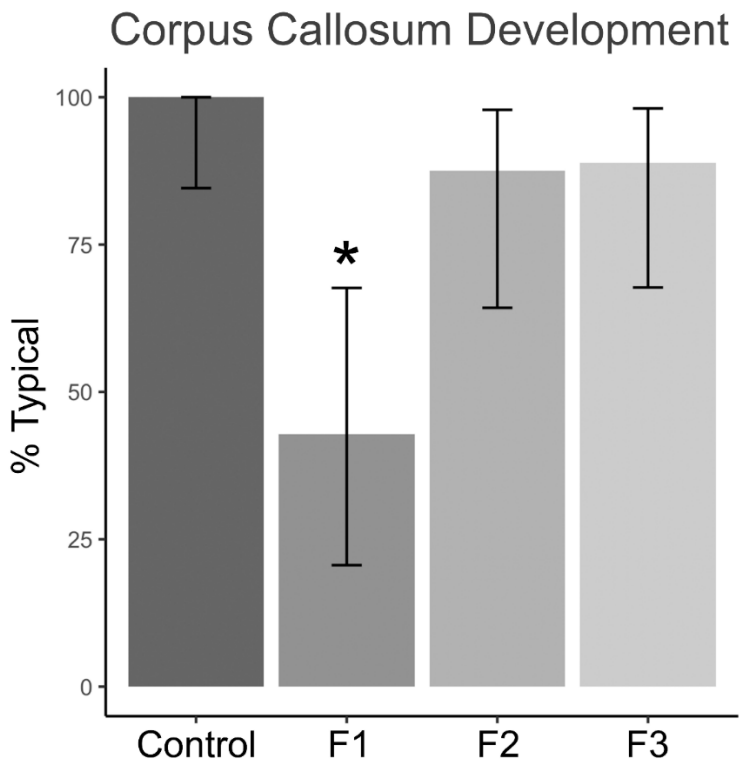


**Figure 2.7. Corpus callosum development chi-squared evaluation.** Percent typical development of corpus callosum development was evaluated using a $\chi 2$ test. We found that the number of pups with typical development varied significantly between the four groups of mice ($p = 0.0087$). This effect was driven by F1 pups since they were significantly different from control pups ($^*p = 0.0345$).

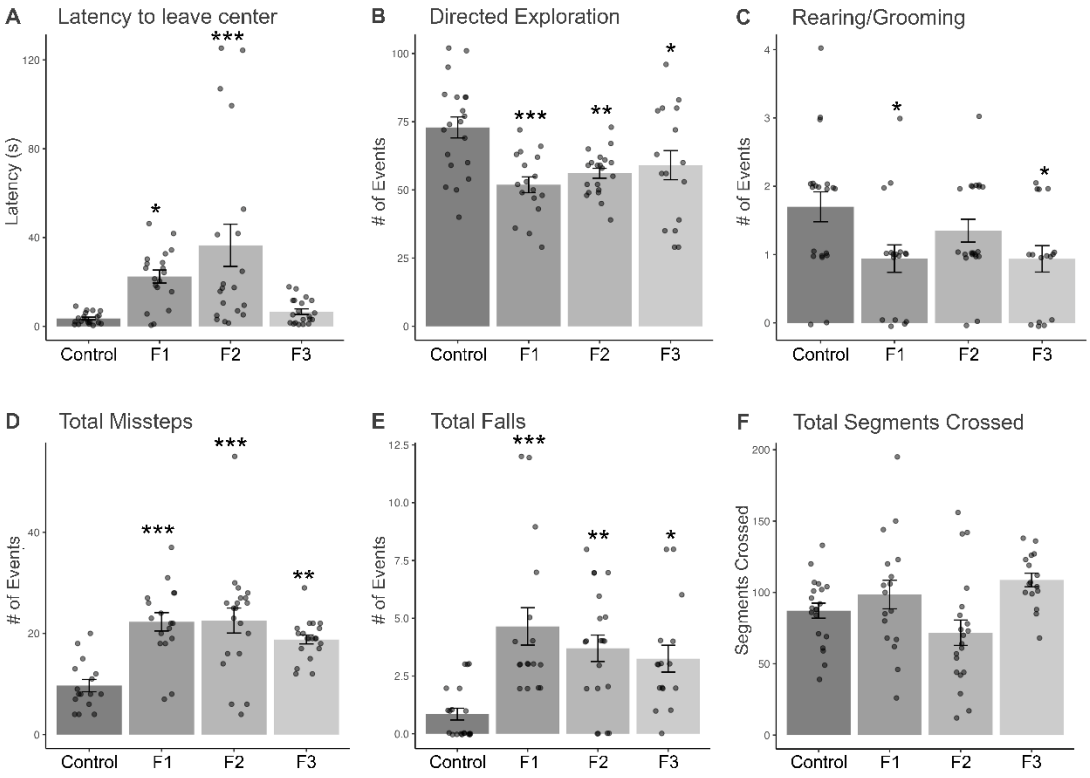


**Figure 2.8. Sensorimotor integration and anxiety-like behaviors at P20.** The Suok test behavioral assay performed on mice at twenty days old. Measures of anxiety: Latency to leave center zone (**A**) directed exploration (**B**), and rearing/grooming (**C**). Significant group differences were seen in the F1 & F2 generation for latency to leave the center. Directed exploration showed altered behavior in all three generations of mice. Rearing/grooming showed altered behavior in the F1 & F3 generation. Motor Measures: missteps (**D**), falls (**E**), and segments crossed (**F**). F1 (n = 19), F2 (n = 34), and F3 (n = 22) mice made significantly more missteps and fell significantly more times than controls (n=24). Interestingly, no differences were observed in segments crossed. Data expressed as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Variations in dot opacity are due to overlap of individual dots.

## REFERENCES


Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K. M., & Huffman, K. J. (2016). The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice. *Alcoholism: Clinical and Experimental Research*, *40*(1), 122-133.

Abbott, C. W., Rohac, D. J., Bottom, R. T., Patadia, S., & Huffman, K. J. (2018). Prenatal ethanol exposure and neocortical development: A transgenerational model of FASD. *Cerebral Cortex*, *28*(8), 2908-2921. https://doi.org/10.1093/cercor/bhx168

Abel, E. L., Jacobson, S., & Sherwin, B. T. (1983). In utero alcohol exposure: functional and structural brain damage. Neurobehav Toxicol Teratol., 5(3), 363-6. PMID: 6877477.

Adhikari, A., Lernet, N. T., Finkelstein, J., Pak, S., Jennings, H. J., Davidson, J. T., Ferenczi, E., Gunaydin, L. A., Mirzabekov, J. J., Ye, L., Kim, S-Y., Lei A. & Deisseroth K. (2015). Basomedial amygdala mediates top-down control of anxiety and fear. *Nature 527*, 179-185. doi: 10.1038/nature15698

Almeida, L., Andreu-Fernández, V., Navarro-Tapia, E., Aras-López, R., Serra-Delgado, M., Martínez, L., Garcia-Algar, O., & Gómez-Roig, M. D. (2020). Murine models for the study of fetal alcohol spectrum disorders: An overview. *Frontiers in Pediatrics*, *8*. https://doi.org/10.3389/fped.2020.00359

Aliyu, M. H., Wilson, R. E., Zoorob, R., Chakrabarty, S., Alio, A. P., Kirby, R. S., & Salihu, H. M. (2008). Alcohol consumption during pregnancy and the risk of early stillbirth among singletons. *Alcohol*, *42*, 369-374. doi:10.1016/j.alcohol.2008.04.003.

Amaral, D. G. (2003). The amygdala, social behavior, and danger detection. *Annals of the New York Academy of Sciences*, *1000*(1), 337-347.

Andreu-Fernández, V., Bastons-Compta, A., Navarro-Tapia, E., Sailer, S., & Garcia-Algar, O. (2019). Serum concentrations of IGF-I/IGF-II as biomarkers of alcohol damage during foetal development and diagnostic markers of Foetal Alcohol Syndrome. *Scientific reports*, *9*(1), 1562.

Archibald, S. L., Fennema-Notestine, C., Gamst, A., Riley, E. P., Mattson, S. N., & Jernigan, T. L. (2001). Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Developmental Medicine and Child Neurology, 43*(3), 148-154.

Astley, S. J., Olson, H. C., Kerns, K., Brooks, A., Aylward, E. H., Coggins, T. E., & Richards, T. (2009). Neuropsychological and behavioral outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders, *Alcohol Clin Exp Res, 16*(1), 178–201.

Autti-Rämö, I., Autti, T., Korkman, M., Kettunen, S., Salonen, O., & Valanne, L. (2002). MRI findings in children with school problems who had been exposed prenatally to alcohol. *Dev Med Child Neurol, 44*, 98–106.

Baculis, B. C., Diaz, M. R., & Valenzuela, C. F. (2015) Third trimester-equivalent ethanol exposure increases anxiety-like behavior and glutamatergic transmission in the basolateral amygdala. *Pharmacol Biochem Behav.*, *137*, 78-85. doi: 10.1016/j.pbb.2015.08.009.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi:10.18637/jss.v067.i01

Bottom, R. T., Abbott, C. W., 3rd, & Huffman, K. J. (2020). Rescue of ethanol-induced FASD-like phenotypes via prenatal co-administration of choline. *Neuropharmacology 168*, 107990. doi:10.1016/j.neuropharm.2020.107990

Bottom, R. T., Kozanian, O. O., Rohac, D. J., Erickson, M. A., & Huffman, K. J. (2022). Transgenerational effects of prenatal ethanol exposure in prepubescent mice. *Front. Cell Dev. Biol. 10*. doi: 10.3389/fcell.2022.812429

Carter, R. C., Jacobson, J. L., Sokol, R. J., Avison, M. J., & Jacobson, S. W. (2013). Fetal alcohol-related growth restriction from birth through young adulthood and moderating effects of maternal prepregnancy weight. *Alcoholism: Clinical and Experimental Research*, *37*(3), 452-462.

Carter, R. C., Jacobson, J. L., Molteno, C. D., Dodge, N. C., Meintjes, E. M., & Jacobson, S. W. (2016). Fetal alcohol growth restriction and cognitive impairment. *Pediatrics*, *138*(2). https://doi.org/10.1542/peds.2016-077

Centers for Disease Control and Prevention, (2022). Alcohol use during pregnancy. U.S. Department of health and human services. Available online at: https://www.cdc.gov/ncbddd/fasd/alcohol-use.html

Cederbaum, A. I. (2012). Alcohol metabolism. *Clinics in liver disease, 16*(4), 667-685.

Coles, C. D., Brown, R. T., Smith, I. E., Platman, K. A., Erickson, S., & Falek, A. (1991). Effects of prenatal alcohol exposure at school age: I. Physical and cognitive development. *Neurotoxicol. Teratol*., *13*, 357-367.

Coles, C. D., Goldstein, F. C., Lynch, M. E., Chen, X., Kable, J. A., Johnson, K. C., & Hu, X. (2011). Memory and brain volume in adults prenatally exposed to alcohol. *Brain Cogn, 75*(1), 67-77. doi: 10.1016/j.bandc.2010.08.013.

Davies, D. L., & Smith, D. E. (1981). A Golgi study of mouse hippocampal CA1 pyramidal neurons following perinatal ethanol exposure. *Neurosci Lett 26*, 49-54.

Driscoll, C. D., Streissguth, A. P., & Riley, E.P. (1990). Prenatal alcohol exposure: Comparability of effects in humans and animal models. *Neurotoxicology and Teratology, 12*(3), 231–237

Dunnett, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association*, *50*(272), 1096-1121. https://doi.org/10.1080%2F01621459.1955.10501294

Dye, C. A., El Shawa H., & Huffman, K. J. (2011). A lifespan analysis of intraneocortical connections and gene expression in the mouse I. *Cerebral Cortex, 21,* 1311-1330. doi:10.1093/cercor/bhq212

El Shawa, H., Abbott, C. W., 3rd, & Huffman, K. J. (2013). Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene expression, and behavior in a mouse model of FASD. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *33*(48), 18893-18905. https://doi.org/10.1523/JNEUROSCI.3721-13.2013

Engin, E., Kiersten S. S., Gao, Y., Nagy, D., Foster, R. A., Tsvetkov, E.,... & Rudolph, U. (2016). Modulation of anxiety and fear via distinct intrahippocampal circuits. *eLife, 5*. doi: 10.7554/eLife.14120

Fagerlund, A., Heikkinen, S., Autti-Rämö, I., Korkman, M., Timonen, M., Kuusi, T., Riley, E. P., & Lundbom, N. (2006). Brain metabolic alterations in adolescents and young adults with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res, 30*, 2097-104. doi: 10.1111/j.1530-0277.2006.00257.x. PMID: 17117975.

Fukuchi-Shimogori, T., & Grove, E. A. (2001). Neocortex patterning by the secreted signaling molecule FGF8. *Science. 294*(5544), 1071-4. doi: 10.1126/science.1064252.

Furtado, E. F., & Roriz, S. T. D. S. (2016). Inattention and impulsivity associated with prenatal alcohol exposure in a prospective cohort study with 11-years-old Brazilian children. *European child & adolescent psychiatry*, *25*, 1327-1335.

Gangisetty O., Wynne O., Jabbar S., Nasello C., & Sarkar D. K. (2015). Fetal alcohol exposure reduces dopamine receptor d2 and increases pituitary weight and prolactin production via epigenetic mechanisms. *PLoS One.* 10:e0140699. 10.1371/journal.pone.0140699

Gangisetty, O., Palagani, A., & Sarkar, D. K. (2020). Transgenerational inheritance of fetal alcohol exposure adverse effects on immune gene interferon-Y. *Clinical Epigenetics*, *12*(1), 70. https://doi.org/10.1186/s13148-020-00859-9

Gapp K, & Bohacek J. (2018). Epigenetic germline inheritance in mammals: Looking to the past to understand the future. *Genes, Brain and Behavior*. *17*(3). DOI: 10.1111/gbb.12407

Gautam, P., Warner, T. D., Kan, E. C., & Sowell, E. R. (2015). Executive function and cortical thickness in youths prenatally exposed to cocaine, alcohol and tobacco. *Developmental cognitive neuroscience*, *16*, 155-165.

Glajch, K. E., Fleming, S. M., Surmeier, D. J., & Osten, P. (2012) Sensorimotor assessment of the unilateral 6-hydroxydopamine mouse model of Parkinson's disease. *Behav Brain Res., 230*, 309–316.

Govorko, D., Bekdash, R. A., Zhang, C., & Sarkar, D. K. (2012). Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene cross generations. *Biological Psychiatry*, *72*(5), 378–388. https://doi.org/10.1016/j.biopsych.2012.04.006

Haley, D. W., Handmaker, N. S., & Lowe, J. (2006). Infant stress reactivity and prenatal alcohol exposure. *Alcohol Clin Exp Res, 30*, 2055–64.

Harms, M. B., Zayas, V., Meltzoff, A. N., & Carlson, S. M. (2014). Stability of executive function and predictions to adaptive behavior from middle childhood to pre-adolescence. *Frontiers in Psychology*, *5*, 1-11. https://doi.org/10.3389/fpsyg.2014.00331

Hellemans, K. G., Sliwowska, J. H., Verma, P., & Weinberg, J. (2010a). Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neurosci Biobehav Rev., 34*, 791-807.

Hellemans, K. G., Verma, P., Yoon, E., Yu, W. K., Young, A. H., & Weinberg, J. (2010b). Prenatal alcohol exposure and chronic mild stress differentially alter depressive- and anxiety-like behaviors in male and female offspring. *Alcohol Clin Exp Res 34*, 633– 645.

Hellström, A., Ley, D., Hansen-Pupp, I., Hallberg, B., Ramenghi, L. A., Löfqvist, C., Smith, L. E., & Hård, A. L. (2016). Role of insulin like growth factor 1 in fetal development and in the early postnatal life of premature infants. *Am J Perinatol, 33*(11), 1067-71. doi: 10.1055/s-0036-1586109.

Hen-Herbst, L., Jirikowic, T., Hsu, L. Y., & McCoy, S. W. (2020). Motor performance and sensory processing behaviors among children with fetal alcohol spectrum disorders compared to children with developmental coordination disorders. *Research in Developmental Disabilities*, *103*(January), 103680. https://doi.org/10.1016/j.ridd.2020.103680

Huang, X., Du, X., Song, H., Zhang, Q., Jia, J., Xiao, T., & Wu, J. (2015). Cognitive impairments associated with corpus callosum infarction: A ten cases study. *Int J Clin Exp Med., 8*(11) 21991-8. PMID: 26885171

Huffman, K. J., Garel, S., & Rubenstein, J. L. (2004). Fgf8 regulates the development of intraneocortical projections. *J Neurosci, 24*.

Hwang, H. M., & Hashimoto-Torii, K. (2022). Activation of the anterior cingulate cortex ameliorates anxiety in a preclinical model of fetal alcohol spectrum disorders. *Transl Psychiatry, 12*(1), 24. doi: 10.1038/s41398-022-01789-1.

Jirikowic, T. L., Thorne, J. C., McLaughlin, S. A., Waddington, T., Lee, A. K., & Hemingway, S. J. A. (2020). Prevalence and patterns of sensory processing behaviors in a large clinical sample of children with prenatal alcohol exposure. *Research in Developmental Disabilities*, *100*, 103617.

Jirtle, R. L., & Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nat Rev Genet 8*, 253–262.

Johnson, S. C., Pinkston, J. B., Bigler, E. D., & Blatter, D. D. (1996). Corpus callosum morphology in normal controls and traumatic brain injury: Sex differences, mechanisms of injury, and neuropsychological correlates. *Neuropsychology, 10*(3), 408–415. https://doi.org/10.1037/0894-4105.10.3.408

Johnston, D., Branton, E., Rasmuson, L., Schell, S., Gross, D. P., & Pritchard-Wiart, L. (2019). Accuracy of motor assessment in the diagnosis of fetal alcohol spectrum disorder. *BMC Pediatrics*, *19*(1), 1–9. https://doi.org/10.1186/s12887-019-1542-3

Jones, K. L., & Smith, D. W. (1973a) Recognition of the fetal alcohol syndrome in early infancy. *Lancet, 302*(7836), 999-1001. doi: 10.1016/s0140-6736(73)91092-1.

Jones, K. L., Smith, D. W., Ulleland, C. N., & Streissguth, P. (1973b). Pattern of malformation in offspring of chronic alcoholic mothers. *The Lancet, 301*(7815), 1267-1271.

Jones, K. L., Hoyme, H. E., Robinson, L. K., Del Campo, M., Manning, M. A., Prewitt, L. M., & Chambers, C. D. (2010). Fetal alcohol spectrum disorders: Extending the range of structural defects. *American Journal of Medical Genetics Part A*, *152*(11), 2731-2735.

Kadakia R. & Josefson J. (2016). The relationship of insulin-like growth factor 2 to fetal growth and adiposity. *Hormone Research in Pediatrics, 85*(2), 75-82. https://doi.org/10.1159/000443500

Kalberg, W. O., Provost, B., Tollison, S. J., Tabachnick, B. G., Robinson, L. K., Eugene Hoyme, H., Trujillo, P. M., Buckley, D., Aragon, A. S., & May, P. A. (2006). Comparison of motor delays in young children with fetal alcohol syndrome to those with prenatal alcohol exposure and with no prenatal alcohol exposure. *Alcohol Clin Exp Res, 30*, 2037–2045.

Kalueff, A. V., Keisala, T., Minasyan, A., Kumar, S. R., LaPorte, J. L., Murphy, D. L., & Tuohimaa, P. (2008). The regular and light-dark Suok tests of anxiety and sensorimotor integration: utility for behavioral characterization in laboratory rodents. *Nat Protoc, 3*, 129 –136.

Kerns, K. A., Don, A., Mateer, C. A., & Streissguth, A. P. (1997). Cognitive deficits in nonretarded adults with fetal alcohol syndrome. *J Learn Disabil, 30*(6), 685-93. doi: 10.1177/002221949703000612.

Kerr, W. C., Ye, Y., Martinez, P., Karriker-Jaffe, K. J., Patterson, D., Greenfield, T. K., & Mulia, N. (2022). Longitudinal assessment of drinking changes during the pandemic: The 2021 COVID-19 follow-up study to the 2019 to 2020 National Alcohol Survey. *Alcohol Clin Exp Res*, *46*(6),1050-1061. doi: 10.1111/acer.14839.

Kozanian, O. O., Rohac, D. J., Bavadian, N., Corches, A., Korzus, E., & Huffman, K.J. (2018). Long-lasting effects of prenatal ethanol exposure on fear learning and development of the amygdala. *Frontiers in Behavioral Neuroscience, 12* (200). https://doi.org/10.3389/fnbeh.2018.00200.

Lam, V. Y. Y., Raineki, C., Wang, L. Y., Chiu, M., Lee, G., Ellis, L., Yu, W., & Weinberg, J. (2019). Role of corticosterone in anxiety- and depressive-like behavior and HPA regulation following prenatal alcohol exposure. *Prog Neuropsychopharmacol Biol Psychiatry. 90*, 1-15. doi: 10.1016/j.pnpbp.2018.10.008.

Lemoine, P., Harousseau, H., Borteyru, J. P., & Menuet, J. C. (1968). Les enfants de parents alcooliques: Anomalies observées à propos de 127 cas. *Ouest méd, 21*(476-482), 2.

Lemoine, P., Harousseau, H., Borteyru, J. P., & Menuet, J. C. (2003). Children of alcoholic parents—observed anomalies: discussion of 127 cases. *Ther. Drug Monit., 25*(2), 132-136.

Lenth, R. V. (2022). *emmeans: Estimated marginal means, aka least-squares means. R package Version 1.7.2*. Available online at: https://CRAN.R-project.org/package=emmeans.

Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P., & Zhou, F. C. (2009). Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics, 4*(7), 500-11. doi: 10.4161/epi.4.7.9925. PMID: 20009564; PMCID: PMC2805036.

Livy, D. J., Miller, E. K., Maier, S. E., & West, J. R. (2003). Fetal alcohol exposure and temporal vulnerability: Effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicology and Teratology, 25*(4), 447-458. doi:10.1016/S0892-0362(03)00030-8

Lucia D., Burgess D., Cullen C. L., Dorey E. S., Rawashdeh O., & Moritz K. M. (2019). Periconceptional maternal alcohol consumption leads to behavioural changes in adult and aged offspring and alters the expression of hippocampal genes associated with learning and memory and regulators of the epigenome. *Behav. Brain Res, 362*, 249–257. 10.1016/j.bbr.2019.01.009

Mattson, S. N., & Riley, E. P. (1998). A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcoholism: Clinical and Experimental Research, 22*(2), 279-294.

Mattson, S. N., Bernes, G. A., & Doyle, L. R. (2019). Fetal alcohol spectrum disorders: A review of the neurobehavioral deficits associated with prenatal alcohol exposure. *Alcoholism: Clinical and Experimental Research*, *43*(6), 1046–1062. https://doi.org/10.1111/acer.14040

May, P. A., Chambers, C. D., Kalberg, W. O., Zellner, J., Feldman, H., Buckley, D…. Hoyme, H. E. (2018). Prevalence of fetal alcohol spectrum disorders in 4 US communities. *Jama, 319*, 474–482. doi:10.1001/jama.2017.21896

May, P. A., Hasken, J. M., Hooper, S. R., Hedrick, D. M., Jackson-Newsom, J., Mullis, C. E., Dobyns, E., Kalberg, W., Buckley, D., Robinson. L., Abdul-Rahman, O., Adam, M. P., Manning. M. A., Jewett. T., & Hoyme, H. E. (2021). Estimating the community prevalence, child traits, and maternal risk factors of fetal alcohol spectrum disorders (FASD) from a random sample of school children. *Drug and Alcohol Dependence*, *227*, https://doi.org/10.1016/j.drugalcdep.2021.108918

Molteno, C. D., Jacobson, J. L., Carter, R. C., Dodge, N. C., & Jacobson, S. W. (2014). Infant emotional withdrawal: A precursor of affective and cognitive disturbance in fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research*, *38*(2), 479–488. https://doi.org/10.1111/acer.12240

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology, 38*, 23-38.

Muralidharan, P., Sarmah, S., Zhou, F. C., & Marrs, J. A. (2013). Fetal alcohol spectrum disorder (FASD) associated neural defects: Complex mechanisms and potential therapeutic targets. *Brain Sciences, 3*(2), 964-991. https://doi.org/10.3390/brainsci3020964

Naik, V. D., Lee, J., Wu, G., Washburn, S., & Ramadoss, J. (2022). Effects of nutrition and gestational alcohol consumption on fetal growth and development. *Nutrition Reviews*, *80*(6), 1568-1579. https://doi.org/10.1093/nutrit/nuab119

Nestler, V., Spohr, H. L., & Steinhausen, H. C. (1981). Mehrdimensionale Studien zur Alkoholembryopathie [Studies on alcohol embryopathy (author's translation)]. *Monatsschr Kinderheilkd, 129*(7), 404-9. PMID: 6167854.

Norman, A. L., Crocker, N., Mattson, S. N., & Riley, E. P. (2009). Neuroimaging and fetal alcohol spectrum disorders. *Developmental Disabilities Research Review, 15*(3), 209-217.

O'Neil, E. (2010). Corpus callosum defects associated with fetal alcohol syndrome. *Embryo Project Encyclopedia.* ISSN: 1940-5030 Retrieved from http://embryo.asu.edu/handle/10776/2062

Paxinos, G., Watson, C., Koutcherov, Y., Halliday, G. M., & Wang, H. (2007). *Atlas of the developing mouse brain at E17.5, P0 and P6.* Amsterdam: Elsevier Science.

R Core Team (2021). *R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.*

Richmond, S. B. & Fling, B. W. (2019). Transcallosal control of bilateral actions. *Exerc Sport Sci Rev*., *47*(4), 251-257. doi: 10.1249/JES.0000000000000202.

Riikonen, R., Salonen, I., Partanen, K., & Verho, S. (1999). Brain perfusion SPECT and MRI in fetal alcohol syndrome. *Developmental Medicine and Child Neurology, 41*(10), 652-659.

Riley, E. P., Barron, S., Driscoll, C. D., & Chen, J. S. (1984). Taste aversion learning in preweanling rats exposed to alcohol prenatally. *Teratology, 29*, 325-331.

Riley, E. P., Mattson, S. N., Sowell, E. R., Jernigan, T. L., Sobel, D. F., & Jones, K. L. (1995). Abnormalities of the corpus callosum in children prenatally exposed to alcohol. *Alcohol Clinical Exp Res, 19*,1198–1202.

Riley, E. P., McGee, C. L., & Sowell, E. R. (2004). Teratogenic effects of alcohol: a decade of brain imaging. *American Journal of Medical Genetics. Part C: Seminars in Medical Genetics, 127C* (1), 35–41.

Sarkar, D. K. (2015). Male germline transmits fetal alcohol epigenetic marks for multiple generations: a review. *Addiction Biology*, doi:10.1111/adb.12186

Streissguth, A. P., Barr, H. M., Kogan, J., & Bookstein, F. L. (1996). Understanding the occurrence of secondary disabilities in clients with fetal alcohol syndrome (FAS) and fetal alcohol effects (FAE): Final report to the Centres for Disease Control and Prevention. Seattle, WA: University of Washington Fetal Alcohol and Drug Unit.

Strandberg-Larsen, K., Nielsen, N. R., Grønbaek, M., Andersen, P. K., Olsen, J., & Andersen, A. M. N. (2008). Binge drinking in pregnancy and risk of fetal death. *Obstetrics and Gynecology*, *111*(3), 602-609. doi:10.1097/AOG.0b013e3181661431.

Swayze, V. W., Johnson, V. P., Hanson, J. W., Piven, J., Sato, Y., Giedd, J. N., ... & Andreasen, N. C. (1997). Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. *Pediatrics*, *99*(2), 232-240.

Uecker, A., & Nadel, L. (1996). Spatial locations gone awry: Object and spatial memory deficits in children with fetal alcohol syndrome. *Neuropsychologia, 34*, 209-223

Vannucci, R. C., Barron, T. F., & Vannucci, S. J. (2017) Development of the corpus callosum: an MRI study. *Dev Neurosci, 39*, 97-106 doi:10.1159/000453031 pmid:28013305

Wieczorek, L., Fish, E. W., O'Leary-Moore, S. K., Parnell, S. E., & Sulik, K. K. (2015). Hypothalamic-pituitary-adrenal axis and behavioral dysfunction following early binge-like prenatal alcohol exposure in mice. *Alcohol. 49*, 207-17.

Willoughby, K. A., Sheard, E. D., Nash, K., & Rovet, J. (2008). Effects of prenatal alcohol exposure on hippocampal volume, verbal learning, and verbal and spatial recall in late childhood. *Journal of the International Neuropsychological Society, 14*(6), 1022–1033.

Windham, G. C., Von Behren, J., Fenster, L., Schaefer, C., & Swan, H. (2015). Moderate maternal alcohol consumption and risk of spontaneous abortion. *Epidemiology, S., & Sep, N, 8*(5), 509-514.

Yang, M., Silverman, J. L., & Crawley, J. N. (2011). Automated three-chambered social approach task for mice. *Current Protocols in Neuroscience*, (SUPPL. 56). https://doi.org/10.1002/0471142301.ns0826s56

Zhou, D., Lebel, C., Lepage, C., Rasmussen, C., Evans, A., Wyper, K., ... & Beaulieu, C. (2011). Developmental cortical thinning in fetal alcohol spectrum disorders. *Neuroimage*, *58*(1), 16-25.

## Chapter 3: Transgenerational effects of paternal alcohol consumption on offspring brain and behavioral development


### ABSTRACT

Fetal alcohol spectrum disorders (FASD) describe the wide array of long-lasting developmental abnormalities in offspring due to prenatal alcohol (ethanol [EtOH]) exposure via maternal gestational drinking. Although the teratogenic consequences of prenatal EtOH exposure are apparent, the effects of preconception paternal EtOH exposure (PatEE) are still unclear. In previous rodent models, PatEE has been shown to disturb gene expression in the brain (Finegersh & Homanics, 2014; Rompala et al., 2017) and liver (Chang et al., 2017, 2019) of offspring. Our previous research suggests that PatEE can induce molecular changes and abnormal behavior in the offspring in the first filial generation (F1) (Conner et al., 2020). However, it is not known whether the effects of PatEE can be passed transgenerationally.

In this study, we utilized a novel mouse model of PatEE where male mice self-administered 25% EtOH for 15-20 days prior to conception, generating indirect exposure to the offspring (F1) through the paternal germline. PatEE (F1) males were paired with EtOH naïve females to generate offspring for the second generation (F2). The impact of paternal ethanol exposure on newborn offspring neocortical development was evaluated using neuroanatomical and molecular

techniques at postnatal day (P) 0, as well as a battery of behavioral assays in pre- and peri-pubescent mice.

We observed no differences between control and PatEE (F1 and F2) mice in measures of neocortical length. Abnormal patterns of *Id2* and *RZRβ* gene expression were observed in the F1 generation but not the F2 at P0. Additionally, PatEE may generate sex-specific effects on offspring behavior that can last up to two generations after the sire's initial exposure.

Overall, these findings suggest that ethanol exposure in males prior to conception may induce long-lasting, transgenerational changes in the brain and behavior of offspring. This study provides additional insight into the biological mechanisms that potentially underlie atypical behavior observed in children of alcoholic fathers.

## INTRODUCTION

Prenatal ethanol (EtOH) exposure (PrEE) can result in fetal alcohol spectrum disorders (FASD). This designation incorporates a variety of long-lasting cognitive and behavioral deficits (Hoyme et al., 2016) and has incidence rates as high as 5% in the United States (May et al., 2018). Much less is known about the impact of preconception paternal EtOH exposure (PatEE), despite a growing body of preclinical evidence indicating that offspring sired by males exposed to EtOH prior to conception display–altered brain and behavioral development similar to maternal-mediated prenatal EtOH exposure (Chang et al.,

2017, 2019; Finegersh & Homanics, 2014; Jamerson et al., 2004; Kim et al., 2014; Meek et al., 2007; Rompala et al., 2016, 2017). Additionally, clinical research in humans has found associations among heavy paternal EtOH consumption and adverse developmental outcomes in offspring (reviewed in Finegersh et al., 2015; Xia et al., 2018; Zuccolo et al., 2017), providing further support for the deleterious impact of paternal drinking. Although research investigating the impact of PatEE is on the rise, it remains greatly understudied compared to models of FASD generated from EtOH exposure via maternal drinking.

The neocortex, the largest part of the human brain, has many emergent properties that mediate complex, higher order functions and behaviors. The neocortex relies on a tightly regulated temporal and spatial orchestration of genetic and environmental cues for proper development, a process that seems particularly susceptible to prenatal EtOH insult. Animal studies focusing on maternal EtOH exposure have found a plethora of atypical cortical phenotypes present in offspring including increased apoptosis (Ikonomidou et al., 2000), altered pyramidal cell morphology (Granato et al., 2003), modified development of anatomical regions or structures (Abbott et al., 2016), and atypical development of the intraneocortical circuitry (El Shawa et al., 2013). Human neuroimaging studies in children with FASD have also demonstrated abnormalities in neocortical development (Zhou et al., 2011), suggesting that

irregular cortical phenotypes may underlie some PrEE-induced behavioral alterations.

One particular aspect of neocortical development affected by prenatal EtOH exposure is arealization or the patterning of neurons into functionally and spatially distinct areas (Dye et al., 2011a, b). Specifically, prenatal EtOH exposure results in aberrant intraneocortical connections (INCs), as well as altered expression of genes critical for proper patterning of the neocortex in mice (El Shawa et al., 2013). Recently, we have demonstrated that these phenotypes pass to second and third filial generations after an initial prenatal EtOH exposure (Abbott et al., 2018), suggesting EtOH may have potent transgenerational effects. Despite a growing body of research on how PrEE impacts the neocortex, there is a paucity of data regarding how paternal EtOH exposure may alter cortical development. Preclinical studies focusing on PatEE's effects on the neocortex are sparse but have shown that affected offspring have increased cortical thickness (Jamerson et al., 2004) and altered expression and epigenetic regulation of the dopamine transporter in frontal cortex (Kim et al., 2014). Importantly, to our knowledge, no study exists examining the effect of preconception paternal EtOH consumption on development of neocortical connections. Due to the ability of PatEE to disrupt normal development in the neocortex, as well as EtOH's ability to modify INCs in the absence of direct exposure, we hypothesized that PatEE offspring could also demonstrate abnormal neocortical development.

Since many prenatal EtOH exposure phenotypes have been shown to pass transgenerationally (Abbott et al., 2018; Gangisetty et al., 2020; see review by Chastain and Sarkar 2017), we investigated whether the observed phenotypes from preconception PatEE also persist into future generations beyond the F1 generation. The goal of this study was to characterize the impact of a paternal binge of EtOH prior to conception on offspring cortical and behavioral development and to investigate if these effects spread transgenerationally after initial EtOH insult. Specifically, we analyzed neocortical thickness, gene expression, and development of INCs in newborn mice. We also examined behavioral effects of PatEE in pre- and peri-pubescent mice at ages P20 and P30 using a battery of assays. Results from this study suggest that the paternal environment before conception is critical for healthy offspring development.

## MATERIALS AND METHODS

### *Animal Care*

All studies were conducted under research protocol guidelines approved by the Institutional Animal Care and Use Committee at UCR. CD-1 mice were initially purchased from Charles River Laboratories (Wilmington, MA). All subjects were housed in UCR animal facilities under a standard 12-hour/12-hour light/dark cycle. All efforts were made to minimize animal discomfort and the number of mice utilized.

*EtOH Administration and Breeding*

Male mice, aged 3 to 6 months, were separated into control and EtOH-exposed groups. Initially, experimental EtOH-treated male mice (n = 10) were provided a 25%EtOH in water solution, *ad libitum*, for 15 days and standard mouse chow (Fig. 3.1). Control males (n = 10) were provided *ad libitum* water and standard mouse chow. After the binge period, P90 female mice were paired with control or EtOH-treated sires at the beginning of the dark cycle for breeding. The day of conception was determined by the presence of a vaginal plug, after which males were removed from the dam's cage. If no vaginal plug was observed, the male was returned to his home cage for continued treatment of EtOH or water for the remainder of the day and then placed back into the breeding cage at the start of the dark cycle. The average time to conception was 3.5 days for EtOH sires and 2.8 days for control sires. Each group had a time to conception of 0 to 8 days with total length of treatment being 15 to 23 days. All pregnant female mice were housed individually and provided standard mouse chow and water *ad libitum*. All female dams were EtOH-naïve and did not have access to EtOH. The male germline was used to produce the next generation (F2). To do this, P90 F1 PatEE males were bred with EtOH-naïve P90 females.

*Sire Daily Intake and Blood EtOH Content Measurements*

Daily measurements of food and liquid intake of male mice were recorded at 1,200 hours to assess confounding nutritional differences between EtOH-exposed and control sires. Each male was provided 100 g of food, and the chow was reweighed daily at noon and replenished to 100 g. Daily liquid intake (25% EtOH in water or water alone) for F1 and control sires was measured using a graduated drinking bottle. Average daily values for food and liquid intake of experimental mice and weight gain were compared with control mice using t-test analyses. Also, body weights, in grams, were recorded for all mice at the beginning of exposure when the EtOH solution was provided and at the end when EtOH was removed, to eliminate weight gain differences as a potential confound. Blood EtOH concentration (BEC) of a separate subset of males, resulting from treatment of 25%EtOH in water (n = 7) or water alone (n = 7), was determined using an alcohol dehydrogenase–based enzymatic assay (Pointe Scientific, Canton, MI). Briefly, whole blood samples from a separate subset of males (n = 7) were collected via cardiac puncture after 20 days of treatment with 25% EtOH in water. Blood samples from control males (n = 7) receiving only water treatment for 20 days were also collected and included in analysis as negative controls. Following collection at 1000h, whole blood samples were set at room temperature for 30 minutes to allow clotting. After clotting, samples were centrifuged for 10 minutes at 2,000 x g at 4°C to obtain serum. 5 µL of serum samples were mixed with 1 mL of alcohol reagent and assayed in duplicate immediately using a Nanodrop 2000 spectrophotometer at 340 nm wavelength.

An ethanol standard (100 mg/dL) was also reacted with alcohol reagent to create a BEC standard. BEC levels were quantitatively determined by comparing test samples with the ethanol standard.

*Pup Measurement and Tissue Preparation*

On the day of birth, P0, the litter sizes were recorded. Pups born to EtOH naïve dams bred with EtOH-treated sires were designated as PatEE F1 pups, while pups bred from PatEE F1 sires and EtOH naïve dams were designated as the PatEE F2 pups. Pups born to dams bred with water-treated sires were designated as controls. For each litter (n = 10, all groups), subsets of offspring were randomly designated for P0 analyses, P20 behavioral assessment, or P30 behavioral assessment to reduce potential litter effects and ensure an even sampling for each experimental endpoint. Experimental/control subsets per litter were limited to 2 ± 2 pups for P0 endpoints, 5 ± 2 pups for P20 endpoints, and 6 ± 2 pups for P30 endpoints, depending on total litter size. Larger subsets were reserved for P20 and P30 behavioral assessment due to the statistical power needed for accurate sex-specific analysis; all efforts were made to limit three animals/per sex/per litter for each experimental endpoint. P0 pups used for dye tracing (see Conner et al., 2020), anatomy, and gene expression studies were weighed, sacrificed via hypothermia and exsanguination, and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4.

Due to the absence of distinguishing sexual characteristics at P0, the relative inaccuracy of using anogenital distances at this age (Greenham & Greenham, 1977), and the absence of pigment in albino CD-1 mice (Wolterink-Donselaar et al., 2009), no sex differences were assessed at P0. Next, brains were removed from the skull and weighed. Dorsal views of whole brains were imaged using a Zeiss (Oberkochen, Germany) Axio high-resolution (HRm) camera attached to a dissecting microscope. Cortical lengths were measured with a digital micrometer in ImageJ (NIH, Bethesda, MD, USA) using dorsal whole-brain images. After postfixation in 4% PFA, brains were hemisected and hemispheres were designated for either anatomy, gene expression assays, or dye tracing. Designated subsets of each litter were kept with their mothers until P20 where they were either used immediately in behavioral analysis or were weaned until P30 where separate, alternate behavioral analyses took place. Some male PatEE F1 mice were used to generate the F2 generation. Offspring weights were documented at P0, P20, and P30 to assess for changes throughout development.

*Neuroanatomical Measurements*

For anatomical measurements of cortical and subcortical areas, P0 hemispheres (PatEE F1: n = 8; PatEE F2: n =4; P0 control: n = 8) were cryoprotected in 30%sucrose solution for 1 to 3 days. Tissue was sectioned at 40 µm on the coronal plane via Leica cryostat, mounted, and then stained for Nissl

substance. Brain sections were imaged using a Zeiss Axio Upright Imager microscope equipped with a Zeiss Axio HRm camera. To make comparisons between groups, strict matching of sections took place using the Paxinos developing mouse brain atlas (Paxinos et al., 2007) as a guide and a number of anatomical landmarks including corpus callosum, hippocampus, and subcortical structures. Due to the stringent nature of such section matching, individual cases were only included in quantitative analysis for particular cortical areas if they met the exact-match criteria. Once matched, regions of interest (ROIs) were measured using an electronic micrometer in ImageJ (NIH) by a trained researcher blind to treatment, as previously reported (Abbott et al., 2016). Briefly, cortical thickness measurements were determined from electronic lines perpendicular to the cortical sheet, drawn from the most superficial region of layer I to the deepest region of layer VI. Regions measured included putative frontal cortex, prelimbic cortex, auditory cortex, putative somatosensory cortex (S1), and putative visual cortex (V1). Additionally, measurements of the corpus callosum, CA3 of the hippocampus, and areal measurements of the amygdala were also taken.

*Gene Expression Assays*

Analysis of gene expression within P0 brain hemispheres was carried out via *in situ* RNA hybridization (ISH). Standard protocols for free-floating nonradioactive ISH were used to visualize neocortical *Id2* and *RZRβ* gene

expression of P0 control and PatEE brains, as previously described (Dye et al., 2011a, 2011b; El Shawa et al., 2013). Briefly, hemispheres were first embedded in gelatin–albumin and sectioned at 100 μm via vibratome. After hybridization to probes for *Id2* and *RZRβ* synthesized from cDNA (gifts from John Rubenstein, UCSF; see Abbott et al., 2018 for details), sections were mounted in a 50% glycerol solution onto glass slides, coverslipped, and imaged using a Zeiss Axio HRm camera attached to a dissecting microscope. Anatomically matched ISH sections from PatEE and control P0 brains are presented to highlight the effects of altered gene expression of *Id2* and *RZRβ* associated with PatEE.

*Behavioral Assays*

Behavioral assessment of PatEE and control offspring occurred via a battery of behavioral tests at pre- and peri-pubescence. All P20 control and PatEE mice were evaluated on the Suok assay, and P30 mice were tested on the accelerated rotarod (AR), forced swim test (FST), elevated plus maze (EPM), and 3-chambered sociability test. For P30 behavior, all mice were randomly assigned to 2 of 3 assessments on the day of behavioral testing to reduce handling and stress.

The Suok assay was used to assess anxiety and sensorimotor integration (Kalueff et al., 2008). The apparatus consists of a smooth 2-meter-long aluminum rod, 3-centimeters in diameter, elevated to a height of 20 centimeters. The tube is divided into 10-centimeter segments by colored markings and held in place

between two clear acrylic walls. A 20-centimeter zone is marked at the center most point of the rod and serves as the placement location when starting the assay. At the start of each five-minute testing period, animals will be placed on the center of the bar with their body parallel to the rod. Mice that fall off the apparatus will be quickly placed back onto the rod in the position they fell from. Two measures of sensory motor integration, missteps and falls, will be evaluated. Anxiety-like behaviors including rearing/grooming events, directed exploration, and latency to leave the center of the bar will also be examined.

An accelerated rotarod (AR) (Ugo Basile; Gemonio, Italy) was used to assess motor ability, learning, and coordination (Buitrago et al., 2004; Rustay et al., 2003). A mouse will be placed on the rotarod, a cylindrical rod (3 cm diameter) that rotates and increases speed from 4 rpm to 40 rpm as time progresses, for five minutes. Each mouse will complete 4 trials with a ten-minute break in between each trial. Once the mouse falls off the rod the trial is over, and time is recorded. If the mouse lasts the full five minutes without falling, then it is given a full score of 300 seconds.

The forced swim test (FST) was originally used to assess the effects of antidepressant drugs in the late 1970s (Porsolt et al., 1978) Therefore the forced swim test was used to screen for depressive-like behavior (Lucki et al., 2001) as well as active/passive coping styles. The apparatus consists of a clear acrylic cylinder, 30 cm tall by 13 cm in diameter, which will be filled with room-temperature water to a height of 20 cm. Mice will be placed in the center of the

water and video recorded for 6 minutes. The first 2 minutes of swimming will be considered as an adaptive period and the last 4 minutes will be scored. Time spent immobile will be scored. Actively swimming/attempting to climb or floating immobile has been viewed as potential responses to this stressful environment. The mice could respond with a passive coping style (immobility) or an active stress-coping style (swimming/climbing). Animals that are immobile for greater periods of time signify hopeless behaviors, which are thought to be indicative of increased depression. The active stress coping has also been hypothesized to be related to hyperactivity (Commons et al., 2017; Conner et al., 2020; Armario, 2021). FST was always reserved as the final test due to the high-stress nature of the test.

Anxiety-like behavior (Hadley & Mithani, 1984) as well as risk taking (Macri et al., 2002) will be assessed using the elevated plus maze (EPM). The testing apparatus is plus “+” shaped and consists of four 5cm x 29.5cm arms, with two arms enclosed by 20cm high non-transparent walls. These arm types are arranged adjacently to one another on the apparatus in a manner that allows the mouse to travel directly across in a straight path to the same environment or the animal can turn to enter the opposing environment. Additionally, the apparatus is lifted 50 cm above the ground using stilts. mice were subjected to a single 10-minute trial on the EPM where the mouse was placed in the center of the apparatus and could move freely for the entire testing period. Video recordings were made for each session and the total number of entries and time

spent in closed and open arms was recorded. Anxiety-reduction and increased risk taking is defined by an increased duration of time spent in open arms, while total number of arm entries will be used as a measure of locomotor activity.

Lastly, the 3-chambered sociability test was used to examine social interaction behavior or sociability (Yang et al., 2011). Apparatus design includes a clear Plexiglas chamber (610 mm long, 406 mm wide) with three rectangular areas divided evenly (203.3 mm long and 406 mm wide) by clear walls inside the box. During the test, two wire-mesh cups (to allow for visualization of what was inside) were placed on opposite ends of the chamber. Following a 10-minute acclimation trial and a 10-minute trial to assess natural side bias, social interaction is scored during a final 10-minute trial as the time the subject mouse spends in the chamber containing a novel target mouse compared to the chamber containing an empty cup (Yang et al., 2011). Novel target mice were docile, sex-matched, and of similar age. By comparing how much time the test mouse spends with the novel target mouse versus the empty cup, we gained insight into its social behavior (Nadler et al., 2004). Only mice who did not display significant side bias were included for final social behavior analysis.

All behavioral analyses and scoring were accomplished by trained researchers blind to experimental condition.

*Statistical Analysis*

All statistical analyses were completed using GraphPad Prism 10 (La Jolla, CA) and Jamovi (version 2.3.28). For all sire measurements, pup measurements, and P0 pup brain analyses (ISH, anatomy), a one-way ANOVA was used to compare results between control and experimental groups. In all behavioral measures except for P30 AR analysis, 2-way analyses of variance (ANOVAs; factors: sex and treatment) followed by Tukey's post hoc test were used to assess group differences. For AR analysis, a 3-way repeated-measures ANOVA (factors: sex, trial, treatment) was used to directly examine potential sex differences and 3-way interactions. For all statistical measures, a p value of $<0.05$ was set for significance. All data are expressed as mean ± SEM.

## RESULTS

*Paternal Measurements*

To assess potential differences in liquid intake among EtOH-exposed and control fathers, we measured liquid intake daily for the length of exposure (Fig. 3.3). We found no significant differences in daily liquid intake between control and EtOH-exposed sires (Fig. 3.3A, $p = 0.560$). We also found no significant differences in total weight gain during the exposure period between control and EtOH-exposed sires or in daily average food intake (Control: $M = 7.38 \pm 0.97$, $p = 0.7748$, EtOH-exposed: $M = 5.49 \pm 0.64$, $p = 0.1191$). To confirm EtOH exposure, as well as measure the level of EtOH intoxication, we measured BECs

in 7 males exposed to 25% EtOH for 20 days, as well as 7 control males (Fig. 3.3B). The 20-day exposure time point was chosen to measure BECs due to its near-average position in the 15 to 23-day exposure paradigm used. As expected, sires exposed to 20 days of 25%EtOH showed elevated BEC levels and control (water exposed) sires did not display any detectable BEC levels (Fig. 3.3B, $^{**}p < 0.01$). Overall, these results suggest that no disparity in liquid intake, food intake, or weight gain occurs due to EtOH exposure in sire mice and that sufficient levels of EtOH intoxication occur in male mice following a 20-day exposure period.

*P0 Pup Measurements*

Body weights were documented at P0 (Fig. 3.4A), P20 (Fig. 3.4B), and P30 (Fig. 3.4C) to assess gross anatomical differences between pups. Specifically, PatEE F1 mice weighed more than controls at P0 (Control: $M$ = 1.729g, F1: $M$ = 1.841g, $p$ = 0.0151) P20 (Control: $M$ = 11.59g, F1: $M$ = 15.96g, $p$ < 0.0001) and P30 (Control: $M$ = 22.19g, F1: $M$ = 27.27g, $p$ = 0.0001). At P0, PatEE F2 mice (F2: $M$ = 1.653g, $p$ = 0.0425) weighed less than controls, but PatEE F2 mice weighed more than controls at P20 (Control: $M$ = 11.59g, F2: $M$ = 13.93g, $p$ < 0.0001) and P30 (Control: $M$ = 22.19g, F2: $M$ = 27.08g, $p$ < 0.0001). PatEE F1 mice were heavier than PatEE F2 mice at P0 (F1: $M$ = 1.841g, F2: $M$ = 1.653g, $p$ < 0.0001) and P20 (F1: $M$ = 15.96g, F2: $M$ = 13.93g, $p$ = 0.0013) but no significant differences were observed at P30 (F1: $M$ = 27.27g, F2: $M$ = 27.08g, $p$ = .9842).

To examine the ability of PatEE to produce gross alterations in pup CNS development, we evaluated brain weights and total cortical lengths in control and PatEE F1 and F2 offspring at P0. No significant differences in cortical lengths (Fig. 3.5B, $p = 0.3401$) were observed between PatEE F1, F2, and control mice. No significant differences were present in newborn brain weights between controls and PatEE F1 mice; however, a significant reduction in brain weight was observed in PatEE F2 mice compared to controls (Fig. 3.5C, $p = 0.0221$). Additionally, due to EtOH's ability to reduce overall litter sizes in CD-1 mice following maternal EtOH exposure (El Shawa et al., 2013), we measured control and PatEE litter sizes and found no differences in litter size due to PatEE (Control: $M = 10.25$, PatEE F1: $M = 10.90$, PatEE F2: $M = 14.40$, $p = 0.1754$). Overall, these results suggest that a moderate paternal exposure does not produce gross alterations in CNS development in pups at P0, as well as differences in litter size/viability in non-directly EtOH-exposed dams.

*Cortical Anatomical Measurements*

PatEE in rats has been shown to generate altered cortical thickness in P28 offspring (Jamerson et al., 2004). To assess the effects of a 15- to 23-day PatEE on cortical thickness development at P0, we measured from 5 distinct regions in Nissl-stained coronal sections in both control and PatEE mice (Fig. 3.6). These regions included putative frontal, prelimbic, somatosensory, auditory, and visual cortices. No significant differences in cortical thickness were observed

in PatEE F1 or PatEE F2 mice compared to controls in the frontal cortex (Fig. 3.6A, Control: *M* = 0.5197mm, PatEE F1: *M* = 6344mm, PatEE F2: *M* = 0.5555mm, *p* = 0.2993), prelimbic cortex (Fig. 3.6B, Control: *M* = 0.7360mm, PatEE F1: *M* = 0.7356mm, PatEE F2: *M* = 0.7635mm, *p* = 0.8045), somatosensory cortex (Fig. 3.6C, Control: *M* = 0.6913mm, PatEE F1: *M* = 0.8204mm, PatEE F2: *M* = 0.7300mm, *p* = 0.1356), auditory cortex (Fig. 3.6D, Control: *M* = 0.5403mm, PatEE F1: *M* = 0.5791mm, PatEE F2: *M* = 0.6133mm, *p* = 0.1205), or visual cortex (Fig. 3.6E, Control: *M* = 0.4643mm, PatEE F1: *M* = 0.4950mm, PatEE F2: *M* = 0.5035mm, *p* = 0.5012).

Additionally, subcortical areas were measured to test for differences in anatomical structure. No significant differences were observed in corpus callosum thickness (Fig. 3.7A, Control: *M* = 0.4518mm, PatEE F1: *M* = 0.4732mm, PatEE F2: *M* = 0.4538mm, *p* = 0.7878), CA3 of the hippocampus (Fig. 3.7B, Control: *M* = 0.1813mm, PatEE F1: *M* = 0.1813mm, PatEE F2: *M* = 0.1795mm, *p* = 0.9981), and amygdala (Fig. 3.7C, Control: *M* = 0.1447mm, PatEE F1: *M* = 0.1342mm, PatEE F2: *M* = 0.1331mm, *p* = 0.4349) compared to controls (Fig. 3.7). Together, these results suggest that a moderate 15 to 23-day paternal EtOH exposure does not significantly impact cortical thickness, corpus callosum or CA3 thickness, or amygdala area in mice at P0.

*Cortical Gene Expression Analyses*

*Id2* and *RZRβ* are two genes important for neocortical patterning and arealization (Rubenstein et al., 1999). Previously, we demonstrated that cortical expression of these two genes is altered by maternal prenatal EtOH exposure at P0 (Abbott et al., 2018; El Shawa et al., 2013). Accordingly, we investigated expressions of *Id2* and *RZRβ* in the neocortex to determine whether this patterning is affected by PatEE in mice at P0. Using ISH, we examined patterns of gene expression in P0 coronal hemisections via side-by-side analyses of anatomically matched sections from both control and PatEE brains (Fig. 3.8). In control mice, *Id2* expression is highly complex, spanning multiple cortical layers including II/III, V, and VI (Fig. 3.8 A1-5). Additionally, a distinct boundary of the most superficial *Id2* expression occurs in control mice at P0 (arrows, Fig. 3.8 A2-3), which marks the border of absent lateral *Id2* expression. In anatomically matched sections of PatEE mice, however, this border appears to be visually shifted to further lateral cortical regions (arrows, Fig. 3.8 B2- 3), and thus, *Id2* expression also extends further laterally compared to controls. This shift seen in F1 PatEE mice appears to be rescued in the F2 generation (Fig 3.8 C2-3).

*RZRβ* expression in control animals is largely limited to cortical layer IV (Fig. 3.9 A 1-5). However, a typical border occurs that delineates an absence of expression in the medial cortical wall (arrow, Fig. 3.9 A3). In contrast, PatEE brains exhibit a medial shift in this boundary (arrow, Fig. 3.9 B3), and *RZRβ*

expression extends medially as a result. This shift seen in F1 PatEE mice appears to be rescued in the F2 generation (Fig 3.9 C2-3).

*P20 Behavioral Analysis*

To assess PatEE's impact on behavioral development, we used a battery of behavioral tests at two ages in PatEE F1, F2, and control mice. At P20, we assessed behavior using the Suok test, a succinct behavioral test that can assess both sensorimotor integration/function and anxiety-like behavior (Kalueff et al., 2008). Two measures of sensory motor integration, missteps and falls, were evaluated during the singular 5-minute trial of the Suok. There was a significant main effect in treatment ($F(2,101) = 5.475$, $p = 0.0055$) when investigating falls. However, there was no significant difference in falls when comparing the sex of the animal ($p = 0.4501$) and no treatment x sex interaction. A 2-way ANOVA revealed a significant main effect of treatment for missteps, $F(2, 99) = 9.606$, $p < 0.0002$, but no main effect of sex. Post hoc analyses revealed an increase in missteps in female PatEE F1 mice compared to female controls (Fig. 3.10B, $p < 0.0195$), as well as increased missteps for male PatEE F1 versus control males (Fig. 3.10B, $p = 0.0444$). Additionally, there was no sex x treatment interaction for missteps. There were no main effects of sex or treatment for falls and no sex x treatment interaction (Fig. 3.10A). Additionally, there was a significant main effect of treatment on segments crossed, $F(2, 101) = 12.17$, $p < 0.0001$, and post hoc analyses revealed an increase in the number of segments

crossed in PatEE F1 versus control males (Fig. 3.10C, $p = 0.0007$) and PatEE F2 versus control males (Fig. 3.10C, $p = 0.0188$). However, there was no significant difference in PatEE F1 or F2 females versus control females. Additionally, there was no main effect of sex for segments crossed and no sex x treatment interaction.

Anxiety-like behaviors including rearing/grooming events, directed exploration, and latency to leave the center of the bar were also evaluated using the Suok test. Two-way ANOVAs revealed no significant main effects of sex ($F(1,86) = 0.6838$, $p = 0.4106$) or treatment ($F(2,86) = 1.123$, $p = 0.3299$) or sex x treatment interactions ($F(2,86) = 0.3141$, $p = 0.7313$) for rearing/grooming events (Fig. 3.10D). Directed exploration events also exhibited no significant main effects for sex ($F(1,99) = 0.3660$, $p = 0.5466$) and no treatment x sex interaction ($F(2,99) = 0.0.5415$, $p = 0.5836$, Fig. 3.10E). However, there was a significant main effect in treatment ($F(2,99) = 13.28$, $p < 0.0001$) for directed exploration with post hoc analysis revealing increased exploration in male F1 and F2 PatEE mice compared to controls (Fig. 3.10E). There was a main effect of treatment ($F(2,96) = 5.533$, $p = 0.0053$) in latency to leave center with post hoc analysis showing increased latency in female F2 mice compared to female F1 mice. However, there was no main effect of sex ($F(1,96) = 0.5973$, $p = 0.4415$) or treatment x sex interaction ($F(2,96) = 1.729$, $p = 0.1830$, Fig. 3.10F). Overall, results suggest that 15 to 23 days of PatEE can produce altered behavior at P20

in a sex-specific manner, including increased activity in males and perturbed sensorimotor integration in both males and females.

*P30 Behavioral Analysis*

We examined behavior at P30 in control and PatEE mice using several different tasks including the AR for motor learning (Rustay et al., 2003), FST for depressive-like behavior (Lucki et al., 2001), the elevated plus maze (EPM) for anxiety-like (Hadley & Mithani, 1984) as well as risk taking (Macri et al., 2002) behaviors, and 3-chambered sociability test for social behavior and interaction (Yang, et al., 2011; Figs. 3.11, 3.12).

A 3-way repeated-measures ANOVA showed significant main effects of trial, $F(3, 168) = 19.023$, $p < 0.001$ with post hoc tests revealing an increase in amount spent on the rod between trial 1 and trials 2 ($p$ <.001), 3 ($p$ <.001), and 4 ($p$ <.001) indicating overall improvement in performance as trials went on. However, further analysis revealed that when comparing trial and treatment controls showed significant improvement in the amount of time spent on the rod between trial 1 and 2 ($p = 0.002$), whereas F1 ($p = 0.835$) and F2 ($p = 0.647$) PatEE mice did not show significant improvement between trials 1 and 2 suggesting that motor learning may be acutely hindered due to PatEE. Furthermore, there was a trial x sex interaction, $F(3, 168) = 2.898$, $p = 0.037$, with post hoc analysis revealing that females performed better than males in trial 1 ($p = 0.044$) and trial 2 ($p = 0.040$) but not in trials 3 ($p = 0.310$) or 4 ($p = 0.997$).

Between subjects effects included a main effect of sex ($F(1, 56) = 17.55$, $p < 0.001$) and a sex x treatment interaction ($F(2, 56) = 3.81$, $p = 0.028$). Overall, females performed better than males on the AR ($p < 0.001$). Tukey post hoc analysis revealed significant differences between male PatEE F1 mice and female PatEE F1 ($p = 0.047$), F2 ($p = 0.006$) and control ($p = 0.034$) mice. Additionally, differences between PatEE F2 males and control ($p = 0.010$), F1 ($p = 0.023$) and F2 ($p = 0.001$) females were observed (Fig 3.11A, B).

In the FST, time spent immobile was used as a measure for depressive-like behaviors (Fig. 3.12A) and a 2-way ANOVA revealed significant main effects of treatment ($F(2,50) = 6.280$, $p = 0.0037$) but no main effect of sex ($F(1,50) = 0.8239$, $p = 0.3684$) and no sex x treatment interaction ($F(2,50) = 0.1130$, $p = 0.8934$).

The elevated plus maze (EPM) was used to assess anxiety-like and risk-taking behaviors by investigating time spent in the open arms (Fig. 3.12C). A main effect of treatment ($F(2,31) = 21.48$, $p < 0.0001$) on time spent in the open arms was observed. Post hoc analysis revealed a significant difference between control female ($p = 0.0022$) and PatEE F2 mice as well as between control male and PatEE F1 ($p = 0.0039$) and F2 ($p = 0.0007$) mice. However, no main effect of sex ($F(1,31) = 0.2237$, $p = 0.2237$) or treatment x sex interaction ($F(2,31) = 0.02971$, $p = 0.9708$) was observed. Additionally, entries into the open and closed arms were recorded to investigate motor activity. There was a significant main effect of treatment on entries into the open arms ($F(2,31) = 6.948$, $p =$

0.0032) with post hoc tests revealing trends exhibiting increased entries in PatEE F1 ($p$ = 0.0658) and F2 ($p$ = 0.0540) mice compared to controls (Fig. 3.12D).

Lastly, a 2-way ANOVA for the sociability test revealed no significant main effects of treatment or sex and no sex x treatment interaction between control and PatEE mice in the amount of time spent with the novel mouse (Fig. 3.12B), indicating social interaction abnormalities do not result from 15 to 23 days of PatEE in our model. Together, these data suggest that PatEE also generates behavioral abnormalities at P30, including sensorimotor and short-term motor learning impairments.

## DISCUSSION

In this study, we demonstrate that paternal EtOH exposure can induce dramatic alterations in PatEE F1 offspring neocortical gene expression. Additionally, behavioral testing indicated that PatEE F1 and F2 male mice display increased activity, as well as impaired coordination and short-term motor learning abilities. Furthermore, female PatEE mice displayed deficits in sensorimotor integration. Increased risk-taking behavior was also observed in female and male PatEE F1 and F2 mice. Overall, these results suggest that 15 to 23 days of PatEE prior to conception can alter gross anatomical, neuroanatomical, and behavioral developmental trajectories in rodent offspring with changes potentially lasting to the second generation.

*PatEE and General Offspring Development: Human and Rodent Models*

The deleterious effects of PatEE, while markedly understudied in comparison with PrEE via maternal EtOH consumption during pregnancy, have been examined for over 100 years (Stockard & Papanicolaou, 1918). Children of alcoholic fathers have been shown to display cognitive impairments (Tarter et al., 1989) and lower birth weights (Little & Sing, 1987). They also have an increased risk of congenital defects (Zuccolo et al, 2017) and altered reproductive development (Xia et al., 2018), Rodent studies, using a variety of species, have corroborated these findings as lower birth weights (Bielawski et al., 2002), increased incidence of runts in litters (Bielawski and Abel, 1997), smaller litter sizes (Liang et al., 2014; Meek et al., 2007), and congenital CNS anomalies (Lee et al., 2013). However, some studies report that PatEE offspring have increased weight after weaning at P20 (Finegersh & Homianics, 2014) which could be due to epigenetic modifications that affect metabolism. In the current study, we report an increase in PatEE weight regardless of generation compared to controls at all ages, with the exception of a reduction in PatEE F2 weight at P0. We do not report any significant differences in litter size.

Additionally, there were no significant changes in cortical lengths between PatEE F1, F2, and control mice at birth due to PatEE. However, brain weights in PatEE F2 mice were significantly lower compared to controls but no changes were observed in PatEE F1 mice compared to controls. These apparent differences may be related to the length or dosage of exposure, as some

previous studies were designed to encapsulate at least one cycle of spermatogenesis within sires (~35 days; Adler, 1996). However, duration of PatEE has not always predicted litter size alterations, as a 6-month exposure did not result in any significant differences in PatEE offspring litter size (Ceccanti et al., 2016). An alternative explanation is that differences could be species- and strain specific, as Ceccanti and colleagues (2016) also utilized the same strain used within our study (CD-1 mice).

*PatEE and behavioral development*

Children with FASD often have cognitive deficits including learning disabilities, poor judgment and reasoning, and problems with attention and memory which can persist into adulthood. Children of alcoholic fathers may also be susceptible to abnormal behavior and cognition. Studies have demonstrated effects on learning, including deficits in IQ (Ervin et al., 1984; Gabrielli and Mednick, 1983), visual–spatial skills, perceptual–motor skills (Pihl and Bruce, 1994; Schandler et al., 1995), auditory–visual attention span (Tarter et al., 1989), and difficulties in abstraction and problem solving (Ervin et al., 1984). Additionally, PatEE has been shown to negatively impact various aspects of learning in rodent models. Specifically, PatEE impairs spatial learning performance in male rat offspring (Wozniak et al., 1991; Ledig et al., 1998). Offspring of PatEE mice exhibited dose-dependent learning and memory deficits (Liang et al., 2014). Previous studies on effects of chronic paternal ethanol

exposure suggest that children of alcoholic fathers are hyperactive and have reduced cognitive performance (Curley et al., 2011). Specifically, clinical findings indicate that attention-deficit/hyperactivity disorder (ADHD) incidence is increased in the children of alcoholic fathers (Knopik et al., 2005). Interestingly, ADHD prevalence in children prenatally exposed to EtOH via maternal consumption ranges between 49.4% and 94%, exhibiting the highest comorbidity in FASD patients (Bhatara et al., 2006; Fryer et al., 2007). This suggests that PatEE and PrEE may disrupt behavioral phenotypes in comparable manners.

Studies in rodents corroborate these findings of hyperactivity as PatEE can cause expression of ADHD–like behavioral phenotypes, as assessed via increased activity and impulsivity measures (Kim et al., 2014; Ledig, et al., 1998). Specifically, Kim and colleagues (2014) found that male PatEE mouse offspring display increased open-field activity, suggesting that male offspring may be particularly prone to increased activity or hyperactive behaviors imparted by paternal drinking. Our current study found that PatEE male mice display increased activity, which may be reflective of a hyperactive phenotype, compared to male control mice as shown by increased segments crossed in the Suok test. However, rodent models have also reported hypoactivity in PatEE offspring (Abel, 1989), obscuring the push toward a unified PatEE behavioral phenotype.

Impaired social behaviors have been widely described in both rodent (Hamilton et al., 2010) and human offspring (Thomas et al., 1998) due to PrEE. People with FASD may experience emotional and behavioral dysregulation

including symptoms of anxiety and depression, as well as aggressive-irritable and risk-taking behaviors (Mattson & Riley, 1998; Riley & McGee, 2005; Greenbaum et al., 2009). Deficits in fear acquisition (Kozanian et al., 2018) and increased anxiety-like behaviors (Madarnas et al, 2020) are also observed in PrEE mice. To our knowledge, no previous reports have examined social interaction or risk-taking behaviors in PatEE offspring; however, others have found increased aggression in PatEE male offspring (Meek et al., 2007).

Lastly, anxiety-like and depressive-like behaviors were also assessed via the Suok and FST, respectively. In the Suok assay, no significant differences in exploration, except increased exploration in male F1 and F2 PatEE mice compared to controls were observed. There was an increase in latency to leave the center of the bar in female F2 mice compared to female F1 mice, indicating a potential increase in anxiety in F2 mice. However, no differences were found in PatEE mice compared with controls in depressive-like behavior, regardless of sex. A previous report indicated increased anxiety-like and depressive-like behaviors in mice offspring whose fathers were exposed to 4 weeks of EtOH exposure (Liang et al., 2014). However, alternate studies reported no differences in offspring basal anxiety levels due to PatEE (Beeler et al., 2019; Finegersh & Homanics, 2014; Rompala et al., 2017). Collectively, these results suggest that the impact of PatEE on rodent offspring anxiety-like and depressive-like behaviors is complex, and their manifestation may rely on multiple factors including sex, strain, treatment type, length, and dosage.

Although motor coordination and sensorimotor integration are consistently altered by maternal EtOH exposure in humans (Doney et al., 2014), much less is known of the impact of PatEE. Furthermore, one study found decreased balance in early postnatal rat pups due to PatEE (Jamerson et al., 2004). We report sex-specific decreased sensorimotor integration and motor coordination at both P20 and P30 due to PatEE. Specifically, we found that PatEE F1 female and male offspring show increased missteps in the Suok at P20. Additionally, overall improvement in performance on the AR was observed as trials went on in all groups regardless of sex or treatment. However, female mice performed better than male mice overall on the AR. Furthermore, controls showed significant improvement between trial 1 and 2, whereas F1 and F2 PatEE mice did not show significant improvement between trials 1 and 2 suggesting that motor learning may be acutely hindered due to PatEE. These results support the ability of PatEE to negatively impact various aspects of learning (Liang et al., 2014; Wozniak et al., 1991). Together, our data confirm the ability of PatEE to impact both learning in offspring and in general motor coordination and sensorimotor integration, further solidifying the negative impact of PatEE on offspring behavior. However, the sex and age-specific findings of the current study highlight the complexity of the outcomes that can result due to PatEE.

Additionally, tactile sensitivity can also be affected by PrEE. Hyper-responsiveness to mildly painful stimuli has been observed in rats (Rogers et al, 2004). Additionally, high aversion responses to specific textures were seen in

PrEE primates (Schneider et al., 2008). However, no known studies have investigated PatEE's effects on tactile sensitivity or dexterity. Because paternal alcohol consumption studies in both human and murine models are limited, more research is needed to investigate possible detrimental effects of PatEE on offspring development.

*Possible mechanisms involved in PatEE*

Several previous studies have examined potential mechanisms for transmission of PatEE's harmful effects from sperm to offspring (reviewed in Rompala & Homanics, 2019). One potential mechanism could be alterations within the male reproductive system and changes in sperm morphology due to EtOH exposure. Chronic alcohol exposure is associated with reduced seminiferous tubular diameter and germinal epithelium. Seminiferous tubules are located within the testes, and are the specific location of meiosis, and the subsequent creation of male gametes (sperm). The germinal epithelium is also known as the wall of the seminiferous tubules. In addition to these changes in structure within the testes, morphological changes in sperm also occur due to chronic alcohol exposure. These can include curling of the tails which can lead to sperm motility problems (Abel, 1983). Additionally, changes to head structure including macrocephaly (Lwow, 2017), double headed sperm, sperm head breakage, and absence of the acrosome can occur. The acrosome is an organelle that develops over the anterior half of the sperm head which is

essential for fertilization. Release of the acrosomal contents is necessary for sperm entry and penetration of the zona pellucida (membrane surrounding the ovum).

Because EtOH is a known disruptor of epigenetic regulation in both adult consumption (Cervera-Juanes et al., 2017) and prenatal exposure (Garro et al., 1991) contexts, most research has focused on the potential mechanism of PatEE germ cell alteration as one that is epigenetically mediated. This hypothesis is supported by studies that have shown EtOH exposure alters DNA methylation (Bielawski et al., 2002), histone acetylation (Kim & Shukla, 2006), and small noncoding RNA profiles (Rompala et al., 2018) of rodent male sperm cells and/or testis. This is especially compelling considering human sperm cell DNA hypomethylation of typically hypermethylated imprinted genes is associated with EtOH consumption (Ouko et al., 2009).

PatEE rodent model studies have examined this question of mechanism and have found various results revealing EtOH's effect on spermatogenesis. For example, Liang and colleagues (2014) reported DNA methylation decreases at paternally imprinted genes in EtOH-exposed sire sperm. However, other studies have found no evidence for PatEE's effects being mediated through epigenetic regulation of imprinted genes (Chang et al., 2017, 2019), suggesting other epigenetic mechanisms may underlie observed effects on offspring brain and behavior. Because small noncoding RNAs (sncRNAs), such as microRNAs, transfer RNA-derived RNAs, and mitochondrial small RNAs, are present in male

gametes (Krawetz et al., 2011), these have also been hypothesized to play a key role in PatEE. In fact, a recent study has confirmed that paternal exposure alters expression of several of these sncRNAs in male sperm (Rompala, et al., 2018). However, no known study has evaluated potential epigenetic effects within the neocortex.

While the exact mechanisms and molecular players are still unclear, evidence points toward an epigenetically mediated alteration of EtOH-exposed sperm. We hypothesize that PatEE may cause sperm cell epigenetic dysregulation, which in turn may alter gene expression within offspring brain (such as in *Id2* and *RZRβ* expression), resulting in altered cortical patterning and ectopic development of neocortical circuits ultimately underlying any abnormal behavior (Fig. 3.13). This study provides additional insights into the biological mechanisms that may underlie atypical behavior observed in children of alcoholic fathers.

*Significance of a study in PatEE*

The goal of this study is to characterize the impact of a paternal binge of EtOH prior to conception on offspring cortical development across generations. Specifically, we determined if paternal EtOH (alcohol) exposure affects gross anatomy and neuroanatomy of offspring across multiple generations. PatEE in humans has been associated with lower birth weights (Little and Sing, 1987), increased risk of congenital defects (Zuccolo et al., 2017), and altered

reproductive development (Xia et al., 2018), suggesting PatEE may be more impactful on offspring development than previously thought. A previous study in our lab was the first to investigate the effects of PatEE on the development of intraneocortical connections along with studies of cortical morphology (Conner et al., 2020). We also elucidated possible genetic mechanisms of action for paternal EtOH exposure across generations and examined the underlying mechanisms of how PatEE insults the neocortex. Previously, we demonstrated that cortical expression of *Id2* and *RZRβ* genes is altered by maternal prenatal EtOH exposure at P0 (El Shawa et al., 2013; Abbott et al., 2018). Accordingly, we investigated expressions of *Id2* and *RZRβ* in the neocortex to determine whether this patterning is affected by PatEE in mice at P0. Confirming whether altered gene expression due to PatEE contributes to developmental abnormalities in the neocortex is a novel and significant approach as well as a crucial priority due to the lack of information in the current literature. Finally, we investigated the transgenerational effects of paternal EtOH exposure on offspring behavior. We have shown previously that PrEE can result in transgenerational inheritance of exposure-related cortical phenotypes (Abbott et al., 2018), and suggest this disorganization in the neocortex may underlie behavioral alterations associated with FASD. We now know that PatEE, like PrEE, results in a heritable condition. Thus, this study is of critical importance to reveal risks for future generations of alcoholic fathers. This is a highly significant and innovative area of study, as little is known about the effects of paternal drinking on offspring development and how

epigenetics may play a role in transgenerational phenotypes. In conclusion, our data support the notion that the preconception paternal environment is more impactful on offspring development than previously thought and that paternal EtOH exposure may cause harmful consequences in offspring.

## FIGURES

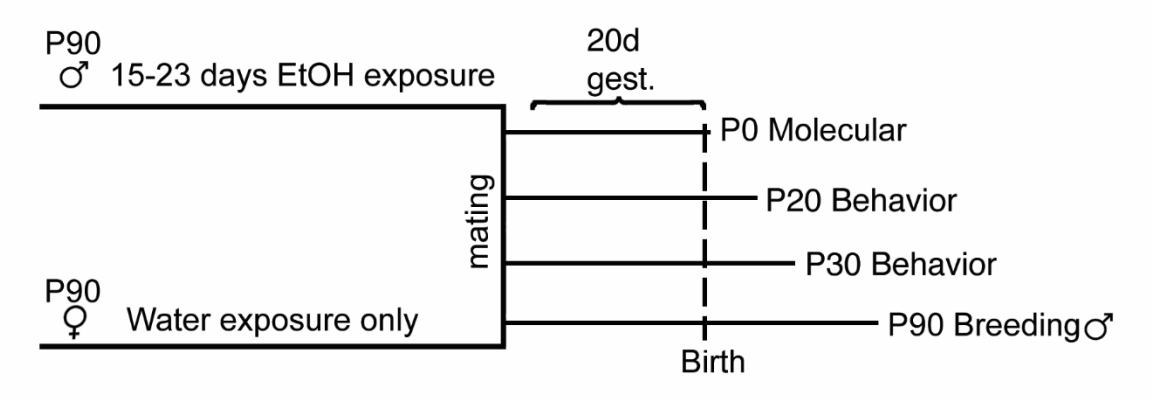


**Figure 3.1. Experimental Timeline for PatEE F1.** Mating between EtOH-exposed males and EtOH-naive females took place following a 15- to 23-day 25% EtOH exposure in sires. Following gestation (~20 days), 3 subsets of pups from each litter were designated for either (1) sacrifice at P0 for anatomical experiments, (2) behavioral assessment at P20, or (3) weaned and assessed for behavior at P30.

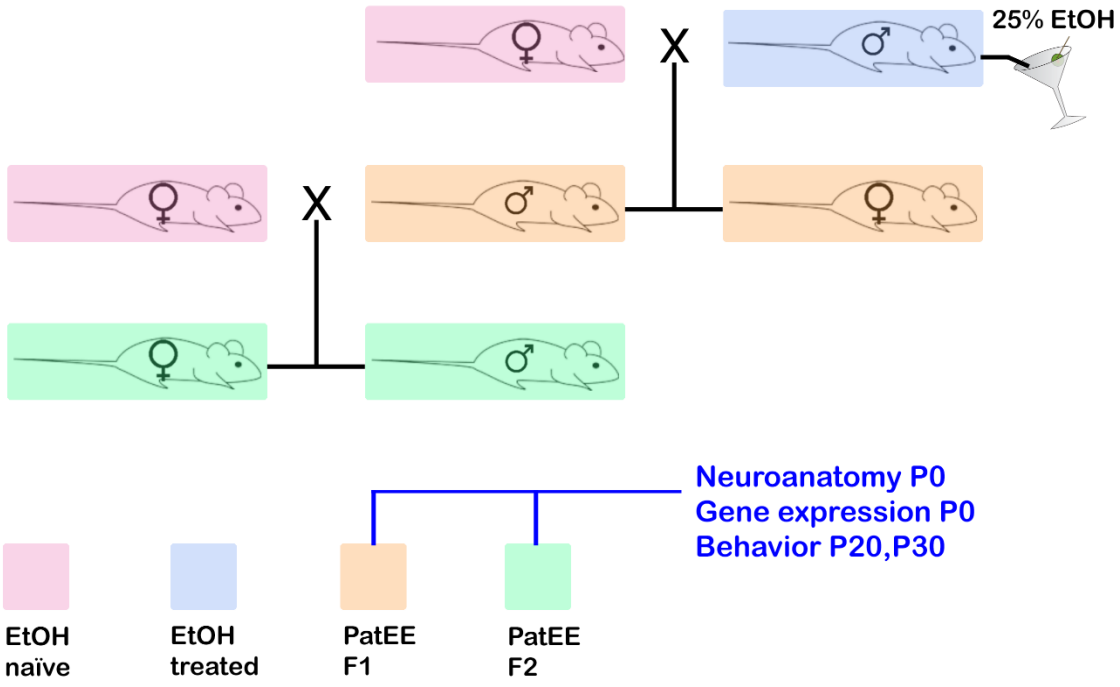


**Figure 3.2. PatEE Breeding Paradigm.** P90 EtOH-treated sires were bred with an ethanol naive P90 female to produce the PatEE F1 generation. Males from the PatEE F1 generation were bred with EtOH naive females to generate the PatEE F2 generation.

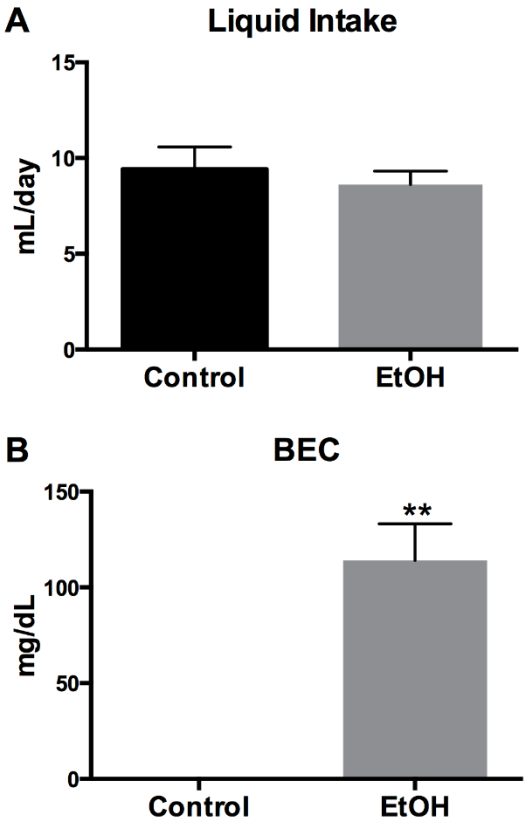


**Figure 3.3. Measurements of EtOH-exposed and control sires.**
Measurements of EtOH-exposed and control sires. (**A**) Average daily liquid intake of sires (ml/d). No differences present between control (n = 10) and EtOH-exposed (n = 10) males. (**B**) BEC measurements in male sires after a 20-day exposure to 25% EtOH or water (control). Sires exposed to 25% EtOH (n = 7) had an average BEC of 125.6 mg/dl compared to controls (n = 7), which had a BEC of 0 mg/dl (****$p < 0.0001$).

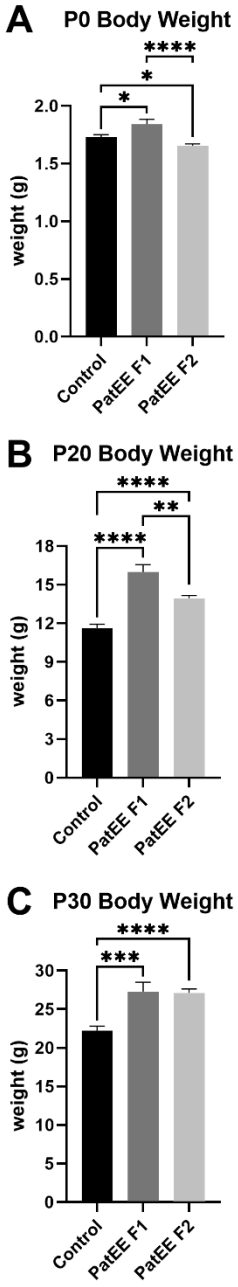


**Figure 3.4. Body weights.** (**A**) At P0, PatEE F1 (n = 32) mice weighed more than controls (n = 67), PatEE F2 (n = 72) mice weighed less than controls. (**B**) At P20, controls (n = 40), weight less than PatEE F1 (n = 23) and PatEE F2 (n = 38) mice. (**C**) At P30, controls (n = 30), weight less than PatEE F1 (n = 13) and PatEE F2 (n = 38) mice. PatEE F2 mice weighed more than controls at P20 (**B**) and P30 (**C**).

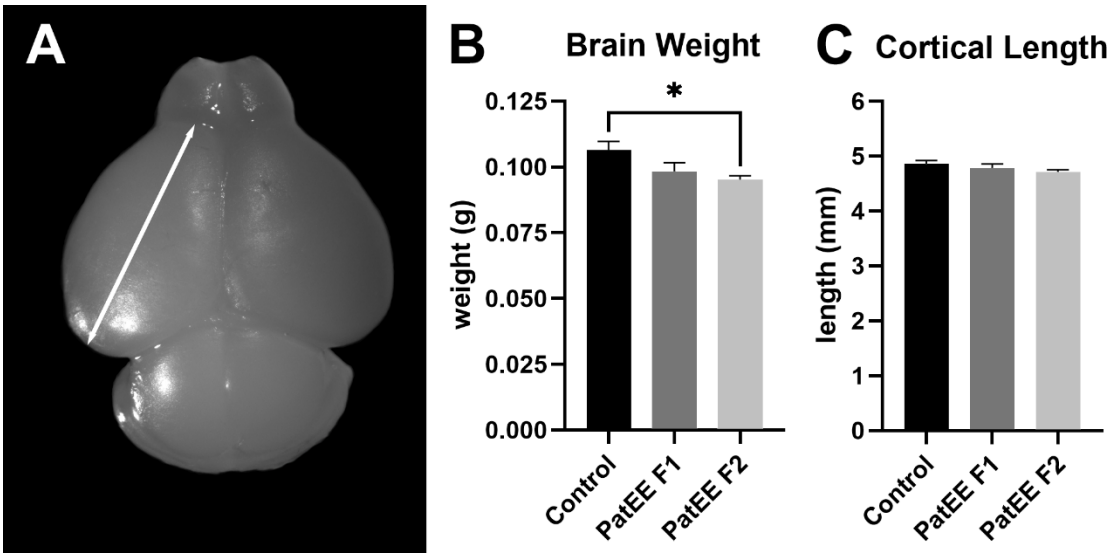


**Figure 3.5. P0 gross cortical measurements.** (**A**) Representative P0 control brain indicating cortical length measurement. (**B**) Brain weights at P0 in control (n = 21), PatEE F1 (n = 19), and PatEE F2 (n =18) offspring. A significant difference in brain weight was observed between control and PatEE F2 mice, but not between control and PatEE F1 or PatEE F1 and PatEE F2 mice at P0. (**C**) Cortical lengths at P0 in control (n = 18), PatEE F1 (n = 23), and PatEE F2 (n =18) offspring. No differences are seen in P0 or cortical length between PatEE and controls. (*$p$ = 0.05).

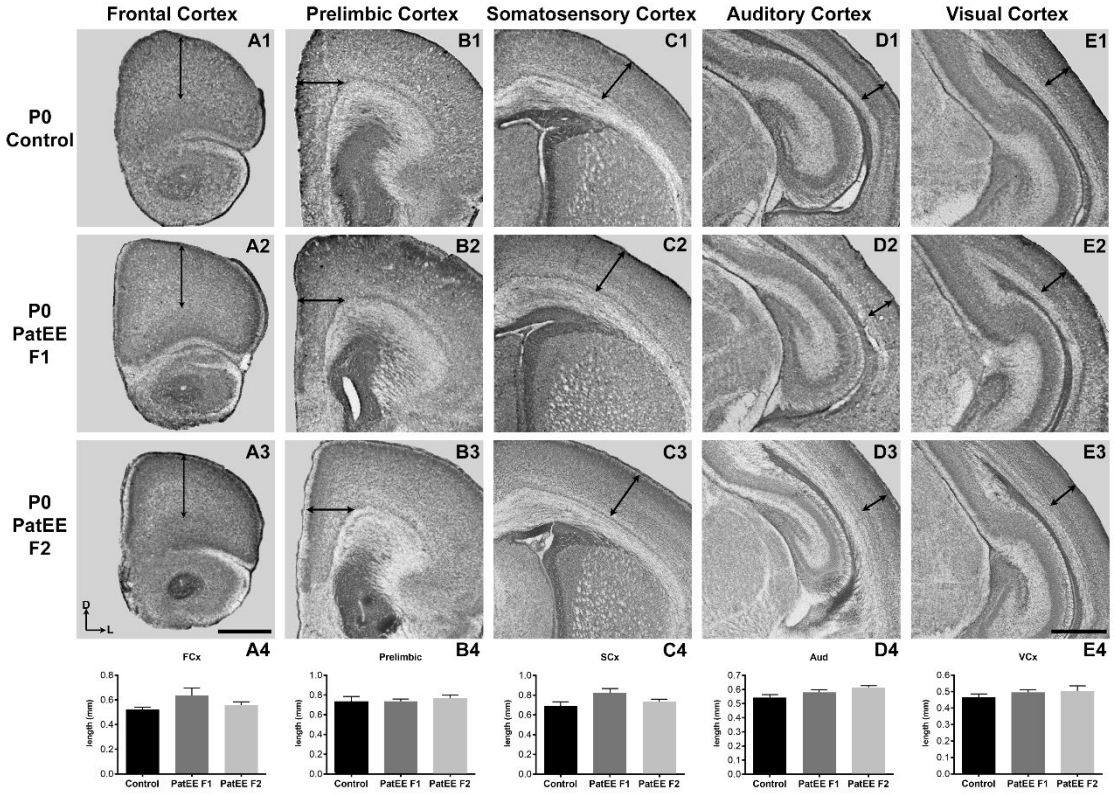


**Figure 3.6. Cortical anatomy.** Measurements of cortical anatomy in newborn (P0) mice. High-magnification coronal sections of Nissl-stained P0 hemisections. Measurements in cortical areas included putative frontal cortex (**A1-3**), prelimbic cortex (**B1-3**), primary somatosensory cortex (**C1-3**), primary auditory cortex (**D1-3**) and primary visual cortex (**E1-3**). No significant differences were observed in any regions between control (n = 4) (**A1-E1**) PatEE F1 (n = 7) (**A2-E2**) and PatEE F2 (n =4) (**A3-E3**) mice. Images oriented dorsal (D) up, lateral (L) to the right. Scale bar, 1000 μm (**A1-3**), 500 μm (**B–E**).

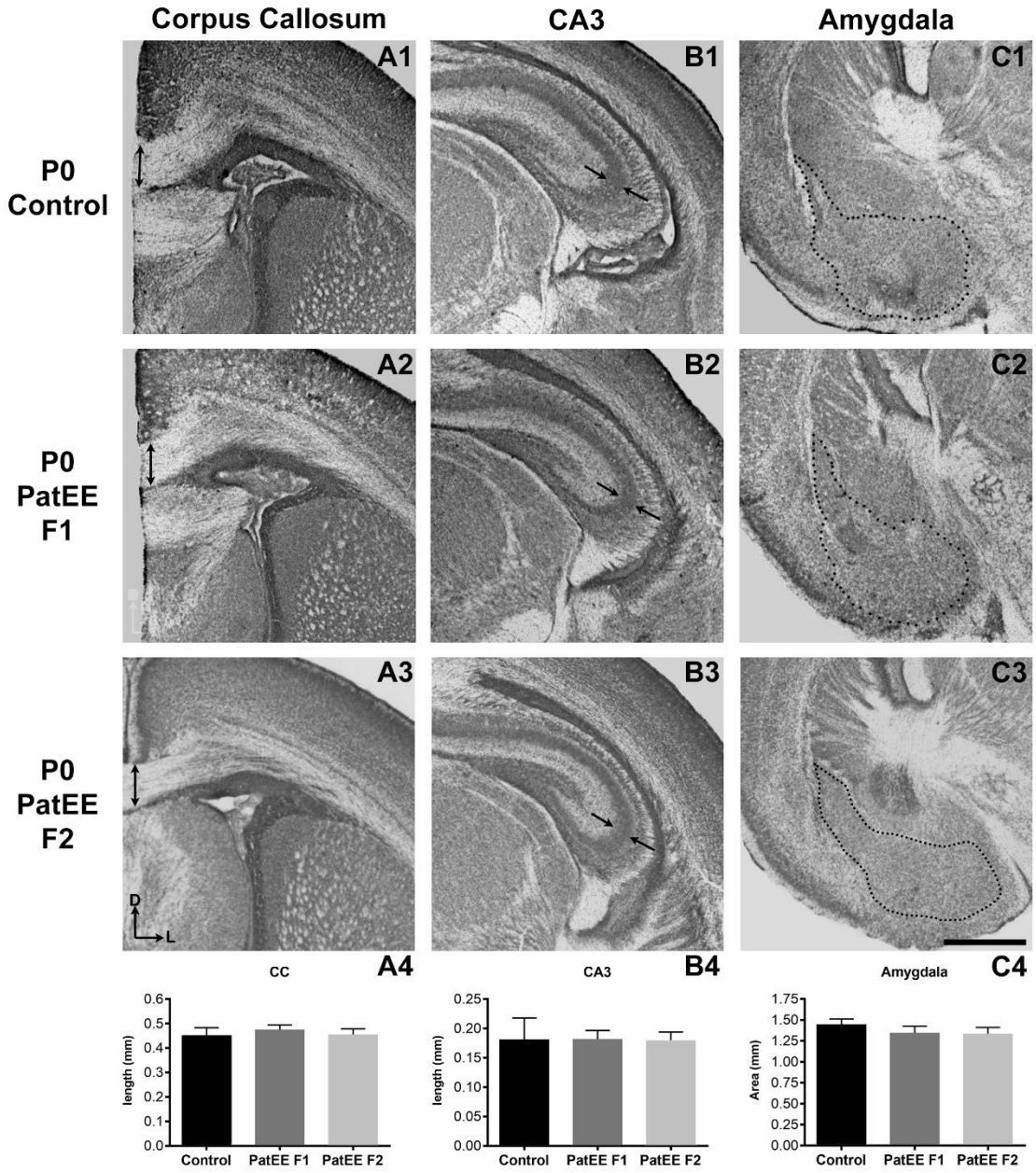


**Figure 3.7. Subcortical anatomy.** Measurements of subcortical anatomy in P0 mice were taken from Nissl stained hemisections. Measurements include corpus callosum (**A1-3**), CA3 of the hippocampus (**B1-3**), amygdala (**C1-3**). No significant differences were observed in any regions between control (n = 4) (**A1-C1**), PatEE F1 (n = 7) (**A2-C2**), and PatEE F2 (n = 4) (**A3-C3**) mice in these regions. Images oriented dorsal (D) up, lateral (L) to the right. Scale bar, 1000 µm.

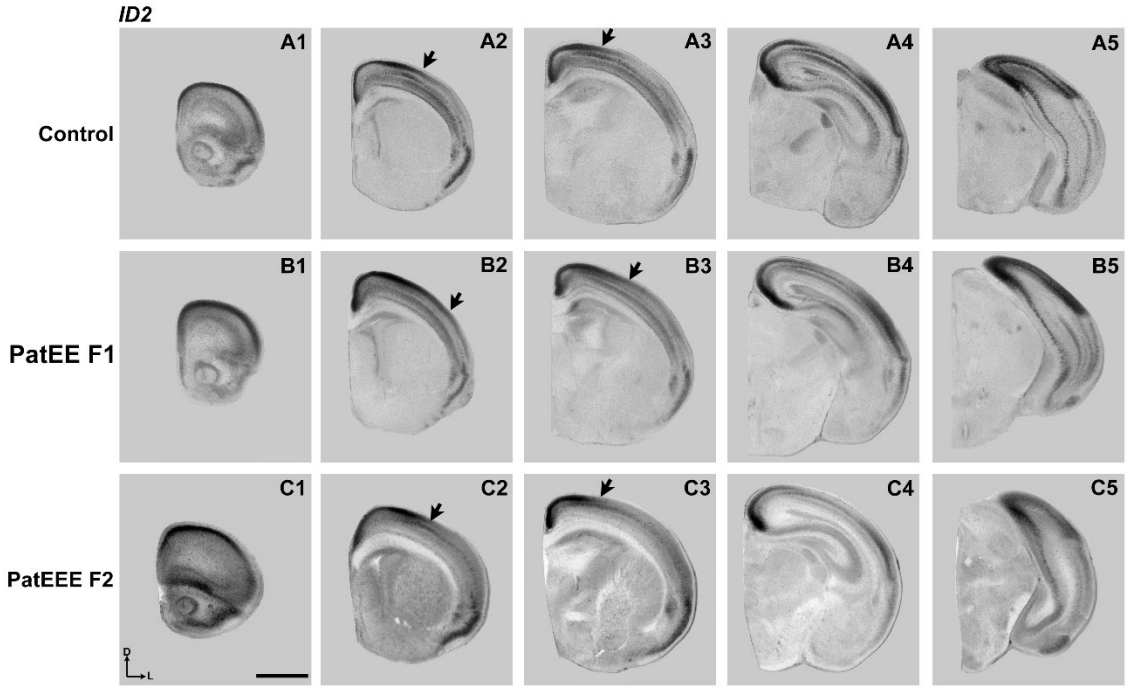


**Figure 3.8. Transgenerational neocortical gene expression of *Id2* in P0 mice.** High magnification of 100-µm coronal sections following free-floating ISH with *Id2* (control: **A1-5**, PatEE F1: **B1-5**, PatEE F2: **C1-5**) probes. Section through the parietal cortex where arrows (**A2-3**) denote the lateral boundary of the most superficial layer of *Id2* expression in a control brain. *Id2* expression extends further laterally in PatEE brains compared to controls as seen by comparing arrow locations (**B2-3**). This shift seen in F1 PatEE mice appears to be rescued in the F2 generation (**C2-3**). Sections oriented dorsal (D) up and lateral (L) to the right. Scale bar, 1000 µm.

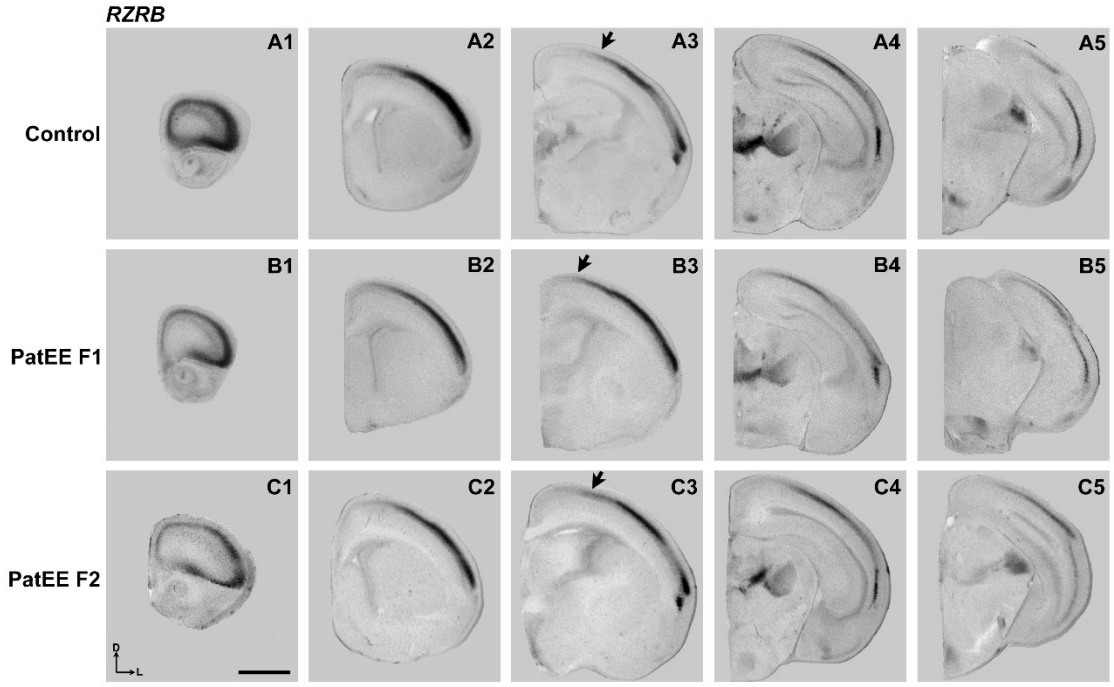


**Figure 3.9. Transgenerational neocortical gene expression of *RZRβ* in P0 mice.** High magnification of 100-lm coronal sections following free-floating ISH with *RZRβ* (control: **A1-5**, PatEE F1: **B1-5**, PatEE F2: **C1-5**) probes. Arrow in A3, medial boundary of *RZRβ* expression in control parietal cortex. In PatEE brains, this medial boundary for *RZRβ* has shifted medially (**arrow in B3**). This shift seen in F1 PatEE mice appears to be rescued in the F2 generation (**C3**). Sections oriented dorsal (D) up and lateral (L) to the right. Scale bar, 1000 μm.

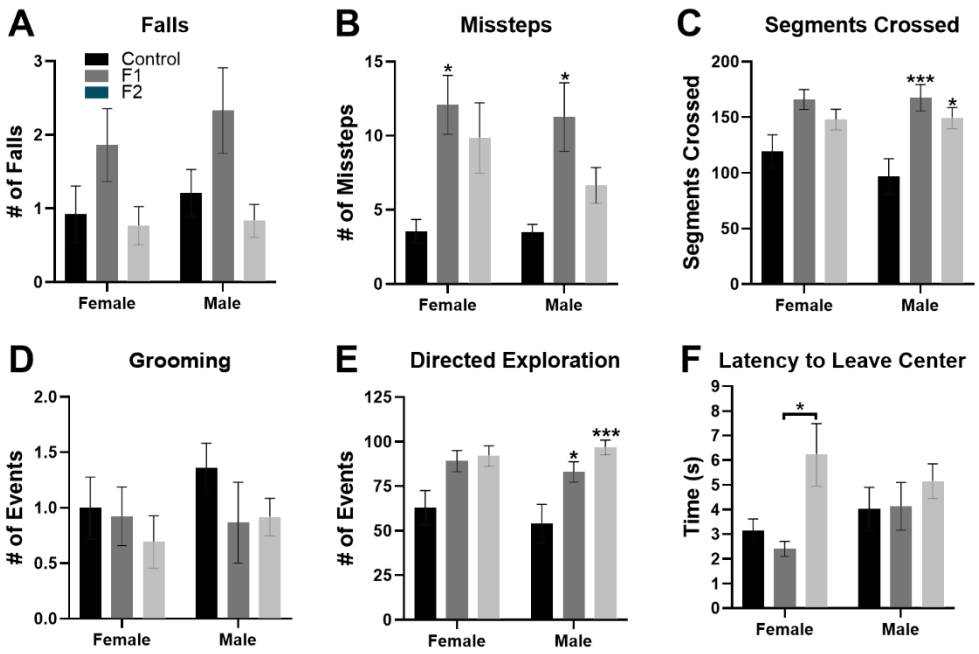


**Figure 3.10. Suok Assay at P20.** Behavioral measures of Suok assay in age P20 mice. (**A**) No significant difference in falls regardless of treatment or sex. (**B**) Increase in missteps in female and male PatEE F1 mice compared to controls. (**C**) Significant main effect of treatment on segments crossed, and post hoc analyses revealed an increase in the number of segments crossed in PatEE F1 and F2 males versus control males, but not for PatEE versus control females. No significant difference in rearing/grooming events (**D**), or latency to leave center (**F**). However, there was an increase in directed exploration events in male F1 and F2 mice (**E**). (*$p < 0.05$, ***$p < 0.001$).

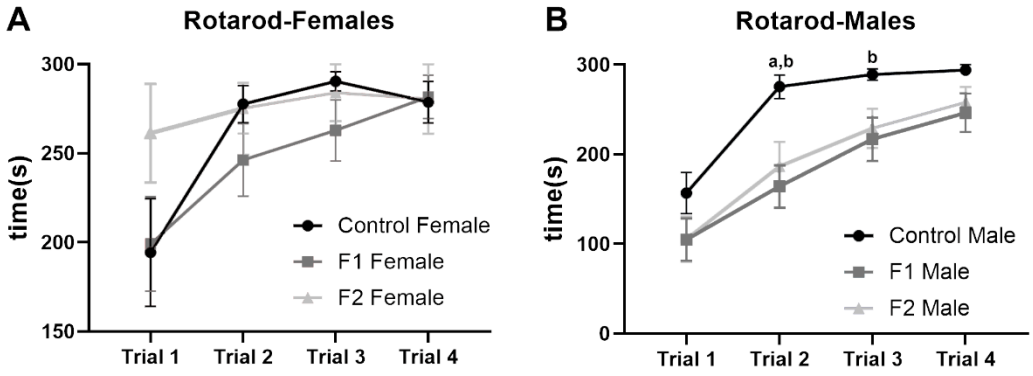


**Figure 3.11. Rotarod at P30.** Although all groups regardless of sex (**A, B**) showed an increase in performance throughout the trials, only control males (**B**) showed a significant difference between trial 1 and 2 (a), indicating a potential impairment in short-term motor learning in males due to PatEE. (**B**) Overall, male control mice performed better than PatEE F1 and F2 in trials 2 and 3 (b).

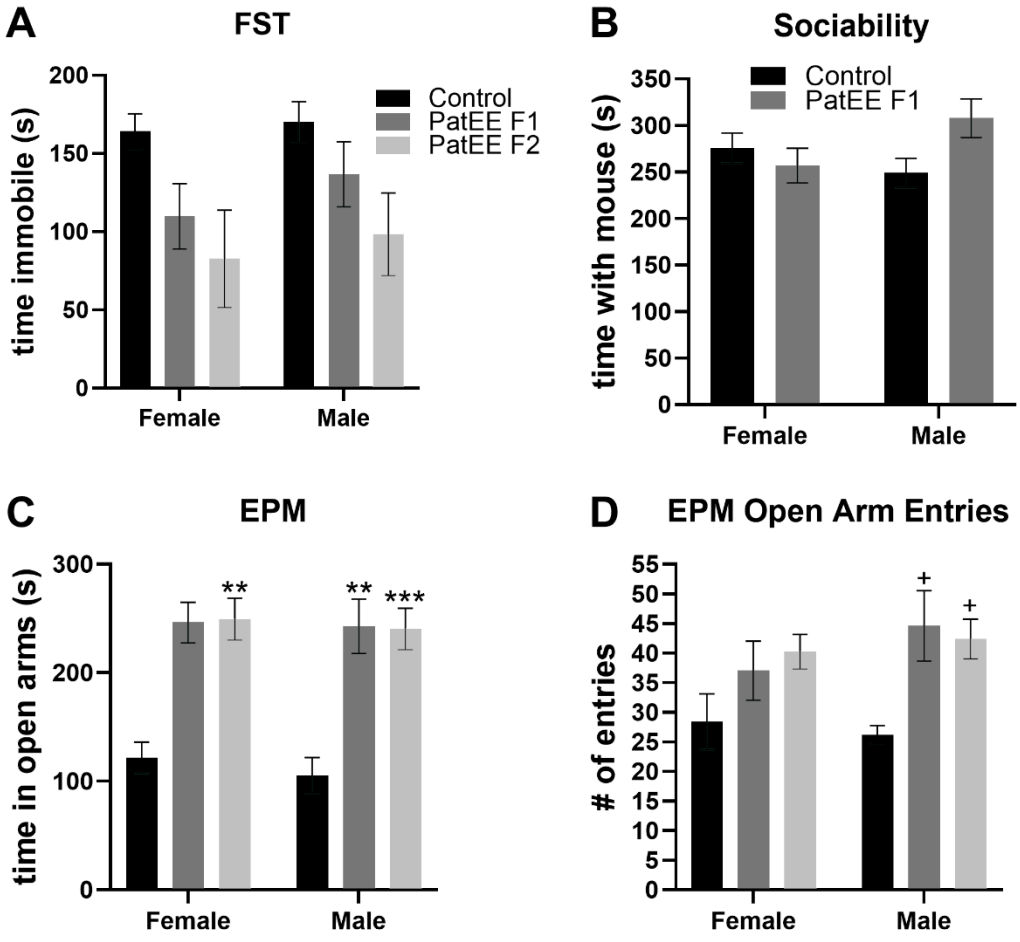


**Figure 3.12. P30 Behavior: Forced Swim Test, Sociability, Elevated Plus Maze.** (**A**) No significant difference in planned comparisons in the Forced Swim Test. (**B**) No significant differences were seen between control and PatEE F1 mice, regardless of sex, in time spent with the novel mouse within the sociability test. (**C**) Significant difference between control female and PatEE F2 mice as well as between control male and PatEE F1 and F2 mice in time spent in open arms. (**D**) Increased open arm entries in PatEE F1 and F2 mice compared to controls with a trend (+) of increased entries specifically in male mice (**$p < 0.01$, ***$p = 0.001$).

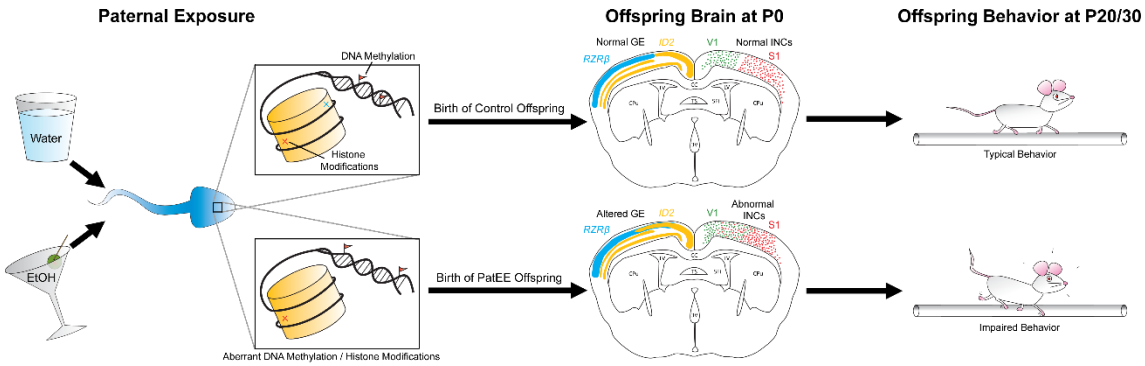


**Figure 3.13. Summary figure.** Paternal EtOH exposure model. Proposed model of how an EtOH-exposed sire's sperm can be negatively affected by EtOH, which may lead to changes in offspring gene expression, intraneocortical connectivity, and behavior compared to the offspring of sires exposed to only water. We propose an initial epigenetic dysregulation in sperm may cause direct changes in expression patterns in genes (overlapping *RZRβ* and *Id2* patterns) who govern connectivity within the cortex, which disrupts INC patterning (overlapping mixed cells in S1 and V1 in PatEE mice) and ultimately results in abnormal later-life behavior.

## REFERENCES


Abbott, C. W., Kozanian, O. O., Kanaan, J., Wendel, K. M., & Huffman, K. J. (2016). The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice. *Alcohol Clin Exp Res, 40,*122–133.

Abbott, C. W., Rohac, D. J., Bottom, R. T., Patadia, S., & Huffman, K. J. (2018) Prenatal ethanol exposure and neocortical development: A transgenerational model of FASD. *Cereb Cortex, 28,* 2908–2921.

Abel, E. L. (1983). *Marihuana, tobacco, alcohol, and reproduction.* CRC Press.

Abel. E. L. (1989). Paternal alcohol consumption: Effects of age of testing and duration of paternal drinking in mice. *Teratology, 40,* 467–474.

Adler, I. D. (1996). Comparison of the duration of spermatogenesis between male rodents and humans. *Mutat Res Mol Mech Mutagen, 352,*169–172.

Armario, A. (2021). The forced swim test: Historical, conceptual and methodological considerations and its relationship with individual behavioral traits. *Neurosci Biobehav Rev, 128*, 74-86. doi: 10.1016/j.neubiorev.2021.06.014.

Bielawski, D. M., & Abel, E. L. (1997). Acute treatment of paternal alcohol exposure produces malformations in offspring. *Alcohol, 14*, 397–401.

Bielawski, D. M., Zaher, F. M., Svinarich, D. M., & Abel, E. L. (2002). Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. *Alcohol Clin Exp Res, 26,* 347–351.

Beeler, E., Nobile, L., & Homanics, G. E. (2019). Paternal preconception every-other-day ethanol drinking alters behavior and ethanol consumption in offspring. *Brain Sci, 9*, 56.

Bhatara, V., Loudenberg, R., & Ellis, R. (2006). Association of attention deficit hyperactivity disorder and gestational alcohol exposure. *J Atten Disord, 9,* 515–522.

Buitrago, M. M., Schulz, J. B., Dichgans, J., & Luft, A. R. (2004). Short and long-term motor skill learning in an accelerated rotarod training paradigm. *Neurobiol Learn Mem, 81*, 211-216.

Ceccanti, M., Coccurello, R., Carito, V., Ciafrè, S., Ferraguti, G., Giacovazzo, G., Mancinelli, R., Tirassa, P., Chaldakov, G. N., Pascale, E., Ceccanti, M., Codazzo, C., & Fiore, M. (2016). Paternal alcohol exposure in mice alters brain NGF and BDNF and increases ethanol-elicited preference in male offspring. *Addict Biol, 21,* 776–787.

Cervera-Juanes, R., Wilhelm, L. J., Park, B., Grant, K. A., & Ferguson, B. (2017). Genome-wide analysis of the nucleus accumbens identifies DNA methylation signals differentiating low/binge from heavy alcohol drinking. *Alcohol, 60,* 103–113.

Chang, R. C., Skiles, W. M., Chronister, S. S., Wang, H., Sutton, G. I., Bedi, Y. S., Snyder, M., Long, C. R., & Golding, M. C. (2017). DNA methylation-independent growth restriction and altered developmental programming in a mouse model of preconception male alcohol exposure. *Epigenetics, 12,* 841–853.

Chang, R. C., Wang, H., Bedi, Y., & Golding, M. C. (2019). Preconception paternal alcohol exposure exerts sex-specific effects on offspring growth and long-term metabolic programming. *Epigenetics Chromatin, 12,* 9.

Chastain, L. G., & Sarkar, D. K. (2017). Alcohol effects on the epigenome in the germline: Role of inheritance of alcohol-related pathology. *Alcohol, 60,* 53-66.

Commons, K. G., Cholanians, A. B., Babb, J. A., & Ehlinger, D. G. (2017). The rodent forced swim test measures stress-coping strategy, not depression-like behavior. *ACS Chem Neurosci, 8*(5), 955-960. doi: 10.1021/acschemneuro.7b00042.

Conner, K. E., Bottom, R. T., & Huffman, K. J. (2020). The impact of paternal alcohol consumption on offspring brain and behavioral development. Alcohol *Clin Exp Res, 44*(1), 125-140. doi: 10.1111/acer.14245.

Curley, J. P., Mashoodh, R., & Champagne, F. A. (2011). Epigenetics and the origins of paternal effects. *Horm Behav, 59*(3), 306–314.doi: 10.1016/j.yhbeh.2010.06.018

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011a). A lifespan analysis of intraneocortical connections and gene expression in the mouse I. *Cereb Cortex, 21,* 1311–1330.

Dye, C. A., El Shawa, H., & Huffman, K. J. (2011b). A lifespan analysis of intraneocortical connections and gene expression in the mouse II. *Cereb Cortex, 21,* 1331–1350.

Doney, R., Lucas, B. R., Jones, T., Howat, P., Sauer, K., & Elliott, E. J. (2014). Fine motor skills in children with prenatal alcohol exposure or fetal alcohol spectrum disorder. *J Dev Behav Pediatr 35*, 598-609.

El Shawa, H., Abbott, C. W., & Huffman, K. J. (2013). Prenatal ethanol exposure disrupts intraneocortical circuitry, cortical gene expression, and behavior in a mouse model of FASD. *J Neurosci, 33,* 18893–18905.

Ervin, C. S., Little, R. E., Pytkowicz Streissguth, A., & Beck, D. E. (1984). Alcoholic fathering and its relation to child's intellectual development: A pilot investigation. *Alcoholism Clinical and Experimental Research, 8*(4). https://doi.org/10.1111/j.1530-0277.1984.tb05681.x

Finegersh, A., & Homanics, G. E. (2014). Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. *PLoS One, 9,* e99078.

Finegersh, A., Rompala, G. R., Martin, D. I. K., & Homanics, G. E. (2015). Drinking beyond a lifetime: New and emerging insights into paternal alcohol exposure on subsequent generations. *Alcohol, 49*, 461–470.

Fryer. S. L., McGee, C. L., Matt, G. E., Riley, E. P., & Mattson, S. N. (2007). Evaluation of psychopathological conditions in children with heavy prenatal alcohol exposure. *Pediatrics, 119*, e733–e741.

Gabrielli, W. F., & Mednick, S. A. (1983). Intellectual performance in children of alcoholics. *Journal of Nervous and Mental Disease, 171*(7), 444–447. https://doi.org/10.1097/00005053-198307000-00009

Gangisetty, O., Palagani, A., & Sarkar, D. K. (2020). Transgenerational inheritance of fetal alcohol exposure adverse effects on immune gene interferon-Ɣ. *Clin Epigenetics, 12*(1), 70. doi: 10.1186/s13148-020-00859-9.

Garro, A. J., McBeth, D. L., Lima, V., & Lieber, C. S. (1991). Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol Clin Exp Res, 15*, 395–398.

Granato, A., Di Rocco, F., Zumbo, A., Toesca, A., & Giannetti, S. (2003). Organization of cortico-cortical associative projections in rats exposed to ethanol during early postnatal life. *Brain Res Bull, 60,* 339–44.

Greenbaum, R. L., Stevens, S. A., Nash, K., Koren, G., & Rovet, J. (2009). Social cognitive and emotion processing abilities of children with fetal alcohol spectrum disorders: a comparison with attention deficit hyperactivity disorder. *Alcohol Clin Exp Res, 33*(10):1656-70. doi: 10.1111/j.1530-0277.2009.01003.x.

Greenham, L. W., & Greenham, V. (1977). Sexing mouse pups. *Lab Anim, 11,* 181-184.

Handley, S. L., & Mithani, S. (1984). Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn Schmiedebergs Arch Pharmacol, 327*(1), 1-5. doi: 10.1007/BF00504983.

Hamilton, D. A., Akers, K. G., Rice, J. P., Johnson, T. E., Candelaria-Cook, F. T., Maes, L. I., Rosenberg, M., Valenzuela, C. F., & Savage, D. D. (2010) Prenatal exposure to moderate levels of ethanol alters social behavior in adult rats: Relationship to structural plasticity and immediate early gene expression in frontal cortex. *Behav Brain Res, 207*, 290–304.

Hoyme, H. E., Kalberg, W. O., Elliott, A. J., Blankenship, J., Buckley, D., Marais, A-S., Manning, M. A., Robinson, L. K., Adam, M. P., Abdul-Rahman, O., Jewett, T., Coles, C.D., Chambers, C., Jones, K. L., Adnams, C. M., Shah, P. E., Riley, E. P., Charness, M. E., Warren, K. R., & May, P. A. (2016). *Updated clinical guidelines for diagnosing Fetal Alcohol Spectrum Disorders. Pediatrics, 138,* e20154256–e20154256.

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, F., Koch, C., Genz, K., Price, M. T., Stefovska, V., Tenkova, T., Dikranian, K., & Olney, J. W. (2000). Ethanol-induced apoptotic neurodegeneration and Fetal Alcohol Syndrome. *Science, 287*,1056–1060.

Jamerson, P. A., Wulser, M. J., & Kimler, B. F. (2004). Neurobehavioral effects in rat pups whose sires were exposed to alcohol. *Dev Brain Res, 149,* 103–111.

Kalueff, A. V., Keisala, T., Minasyan, A., Kumar, S. R., LaPorte, J. L., Murphy, D. L., & Tuohimaa, P. (2008). The regular and light–dark Suok tests of anxiety and sensorimotor integration: Utility for behavioral characterization in laboratory rodents. *Nat Protoc, 3,*129–136.

Kim, J-S., & Shukla, S. D. (2006). Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues. *Alcohol Alcohol, 41,* 126–132.

Kim, P., Choi, C. S., Park, J. H., Joo, S. H., Kim, S. Y., Ko, H. M., Kim, K. C., Jeon, S. J., Park, S. H., Han, S-H., Ryu, J. H., Cheong, J. H., Han, J. Y., Ko, K. N., & Shin, C. Y. (2014). Chronic exposure to ethanol of male mice before mating produces attention deficit hyperactivity disorder-like phenotype along with epigenetic dysregulation of dopamine transporter expression in mouse offspring. *J Neurosci Res, 92*, 658–670.

Knopik, V. S., Sparrow, E. P., Madden, P. A. F., Bucholz, K. K., Hudziak, J. J., Reich, W., Slutske, W. S., Grant, J. D., McLaughlin, T. L., Todorov, A., Todd, R. D., & Heath, A. C. (2005). Contributions of parental alcoholism, prenatal substance exposure, and genetic transmission to child ADHD risk: A female twin study. *Psychol Med, 35,* 25–635.

Kozanian, O. O., Rohac, D. J., Bavadian, N., Corches, A., Korzus, E., & Huffman, K. J. (2018). Long-lasting effects of prenatal ethanol exposure on fear learning and development of the amygdala. *Frontiers in Behavioral Neuroscience, 12*(200). https://doi.org/10.3389/fnbeh.2018.00200.

Krawetz, S. A., Kruger, A., Lalancette, C., Tagett, R., Anton, E., Draghici, S., & Diamond, M. P. (2011). A survey of small RNAs in human sperm. *Hum Reprod, 26,* 3401–3412.

Krubitzer, L., & Huffman, K. J. (2000). Arealization of the neocortex in mammals: Genetic and epigenetic contributions to the phenotype. *Brain Behav Evol, 55,* 322–335.

Ledig, M., Misslin, R., Vogel, E., Holownia, A., Copin, J., & Tholey, G. (1998). Paternal alcohol exposure: developmental and behavioral effects on the offspring of rats. *Neuropharmacology, 37*, 57–66.

Lee, H. J., Ryu, J-S., Choi, N. Y., Park, Y. S., Kim, Y. I., Han, D. W., Ko, K., Shin, C. Y., Hwang, H. S., Kang, K-S., & Ko, K. (2013). Transgenerational effects of paternal alcohol exposure in mouse offspring. *Animal Cells Syst, 17,* 429–434.

Liang, F., Diao, L., Liu, J., Jiang, N., Zhang, J., Wang, H., Zhou, W., Huang, G., & Ma, D. (2014). Paternal ethanol exposure and behavioral abnormities in offspring: Associated alterations in imprinted gene methylation. *Neuropharmacology, 81,* 126–133.

Little, R. E., & Sing, C. F. (1987). Father's drinking and infant birth weight: Report of an association. *Teratology, 36*, 59–65.

Lucki, I., Dalvi, A., & Mayorga, A. J. (2001). Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology, 155,* 315–22.

Lwow, F., Mędraś, M., Słowińska-lisowska, M., Jóźków, P., & Szmigiero, L. (2017). The effect of occasional alcohol drinking on semen quality and sperm morphology among young and healthy polish men. *Journal of Men's Health*, *13*(2),16-24.

Macri, S., Adriani, W., Chiarotti, F., & Laviola, G. (2002). Risk taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. *Animal Behaviour, 64*(4), 542-546. https://doi.org/10.1006/anbe.2002.4004

Madarnas, C., Villalba, N. M., Soriano, D., & Brusco, A. (2020). anxious behavior of adult CD1 mice perinatally exposed to low concentrations of ethanol correlates with morphological changes in cingulate cortex and amygdala. *Front Behav Neurosci, 14,* 92. doi: 10.3389/fnbeh.2020.00092.

Mattson, S. N., & Riley, E. P. (1998). A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcoholism: Clinical and Experimental Research, 22*(2), 279-294.

May, P. A., Chambers, C. D., Kalberg, W. O., Zellner, J., Feldman, H., Buckley, D., Kopald, D., Hasken, J. M., Xu, R., Honerkamp-Smith, G., Taras, H., Manning, M. A., Robinson, L. K., Adam, M. P., Abdul-Rahman, O., Vaux, K., Jewett, T., Elliott, A. J., Kable, J. A., Akshoomoff, N., Daniel, F., Arroyo, J. A., Hereld, D., Riley, E. P., Charness, M. E., Coles, C. D., Warren, K. R., Jones, K. L., & Hoyme, H. E. (2018). Prevalence of fetal alcohol spectrum disorders in 4 US communities. *J Am Med Assoc, 319,* 474–482.

Meek, L. R., Myren, K., Sturm, J., & Burau, D. (2007). Acute paternal alcohol use affects offspring development and adult behavior. *Physiol Behav, 91,* 154–160.

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., Young, N. B., Barbaro, R. P., Piven, J., Magnuson, T. R., & Crawley, J. N. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain Behav., 3,* 303-314

Ouko, L. A., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D. J., & Ramsay, M. (2009). Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: implications for fetal alcohol spectrum disorders. *Alcohol Clin Exp Res, 33,* 1615–27.

Paxinos, G., Halliday, G., Watson, C., Koutcherov, Y., & Wang, H. (2007). *Atlas of the developing mouse brain.* 1st edition. Academic Press/Elsevier, Burlington MA.

Pihl, R. O., & Bruce, K. R. (1995). Cognitive impairment in children of alcoholics. *Alcohol Health Res World, 19*(2).

Porsolt, R. D., Anton, G., Blavet, N., & Jalfre, M. (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol, 47*(4), 379-91. doi: 10.1016/0014-2999(78)90118-8.

Riley, E. P., & McGee, C. L. (2005). Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp Biol Med (Maywood). 230*(6), 357-65. doi: 10.1177/15353702-0323006-03.

Rogers, B. B., Cuddahy, T., Briscella, C., Ross, N., Olszanski, A. J., & Denlinger, C. S. (2019). Oxaliplatin: Detection and management of hypersensitivity reactions. *Clin J Oncol Nurs, 23*(1), 68-75. doi: 10.1188/19.CJON.68-75.

Rompala, G. R., Finegersh, A., & Homanics, G. E. (2016). Paternal preconception ethanol exposure blunts hypothalamic-pituitary-adrenal axis responsivity and stress-induced excessive fluid intake in male mice. *Alcohol, 53*,19–25.

Rompala, G. R., Finegersh, A., Slater, M., & Homanics, G. E. (2017). Paternal preconception alcohol exposure imparts intergenerational alcohol-related behaviors to male offspring on a pure C57BL/6J background. *Alcohol, 60*, 169–177.

Rompala, G. R., Mounier, A., Wolfe, C. M., Lin, Q., Lefterov, I., & Homanics, G. E. (2018). Heavy chronic intermittent ethanol exposure alters small noncoding RNAs in mouse sperm and epididymosomes. *Front Genet, 9,* 32.

Rompala, G. R., & Homanics, G. E. (2019). Intergenerational effects of alcohol: A review of paternal preconception ethanol exposure studies and epigenetic mechanisms in the male germline. *Alcohol Clin Exp Res, 43,*1032-1045.

Rubenstein, J. L. R., Anderson, S., Shi, L., Miyashita-Lin, E., Bulfone, A., & Hevner, R. (1999). Genetic control of cortical regionalization and connectivity. *Cereb Cortex, 9,* 524–532.

Rustay, N. R., Wahlsten, D., & Crabbe, J. C. (2003). Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. *Behav Brain Res, 141*, 237–49.

Schandler, S. L., Brannock, J. C., Cohen, M. J., & Mendez, J. (1993). Spatial learning deficits in adolescent children of alcoholics. *Experimental and Clinical Psychopharmacology, 1*(1-4), 207–214. https://doi.org/10.1037/1064-1297.1.1-4.207

Schneider, M. L., Moore, C. F., Adkins, M., Barr, C. S., Larson, J. A., Resch, L. M. & Roberts A. (2017). Sensory processing in rhesus monkeys: Developmental continuity, prenatal treatment, and genetic influences. *Child Dev, 88*(1), 183-197.

Stockard, C. R., & Papanicolaou, G. N. (1918). Further studies on the modification of the germ-cells in mammals: The effect of alcohol on treated guinea pigs and their descendants. *J Exp Zool, 26*,119-226.

Tarter, R. E., Jacob, T., & Bremer, D. L. (1989). Specific cognitive impairment in sons of early onset alcoholics. *Alcohol Clin Exp Res, 13,* 786–789.

Thomas, S. E., Kelly, S. J., Mattson, S. N., & Riley, E. P. (1998). Comparison of social abilities of children with fetal alcohol syndrome to those of children with similar IQ scores and normal controls. *Alcohol Clin Exp Res, 22,* 528–33.

Wolterink-Donselaar, I. G., Meerding, J. M., & Fernandes, C. (2009). A method for gender determination in newborn dark pigmented mice. *Lab Anim (NY), 38,* 35-38.

Wozniak, D. F., Cicero, T. J., Kettinger, L., & Meyer, E. R. (1991). Paternal alcohol consumption in the rat impairs spatial learning performance in male offspring. *Psychopharmacology, 105,* 289–302.

Xia, R., Jin, L., Li, D., Liang, H., Yang, F., Chen, J., Yuan, W., & Miao, M. (2018). Association between paternal alcohol consumption before conception and anogenital distance of offspring. *Alcohol Clin Exp Res, 42,* 735–742.

Yang, M., Silverman, J. L., & Crawley, J. N. (2011). Automated three-chambered social approach task for mice. *Curr Protoc Neurosci, 56,* 8.26.1-8.26-16.

Zhou, D., Lebel, C., Lepage, C., Rasmussen, C., Evans, A., Wyper, K., Pei, J., Andrew, G., Massey, A., Massey, D., & Beaulieu, C. (2011). Developmental cortical thinning in fetal alcohol spectrum disorders. *Neuroimage, 58,* 16–25.

Zuccolo, L., DeRoo, L. A., Wills, A. K., Smith, G. D., Suren, P., Roth, C., Stoltenberg, C., & Magnus, P. (2017). Erratum: Pre-conception and prenatal alcohol exposure from mothers and fathers drinking and head circumference: results from the Norwegian Mother-Child Study (MoBa). *Sci Rep, 7,* 45877.

## GENERAL CONCLUSION

In a series of studies, our laboratory has identified many deleterious effects of prenatal ethanol exposure (PrEE) as well as paternal ethanol exposure (PatEE) in a mouse model. Overall, our results indicate that ethanol exposure during pregnancy (PrEE) or prior to conception (PatEE) can have substantial effects on the offspring. These effects can potentially be seen generations later in both types of exposure.

Our data suggest that PrEE can result in abnormal brain and behavioral development with heritable effects that persist transgenerationally to subsequent generations of offspring. Through these studies we have visualized changes in gross anatomy and neuroanatomy at various stages during embryonic development (E12.5, E14.5, E16.5, and E18.5) as well as in newborn (P0) mice. Specifically, hippocampal CA3 was significantly thinner in all generations of PrEE mice when compared to controls at P0. Additionally, PrEE resulted in a significant rate of agenesis or partial development of the corpus callosum in the majority of F1 cases, with a less frequent, non-significant, occurrence in F2 and F3 mice. These changes in neuroanatomy may contribute to the behavioral effects we observed due to PrEE including disrupted sensorimotor integration, motor control, and anxiety-like behavior which persisted until at least the F2 generation.

Likewise, PatEE resulted in significant impact on neocortical development, including abnormal patterns of gene expression within the neocortex at P0.

Additionally, PatEE mice exhibited a sex-specific increase in activity and sensorimotor integration deficits at P20, and decreased balance, coordination, and short-term motor learning at P30. Furthermore, increased activity and risk-taking behaviors were observed at P30 suggesting that PatEE may generate long-lasting, sex-specific effects on offspring behavior. These results demonstrate that the developmental impact of preconception PatEE is more harmful than previously thought and provide additional insights into the biological mechanisms that may underlie atypical behavior observed in children of alcoholic fathers. We hypothesize that PatEE may cause sperm cell epigenetic dysregulation, which in turn may result in epigenetic and gene expression alterations within offspring brain (such as in *Id2* and *RZRβ* expression), resulting in altered cortical patterning and ectopic development of neocortical circuits. These changes may contribute to behavioral alterations in PatEE offspring (Fig. 3.12)

We propose that the effects of drinking alcohol during pregnancy may be more serious than previously thought as transgenerational effects were observed in the offspring even though they never consumed alcohol themselves. We suggest that alcohol consumption by fathers prior to conception may have substantial yet unrecognized effects on the health and development of progeny. By promoting this research to the general public, we hope to change society's views on drinking during pregnancy and drinking prior to conception.