UNIVERSITY OF CALIFORNIA
RIVERSIDE

Growth and Behavioral Deficits Associated with Ethanol Consumption During Gestation and Rearing, in a Mouse Model

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

Doctor of Philosophy

in

Psychology

by

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December 2024

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ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Kelly Huffman for her support through my undergraduate and graduate studies. Kelly's guidance, support, and wisdom was instrumental in my tenure at the University of California, Riverside (UCR). During my undergraduate studies I sporadically decided to take an Introductory Psychology course taught by Kelly. This was my first formal experience with Psychology, and I attribute this course as the pivotal moment in my career where I realized I wanted to become a Psychologist. The way that she connected with students and made learning accessible to all is something that I emulate every day in my classes. I am the educator, researcher, and father who I am today thanks to Kelly. I am extremely grateful to consider Kelly a mentor and a friend. Additionally, I would like to thank Kelly's late husband Bruce Neal. His many words of wisdom still guide my life to this day, and I promise to continue being "unapologetically Roberto".

I would also like to thank my committee members Dr. Khaleel Abdulrazak and Dr. Edward Zagha for their support throughout my time at UCR. I very much appreciate all the insights, guidance, and interactions that we've had. Your expertise in your field, and with teaching, have elevated my own pedagogy and have allowed me to grow into the educator that I am now.

I would like to thank all the faculty and staff at UCR. Thank you to all who made me feel welcomed, heard, and at home at UCR. I want to give special thanks to Dr. Michael Erickson. Thank you for all the statistical insights that you've provided

regarding projects and teaching. Additionally, I would like to thank Dr. Elizabeth Davis for the mentorship you've provided throughout the years.

An incredibly special thank you to all Huffman lab members, past and present. Thank you to Dr. Olga Kozanian, Dr. David Rohac, and Dr. Riley Bottom. Your guidance and training during the early stages of my career were invaluable and were instrumental to my success. Thank you Dr. Kathleen Conner, Mirembe Nabatanzi, and Angela Avitua. The memories that we share as lab mates will forever be some of the best times I had at UCR. Thank you to the many undergraduate research assistants. I hope nothing but the best for all Huffman lab members and I can't wait to see what the future holds for us all. Friendships may end, but lab mates are forever! I would also like to thank all the friends that I've made while at UCR. I can't wait to see where we all go.

I would like to thank my family. My parents, Roberto F. Perez and Matilde Perez, thank you for your unending, unconditional support. The education I have received is much more than a diploma, it is a testament to the sacrifice and dedication that our family has endured as immigrants. Thank you to all my relatives (aunts, uncles, cousins, grandparents, and much more) for your support.

I would like to thank my incredible wife Kimberly Leia Perez-Tokuda. Thank you for everything. Your support and love are what kept me sane throughout this entire process. I am excited for the rest of our lives together. Lastly, I want to thank my daughter Estrella Lourdes Perez-Tokuda. You've already grown so much, and I cannot wait to see you blossom into the person you are meant to be.

The chapters of this dissertation, in part or in whole, are reprints of materials as they appear in Frontiers in Neuroscience, March 2023 (V17) (Perez et al., 2023). The co-author Kelly Huffman supervised this research while Kathleen Conner contributed to data collection and analysis. Additional chapters will be published in the future.

ABSTRACT OF THE DISSERTATION

Growth and Behavioral Deficits Associated with Ethanol Consumption During Gestation and Rearing, in a Mouse Model

by

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Doctor of Philosophy, Graduate Program in Psychology
University of California, Riverside, December 2024
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Exposure to alcohol during early development can induce a multitude of long-lasting developmental abnormalities in exposed individuals due to the teratogenicity associated with alcohol exposure. These abnormalities are characterized as Fetal Alcohol Spectrum Disorders (FASD); which range from physical, behavioral, and cognitive impairs due to alcohol exposure before birth. Maintaining up-to-date and extensive characterization profiles of prenatal ethanol exposure (PrEE) and lactational ethanol exposure (LEE) is crucial for understanding the wide ranging impacts of pre-, peri- and postnatal ethanol exposure. Our laboratory has identified many abnormalities associated with PrEE and LEE. In summary, our results indicate that PrEE and LEE measurably impact brain and behavioral development of affected offspring.

In Chapter 1, we explore the heritability of FASD in our mouse model. We previously published on heritable phenotypes in our PrEE mice (Abbott et al., 2018), and this chapter is an extension of the earlier work. Specifically, we document heritable,

transgenerational changes in the gross development and behavior in PrEE offspring, in the absence of subsequent alcohol exposure. Our study investigates PrEE-induced effects in directly exposed first- (F1), indirectly exposed second- (F2), and non-exposed third-filial (F3) generations. We report reduced brain and body weights in F1-F3 due to PrEE. In addition, we report thinning of the cortex in prelimbic, auditory, and visual cortices. Lastly, we report altered social behavior for F1 and F2 PrEE mice with a return to control levels at F3.

In Chapter 2, we established and validated a novel mouse model of LEE where we exposed mice via ethanol contaminated breast milk during the brain growth spurt. This novel model of LEE demonstrated sustained deficits in gross neural development and behavior. Specifically, we note a thinning of the frontal cortex compared to age matched controls, potential reductions in dendritic density within the medial frontal cortex and altered behavior due to the exposure during lactation.

Furthermore, Chapter 3 explores the genomic, epigenomic, and behavioral associated with LEE. This chapter is an extension of our previous work in LEE. We explored mRNA expression of cortical patterning genes, global DNA methylation via 5-methylcytosine (5mC) levels, and further characterized behavior in LEE. Through our approaches we report reductions in gross development, increased gene expression of *Id2*, and altered behavior due to LEE.

In summary, this dissertation includes three projects, presented as chapters. Chapter 2 has been published previously; chapters 1 and 3 will each be published as separate papers. In the body of work represented in these three chapters, we report

sustained, observable neural and behavioral deficits in the affected offspring associated with prenatal or lactational ethanol exposure during early development. Prenatal ethanol exposure not only induces significant deficits in the directly exposed offspring, as shown in many reports from the Huffman Laboratory. Our data demonstrate heritable, transgenerational change to at least the third filial generation in PrEE mice. While early postnatal exposure to ethanol via breast milk during lactation and nursing does not produce the severity of phenotypes associated with PrEE, LEE does cause both neuroanatomical and behavioral effects in the affected offspring. Although this research is not directly applied science, our data strongly suggests that both pregnant and nursing women should avoid alcohol consumption.

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General Introduction

Alcohol exposure during critical developmental periods, both prenatal and postnatal, is a significant public health concern. The effects may have far reaching consequences for offspring health and behavior. Prenatal ethanol exposure (PrEE) is a well-documented cause of Fetal Alcohol Spectrum Disorders (FASD). Characterized by cognitive, sensory, behavioral, motor, and physical impairments in PrEE offspring, which are dependent on the amount of alcohol consumed, the timing of exposure, maternal health metrics, and genetic predisposition (Carter et al., 2016). Despite the complexities associated with PrEE, research has established hallmark features such as sensory processing deficits, impaired motor skills, spatial cognition difficulties, and heightened anxiety and depression (Lemoine et al., 1968; Jones et al., 1973a; 1973b; Kalberg et al., 2006; Hellemans et al., 2010a; 2010b).

The Centers for Disease Control and Prevention (CDC) states that there is "no safe amount" of alcohol consumption during pregnancy and when attempting to conceive (CDC, 2024). Further, the CDC recommends that a woman should avoid alcohol if she is pregnant or might be pregnant due to not knowing pregnancy status for up to 6 weeks post-fertilization (CDC, 2024). Despite this recommendation, the estimated incidence of FASDs in the United States (US) is approximately 5% (May et al., 2018) with prenatal alcohol consumption rates peaking during the first trimester at around 14% (Gosdin et al., 2022). It is worth noting that actual incidence rates may be even higher in the US and globally due to the reliance on self-reporting by expectant mothers. Additionally, FASDs exhibit similar phenotypes to Williams syndrome, Velocardiofacial syndrome (VCFS),

and attention-deficit/hyperactivity disorder (ADHS) (Hoyme et al., 2016; CDC, 2024) which may contribute to the underreporting of FASDs. In conjunction, the spectrum of FASDs is wide-ranging, and proper identification of the specific syndrome may be challenging. For example, Fetal Alcohol Syndrome (FAS) is characterized by central nervous system (CNS) problems, minor facial features, growth retardation, and cognitive abnormalities (CDC, 2024) which may be easier to diagnose compared to Neurobehavioral Disorders associated with Prenatal Alcohol Exposure (ND-PAE) which is characterized by the memory issues, cognitive delays, behavioral problems such as tantrums, and issues with day-to-day functioning (CDC, 2024). This places much of the responsibility for proper identification and diagnosis on the primary care physician, emphasizing the need for up-to-date training for healthcare professionals. In addition, the stigma related to the diagnosis of FASD may negatively impact the patient and their family; therefore, leading to potential reductions in seeking of medical care in women who drank during pregnancy (Bell et al., 2015; Bell et al., 2016).

Adding to the complexity of ethanol exposure during early development, postnatal exposure through breastfeeding introduces a new layer of risk for offspring. Interestingly, the likelihood of breastfeeding was increased in women who had a history of alcohol use compared to women without a history of alcohol use (Washio et al., 2024). Suggesting that offspring may be exposed to both pre- and post-natal ethanol exposures in some populations. In addition, Prior and colleagues (2024), identified that maternal anxiety, coping-with-anxiety, social-drinking motives, and reduced social support were important predictors towards increased postpartum alcohol misuse. Other studies

identified a significant impact of partner influence on the use of alcohol during pregnancy (Kautz-Turnbull et al., 2021). Therefore, it is critically important to evaluate the socio-cultural aspects surrounding safe maternal practices following birth. Shockingly, one in seven women suffers from a specific form of perinatal depression post birth referred to as Postpartum Depression (U.S. Department of Health and Human Services, Health Resources and Services Administration, Office of Maternal and Child Health; HRSA, 2019). The causes of perinatal depression can range from hormonal changes due to pregnancy and child rearing, altered sleeping schedules, and societal pressures to be the "perfect mother" (HRSA, 2019). Interestingly, a strong correlation between perinatal depression and the development of Postpartum Post Traumatic Stress Disorder (PP-PTSD) was identified with higher depression scores corresponding to more severe PP-PTSD symptoms (reviewed in Zhu et al., 2024). High amounts of emphasis is placed on the maternal responsibilities during early postpartum child development, which further emphasizes the importance of identifying the impacts of maternal stressors and appropriate infant care. Overall seeking medical assistance from a health care provider is the first step towards addressing perinatal depression. In addition, it is recommended by the HRSA (2019) to find a support group and lean on friends/family for support. Further emphasizing the psychosocial influence of maternal wellbeing and safe practices during child rearing since increased stress may lead to increase in the consumption of alcohol, for certain populations (Guinle & Sinha, 2020).

This dissertation explores the multifaceted impacts of alcohol exposure in a mouse model, with a focus on the transgenerational inheritance and postnatal effects

through lactation. Animal models have been instrumental in advancing our understanding of prenatal alcohol exposure and its effects on fetal development. These models have provided valuable insights into the teratogenic mechanisms of alcohol, which involve nutrient deficiencies, fetal hypoxia, and alterations in enzyme activities crucial for cell division and membrane integrity (reviewed in Zajac & Abel, 1992). The interaction between animal and human research has been particularly fruitful, with animal studies confirming the teratogenicity of alcohol observed in human cases of Fetal Alcohol Syndrome (Connor, 2001). Animal models have also been essential in addressing basic questions about developmental alcohol exposure, improving identification of affected individuals, and developing interventions to reduce the impact of prenatal alcohol exposure (Wilson & Cudd, 2011; El Shawa et al., 2013; Abbott et al., 2018; Bottom et al., 2020). The multifactorial nature of alcohol's effects is evident, with factors such as genotype, nutritional status, exposure patterns, and concurrent drug use playing significant roles (reviewed in Zajac & Abel, 1992).

Chapter 1 examines the transgenerational impacts of PrEE, an area that remains underexplored despite its importance in understanding the full scope of alcohol’s effects. There is growing evidence that PrEE can result in epigenetic modifications that persist into subsequent generations, impacting gene expression, behavior, and physiological trains (Govorko et al., 2012; Knezovich & Ramsay, 2012; El Shawa et al., 2013; Abbott et al., 2018). Specifically, this chapter investigates the PrEE-induced effects in the second (F2) and third (F3) filial generations.

Chapter 2 shifts focus onto the postnatal exposure of alcohol via a novel breastfeeding model. This is a critical, yet understudied, period of postnatal development. In general, breastfeeding provides a multitude of benefits for both offspring and mother; however, many women consume alcohol after birth, whether they abstained from drinking during their pregnancy or not. We are interested in whether or not maternal alcohol consumption during breastfeeding, leading to offspring lactational ethanol exposure, or LEE, negatively impacts brain and behavioral development. Previous models focused on pre- and (later) post-natal exposure; therefore, we developed a novel exposure paradigm to explore the impacts of Lactational Ethanol Exposure (LEE) beginning on postnatal day 6, a close approximation in age to human birth (human birth is estimated as postnatal day 7-9 in the mouse). The research described in chapter 2 utilizes a multi-method approach to examine offspring phenotypes and outcomes related to LEE. In chapter 3, we extend the study published previously (chapter 2) by examining molecular and behavioral outcomes associated with LEE. Specifically, we investigate the expression of genes known to be involved in neocortical patterning, in the brains of LEE and control mice and further characterize the behavioral profile of offspring exposed to ethanol during the lactation period. This chapter establishes the impact of LEE on cortical patterning genes and potential alterations of behavior, compared to non-exposed offspring.

In summary, both PrEE and LEE models of ethanol exposure revealed widespread changes in offspring's gross, neuroanatomical, and behavioral development. Our results indicate that the deleterious effects of PrEE are likely to be heritable and that postnatal

ethanol exposure via breast milk may introduce a wide range of developmental effects. In the following chapters we explore these changes in depth and introduce potential mechanisms that may influence the observed phenotypes.

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Chapter 1: Transgenerational Behavioral and Neuroanatomical Effects of Prenatal Ethanol Exposure in Newborn and Peri-pubescent Mice

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 the 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 the 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 neocortical areas 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

neocortical thickness. Specifically, prelimbic, auditory, and visual cortical thickness was reduced in F1 mice but not in the subsequent generations, when compared to controls. Finally, we found that altered social 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 offspring generations.

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 peri-pubescent 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 neocortical regions in offspring, beyond the directly-exposed first generation. Additionally, social behavior was analyzed using the Three-Chambered Sociability 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, bred alongside each generation, 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, gestational day (G) 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 the control dams *ad libitum*. Beginning on G 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 1.1.

Dam Data

Food intake, weight gain and litter size:

We measured food consumption each gestational 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 (Figures 1.2, 1.3) 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 (Figure 1.2C). 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 Figure 1.3, 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). Cortical thickness measurements included the prelimbic, somatosensory, auditory, and visual cortices.

Behavior analyses

To evaluate behavioral phenotypes associated with transgenerational PrEE, we examined social behavior in P20 control, F1, F2, and F3 mice through the use of the

behavioral assay, Three-Chambered Sociability Test. Before the commencement of behavioral tests, all mice were acclimated to the dimly lit behavioral room for one hour minimum. 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.

To evaluate altered social behavior we employed the Three Chambered Sociability Test (SOC). The apparatus design included a clear Plexiglas chamber with three rectangular areas divided by clear walls inside the box. During the test, two cups were placed at opposite ends of the chamber; one cup was empty, while the other contained a docile, target mouse (a novel mouse of similar age and sex). To gain insight into social behavior, we compared how much time the test mouse spent with the target mouse, versus the empty cup (Nadler et al., 2004; Yang et al., 2011). Due to the social nature of these animals, a typically behaving control mouse should spend significantly more time near the cup with the target mouse. Prior to the test, the target mice were acclimated to the testing chamber and cups. Each test mouse was habituated to the middle chamber for 10 minutes. Next, the mouse was allowed free access to all three chambers for 10 minutes. A final, 10-minute, trial introduced the enclosed, target mouse to a side chamber. The behavior was recorded and time spent in each chamber was blind scored by trained research assistants.

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 carried out 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 (Figures 1.2A, 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 (Figure 1.2A, n=8; M=7.75 g/day, SD=1.34 g/day, 95% CI [6.63, 8.87]) and in the F1 condition (n=8; 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 (n=10; 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 (n=10; 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, Figure 1.2B, $F(3,32)$=2.03, p=0.1297. 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 (Figure 1.2B, n=8; M=13.69 mL/day, SD=0.70 mL/day, 95% CI [13.10, 14.28]) and in the F1 condition (n=8; 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 (n=10; 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 (n=10; 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, ethanol exposed dams had greater blood ethanol concentrations (BEC; n=6; M=138.7 mg/dL, SD=8.0 mg/dL, 95% CI [130.3, 147.1]) than control dams (Figure 1.2C, *n=6; M*=0.0 mg/dL, SD=0.0 mg/dL, 95% CI [0.0, 0.0]), $t(5)$=42.48, p<.001. To ensure that the exposure paradigm did not result in dehydration, blood plasma osmolality (pOsm) was measured at wean (Figure 1.2D). There was no evidence of a difference in blood plasma osmolality at wean between ethanol exposed (n=16; M=311.81 mOsm/kg, SD=8.44 mOsm/kg, 95% CI [307.32, 316.31]) and control

dams (n=14; 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 F0 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 (Figure 1.3A). Planned comparisons between the size of the control litters and those of each filial generation showed greater litter sizes for control mice (n=10; M=11.70 pups, SD=2.87 pups, 95% CI [9.65, 13.75]) than F1 litters (n=9; M=8.33 pups, SD=1.58 pups, 95% CI [7.12, 9.55]), t(35)=3.39, p=0.005, F2 litters (n=10; M=8.80 pups, SD=1.87 pups, 95% CI [7.46, 10.14]), t(35)=3.00, p=0.0139, and F3 litters (n=10; 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, 2018; Bottom et al., 2022). Ethanol-exposed dams (producing F1 offspring), as well as ethanol-naïve dams that produced F2 and F3 offspring, gained less weight over the course of the pregnancy when compared to control dams (Figure 1.3B). 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 (n=8; M=24.39 g, SD=2.75 g, 95% CI [22.09, 26.69]) and the dams that produced F1 offspring (n=8; M=18.15 g, SD=5.21 g, 95% CI [13.79, 22.51]), t(32)=2.72, p=0.0288, the dams that produced F2 offspring (n=10; M=17.77 g, SD=4.52 g, 95% CI [14.54, 21.00]), t(32)=3.04, p=0.0131, and the dams that produced F3

offspring (*n*=10; *M*=18.18 g, *SD*=5.24 g, 95% CI [14.43, 21.93]), *t*(32)=2.85, *p*=0.0209. This effect was likely 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 (Figure 1.3C). 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 (*n*=8; *M*=2.09 g, *SD*=0.60 g, 95% CI [1.58, 2.59]) and in the F1 condition (*n*=8; *M*=2.28 g, *SD*=0.93 g, 95% CI [1.50, 3.06]), *t*(32)=0.48, *p*=0.9036, the F2 condition (*n*=10; *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 (*n*=10; *M*=2.19 g, *SD*=0.71 g, 95% CI [1.68, 2.69]), *t*(32)=0.27, *p*=0.9707. The dam data presented here, along with data from our prior work in the area demonstrate the reliability of the exposure model (El Shawa et al., 2013; Abbott et al., 2016; 2018; Bottom et al., 2020; 2022). Dam data collected for the current transgenerational project (Figures 1.2, 1.3) did not differ significantly from data presented previously in our transgenerational PrEE publications. For instance, in Abbott and colleagues (2018), we reported no significant differences in food intake or hydration (blood plasma osmolality) in the control dams and the dams used to generate the F1-3 generations. As expected, we found moderate BEC levels in ethanol-treated dams only, and zero BEC in ethanol-naïve dams (Figure 1.2C). The consistent reduction in maternal weight gains in dams used to

generate the F1-3 generations (here and in prior work) was correlated with a reduction in litter size for all three generations of PrEE offspring (see Figure 3, Abbott et al., 2018).

Pup Measures

Body weights, brain weights, brain-body weight ratios, and cortical lengths were measured in newborn (P0) control, F1, F2, and F3 mice (Figures 1.4, 1.5). A one-way ANOVA identified differences in body weight at P0 among the four conditions, $F(3,34)=10.04$, $p<.001$ (Figure 1.4A). Additionally, planned comparisons provided evidence that P0 control pups ($n=10$; $M=1.78$ g, $SD=0.12$ g, 95% CI [1.70, 1.87]) weighed more than F1 mice ($n=9$; $M=1.44$ g, $SD=0.17$ g, 95% CI [1.31, 1.56]), $t(34)=4.93$, $p<.001$, F2 mice ($n=9$; $M=1.48$ g, $SD=0.12$ g, 95% CI [1.39, 1.57]), $t(34)=4.35$, $p<.001$, and F3 mice ($n=10$; $M=1.53$ g, $SD=0.19$ g, 95% CI [1.39, 1.67]), $t(34)=3.70$, $p=0.002$. These effects persist to P20, as reported previously (Bottom et al., 2022). Thus, the prenatal ethanol exposure of F1 pups affected the body weights across all three filial generations.

Similarly, we identified differences in the weight of pups' brains across the four conditions, $F(3,34)=7.58$, $p<0.001$ (Figure 1.4B). Planned comparisons between control pups and each filial generation showed the brain weight of the control pups ($n=10$; $M=0.108$ g, $SD=0.0141$ g, 95% CI [0.097, 0.118]) was greater than than PrEE pups: F1 pups ($n=9$; $M=0.087$ g, $SD=0.0071$ g, 95% CI [0.081, 0.092]), $t(34)=4.12$, $p=0.0007$, F2 pups ($n=9$; $M=0.094$ g, $SD=0.0108$ g, 95% CI [0.085, 0.102]), $t(34)=2.78$, $p=0.0241$, and F3 pups ($n=10$; $M=0.088$ g, $SD=0.0102$ g, 95% CI [0.080, 0.095]), $t(34)=4.09$, $p=0.0007$.These effects persist to P20, as reported previously (Bottom et al., 2022). These

findings provide evidence that prenatal ethanol exposure of F1 pups 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 pups in each condition (Figure 1.4C). A one-way ANOVA failed to identify significant differences in brain-body weight ratio at P0 between the four conditions, $F(3,34)=2.39$, $p=0.0862$. Planned comparisons between control mice and each filial generation, likewise, failed to show significant differences between the brain-body weight ratio of control mice ($n=10$; $M=0.060$, $SD=0.0056$, 95% CI [0.056, 0.064]) and F1 mice ($n=9$; $M=0.061$, $SD=0.0045$, 95% CI [0.057, 0.064]), $t(34)=0.25$, $p=0.9740$, F2 mice ($n=9$; $M=0.063$, $SD=0.0053$, 95% CI [0.059, 0.067]), $t(34)=1.4$, $p=0.3755$, and F3 mice ($n=10$; $M=0.057$, $SD=0.0039$, 95% CI [0.055, 0.060]), $t(34)=1.29$, $p=0.4409$. 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.

There were also differences in pups' cortical lengths across the four conditions, $F(3,36)=10.25$, $p<.001$ (Figure 1.5, dorsal images of exemplar brains shown in A-D; measurement data graphed in E). Again, planned comparisons between control mice and each filial generation showed that the cortical lengths of control mice ($n=10$; $M=4.48$ mm, $SD=0.38$ mm, 95% CI [4.21, 4.76]) were greater than F1 mice ($n=10$; $M=3.84$ mm, $SD=0.22$ mm, 95% CI [3.68, 3.99]), $t(36)=5.39$, $p<.001$, F2 mice ($n=10$; $M=4.06$ mm, $SD=0.12$ mm, 95% CI [3.98, 4.15]), $t(36)=3.52$, $p=0.0035$, and F3 mice ($n=10$; $M=4.04$ mm, $SD=0.28$ mm, 95% CI [3.84, 4.24]), $t(36)=3.68$, $p=0.0022$.These effects persist to

P20, as reported previously (Bottom et al., 2022). These results show that prenatal ethanol exposure of F1 pups affected neocortical lengths across all three filial generations.

Anatomical measures

Analysis of anatomical measures identified additional changes in F1, F2, and F3 mice compared to controls on the day of birth. Measures taken from Nissl stained sections at age P0 showed some differences in cortical thickness (Figure 1.6). Specifically, the thickness of the prelimbic cortex across conditions showed a significant main effect, $F(3,22)=6.07$, $p=0.0036$ (Figure 1.6A1-5). Planned comparisons between control mice and each filial generation showed that the thickness of the prelimbic cortex in control mice ($n=8$; $M=0.229$ mm, $SD=0.01429$ mm, 95% CI [0.217, 0.241]) was greater than in F1 mice ($n=5$; $M=0.208$ mm, $SD=0.00610$ mm, 95% CI [0.201, 0.216]), $t(22)=3.19$, $p=0.0117$, but the difference between control and F2 mice ($n=5$; $M=0.227$ mm, $SD=0.01388$ mm, 95% CI [0.210, 0.244]) failed to reach significance, $t(22)=0.32$, $p=0.9583$, as did the difference between control and F3 mice ($n=8$; $M=0.235$ mm, $SD=0.00838$ mm, 95% CI [0.228, 0.242]), $t(22)=1.14$, $p=0.5329$. Thus, the primary phenotype in the prelimbic cortex is the thinning of the cortex for F1 pups relative to controls.

A one-way ANOVA failed to find evidence of differences in the thickness of the pups' somatosensory cortex across the four conditions, $F(3,21)=1.54$, $p=0.2326$ (Figure 1.6B1-5). Planned comparisons between control mice and each filial generation likewise failed to show differences between the cortical thickness of control mice ($n=7$; $M=0.339$

mm, *SD*=0.0248 mm, 95% CI [0.316, 0.362]) and F1 mice (*n*=6; *M*=0.347 mm, *SD*=0.0298 mm, 95% CI [0.316, 0.379]), *t*(21)=0.51, *p*=0.8934, F2 mice (*n*=6; *M*=0.371 mm, *SD*=0.0353 mm, 95% CI [0.334, 0.408]), *t*(21)=1.94, *p*=0.1642, or F3 mice (*n*=6; *M*=0.340 mm, *SD*=0.0283 mm, 95% CI [0.311, 0.370]), *t*(21)=0.07, *p*=0.9980.

An examination of the thickness of the pups' auditory cortex across the four conditions, however, provided evidence of differences among the four conditions, *F*(3,26)=6.34, *p*=0.0023 (Figure 1.6 C1-5). Planned comparisons between control mice and each filial generation showed that the auditory cortex of control mice (*n*=8; *M*=0.341 mm, *SD*=0.0375 mm, 95% CI [0.310, 0.372]) was thicker than that of F1 mice (*n*=8; *M*=0.266 mm, *SD*=0.0293 mm, 95% CI [0.242, 0.291]), *t*(26)=4.20, *p*=0.0008, and F3 mice (*n*=7; *M*=0.292 mm, *SD*=0.0358 mm, 95% CI [0.258, 0.325]), *t*(26)=2.69, *p*=0.0335, but was not significantly thicker than that of F2 mice (*n*=7; *M*=0.314 mm, *SD*=0.0393 mm, 95% CI [0.277, 0.350]), *t*(26)=1.49, *p*=0.3342. Thus, in the auditory cortex, there is evidence of cortical thinning in F1 and F3 mice relative to controls.

An examination of thickness of the pups' visual cortex across the four conditions failed to find overall evidence of differences, *F*(3,24)=2.22, *p*=0.1117 (Figure 1.6 D1-5). Nevertheless, planned comparisons between control mice and the three filial PrEE generations did show that the thickness of visual cortex of control mice (*n*=7; *M*=0.294 mm, *SD*=0.0195 mm, 95% CI [0.276, 0.312]) is greater than F1 mice (*n*=7; *M*=0.255 mm, *SD*=0.0281 mm, 95% CI [0.229, 0.281]), *t*(24)=2.55, *p*=0.0470 (Figure 6D1-5). Although the visual cortices in the two subsequent filial generations are numerically thinner, neither the difference between control mice and F2 mice (*n*=7; *M*=0.271 mm,

SD=0.0406 mm, 95% CI [0.234, 0.309]), $t(24)=1.49$, $p=0.3381$, nor F3 mice ($n=7$; *M*=0.277 mm, *SD*=0.0229 mm, 95% CI [0.256, 0.298]), $t(24)=1.09$, $p=0.5649$, reached significance. For visual cortex, thinning is again found in the F1 pups relative to controls.

Behavior

We evaluated social interactions between the mice from the four different conditions using the sociability test (Figure 1.7). Because this test uses repeated measures, the data were analyzed using a multilevel model in which condition (Control, F1, F2, F3) and side (empty vs. mouse) were fixed factors, and mouse was included as a random factor. The analysis identified no significant main effects but did identify an interaction between condition and side, $\chi^2(3)=11.646$, $p=.0087$. Planned contrasts examining the interaction effect between the control mice and each filial generation showed that compared to the control mice (*M*=−130.4s, 95% CI [−221.8, −39.0]), the F1 mice (*M*=76.0s, 95% CI [−29.5, 182.0]), $t(24)=3.051$, $p=.0152$, and the F2 mice (*M*=35.2s, 95% CI [−62.5, 133.0]), $t(24)=2.554$, $p=.0467$ spent more time in the empty side. The interactions for the control mice compared to the F3 mice (*M*=25.1s, 95% CI [−72.7, 123.0]), however, failed to reach significance, $t(24)=2.397$, $p=.0651$. Data from these behavioral studies suggest 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, brain weights, and neocortical lengths compared to controls. Neocortical thickness in F1, F2, and F3 newborn PrEE mice were impacted by the initial exposure. Notably, prelimbic, auditory, and visual cortical thicknesses were only reduced in F1 mice. Social behavior 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 have 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 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 gestational day (GD) 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. In order 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; Kozanian 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 is able to slow fetal growth (Andreu-Fernandez et al., 2019).

Smaller brains and short cortical lengths: 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 and cortical lengths 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.

Thinner cortex: Cortical thinning is a documented effect of FASD in humans (Zhou et al., 2011; Gautam et al., 2015). In the current study, we observed cortical thinning in directly exposed F1 PrEE in prelimbic, auditory, and visual cortex regions at P0. For transgenerational transfer, we only observed significant thinning in the auditory cortex of F3 PrEE mice. Delays in neurogenesis or increases in apoptosis have been suggested as possible mechanisms for cortical thinning in newborns directly exposed to ethanol via maternal consumption during pregnancy (Dunty et al., 2001; Green et al., 2007; Livy &

Elberger, 2008; Dong et al., 2010; Goodlett et al., 2014). Work published by our laboratory demonstrated altered cortical connectivity in F1, F2, and F3 PrEE mice at P0 (Abbott et al., 2018). Abnormal, ectopic neural connections may play a role in the cortical thinning observed at P0 (Abbott et al., 2018), although these circuit phenotypes seem to recover by P20 in all three generations (Bottom et al, 2022). Potential compensatory mechanisms, such as parental care may account for the recovery at P20. Our PrEE mice are always cross fostered so parental care is superior to what it would have been with the alcoholic mother. Environmental enrichment has also been successful in ameliorating issues in declarative memory in models of continuous alcohol drinking (Brancato et al., 2020). Future experimental models could vary the home cage environment to investigate whether changes in parental care or cage structure impacted the degree of recovery.

PrEE and behavioral development: transgenerational effects.

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 the 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 *RZR*β 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 the 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, this study is limited in that we did not investigate potential mechanisms that mediate the anatomical and behavioral phenotypes associated with PrEE. The examination of potential mechanisms is the focus of additional ongoing research in our laboratory, where we explore molecular and epigenetic candidate mechanisms that lead to the phenotypic variation observed in PrEE mice transgenerationally.

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

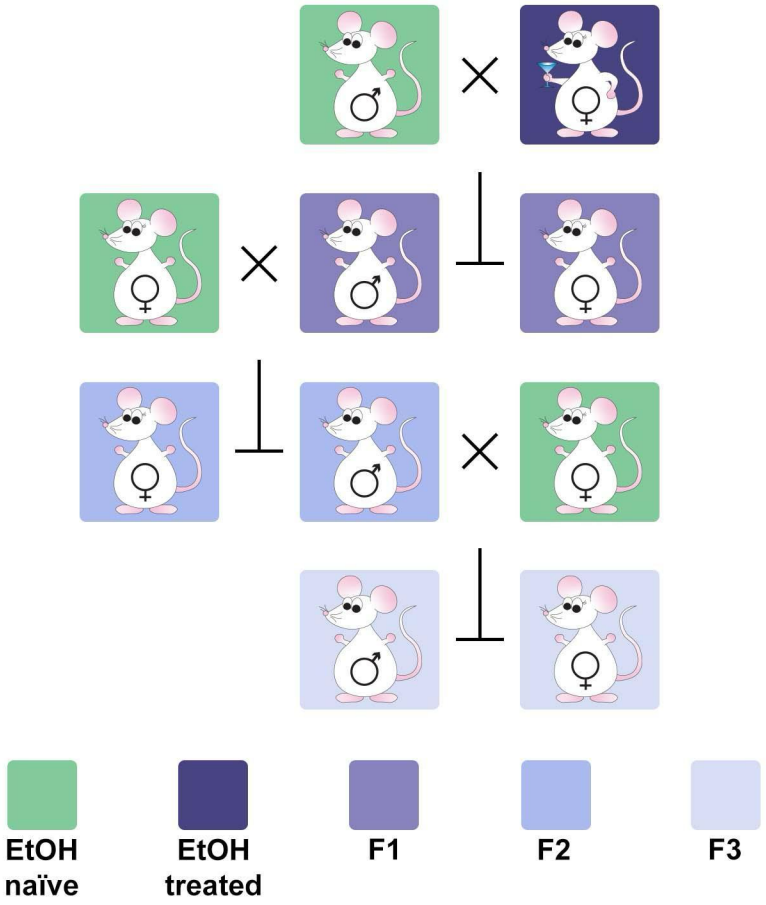

Figure 1.1 Breeding paradigm. Summarization of the breeding paradigm used to generate first, second, and third generation FASD mice. Alcohol exposure occurs during pregnancy of the first filial (F) generation mice with 25% (v/v) ethanol consumed *ad libitum*. Breeding of second and third generation mice is done by pairing up first and second generation male mice with control, ethanol naive, and females respectively.

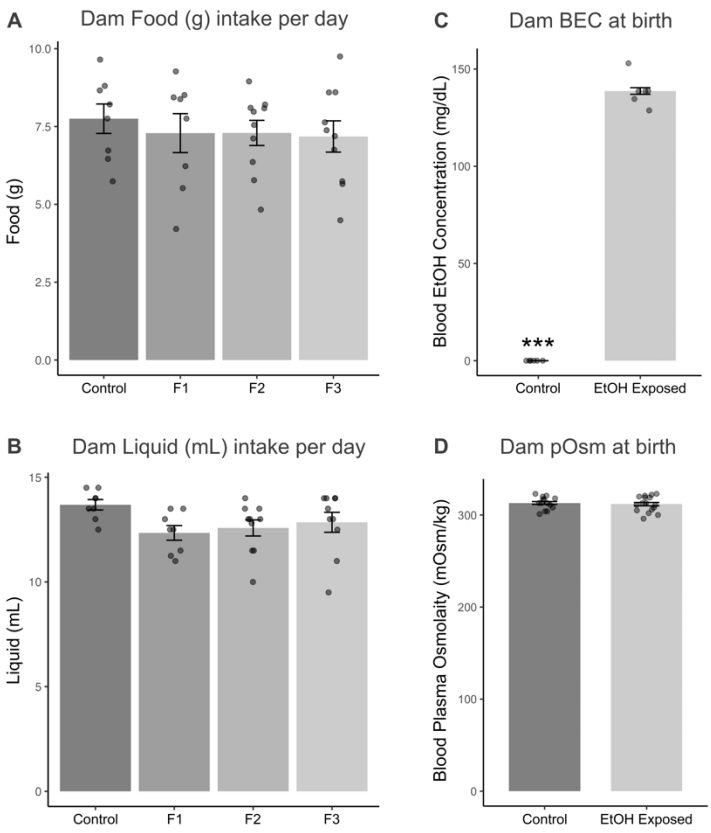

Figure 1.2 Dam metrics at birth. Evaluation of maternal metrics at birth of pups. A. No significant difference in food consumption between control, F1, F2, and F3 dams. B. No significant differences in liquid intake were observed between control, F1, F2, and F3 dams. C. Dam blood ethanol concentration (BEC) in mg/dL at birth. Elevated BEC levels were detected for ethanol exposed dams. D. Dam plasma osmolality (pOsm) in milliosmole per kilogram (mOsm/kg). No significant differences were observed between control and ethanol exposed dams. Data expressed as mean ± S.E.M. Dots have been jittered along the x-axis to show individual points and increase visibility. *** $p<0.001$

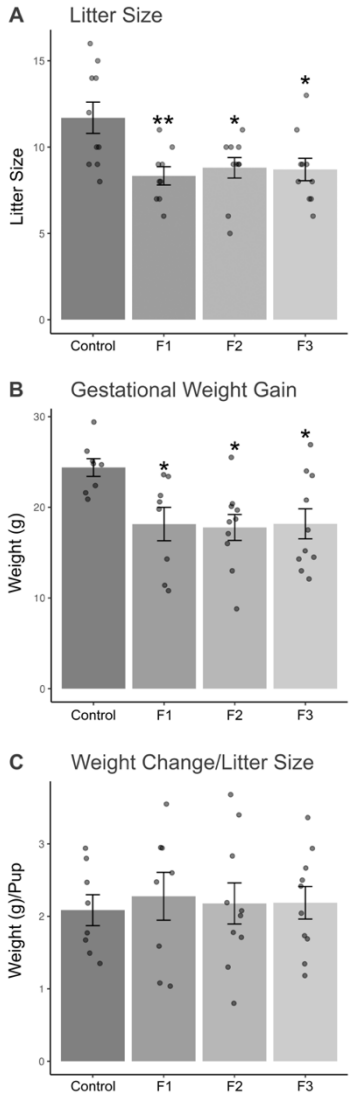

Figure 1.3. Gestational metrics. A. Litter sizes at birth for F1, F2, and F3 were significantly reduced compared to controls. B. Gestational weight gain was significantly reduced in F1, F2, and F3 dams compared to controls. C. No significant difference in weight change with respect to litter size for control, F1, F2, and F3 dams. Data expressed as mean ± S.E.M. Dots have been jittered along the x-axis to show individual points and increase visibility. * $p<0.05$ and ** $p<0.01$.

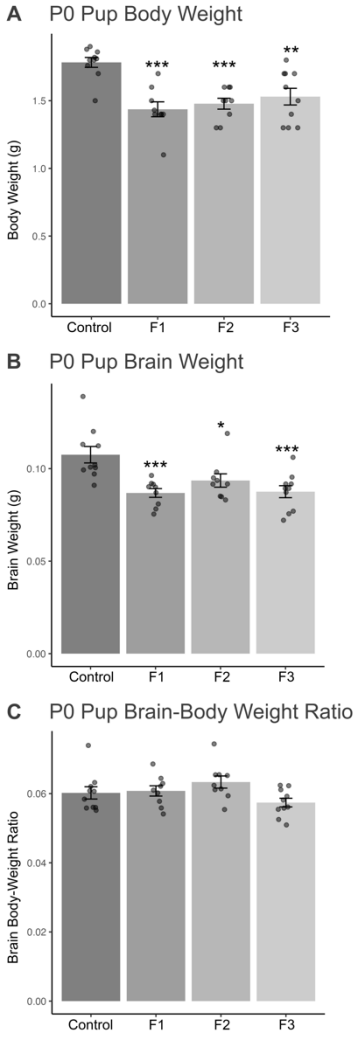

Figure 1.4. Pup metrics at birth. A. Significant decrease in F1, F2, and F3 body weights of offsprings at P0, compared to controls at P0. B. Significant reductions in F1, F2, and F3 brain weights compared to controls were observed at P0. C. P0 Brain Weight/Body Weight ratios were calculated for each experimental condition. No significant differences observed between F1, F2,and F3 ratios compared to controls. Data expressed as mean ± S.E.M. Dots have been jittered along the x-axis to show individual points and increase visibility. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

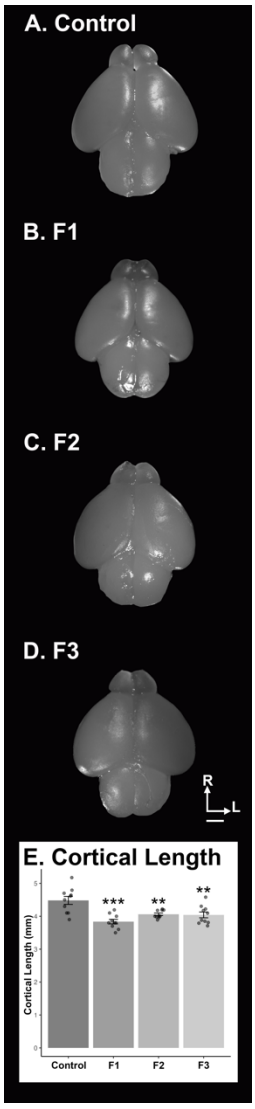

Figure 1.5. Representative dorsal views of mouse brains at P0. Representative dorsal images of control (A), F1 (B), F2 (C), and F3 (D) mouse brains. Notably, F1, F2, and F3 mice had significantly reduced cortical lengths at P0, compared to controls. E. Graphs showing the mean ± S.E.M. of each condition. Dots have been jittered along the x-axis to show individual points and increase visibility. Images oriented rostral (R) up and lateral (L) to the right. Scale bar, 2 mm. ** $p<0.05$ and *** $p<0.001$.

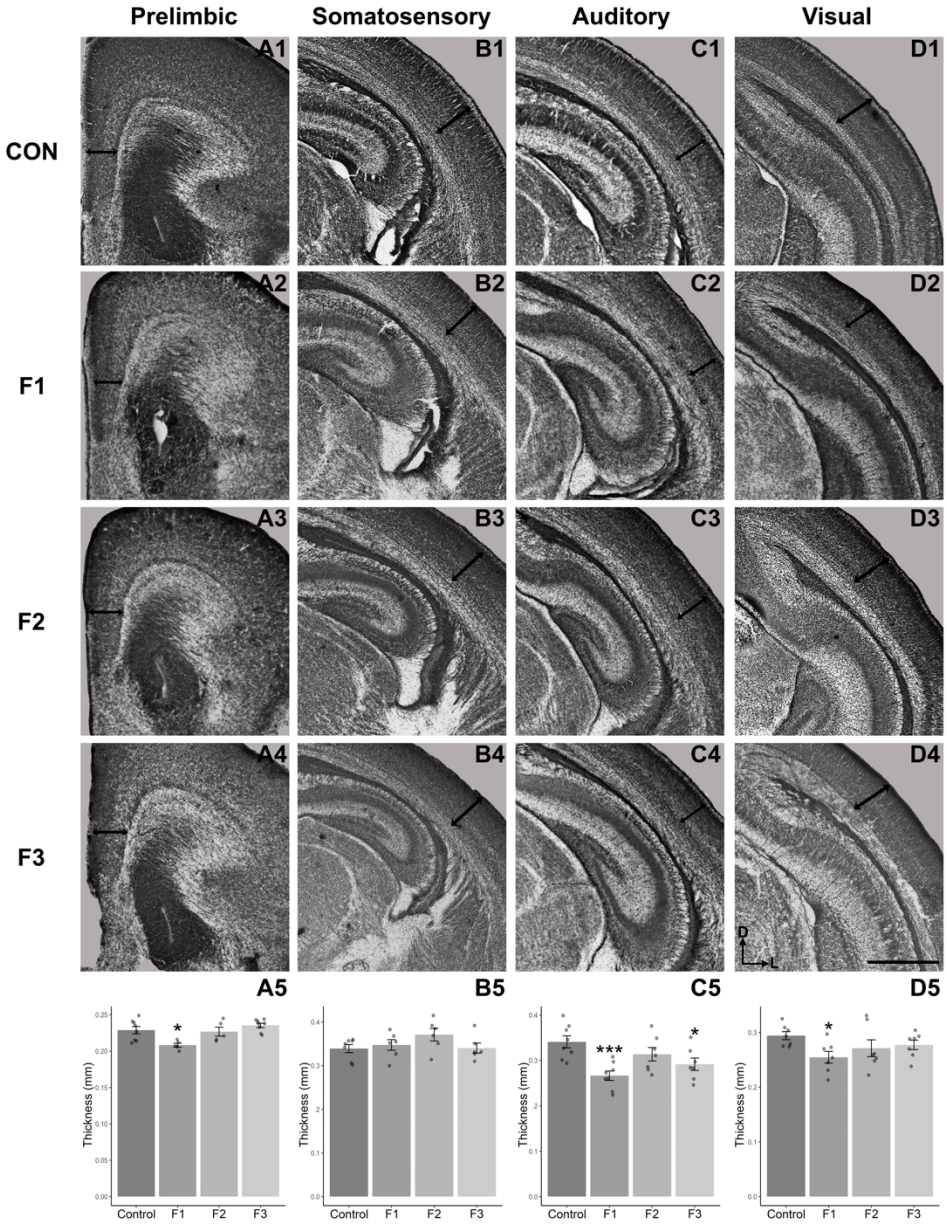

Figure 1.6. Cortical sheet thickness measurements at P0. Representative Nissl stained coronal sections of P0 mouse brain tissues for all experimental groups (Control, F1, F2, and F3). Cortical areas evaluated include prelimbic cortex (A1–A5), somatosensory cortex (B1–B5), auditory cortex (C1–C5), and visual cortex (D1–D5). Significant decreases in cortical thickness were observed for F1 mice in prelimbic, auditory, and visual cortices, compared to controls. Significant decreases in cortical thickness were observed for F3 mice in the auditory cortex, compared to controls. Data expressed as mean ± SEM. Dots have been jittered along the x-axis to show individual points and increase visibility. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar, 1 mm. * $p<0.05$ and *** $p<0.001$.

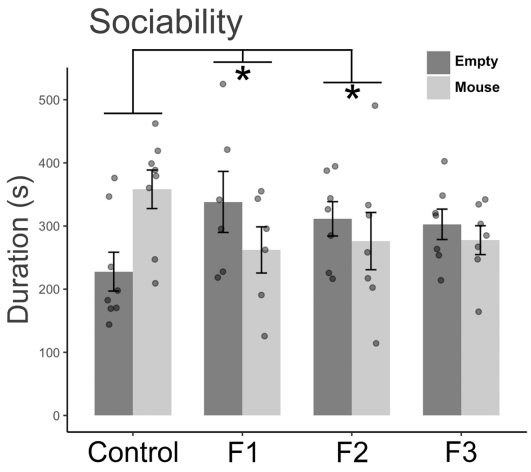

Figure 1.7. Social behavioral assessment at P20. Control mice spent significantly more time with the novel mouse than in the empty chamber during the ten-minute testing phase of the three-chambered sociability test. F1 and F2 mice did not spend more time with the novel mouse versus time spent in the empty chamber, suggesting lowered social interaction. However, F3 mice did not show any alterations in social interaction, compared to controls. Data expressed as mean ± S.E.M. Dots have been jittered along the x-axis to show individual points and increase visibility.* $p<0.05$.

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Chapter 2: Alcohol and Lactation: Developmental Deficits in a Mouse Model

ABSTRACT

It is well documented that prenatal ethanol exposure via maternal consumption of alcohol during pregnancy alters brain and behavioral development in offspring. Thus, the CDC advises against maternal alcohol consumption during pregnancy. However, little emphasis has been placed on educating new parents about alcohol consumption while breastfeeding. This is partly due to a paucity of research on lactational ethanol exposure effects in children; although, it has been shown that infants exposed to ethanol via breastmilk frequently present with reduced body mass, low verbal IQ scores, and altered sleeping patterns. As approximately 36% of breastfeeding mothers in the US consume alcohol, continued research in this area is critical. Our study employed a novel murine lactational ethanol exposure (LEE) model, where offspring were exposed to ethanol via nursing from postnatal day (P) 6 through P20, a period correlated with infancy in humans. Compared to controls, LEE mice had reduced body weights and neocortical lengths at P20 and P30. Brain weights were also reduced in both ages in males, and at P20 for females, however female brain weights recovered to control levels by P30. We investigated neocortical features and found that frontal cortex thickness was reduced in LEE males compared to controls. Analyses of dendritic spines in the prelimbic subdivision of medial prefrontal cortex revealed a trend of reduced densities in LEE mice. Results of behavioral tests suggest that LEE mice engage in higher risk-taking behavior, show abnormal stress regulation and exhibit increased hyperactivity. In summary, our data describe potential adverse brain and behavioral developmental

outcomes due to LEE. Thus, women should be advised to refrain from consuming alcohol during breastfeeding until additional research can better guide recommendations of safe maternal practices in early infancy.

INTRODUCTION

Alcohol is known as a developmental teratogen in mammalian systems. However, research in this area has primarily focused on exposures during the prenatal period. Maternal consumption of alcohol during pregnancy can result in Fetal Alcohol Spectrum Disorders (FASD) in offspring and children with FASD may exhibit physical, cognitive, emotional, and behavioral phenotypes related to the exposure (Hoyme et al., 2016; May et al., 2009, 2014). Thus, Centers for Disease Control (CDC) have released a statement that no amount of alcohol is safe to consume during pregnancy. Generally, these recommendations are followed, as demonstrated by a reduction in alcohol consumption during pregnancy. However, consumption levels approach preconception levels shortly after birth in some populations (Little, Lambert, and Worthington-Roberts, 1990; Giglia, 2006). The prevalence of breastfeeding mothers consuming alcohol is high, ranging from 20% in Canada (Popova et al., 2013), 36% in the United States (May et al., 2016), and 60% in Australia (Tay et al., 2017). For a specific example, in Seattle, Washington, 80% of women consumed alcohol during the month before conception, 40% consumed alcohol during the last trimester of pregnancy, and 70% were drinking 3 months postpartum. Notably, this study also reported that 10% of breastfeeding mothers reported drinking more than once a day (>15g alcohol) (Little, Lambert, and Worthington-Roberts, 1990).

Given the prevalence of maternal alcohol consumption during breastfeeding, it is important to understand how this can represent a teratogenic exposure for infants. Studies have shown that the levels of alcohol in the breast milk mirror the amount of alcohol in the blood (Lawton, 1985; Chien et al., 2005). These levels peak at 30-60 minutes after ethanol consumption and continue to be detected 2-3 hours after consumption (Chien et al., 2005; CDC, 2019). Although these levels are lower than the percentage in alcoholic beverages, they are non-zero values. In infants, exposure to breast milk containing alcohol may result in reduced body mass and verbal IQ scores (May et al., 2016). Congruently, exposure to alcohol via breast milk may result in a dose-dependent reduction of cognitive functions as seen when testing exposed children aged 6-7 years (Gibson and Porter, 2018) and dose-dependent reductions in children's academic abilities up to grade 5 (Gibson and Porter, 2020). Additionally, deficits in abstract reasoning skills are observed at age 7 in lactational-exposed children (Oei, 2019). Changes in sociability can also occur as exposed infants scored below, or within the monitoring zone, on the scale of the personal-social interactions at 12 months of age (Tay et al., 2017). Despite these potential negative effects of alcohol compromised breast milk on offspring development, there is a disconnect between conclusions drawn from scientific literature and behaviors in many new mothers.

In humans, there is variability in maternal behavior in terms of infant feeding preferences. In the US from 2012-2019, around 80% of mothers breastfed their infants, with just over half of them breastfeeding exclusively (from the National Immunization Survey, CDC, 2019). Additionally, there is variability among women in their ability to

metabolize alcohol and to respond to stressors, which can moderate infant exposure. Indeed, higher tolerance and stress may result in the increase of the consumption of alcohol, for certain populations (Guinle and Sinha, 2020). Women who consume alcohol during pregnancy are also more likely to drink while breastfeeding (May et al., 2016), suggesting certain populations may be considered high-risk for breast milk contamination. Additionally, unplanned, and drastic lifestyle changes may influence alcohol consumption levels. For example, the COVID-19 pandemic and subsequent "stay-at-home" orders, rapidly emerged as a public and/or personal health concern for many. In response to this novel stressor, women in the United States showed an increase in their Alcohol Use Disorders Identification Test scores during the Covid-19 "stay-at-home" order (Boschuetz et al., 2020). These results translate to an increase in frequency and quantity of alcohol ingested in those who already used alcohol; congruently, factors such as having children at home and a history of substance abuse were positively associated with an increase in alcohol use during the pandemic (Boschuetz et al., 2020). Similar results were observed in Australia (Bramness et al., 2021), Norway (Rossow et al., 2021), and Belgium (Vanderbruggen et al., 2020), and thus, the pandemic and "stay-at home" orders may have unintentionally increased infant alcohol exposure via increased maternal consumption. These studies show an increase in alcohol consumption in certain child rearing populations, elucidating the deleterious effects of postnatal ethanol exposure via breast milk, and bolster the importance of alcohol abstinence during breastfeeding. However, published postnatal alcohol exposure paradigms (via breast milk) tend to be uncontrolled, unstandardized and often limited to humans. Much of the existing data

leave questions of dosing, timing, and how the developing nervous system is affected by lactational ethanol exposure (LEE). Data from animal models are not always consistent, most likely due to the variability in postnatal ethanol exposure methods, ranging from direct ethanol exposure to combined prenatal and postnatal exposure. In one study, researchers exposed rat pups to ethanol via intragastric intubation from postnatal (P) day 4 to 8 and reported increased male body weights but no increases in cerebral cortex weight (Light et al., 1989). Another direct exposure study reported a reduction of stem cell progenitor cells in the hippocampus and reduced adult neurogenesis after a singular subcutaneous injection of alcohol at P7 (Ieraci and Herrera, 2007). A study from Vilaró and colleagues (1987) exposed rat pups to alcohol via an alcohol-treated mother and reported a reduction in weight of rat pups at age P15 compared to controls; however, this study exposed rats to ethanol during gestation as well as postnatally. These studies provide much-needed evidence towards the damaging effects of postnatal ethanol exposure; however, they do not target a particular time window in mammalian brain development. Hence, many of their results are contradictory. To combat this, an analogous age range for exposure must be established between mice and humans. To begin, the brain growth spurt (BGS) is a time window where the mammalian brain undergoes rapid growth (Dobbing and Sands, 1979). In humans this period ranges from the third trimester of pregnancy to about the first two years of life, peaking at the birth (Dobbing and Sands, 1979). In murine models, this period ranges from the first week postnatal to the third week, peaking around P7 (Dobbing and Sands, 1979). A study has shown that exposure to alcohol during the BGS induces deficits such as a reduction in

long-term cerebellar growth and altered rotarod performance in a rat model (Goodlett et al., 1991). However, this study used artificial-rearing procedures to directly expose pups to ethanol during the P4-P9 time window and was a binge model (Goodlett et al., 1991). Furthermore, ethanol exposure has been shown to cause alterations in synaptic pruning (Kyzar et al., 2016). In mice, synaptic pruning reaches its peak 2-3 weeks postnatal (Lewis, 2011), this is within the BGS, providing further evidence of sensitivity towards perturbations early in postnatal development. Clearly, additional research is needed to illuminate the specific details of risk including dose-dependencies and the interaction of developmental time and exposure. Here, we are specifically interested in how maternal drinking while breastfeeding impacts brain and behavioral development of offspring. The period of time we targeted is within the BGS but begins on a postnatal day roughly equivalent to the day of human birth, to better mimic the time when breastfeeding would begin in humans.

In the current study, we targeted early lactational ethanol exposure in our mouse model by estimating typical human birth in murine time. When making cross-species comparisons for developmental stage, the first postnatal week in mice relates to the third trimester in humans (Clancy et al., 2007). As our study did not aim to model human *prenatal* alcohol exposure, or FASD, we began our maternal dosing of ethanol at the end of the first week of murine life (evening of postnatal day 6). This way, offspring will have consumed alcohol via breastmilk by P7. Estimates of human day of birth (full term) is between 245-265 days post conception with the mouse equivalent between 7-9 days postnatal (Clancy et al., 2007, Jukic et al., 2013). Specifically, we exposed CD-1 pups to

breast milk contaminated with ethanol, via maternal consumption, at the end of the postnatal week until weaning. By mimicking human postpartum drinking behavior, our results revealed potential effects of LEE on offspring outcomes. We measured maternal blood ethanol content to assure exposure validity and blood osmolality to assess hydration. We analyzed several outcome measures in offspring to determine to what degree ethanol exposure via lactation altered key features of neuroanatomical development and whether these phenotypes were read out in behavior. As predicted, LEE resulted in abnormal brain and behavioral development.

MATERIALS AND METHODS

Animal Care

All breeding and experimental studies were conducted in accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside (UCR). CD-1 mice, initially purchased from Charles River Laboratories (Wilmington, MA/USA), were used for breeding. We chose to use the outbred CD-1 mouse strain in this lactational model because these mice show superior maternal care compared to inbred strains and because we had validated them as a model for prenatal ethanol exposure in our prior work (El Shawa et al., 2013). Mice were housed in animal facilities located at UCR that were kept at approximately 22°C on a 12-hour light/dark cycle. Mouse chow and water (for controls), or mouse chow and a 25% ethanol solution in water, were provided *ad libitum* to the dams according to the dosing schedule.

Breeding and Lactational Ethanol Exposure Paradigm

Adult female and male mice, aged P90-150, were paired just before the start of the dark cycle. Once a vaginal plug was detected, the male was removed from the cage. Throughout pregnancy, mouse chow and water were provided *ad libitum* to all dams. Dams were undisturbed through pregnancy and birth until the pups were 6 days old, when litter sizes were recorded (Fig. 2.1). During this time, we pseudo-randomly assigned each dam to the control or experimental group (Lactational Ethanol Exposed, LEE group). LEE dams had their water replaced with a 25% v/v ethanol in water solution throughout the exposure period from the evening of P6 to P20, while control dams remained on water. The liquid bottle tip was placed high in the cage so that developing pups could not reach it, thus, their only liquid intake was via dam breast. There were no alterations to the dam's food supply through the exposure period for any experimental condition. Measurements were taken daily for maternal liquid and food consumption during the exposure period for both conditions. At wean (P20), litter size was assessed, control and LEE pups were weighed and divided into two subsets. Subsets A and B had different sacrificial end dates of P20 and P30, respectively. Subset B control and LEE pups were weighed and subjected to no more than two behavioral assays. The division of the litters into subsets allowed us to evaluate the short and long-term effects of lactational ethanol exposure with an array of techniques. To avoid litter effects, we distributed pups from multiple litters for each assay tested.

Dam and Pup Blood Ethanol Concentration and Plasma Osmolality Measurements

To measure dam and pup blood ethanol concentration (BEC) and blood plasma osmolality (pOsm), a measure of hydration, animals from control and LEE groups were subjected to a whole blood collection protocol. Whole blood was collected at the time of weaning for dams and pups via cardiac puncture. After collection, blood was placed in an untreated 1.5mL centrifuge tube and allowed to clot for 30 minutes at room temperature. The entire sample was then centrifuged at 4,000 × g for 15 minutes at 4^{o}C to separate serum from whole blood. To determine BEC in control and LEE groups, an alcohol dehydrogenase (ADH) based enzymatic assay (Pointe Scientific, Canton, MI) was employed. In brief, ethanol, and nicotinamide adenine dinucleotide (NAD+) become catalyzed by ADH and this interaction causes the oxidation of ethanol to acetaldehyde and reduces NAD+ to NADH. The modified sample was read on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) at 340 nm. To determine pOsm, freshly extracted serum from control and LEE groups were subjected to testing using an osmometer.

Brain Tissue Preparation and Collection

Pups from all conditions were randomly assigned for gross anatomical studies. Mice were weighed then sacrificed using a lethal dose of sodium pentobarbital (100 mg/kg) administered via intraperitoneal injection. Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in PBS (PFA, pH: 7.4) for fixation. The skulls were post-fixed in a 4% PFA solution overnight, then the brains were extracted,

weighed, and imaged. Dorsal views of whole brains were imaged using a Zeiss (Oberkochen, Germany) Axio high-resolution (HRm) camera attached to a dissecting microscope. Extracted brains were stored in 4% PFA for later use.

Anatomical Measurements

Brain and body weights were assessed at P20 and P30 for both sexes and conditions. They were compared using statistical analyses and a brain/body weight ratio was computed to determine if any changes in brain or body weight were independent of one another. Typically, in normal development, brain and body size/weight are related. Larger animals within the same species tend to have larger brains. We calculated the ratio able to differentiate whether the exposure was causing a decrease in brain size alone, or whether decreases in brain size from our perturbation could be related to overall decrease in body size. Next, to measure cortical length of all brains, we used a digital micrometer in Image J (NIH, Bethesda, MD, USA), using the dorsal whole-brain images. To examine anatomical cortical areas, perfused brain tissues were hemisected and cryoprotected using a 30% Sucrose (w:v) in PBS solution. Tissue was then sectioned using a Leica cryostat at 40µm thick in the coronal plane, mounted on subbed slides, and stained for Nissl bodies using a 0.1% Cresyl Violet solution staining protocol then imaged using a Zeiss Axio Upright Imager microscope equipped with a Zeiss Axio HRm camera. To control for comparisons between groups, the Allen Mouse Brain Atlas (brain-map.org) and the Paxinos Developing Mouse Brain Atlas (Paxinos et al., 2007) were used to determine matching planes of section between groups (anatomical landmarks used:

corpus callosum, hippocampus, and subcortical structures). Once images were selected, regions of interest (ROIs) were measured using the ImageJ (NIH) electronic micrometer function by trained researchers blind to treatment conditions, as previously reported in (Abbott et al., 2016). In brief, cortical thickness was measured with respect to the cortical sheet, by drawing perpendicular lines from the most superficial region of layer I to the deepest region of layer VI. Cortical regions measured include the frontal cortex (the boundary of layer ⅔ of the secondary motor area to boundary of layer ⅔ of the orbital area), prelimbic cortex, primary somatosensory cortex (S1), primary auditory cortex (A1), and primary visual cortex (V1).

Dendritic Spine Density Measurements

P20 and P30 brains were hemisected and placed into a modified Golgi-Cox solution (Bayram-Weston et al., 2016; Zaquot and Kaindl, 2016) for 14 days in the dark at room temperature. Brains were then removed from the solution and placed in 30% sucrose in PBS for 2 days. Brains were then embedded in 5% agarose and sliced on a vibratome at 100µm and mounted on subbed slides. Slides were allowed to dry for 2-3 days before developing. Slides were dipped in distilled water for 10 minutes, then 20% ammonia for 10 minutes, then distilled water for 10 minutes, then 70%, 95%, and 100% ethanol (EtOH) for 5 mins each, and xylenes for 40 minutes. Slides were then immediately coverslipped with permount solution. Images of dendritic spines, of pyramidal cells in layer IV/V of the Prelimbic and Frontal cortices, were then imaged using a 630X oil immersion objective on a Leica Dmi8 bright field stereoscope using an

attached Leica DFC 450C camera. Dendritic spine density was calculated for the entire length of the dendrites using Image J by an experimenter blind to condition. Counted spines were then divided by the length of the dendrite measured, then an average of dendritic spines was taken for each mouse as multiple neurons were sampled from each individual subject. In depth dendritic spine staining methodology has been previously described elsewhere (Bottom et al., 2022).

Behavioral Assays

Due to higher than zero BEC levels in LEE pups at wean, behavioral assays were only performed at P30. Therefore the 10-day post wean period was considered a "wash out" period in the paradigm. Mice were subjected to a maximum of two behavioral tests during the testing period with the forced swim test (FST) always being last due to the high-stress nature of the test. All behavioral analyses and scoring were performed and analyzed by trained researchers blind to experimental conditions. All apparatuses were cleaned using Virkon before and after each testing session.

Elevated Plus Maze (EPM)

The Elevated Plus Maze has been historically employed to measure anxiety-like behaviors in rodents (Handley and Mithani, 1984; Rodgers and Dalvi, 1997). Notably, young CD-1 mice are known to contradict this measure of anxiety-like behaviors and they typically interact with the lower anxiety-associated metrics of this assay at higher portions; therefore, this behavior is thought to be considered risk-taking behavior (Macrì,

et al., 2002). This test has been used in our laboratory's prenatal ethanol exposure (PrEE) mouse model (Bottom et al., 2022). In a dimly lit room, we employed the use of a plus "+" shaped apparatus that is designed to provide test mice with two different arm environments (arm specifications; 54cm wide and 30cm long). The first arm type (closed arms) shields the mouse from the testing room using 15cm high non-transparent panels that laterally enclose the mouse, with an opening on top of the apparatus. This provides the mouse with a shaded semi-enclosed space. The second arm type (open arms) exposes the mouse to the testing room through omission of the non-transparent panels. These arm types are arranged adjacently to one another on the apparatus, such that each environment is flanked by the opposing environment. Additionally, the apparatus is lifted 50 cm above the ground using stilts. In sum, mice were subjected to a single five-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. The amount of time spent in each arm, as well as entries and total time was recorded. Video recordings were made of each testing session. A longer time spent in the open arms may indicate increased risk-taking behavior or active exploratory behavior.

Forced Swim Test (FST)

Designed to assess the effects of antidepressant drugs in the late 1970s (Porsolt et al., 1978), the forced swim test was originally used to measure depressive-like behaviors (Lucki et al., 2001). More recently, studies have re-evaluated the interpretation of the test. Mouse performance in the water (either actively swimming/attempting to climb or

floating immobile) has been viewed as a response to the stressful environment; the mice could respond with a passive coping style (immobility) or an active stress-coping style (swimming/climbing). The active stress coping has also been hypothesized to be related to hyperactivity (Commons et al., 2017; Conner at al., 2020; Armario, 2021). This technique has been used in our laboratory previously in our PrEE mice (Abbott et al., 2018; Conner et al., 2020; Bottom et al., 2022). Mice were placed in an acrylic glass cylinder (30 cm in height and 12 cm in diameter) filled to two-thirds total volume with room temperature (27°C) water for six minutes. The initial two minutes were an acclimation period and the remaining four minutes (240 seconds) were video-recorded and the time in which the animal was immobile in the water was recorded. Mice had light placed directly above them throughout the testing period and no more than two experimenters were allowed to be present during the testing period. Percentage of time spent immobile was calculated for each mouse.

Accelerated Rotarod

The accelerated rotarod (AR) test was used to examine motor ability, learning, grip strength, and coordination (Rustay et al., 2003, Buitrago et al., 2004). This test has been used in our laboratory's prenatal ethanol exposure (PrEE) mouse model (Abbott et al., 2018; Bottom et al., 2022). Briefly, the mice were subjected to four, five-minute trials on the rotarod apparatus with each trial separated by a ten-minute interval. The AR (Ugo Basile; Germonio/Italy) consists of a rod (diameter 28.5mm) that rotates and gradually increases speed from 4 RPM to 40 RPM. Mice are scored for the amount of time they are

able to stay balanced on the accelerated rotarod. If they are able to maintain balance for the entire trial length, they are given a perfect score of 300 seconds.

Statistical analyses

All statistical analyses were completed using R (v4.1.2; R Core team, 2021). Between-subjects tests were carried out using ANOVA with Type III sums of squares (via the car package, v3.0.12; Fox & Weisberg, 2019). 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 carried out using the emmeans R package (v1.7.2, Lenth, 2022).

RESULTS

Model verification: Blood Ethanol Concentration and Blood Plasma Osmolality in Dams and Pups

To ensure adequate maternal intake of ethanol, we measured blood ethanol concentrations (BEC) at wean. As expected, at wean, LEE dams had significantly greater BEC when compared to control dams, $t(4)=33.30$, $p<.001$ (Fig. 2.2A). Additionally, to assess maternal hydration during the ethanol self-administration period, we measured dam blood plasma osmolality (pOsm). No significant differences in pOsm were found between LEE and control dams at wean, $t(7.95)=1.66$, $p=.1366$, suggesting similar levels of hydration in dams across conditions (Fig. 2.2B). Ethanol treated dams showed lower caloric consumption and body weights when compared to control dams. From P6 through

P20, LEE dams consumed fewer calories from food and ethanol combined (M=75.1, SD=9.6, 95% CI [68.3, 82.0]) than control dams (from food alone; M=98.5, SD=11.6, 95% CI [87.8 109.2]), t(11.37)=4.39, p=.001. At wean, LEE dams (M=37.9 g, SD=4.4 g, 95% CI [35.5, 40.7]) weighed less than control dams (M=46.0 g, SD=4.7 g, 95% CI [42.7, 49.2]), t(12.53)=3.59, p=.003. In the case of the ethanol treated dams in the current study, they engaged in higher rates of infanticide and cannibalism (From P6 through P20, more of the LEE dams' pups died (M=5.2, SD=3.3, 95% CI [3.0, 7.4]) than control dams' (M=1.3, SD=1.4, 95% CI [0.2, 2.4]), t(14.08)=3.49, p=.004.), which would reduce their requirements to produce milk, and, to some degree, compensate for lower food intake.

LEE pups at wean, as anticipated, had greater BEC than control pups, t(6)=3.41, p<.014, although considerable variation was observed between individual measures (Fig. 2.2C). We endeavored to account for this variation by examining the relationship between both litter size and pups' sex on LEE pups' BEC at wean. Neither litter size (t(6)=0.34, p=.742) nor sex (t(6)=0.49, p=.642), however, was a significant predictor of BEC. Nevertheless, additional possible explanations for the increased variability are discussed in the section on study limitations and future directions.

There were no significant differences in blood plasma osmolality (pOsm) found between LEE and control pups at wean, t(8.38)=0.40, p=.700, also suggesting similar levels of hydration in pups across conditions (Fig. 2.2D). These results confirm that non-zero levels of EtOH intoxication occur in LEE dams and pups at wean. Furthermore, these results indicate no disparity in dam or pup pOsm due to the exposure paradigm.

P20 and P30 Pup Gross Measurements

To examine the ability of our exposure paradigm to produce gross alterations in pup central nervous system (CNS), and overall development, we evaluated body and brain weights, body-brain weight ratio (Fig. 2.3), and cortical length measurements (Figs. 2.4, 2.5) at P20/P30 and by sex.

A three-way, condition (Control vs. LEE) × age (P20 vs. P30) × sex ANOVA identified a three-way condition × age × sex interaction on pups' weight, $F(1,302)=4.22$, $p=.0409$ (Fig. 2.3A). In light of this three-way interaction, lower order interactions and main effects should be considered with caution. Nevertheless a two-way age × sex interaction was also present, $F(1,302)=31.02$, $p<.001$, as was a main effect of condition, $F(1,302)=58.45$, $p<.0001$, and age $F(1,302)=434.23$, $p<.0001$. To examine the three-way interaction, the two-way condition × age interactions were examined separately for male and females. For males, the two-way interaction was significant, $F(1,302)=12.36$, $p=.005$, indicating that the effect of condition was greater at P30 than at P20. For females, the two-way interaction failed to reach significance, $F(1,302)=0.22$, $p=.637$. Sidak corrected planned comparisons were carried out to examine the difference between the weight of the control and LEE pups at each combination of age and sex. These indicated that control pups weighed more in all four combinations: P20 male, $t(302)=7.343$, $p<.0001$, P30 male, $t(302)=10.602$, $p<.0001$, P20 female, $t(302)=7.646$, $p<.0001$, P30 female, $t(302)=6.296$, $p<.0001$.

Next, a three-way, condition × age × sex ANOVA identified main effects of condition, $F(1,62)=10.26$, $p=.002$, and age $F(1,62)=9.12$, $p=.004$ on the weight of pups'

brains (Fig. 2.3B). As described previously, Sidak-corrected planned-comparisons were carried out to examine differences between control and LEE pups weights at each combination of age and sex. Results indicated that control pups' brains weighed more in three of the four combinations: P20 male, $t(62)=4.24$, $p=.0003$, P30 male, $t(62)=2.72$, $p=.0332$, and P20 female, $t(62)=3.20$, $p=.0085$, but not P30 female, $t(62)=2.207$, $p=.1184$.

Lastly, to consider the relationship between body and brain weight, we examined the ratio of pups' brain to body weight via a three-way, condition × age × sex ANOVA. This analysis identified main effects of condition, $F(1,62)=128.75$, $p<.001$, and age $F(1,62)=243.95$, $p<.0011$ on the on the ratio of pups' brain to body weight (Fig. 2.3C). These main effects, however, should be considered in light of interactions between age and condition, $F(1,62)=61.82$, $p<.001$, and sex and condition, $F(1,62)=8.47$, $p=.005$. The age × condition interaction provided evidence that the effect of exposure to EtOH diminished between P20 ($M = 0.022$, 95% CI [0.018, 0.023]) and P30 ($M = 0.004$, 95% CI [0.002, 0.007]), and the sex × condition interaction provided evidence that the effect of exposure to EtOH was greater for male ($M = 0.016$, 95% CI [0.014, 0.019]) than female ($M = 0.009$, 95% CI [0.007, 0.012]) pups. As described previously, we carried out Sidak corrected planned comparisons to examine the difference between the ratio of pups' brain to body weight in the control and LEE pups at each combination of age and sex. These indicated that LEE pups' brain-body weight ratio was greater in three of the four combinations: P20 male, $t(62)=15.88$, $p<.001$, P30 male, $t(62)=5.03$, $p<.001$, and P20 female, $t(62)=11.35$, $p<.001$, but not P30 female, $t(62)=0.49$, p=.981.

To examine cortical length (photographs in Fig. 2.4 and graphs reflecting measurements in Fig. 2.5), we performed a three-way, condition × age × sex ANOVA that identified main effects of condition, $F(1,68)=5.52$, $p=.022$, and age $F(1,68)=12.69$, $p=.001$ on the length of pups' brains. As described previously, Sidak corrected planned comparisons were used to examine the difference between the weight of the control and LEE pups at each combination of age and sex. None of these comparisons indicated a significant difference between the lengths of control and LEE pups' brains: P20 male, $t(68)=1.00$, $p=.7860$, P30 male, $t(68)=2.27$, $p=.1015$, P20 female, $t(68)=2.35$, $p=.0843$, and P30 female, $t(68)=1.37$, $p=.5370$.

Together, these results suggest that our exposure paradigm produces long-lasting gross alterations in CNS and general development in the LEE pups.

P20 and P30 Pup Cortical Neuroanatomical Measurements

To assess the effects of the exposure paradigm on cortical thickness development, we measured from 5 distinct regions (frontal, prelimbic, somatosensory, auditory, and visual cortices) in Nissl-stained coronal sections in both LEE and control pups at both milestone dates (Figures 2.6 males and 2.7 females). We carried out a three-way condition × age × sex ANOVA on cortical thicknesses of pups' brains in each region. In these analyses, none of the main effects or interactions were significant although the main effect of age in the visual cortex trended toward greater thickness at age P30 ($M = 0.664 \pm 0.0173$; Figure 2.6 A5, B5, C5, D5, E5; Figure 2.7 A5, B5, C5, D5, E5) than at age P20 ($M = 0.590 \pm 0.0171$), $F(1, 33) = 3.22$, $p = 0.0820$ (Figure 2.6 A5, B5, C5, D5, E5; Figure

2.7 A5, B5, C5, D5, E5). The corresponding Sidak-corrected planned comparisons we carried out to examine the difference between the cortical thickness in the control and LEE pups at each combination of age and sex also failed to show significant differences (Figure 2.6 B5, C5, D5, E5; Figure 2.7 A5, B5, C5, D5, E5) with the exception of the frontal cortex in the P20 male pups, $t(34) = 2.94$, $p = 0.0235$ (Fig. 2.6 A5).

These results suggest that there were only modest alterations to frontal cortical thickness in the development of the LEE mice, most notably, frontal cortex in LEE P20 mice was reduced when compared to controls

Dendritic Spine Measurements

An analysis on dendritic spine density (spines/um) was employed to explore the impact of our exposure paradigm on spine density at both milestone dates via Golgi-Cox-stained coronal sections (Fig. 2.8 males and 2.9 females). Because we measured spinal density on multiple dendrites from individual mice, the data were analyzed using a multilevel model in which condition × age × sex were fixed factors and mouse was included as a random factor. In prelimbic cortex, this analysis indicated a main effect of sex on spinal density (male, $M = 0.662$ spines/μm ± 0.0305; female, $M = 0.719$ spines/μm ± 0.0304), $t(26.24) = 2.26$, p=.0326 (Figure 2.8 A5, B5; Figure 2.9 A5, B5). There was also a trend toward an effect of condition (control, $M = 0.708$ spines/μm ± 0.0307; LEE, $M = 0.673$ spines/μm ± 0.0302), $t(23.73) = 2.05$, p= 0.0517, and an interaction between sex and condition (male LEE – control, $M = 0.0308$ spines/μm ± 0.0610; female LEE – control, $M = –0.1011$ spines/μm ± 0.0607), $t(24.97) = 1.73$, p=0.0954 (Figure 2.8 A5,

B5; Figure 2.9 A5, B5). Sidak-corrected planned comparisons failed to show significant differences between the spinal densities of neurons in the prelimbic cortex of control and LEE pups for either male or female pups at either age. In frontal cortex, this analysis did not indicate any significant effects or interactions, nor did any of the planned comparisons show significant differences at any combination of sex and age. Overall, these results suggest a possible modest difference between the experimental group and controls moderated by sex in prelimbic cortex (Figure 2.8 B5; Figure 2.9 B5), but provided no evidence of differences in dendritic spine density in frontal cortex (Figure 2.8 A5; Figure 2.9 A5).

P30 Behavioral Analyses

To assess the impact of the exposure paradigm on behavioral development, we employed a number of behavioral tests to investigate potential differences. The included tests were: Elevated Plus Maze (EPM), Forced Swim Test (FST), and Accelerated Rotarod (AR).

The EPM provides a measure of anxiety-like and risk-taking behaviors. We investigated the risk-taking behaviors by recording the percent of time mice spent in the open arms of the maze (Fig. 2.10). A two-way, condition × sex ANOVA failed to identify a significant effect of condition or sex on the time pups spent in the open arms of the maze. There was, however, a trend toward LEE pups (23.0% ± 1.63) spending more time in open arms than control pups (17.2% ± 1.59), $F(1, 37) = 3.40$, $p=.0733$ (Fig. 2.10A). Sidak corrected planned comparisons were used to examine the difference between the

percent of time the control and LEE pups spent in open arms for male and female pups separately. These comparisons similarly failed to indicate significant differences (Fig. 2.10B). The results suggest that LEE mice may spend more time on the uncovered arms of the apparatus compared to controls, regardless of sex (Fig. 2.10A) suggesting the possibility of increased risk-taking behavior.

In the FST, immobility may be understood as a measure of passive coping behavior. A two-way, condition × sex ANOVA failed to identify a significant effect of condition or sex on the percent of time each mouse was immobile (Fig. 2.11A). We utilized Sidak corrected planned comparisons to examine the difference between the percent of time the control and LEE pups spent immobile for male and female pups separately. Here, it was found that male LEE pups spent less time immobile than male control pups, $t(30)=3.31$, $p=.0049$ (Fig. 2.11A). For female pups, however, the difference between the time spent immobile in the two groups was not significant, $t(30)=1.31$, $p=.3588$.

The AR test measures motor ability, balance, coordination, and learning through repeated measures. Because the AR task extends across four trials for each mouse, the data were analyzed using a multilevel model in which condition × sex × trial (1–4) were fixed factors, and mouse was included as a random factor. The analysis indicated a main effect of trial, $\chi 2(3)=104.67$, $p<0.001$. Planned polynomial contrasts showed significant linear ($t(114)=3.54$, $p=0.0006$) and quadratic ($t(114)=2.15$, $p=0.0338$) effects of trial as well as a three-way interaction between condition, sex, and the quadratic trial contrast ($t(114)=2.07$, $p=0.0405$). This interaction can be understood by considering the pattern of

the effect of condition across trials for males (trial 1 M=12.576, 95% CI [−71.04,96.17]; trial 2 M=3.48, 95% CI [–80.13, 87.09]; trial 3 M=35.05, 95% CI [−48.56,118.66]; trial 4 M=−29.40, 95% CI [–109.45, 50.65]) and for females (trial 1 M=75.76, 95% CI [−4.29, 155.81]; trial 2 M=−74.39, 95% CI [–154.44, 5.66]; trial 3 M=−31.77, 95% CI [–111.82, 48.28]; trial 4 M=−3.58, 95% CI [−87.19, 80.03]). Other main effects and interactions did not reach significance (Fig. 2.11B, 2.11C). Additional Sidak corrected planned comparisons between adjacent trials within each combination of sex and condition yielded significant differences between trials 1 and 2 for Control, $t(114)$=2.68, p=0.0417, and LEE, $t(114)$=2.97, p=.0191, females (Fig. 2.11C) and for LEE males, $t(114)$=6.61, p<.0001, (Fig. 2.11B).

Overall, these data suggest that our exposure paradigm generates behavioral aberrations at P30 including increased risk-taking behaviors in LEE mice regardless of sex as well as abnormal stress regulation, active stress-coping styles and/or hyperactivity in male LEE mice.

DISCUSSION

Fifty years ago, several physicians at the University of Washington Medical School studied a small group of children who exhibited a particular set of developmental delays. The commonality among the children was that they were all born to alcoholic mothers. This was the first of many studies that aimed to identify and understand the condition that would be later known as Fetal Alcohol Syndrome (FAS) (Jones et al.,1973). Our laboratory has studied the effects of prenatal ethanol exposure for over 10

years now and although we have gained insight on FAS, or its spectrum disorder, FASD, our work was limited to prenatal exposures. Unfortunately, maternal alcohol consumption may continue during pregnancy, or if the mother abstained from drinking while pregnant, it may begin in the early postnatal period. Many new mothers report that after 9 months of abstinence, they begin to drink again after the baby is born (Jagodzinski and Fleming, 2007). The advice by physicians for drinking alcohol while breastfeeding is quite variable, and this presents a possible health issue for infants of drinking mothers. In fact, the Centers for Disease Control warn against heavy drinking during breastfeeding, but suggest that 'moderate consumption of alcohol' is not harmful to offspring (CDC, 2022). Compared to research on prenatal alcohol exposure, studies examining the effects of maternal drinking during lactation are mostly limited to epidemiological reports with a paucity of papers in animal models where changes in the developing nervous system are investigated. Thus, we developed a novel postnatal alcohol exposure model in breastfeeding mice, using the murine strain utilized in our prenatal ethanol exposure (PrEE) studies. In this lactational ethanol exposure model (LEE), we demonstrate that maternal consumption of alcohol while breastfeeding can induce gross developmental deficits in LEE pups including decreased body weights, brain weights, and cortical lengths. Additionally, we discovered some sex-specific, LEE-related phenotypes in the neuroanatomy of the frontal lobe and prelimbic cortex, as well as behavioral deficits in stress-coping styles and risk-taking behaviors in LEE offspring. Our findings that postnatal, indirect ethanol exposure (as modeled by our lactational experimental

paradigm) can negatively impact various aspects of development represents an important advancement in solidifying the significance of conscientious, informed parental care.

A novel murine lactational ethanol exposure (LEE) model: Impact of LEE on Gross Anatomical Changes in offspring

Our results suggest that ethanol exposure via lactation is correlated with reduced body weights in both males and females at P20 and 30. These findings are consistent with human studies where children exposed to ethanol through contaminated breast milk can have consistently lower body weights and growth trajectories (May et al., 2016). Although there is a paucity of rodent data on offspring outcomes after ethanol exposure via lactation, a study from Vilaró and colleagues (1987) reported a reduction in body weight of ethanol-exposed rats after a period of maternal ethanol consumption while nursing her pups. In terms of brain size and morphology, we find some sex-specific effects of LEE in our model. Specifically, while LEE males show sustained low brain weights compared to controls at P20 and P30, LEE females only show deficits in brain weights at P20, with recovery to control weights by P30. Thus, the females show recovery at a faster rate than males.

Few rodent models have examined brain weight changes in LEE mice; however, one study reported a decrease in weights of the forebrain, cerebellum, and brainstem in alcohol treated pups (Chen, et al., 1998). When examining prenatal ethanol exposure (PrEE) paradigms, sustained reductions in body weight and brain weights are observed from P0 to P50 in mice, consistent with findings in LEE offspring (Abbott et al., 2016;

2018). This suggests that LEE and PrEE may impact brain and body growth through similar mechanisms.

Considering the sustained growth retardation in PrEE and LEE mice, the reduction of body and brain weights might be due to the gut's inability to efficiently extract nutrients when alcohol is ingested. Acute and chronic ethanol administration results in a reduction of protein synthesis in the small intestine (Rajendram and Preedy, 2005) and can block absorption of micro- and macronutrients (Seitz and Homann, 2001; Seitz and Suter, 2002). Additionally, nutrient deficiency has the potential to manifest in epigenetic changes, as seen in the populations affected by the Dutch Hunger Winter (Dutch Famine) (Heijmans et al., 2008). We found that, in our PrEE model, epigenetic modifications occurred via changes in DNA methylation, which led to epigenetic and heritable phenotypes spanning three generations of mice (Abbott et al, 2018). It is possible that examination of epigenetic markers in LEE mice could provide further insight into mechanisms underlying LEE-induced phenotypes.

Impact of LEE on Cortical Length

In mammals, much of our sophisticated behavior, including language, sociability, decision making, and even fine motor skills and coordination, originates with complex functions of cells within the neocortex. In FASD or other alcohol-induced conditions, the abnormal phenotypes in humans are often related to presumed dysfunction within the neocortex (El Shawa et al., 2013). Thus, we chose to focus our study of the novel LEE model on development of the neocortex and the behaviors that are mediated, to some

extent, by its function. To begin, we measured cortical length at both P20 and P30 ages in male and female LEE and control mice. We found that while the cortex expanded in length significantly from P20 to P30 in all mice, LEE cortices remained consistently smaller, regardless of sex. Few rodent models have examined the impact of LEE on cortical development and, to our knowledge, there are no studies that specifically measure cortical length after LEE. Similarly, studies from our laboratory demonstrated a reduction in cortical length in PrEE mice (El Shawa et al., 2013, Abbott et al., 2018). As the cortex continues to grow and develop from birth to puberty in mice, we posit here that alcohol exposure via lactation may lead to apoptosis, increased oxidative stress, and interference with the activity of growth factors as is suggested for prenatal exposures (Goodlett and Horn, 2001).

Neocortical Thickness

In mice, neocortical lamination is present by around P5, when barrels become apparent in later IV of the somatosensory cortex. According to a comprehensive set of papers from our laboratory, the areal patterning period ends around this time, P5-6, when cortical areas have adult-like connections and lamination. Beyond P6, cortical thickness continues to increase, although the changes are minimal (Dye et al., 2011A; 2011B). Here, we measured cortical thickness across several neocortical sensory and motor regions at P20 and P30 in LEE and control mice. Given that the frontal cortex develops later than other cortical regions, and that the time of exposure is after the areal patterning period closes, it is not surprising that the only LEE-related phenotype we found was a

reduction in cortical thickness in the frontal cortex of P20 LEE males. This phenotype was recovered by P30 in the LEE male mice. Subsequent measurements in prelimbic, somatosensory, auditory, and visual cortices, at both milestone dates, produced no observable differences. Few rodent models have examined the effects of LEE on neocortex, and to our knowledge there are no studies that examine cortical thickness changes after LEE. There are, however, reports of alcohol-induced changes in cortical thickness measures after prenatal ethanol exposure. Our laboratory demonstrated changes spanning from birth to P50 in cortical thickness measures in the brains of PrEE mice (Abbott et al., 2016). PrEE models impact cortical thickness at a higher extent due to exposure during gestation, as this is the primary time when the cortex develops layer-specific organization of cell types and matures from a simply organized, single layer to a complex 6-layered structure. As the lactational exposure occurs after cortical areas subdivision and lamination, the exposure timing may be too late in development to induce significant changes in neocortical thickness.

LEE and Dendritic Spine Densities in Frontal Cortex

Through Golgi-Cox staining we aimed to evaluate the impact of LEE on dendritic spine densities, as ethanol exposure has the potential to alter synaptogenesis (Adams et al., 2022) and synaptic pruning (Kyzar et al., 2015; 2016). In typically developing mice, cortex wide synaptic pruning has been reported to reach its peak 14-21 days postnatal (Lewis, 2011). In early alcohol exposure models, acute exposures led to increased dendritic pruning in the prefrontal cortex, resulting in significant synapse loss (Socodato

et al., 2020). Also, acute ethanol exposure during synaptogenesis (from P5-P7) led to drastically decreased spine densities in the caudate/putamen, however these densities recovered to normal levels by around P30 (Clabough et al., 2022). Here, we exposed mice to ethanol via lactation within this postnatal sensitive period and conducted intensive spine counts in frontal lobe regions of interest in male and female mice, aged P20 and P30. While we did not find any significant changes in our measured frontal cortex spine densities, we did find a trend towards significance for prelimbic cortex (a subregion of the medial prefrontal cortex) between LEE and control mice. There were no age- or sex-dependent effects observed, but the overall reduction in spine densities observed in the prelimbic cortex of LEE mice could impact later development, and this could be possibly caused by ethanol-induced impairment to synaptogenesis or to increased synaptic pruning as the insult take places during a sensitive period for both. Of note, whether spine densities in the prelimbic cortex decrease or increase is age dependent (Galaj et al. 2020); however, alterations due to alcohol exposure have been associated with altered behavior regardless of the direction of change (Fox et al., 2020). This is not surprising given that the prelimbic cortex is a region shown to play a role in alcohol-drinking reinforcement (Engleman et al., 2020). These data are consistent with other brain areas (basal ganglia) where reductions in spine densities observed immediately after exposure seemed to reverse by one month of age (Clabough et al., 2022). It is possible that alterations occurred in synaptogenesis and/or pruning earlier in the exposure period and recovered by weaning when the first measures were taken.

Impact of LEE on Behavioral Development

While it is important to uncover changes in the developing nervous system that are associated with ethanol exposure through lactation, understanding the potential behavioral effects of the postnatal exposure is critical. In our current study we implemented a battery of behavioral assays to examine LEE's effect on behavioral development. The elevated plus maze is a classic way to measure anxiety in rodents (Walf and Frye, 2007). However, researchers have also looked beyond the initial interpretation of the EPM and created alternative hypotheses about how time spent in open arms versus closed arms can be interpreted. Most importantly, if an animal spends more time in the open arm, it may indicate increased risk taking or increased exploratory behavior (Macrì, et al., 2002, Kozanian et al, 2018). Also, as alcohol exposure impacts fear memory learning, affecting an animal's ability to learn a natural fear response, increased time in open arms could be from inhibited fear learning, as was observed in our PrEE model (Kozanian et al., 2018). Here, we found that, overall, LEE mice spent a significantly longer time in open arms when compared to control mice, without sex-specific effects. This suggests that exposure to ethanol via lactation may increase risk taking or exploratory behavior. This is consistent with exposure to ethanol via lactation in humans, as May and colleagues (2016) found that LEE children exhibited phenotypic variability consistent with FASD, with increased risk taking and cognitive deficits often present in children with FASD (Fast and Conry, 2009).

A hallmark of FASD and alcoholism is depression (Kuria et al., 2012; Pei et al., 2011) and the forced swim test is a classic test used to detect depressive-like behaviors in

animal models (Lucki et al., 2001). Like the EPM, behavioral results associated with the FST have been interpreted differently over time in the literature. Specifically, the FST test has been a successful method used to test for the effects of antidepressant drugs in that they increase the animal's activity in the swim well (Porsolt et al., 1978). Researchers who use the test for other model systems have identified that time immobile may represent a more complex measure than simple depressive behaviors. How the animal responds to being in the swim well, with floating (immobility) or active swimming/climbing can be viewed as different adaptive reactions to the stressful environment. For example, Armario (2021) determined that mice react according to their coping style, either passively or actively, and that the FST may be a more accurate measure of coping style rather than behavioral despair. This may also be correlated with hyperactivity or possibly response to fearful stimuli. Here, we found that LEE males demonstrated reduced time immobile when compared to control males in this task, with the effect not observed in female LEE mice. This indicates that LEE may cause abnormal stress regulation and hyperactivity in males, consistent with findings in humans with FASD (Hellemans et al., 2016). For example, alcohol compromised breast milk has been found to have an activating effect in humans, as behavioral states of infants showed increased variability, such as spending less time in quiet sleep and increased crying (Schuetze et al., 2002). It is also possible that increased time spent immobile during the FST for male LEE mice could indicate alteration in fear responsivity, as we showed abnormal fear learning in our FASD model mice (Kozanian et al., 2018). This behavioral phenotype may be related to reduced frontal lobe thickness in males (Fig. 2.6), as the

frontal cortex is likely to be involved in depression (Zhang et al., 2018) and fear responsivity (Gilmartin et al., 2014).

The accelerated rotarod (AR) apparatus is used to test motor function, balance, coordination as well as learning through repeated presentation of trials. We have found previously that rotarod performance is altered in PrEE mice. Specifically, first generation PrEE mice showed deficits in performance in the first two trials compared to controls at both P20 and P30 (Abbott et al., 2018; Bottom et al, 2022). Additionally, postnatal alcohol exposure in rats can impact accelerated rotarod performance (Goodlett et al., 1991; Cebolla et al., 2009). In our LEE model, male LEE mice showed increased variability in performance in trials 1-2. Specifically, the change in performance was appreciably different from controls: the LEE mice performed worse on trial 1 but showed a significantly greater degree of improvement between trials 1 and 2. After training, LEE mice performed similar to controls on the AR. In summary, male LEE mice show a greater deficit in trial 1 and showed an abrupt learning profile that differs significantly from both controls and female LEE mice.

Collectively, our results from our behavioral studies suggest lactational ethanol exposure may impact offspring in ways similar to prenatal exposures, with increased risk-taking, hyperactivity, active stress-coping responses to environmental stressors, and transient deficits in motor coordination. Additionally, some of these LEE-induced deficits may be sex-specific.

Critical periods, pubescence and plasticity.

Developmental critical periods are described as times when systems are "plastic" or open to change from environmental experience, such as with learning, or insult, such as with early alcohol exposure. For brain development, these are precise time points where neuronal plasticity is heightened and cortical circuits are particularly susceptible to regulation by specific sensory modalities (Jeanmonod et al., 1981). Initial explorational work in somatosensory cortical reorganization found that the removal of mouse vibrissae at birth resulted in an absence of the associated barrels (Van der Loos and Woolsey, 1973). Since then, studies have refined these events and have assigned a critical period range (first week of life in mice) for proper barrel formation (Lo et al., 2017). Additionally, the critical period for the visual system has been extensively studied. A literature review from Hooks and Chen (2007), places the critical period prior to eye opening in mice, at P0-P10. Perturbations in this period may alter cortical retinotopic maps (Hooks and Chen, 2007) along with gene expression and intraneocortical connections (Dye et al., 2012). How perturbations, insults, or changes in input impact a developing animal depends on the critical period for development in the relevant system. If events occur after closure of a critical period, the animal may be protected from detrimental harm. Unfortunately, if these events occur outside the critical period, the ability of the brain to repair itself with plasticity mechanisms may also be reduced. Understanding critical periods when comparing the impact of prenatal versus postnatal alcohol exposure, on the developing nervous system, is critical.

Compared to the effects of prenatal alcohol exposure in our mouse model of FASD, lactational ethanol exposure has more mild phenotypes associated with the exposure, although the changes we observed in our LEE mice could have debilitating consequences if mimicked in human systems. The difference in severity of outcomes between PrEE and LEE is possibly related to critical periods for development. As described previously, much of cortical development (lamination, arealization) in the mouse reaches an adult-like state by the first postnatal week, whereas during the prenatal period and the first few days of life, the developing brain is very susceptible to change. Thus, LEE animals may be somewhat protected, when compared to PrEE, from the more severe effects of the alcohol exposure because the key elements of cortical development, particularly those regulated by gene expression, such as the development of the intricate neuronal circuitry, are near complete.

Interestingly, there are sex differences revealed in our data. Specifically, we found that LEE females recovered brain and body weights more quickly when compared to LEE males, and that frontal cortex phenotypes and atypical behavior on the FST were observed only in LEE males. Also, LEE male rotarod performance demonstrated an abrupt learning pattern that was markedly different.from controls and LEE females. One hypothesis as to why LEE females fare better, when compared to LEE males, related to differences in puberty onset compared to the timing of exposure and dependent measures. Typical onset of puberty for wildtype mice begins around P28 in males, and P25 for females (Ismail et al., 2011; Molenhuis et al., 2014). Alcohol exposure prior to this period may impact the milieu of hormones that regulate onset of puberty. For example, a

gradual increase of Gonadotropin Releasing Hormone (GnRH) is responsible for the typical onset of puberty; its expression is diminished in the presence of alcohol, resulting in a pubertal onset delay (Srivastava et al., 2014; Dees et al., 2017). Therefore, our model can potentially delay puberty onset in LEE mice. Considering that female mice go through puberty earlier than males, it is not surprising that LEE has a greater impact on male behavior at P30.

Study limitation and future directions

With this study, we attempted to model offspring exposure, naturally, via maternal consumption of alcohol during lactation and active breastfeeding in an outbred mouse strain. With this model comes limitations. For example, outbred mice have inherent variability, unlike inbred strains where genetics are controlled. However, inbred mice, such as C57BL/6 are less hardy than CD-1 mice and tend to provide inferior maternal care to their offspring. Additionally, the self-administration design of this experiment leads to variation in maternal ethanol consumption as well as milk production and composition. These factors could play influential roles in offspring outcome in addition to the impact that ethanol provides.

Another limitation is the variability in pup blood ethanol concentration we observed in our data. Although the variability in dam BEC was small, we believe there were several factors besides maternal ethanol levels that influenced pup BEC. The LEE pups were small at P20 and obtaining blood samples in a great enough volume for the assays was difficult. This resulted in a lower sample size. Also, by P20, some pups had

begun eating chow in addition to nursing, possibly reducing ethanol intake and time from the last nursing event was variable from pups selected for analysis. Mice metabolize ethanol quickly, so increased variability in measured BEC is expected when time since the last dose is unknown. Additionally, competition for breastmilk access can result in variability among pups. Also, timing of maternal alcohol consumption relative to the period of nursing that preceded the pup sampling could also introduce variability. Despite the observed variability in pup BEC, the blood ethanol concentrations were non-zero in all LEE pups and the level was significantly higher than controls in all LEE cases.

Future studies could include shorter time periods of exposure, as human mothers sometimes breastfeed for abbreviated periods of time post-partum. Also, additional studies of gene expression analyses in the frontal cortex as well as intraneocortical connectivity would be warranted and behavior tests of fear conditioning and learning as we observed phenotypes in these domains in our prenatal ethanol exposure models. Finally, additional behavioral assays including tests to better assess hyperactivity, such as open field and assays that can detect cognitive deficits such as Morris water maze or radial arm maze.

CONCLUSION

A preponderance of evidence from researchers studying prenatal alcohol exposure and FASD led the CDC to correct its stance on drinking in pregnancy. They now clearly state “There is no known safe amount of alcohol use during pregnancy or while trying to get pregnant” (CDC, 2022). To date, the CDC has not made a similar statement regarding

drinking while breastfeeding, despite research demonstrating high frequency of maternal alcohol consumption while nursing (Lange et al., 2016; Giglia et al., 2008; Giglia, 2010; Parackal et al., 2007; Backstrand et al., 2004). In their review, May and colleagues (2016) make a compelling argument that alcohol consumption during pregnancy can result in poor childhood outcomes. Our data from our novel LEE model supports this notion, as our lactational ethanol exposure model demonstrates similar phenotypes as our prenatal ethanol exposure model; therefore, abstaining from alcohol consumption during BOTH the prenatal period and while breastfeeding is the safest option. Although the effects of LEE are mild compared to PrEE, most likely due to exposure outside critical periods for typical development, offspring exposure to ethanol via breast milk can have deleterious effects on developing brain and behavior and should be avoided.

FIGURES

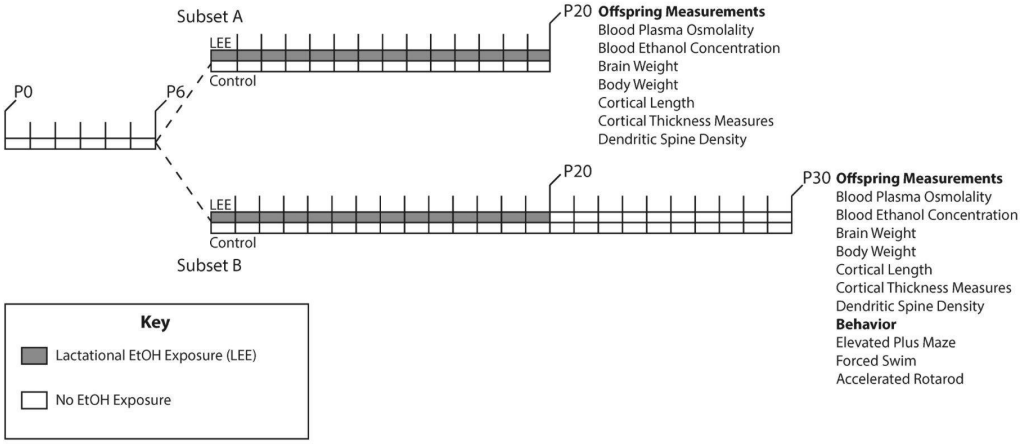

Figure 2.1 ***Experimental paradigm***. Mice were designated as control or LEE at P6. LEE dams received 25% EtOH when pups were P6-P20. At P20 pups were weaned, divided into two subsets, and no longer exposed to EtOH. Subset A was subjected to a variety of measurements at P20. Subset B was subjected to measurements as well as behavioral tests at P30.

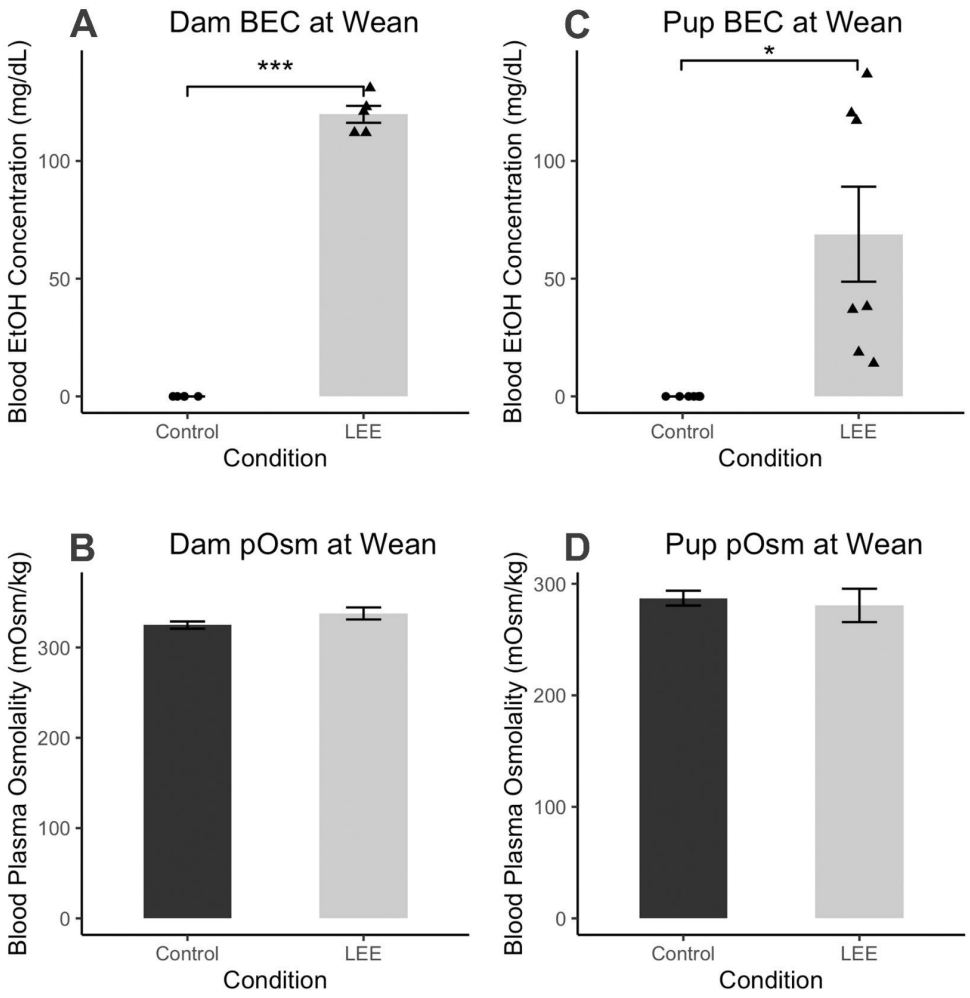

Figure 2.2 ***BEC and pOsm measurements.*** A. BEC measurements in Control and LEE dams at wean after a 14 day exposure to water (control) or 25% EtOH. LEE mice exposed to 25% EtOH had an average BEC of 119.8 mg/dl compared to controls which had a BEC of 0 mg/dl. (N = 10) B. No significant differences observed between control (M= 324.8 mOsm/kg, SD = 8.8 mOsm/kg) and LEE (M = 337.7 mOsm/kg, SD = 16.3 mOsm/kg) dam plasma osmolality (pOsm) at wean. (N=11) C. BEC measurements in Control and LEE pups at wean after dams were exposed to water or 25% EtOH for 15 days. LEE pups had greater BECs (68.9mg/dl on average) compared to controls at 0.0 mg/dl. (N=14). D. No significant differences in pup pOsm at wean between control (M = 287.1 mOsm/kg, SD = 20.0 mOsm/kg) and LEE (M = 280.6 mOsm/kg) offspring. (N=16; *p<0.05, ***p<0.001). A,B, triangles represent individual data points taken for each experimental condition. Data expressed as mean ± S.E.M.

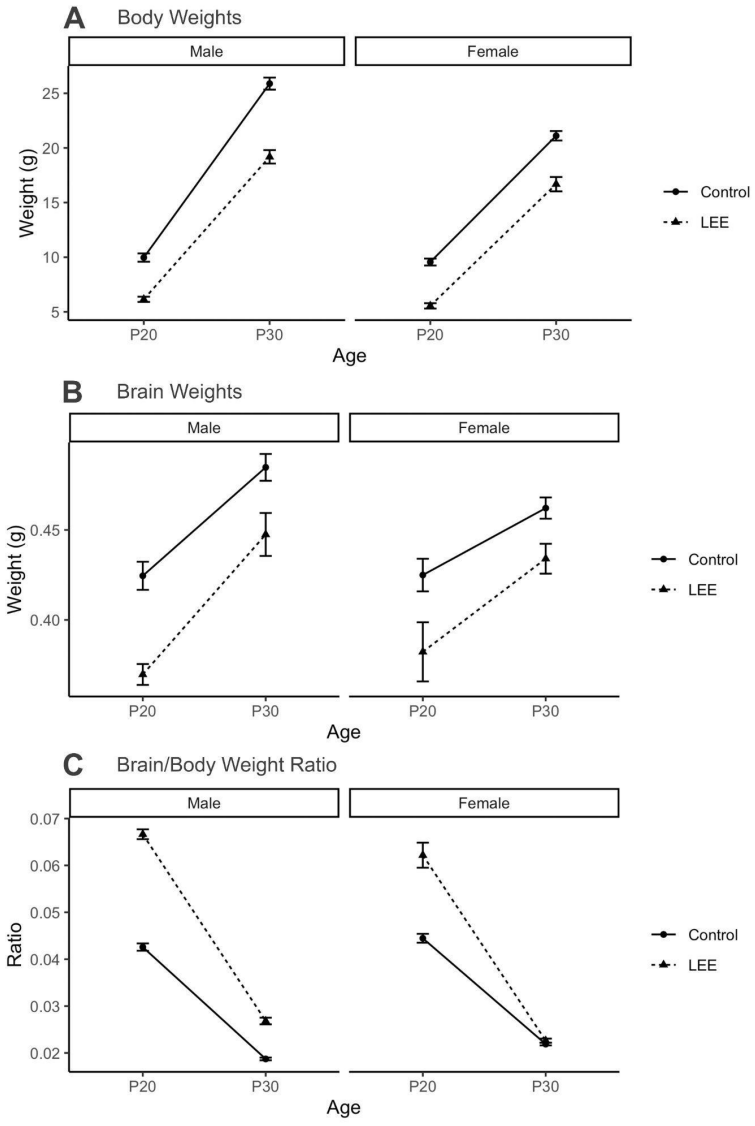

Figure 2.3 ***Offspring Measures: Gross Development.*** A. A significant ($p<0.001$) reduction in body weight for LEE pups was observed in every age and sex group, when compared to controls. (N = 310) B. Significant reductions in brain weights were observed for LEE males at P20 ($p=0.003$) and P30 ($p=0.0332$) developmental time points. However, significant reductions were only observed in P20 LEE females ($p=0.0085$) and no significance is observed in P30 LEE females ($p=.1184$) compared to controls. (N = 70) C. Significant increases to the brain/body ratio are observed in LEE males at both developmental time points. Significant increases to the brain/body ratio were only observed in P20 LEE females and not P30 females as compared to controls. (N = 70) Data expressed as mean ± S.E.M.

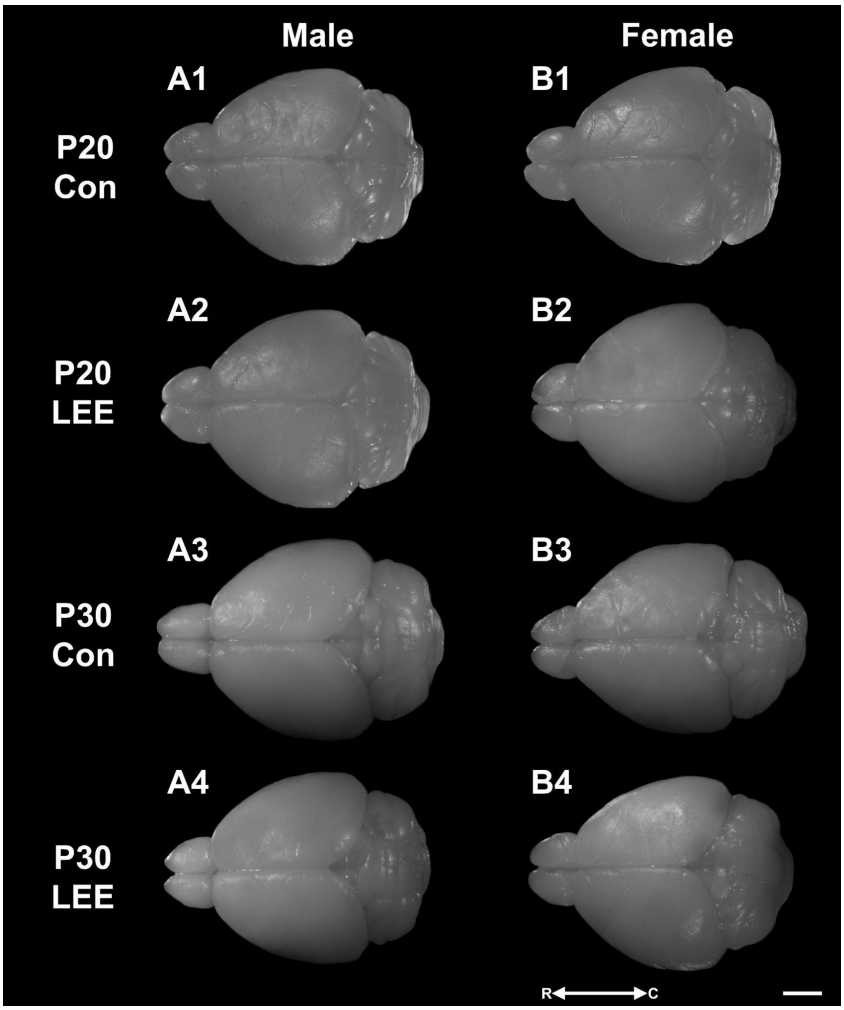

Figure 2.4 ***Dorsal views.*** Representative images of perfused and extracted brains of male (A1-A4) and female (B1-B4) pups at P20 (A1,A2,B1,B2) and P30 (A3,A4,B3,B4) after dams were exposed to water (A1, B1, A3, B3) or EtOH (A2, B2, A4, B4) for 14 days. Images oriented rostral (R) to the left and caudal (C) to the right. Scale bar = 1 cm.

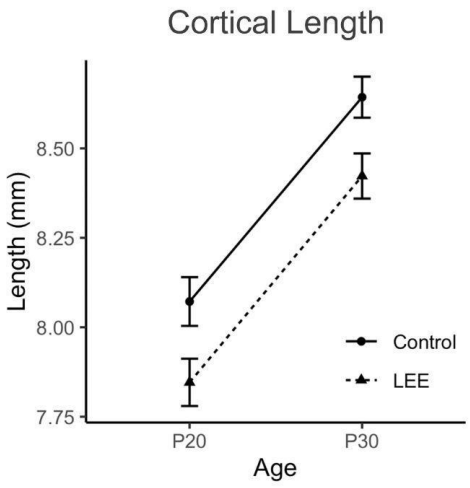

Figure 2.5 ***Cortical lengths.*** As expected, we report significant main effects of age, across groups, indicating that both LEE and control brains grow in length as they offspring ages. However, LEE offspring cortices (at P20 and P30) were significantly shorter compared to controls, regardless of sex and age. Together, our data suggests that LEE results in shorter cortices throughout early postnatal development, regardless of age and sex. (N=70) Data expressed as mean ± S.E.M.

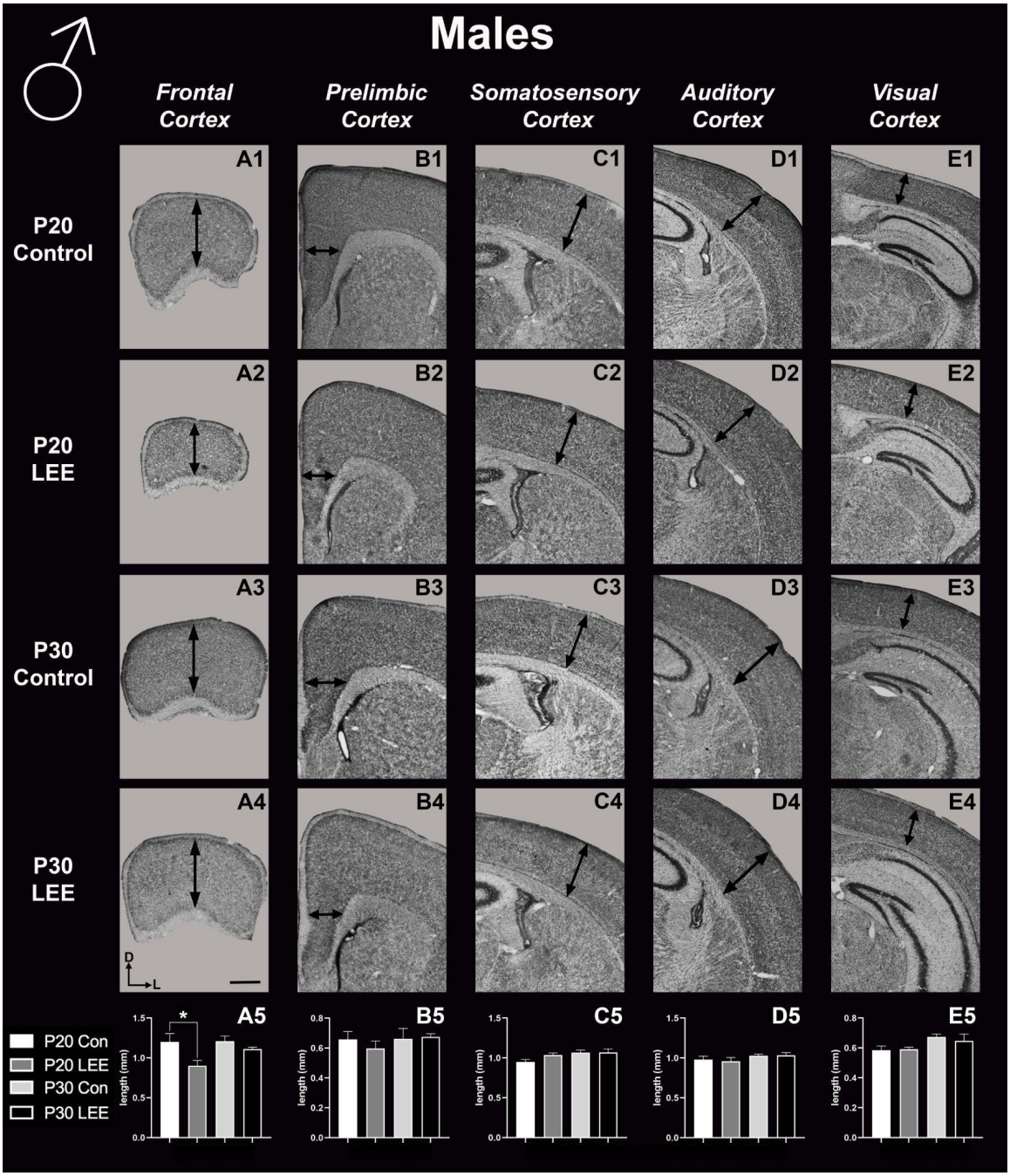

Figure 2.6 ***Cortical thickness measurements - Males.*** High magnification coronal sections of Nissl-stained hemisections. Measurements include frontal cortex (A1-4; N=21), prelimbic cortex (B1-4; N=16), somatosensory cortex (C1-4; N=22), auditory cortex (D1-4; N=19), and visual cortex (E1-4; N=28). No significant differences between control and LEE males, except in the frontal cortex at P20 (p=0.0235). Data expressed as mean ± S.E.M. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar = 1mm

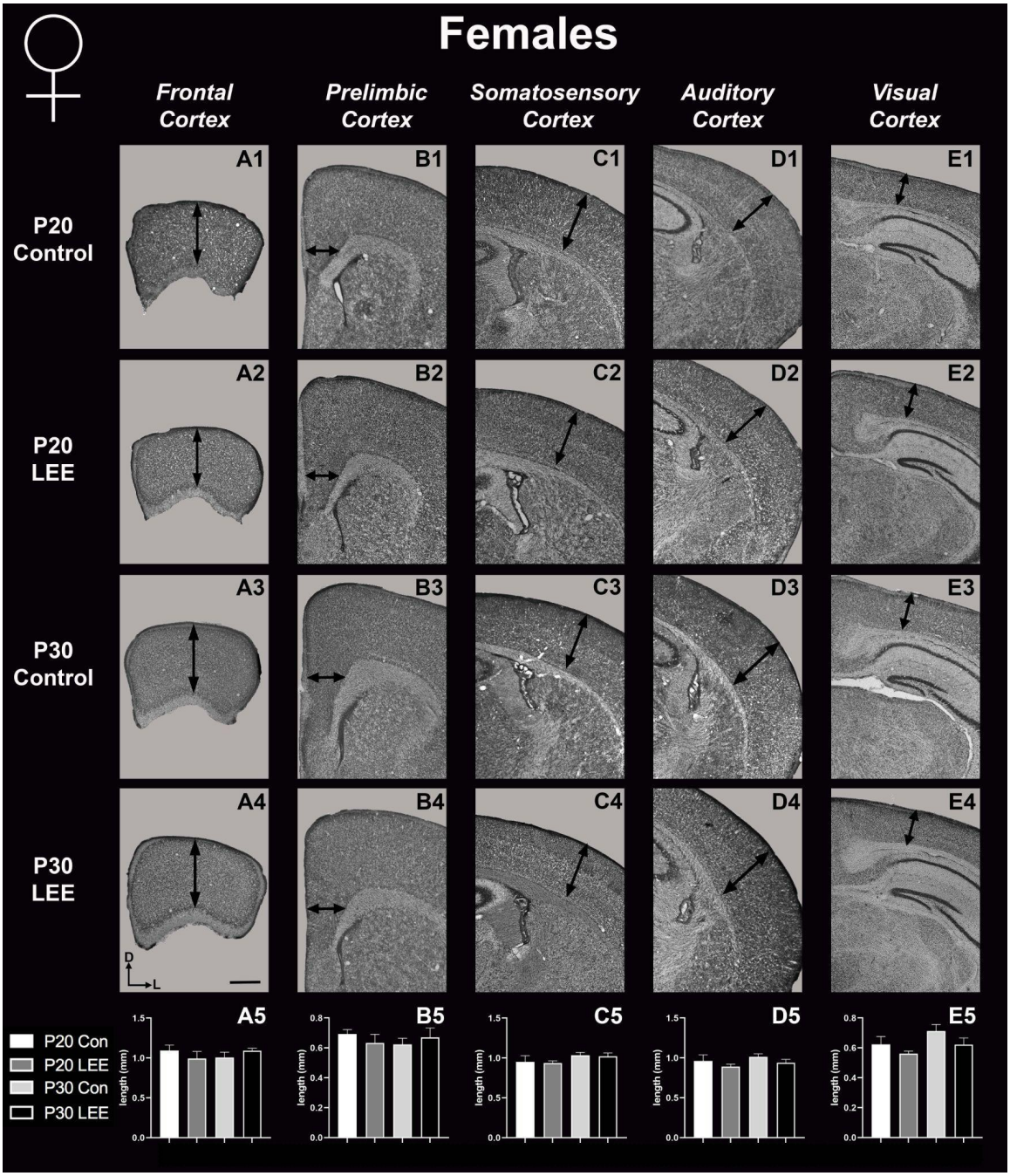

Figure 2.7 ***Cortical thickness measurements - Females.*** High magnification coronal sections of Nissl-stained hemisections. Measurements include frontal cortex (A1-4; N=19), prelimbic cortex (B1-4; N=11), somatosensory cortex (C1-4; N=26), auditory cortex (D1-4; N=20), and visual cortex (E1-4; N=20). No significant differences between control and LEE pups. Data expressed as mean ± S.E.M. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar = 1mm.

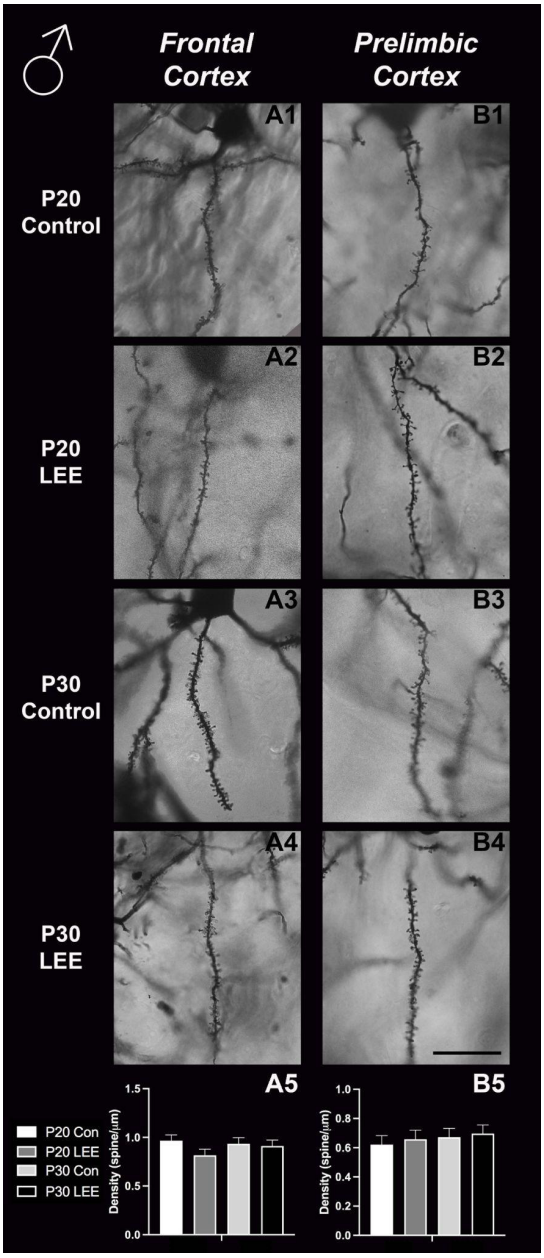

Figure 2.8 ***Dendritic spine density - males.*** Representative images of secondary dendrites of pyramidal cells in layers 4/5 of the frontal and prelimbic cortices of male control (A1, B1, A3, B3) and LEE (A2, B2, A4, B4) pups at P20 and P30. Comparison of dendritic spine density of males indicated no significant differences in frontal (A5; N=14) and prelimbic (B5; N=16) cortices. Data expressed as mean ± S.E.M. Scale Bar = 250 µm.

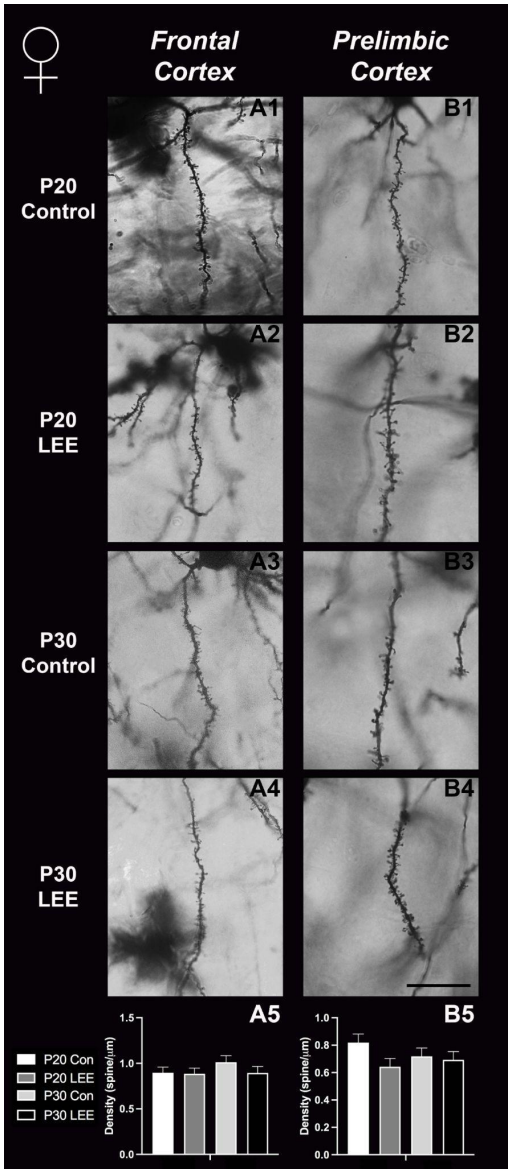

Figure 2.9 ***Dendritic spine density - females*****.** Representative images of secondary dendrites of pyramidal cells in layers 4/5 of the frontal and prelimbic cortices of female control (A1, B1, A3, B3) and LEE (A2, B2, A4, B4) pups at P20 and P30. Comparison of dendritic spine density of males indicated no significant differences in frontal (A5; N=16) and prelimbic (B5; N=16) cortices. Data expressed as mean ± S.E.M. Scale Bar = 250 µm.

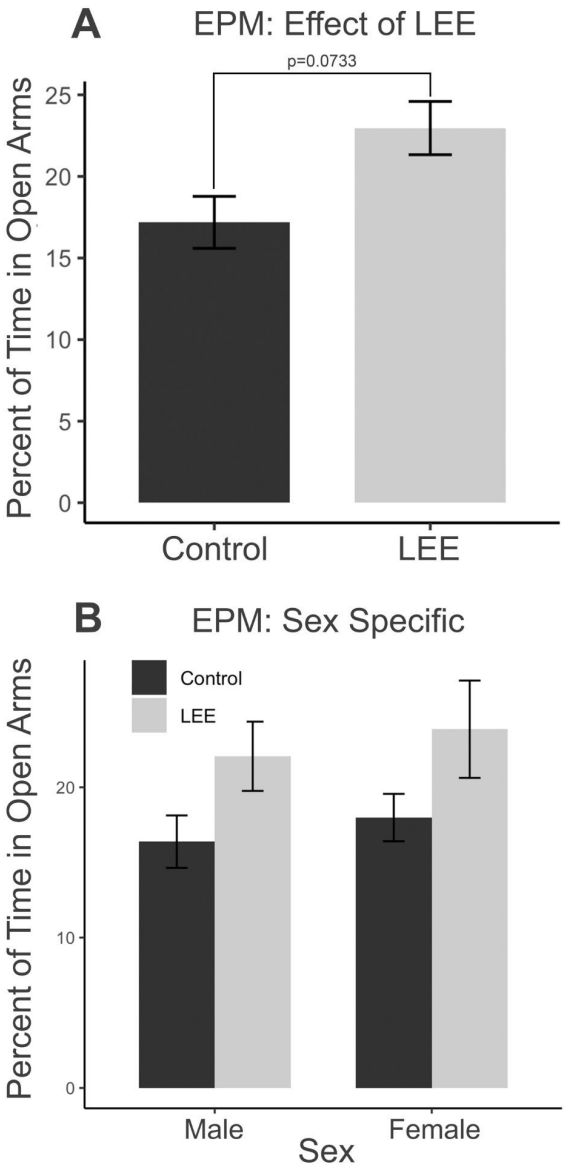

Figure 2.10 ***Behavioral assays at P30: EPM.*** A. No significant differences in time spent in the open arms of the EPM when evaluated by sex and a marginal effect of condition (p=0.07). B. No effects observed in planned comparisons for male and female pups. (N=41) Data expressed as mean ± S.E.M

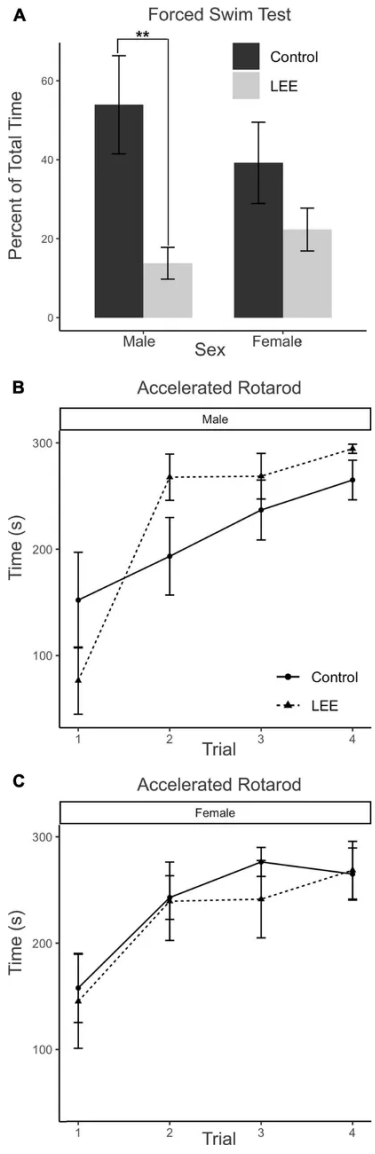

Figure 2.11. ***Behavioral assays at P30: FST, AR.***. A. Male LEE pups (p=0.0049) spent less time immobile than male controls in the FST. No significant differences in time spent immobile for females (p=0.3588) on the FST. (N=34) No significant differences in performance on the AR for males (B.) or females (C.) (N=42) Data expressed as mean ± S.E.M.

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Chapter 3: Lactational Ethanol Exposure: Neocortical Gene Expression and Behavioral Development in a Mouse Model

ABSTRACT

Alcohol exposure during breastfeeding, referred to as lactational ethanol exposure, or LEE, may result in significant alterations to typical behavioral trajectories in exposed offspring (Perez et al., 2023). It is important to characterize the effects of LEE to help us understand the phenotype, and potentially unveil underlying mechanisms that play a role in the effects of LEE. This chapter examines how LEE impacts gross developmental metrics, gene expression patterns, and behavior. Using our LEE mouse model, as described in Perez et al, (2023), we established two critical time points in postnatal development pre-and peri-pubescence, P20 and P30 respectively. We evaluated gross development via body and brain measurements, assessed gene expression patterns using *in situ* RNA hybridization of cortical patterning genes, and evaluated behavior via the Suok bar test and the Three Chambered Sociability test at those ages. Specifically, around the time of wean (postnatal day 20), LEEoffspring had reduced brain weights, body weights and cortical lengths with significantly higher brain:body weight ratios when compared to controls. Gene expression assays showed a medial shift in *Id2* gene expression in LEE mice at the dorsal somatosensory and motor cortex boundary.. Additionally, *Id2* gene expression was significantly elevated in the frontal cortex of LEE mice when compared to controls at wean age. Behavioral assays revealed significantly impaired sensorimotor integration, altered anxiety-like and hyperactive behaviors during peri-pubescence (postnatal day 30) in LEE mice when compared to controls. These

findings, along with those from Perez et al., 2023, demonstrate that ethanol exposure via breast milk generates a distinct phenotype in LEE offspring. Specifically, we identify altered growth metrics and neocortical gene expression patterns that may be acting in a coordinated manner to disrupt typical behavior at pre- and peri-pubescent ages. In sum, our results suggest that LEE results in some developmental deficits that warrant further exploration and emphasize the need for preventative measures to mitigate risks associated with maternal alcohol consumption during lactation and breastfeeding.

INTRODUCTION

Breast milk provides infants with essential nutrients and immunological support and is associated with numerous health benefits for both mother and child. The benefits for infants include, but are not limited to, reduced risk of infection through the transfer of antibodies from the breastfeeding parent (Newburg et al., 2007), reduced rates of childhood obesity (Whaley et al., 2012), and fewer allergies (Gdalevich et al., 2001). Congruently, mothers experience lower risks of breast and ovarian cancers (Victora et al., 2016). The nutritional and emotional support provided by breastfeeding can have enduring positive effects on childhood development, including cognitive and behavioral outcomes (Horta et al., 2015). Despite the extensive benefits, certain lifestyle factors may disrupt breastfeeding's positive impact, particularly maternal alcohol consumption. Alcohol use during breastfeeding is a pertinent issue, as it is relatively common among lactating women and can directly impact both milk composition and infant health outcomes (Anderson, 2018). Alcohol consumption during lactation is a concern because

of the systematic impacts it has throughout the entire body. This is primarily due to the molecular size of alcohol, since it has a molecular weight of 46 Daltons (Da). Therefore, alcohol is water soluble, which means it can readily enter bodily fluids such as breastmilk (Anderson, 2018). Other studies have shown that alcohol levels in the breastmilk mirror the amount of alcohol in the blood (Lawton, 1985; Chien et al., 2005). Therefore, it is critical to increase awareness that alcohol consumed by breastfeeding parents can transfer into breast milk and thereby expose nursing infants to ethanol.

Alcohol exposure may influence infant growth, sleep patterns, and early developmental outcomes (May et al., 2016; Tay et al., 2017, Geiner, 2019). Research indicates that even small amounts of alcohol in breast milk can reduce the infant's milk intake, alter the infant's motor skills temporarily, and may lead to long-term cognitive and behavioral changes (Parackal et al., 2007; May et al., 2016; Tay et al., 2017; Oei, 2019). Therefore, the establishment of an animal model, where high levels of internal validity and a causality, may elucidate the multifaceted impacts LEE has on offspring development. Our laboratory has established a mouse model that is useful for studying the physiological and neurodevelopmental impacts of lactational ethanol consumption on offspring (Perez et al., 2023).

In summary, maternal alcohol consumption during lactation can have significant effects on infant development, particularly in the areas of cognitive and behavioral functioning. While human studies provide correlational data, rodent models offer a controlled setting to investigate these effects more rigorously. This study builds upon our initial research, which established the lactational ethanol exposure (LEE) model and

explored gross development and behavior. Our primary aim in this continuation study was to investigate whether LEE affects gene expression, social behavior, sensorimotor integration, and DNA methylation in pre-pubescent mice. We predict that LEE would alter gene expression patterns of crucial cortical patterning genes, alter typical sensorimotor integration, and alter DNA methylation. Our results provide additional characterization of the phenotype associated with LEE. These outcome domains provide a holistic view of development, assessing both the potential for behavioral changes and molecular impacts. Overall, our research has broader implications for public health, emphasizing the importance of guidance on alcohol use for breastfeeding parents and shedding light on potential mechanisms underlying alcohol-induced developmental risks.

MATERIALS AND METHODS

Animal Care

Experimental studies and breeding were conducted in accordance with the animal use protocol guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside (UCR). CD-1 mice, initially purchased from Charles River Laboratories (Wilmington, MA, USA), were used for breeding. We chose to use the CD-1 mouse strain in this lactational model because these mice show superior maternal care compared to inbred strains; additionally, we validated them as a model for prenatal ethanol exposure (PrEE) in our prior work (El Shawa et al., 2013). Mice were housed in animal facilities located at UCR that were kept at approximately 22◦C on a 12-h light/dark cycle. Mouse chow and water (for controls), or

mouse chow and a 25% ethanol solution in water, were provided *ad libitum* to the dams according to the dosing schedule.

Breeding and Lactational Ethanol Exposure Paradigm

Three to five month old female and male mice were paired just before the start of the dark cycle. Our current study used the validated breeding paradigm from Perez et al., 2023. In sum, once a vaginal plug was detected, the pregnant female was moved to a separate cage. Throughout pregnancy, mouse chow and water were provided *ad libitum*. Dams were undisturbed until the pups were postnatal day (P) 6 days old (Figure 3.1). During this time, we pseudo-randomly selected each dam to be in either the control or experimental (LEE) group. LEE dams had their water replaced with a 25% v/v ethanol in water solution throughout the exposure period from P6 to P20, while control dams remained on water. There were no alterations to the dam's food supply through the exposure period for any experimental condition. Measurements were taken daily for liquid and food consumption during the exposure period for both conditions. At wean (P20), control and LEE pups were weighed and divided into two subsets. Subset A, which had a sacrificial end date of P20, and Subset B, which had a sacrificial end date of P30, which is peri-pubescence. The division of the litters into subsets allowed us to evaluate the short- and long-term effects of lactational ethanol exposure with an array of techniques. To avoid litter effects, we distributed pups from multiple liters for each assay tested.

Brain Tissue Preparation and Collection

Pups from all conditions were randomly assigned for *in situ* hybridization studies. Mice were weighed and then sacrificed using a lethal dose of sodium pentobarbital (100 mg/kg) administered via intraperitoneal injection. Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in PBS (PFA, pH: 7.4) for fixation. The skulls were sunk in a 4% PFA solution, and after an overnight post-fixation, the brains were extracted, weighed, and imaged. Dorsal views of whole brains were imaged using a Zeiss (Oberkochen, Germany) Axio high-resolution (HRm) camera attached to a dissecting microscope (data not shown). The cortical length was measured using a digital micrometer in Image J (NIH, Bethesda, MD, USA), using the dorsal whole-brain images (see Fig. 2.4 from Chapter 2 for example). Extracted brains were stored in 4% PFA for later use.

Gene Expression Assays, Gene Expression Shift and Transcript Density Analyses.

Gene expression assays were performed at P20 using hemisected brains via *in situ* RNA hybridization (ISH). Standard protocols for free-floating nonradioactive ISH were implemented to visualize and assess gene expression patterns in mice as previously described (Dye et al., 2011a, 2011b; El Shawa et al., 2013; Conner et al., 2020). Specifically, *RZRβ* and *Id2* probes (plasmids courtesy of John Rubenstein, UCSF) were applied to coronal sections (100 μm thick) after embedding in gelatin-albumin and sectioning at 100 μm . Once the sections showed adequate expression, sections were mounted, then digitally imaged using a Zeiss Stereo Discovery V.12 microscope. To

quantify shifting of gene expression in the somatosensory and motor cortices, we measured the position of the most medially labeled cells resulting from RNA ISH, as the distance from the cortical midline. This was done using a high-precision electronic micrometer (NIH; ImageJ), see Dye et al., 2012 for more details. Next, transcript density was measured in ISH-stained coronal sections. Specifically, RZR*β* and *Id2* expression was quantified using ImageJ (NIH, Bethesda, MD, USA) software. First, digital images were converted into binary and were adjusted to fit a standardized threshold for all regions of interest (ROI). Anatomical landmarks were used to match sections between all replicates. An ROI-1 of identical, static size was placed in notable areas for each gene (*RZRβ*: Layer IV in medial frontal cortex; *Id2*: Layers II/III in medial frontal cortex) for each condition. A second ROI (ROI-2) of identical size was placed lateral to ROI-1 to serve as an internal control. Each sample was individually measured to determine the amount of expression in the specific ROI, which was reported as the percent of tissue (area fraction) expressing the specific gene within the ROI. The technique was described in further detail previously, (Dye et al. 2012).

Cortical DNA extraction and Global DNA 5mc Methylation Assay

Randomly selected P20 pups were examined for epigenetic change via global 5-methylcytosine (5mc) levels in cortex. To do this, mice were sacrificed using cervical dislocation, the brain was extracted from the skull, the cortex was removed from the subcortex, divided into rostral and caudal cortex portions, and DNA was isolated following the instructions provided in the AllPrep DNA/RNA Mini Kit (QIAGEN,

Germantown, MD, USA; Category Number: 80204). The remaining brain tissues were hyper-cooled using 2-methyl butane and stored at -80C. To measure specific epigenetic change, the levels of 5-methylcytosine (5-mc) were measured by the ELISA-based Methylflash Methylated DNA kit (Epigentek, Brooklyn, NY, USA; Category Number: P-1030-96). In brief, 100 ng of DNA was bound to well plates that were treated to have a high binding affinity for DNA. Through the introduction of detection and reporter antibodies, visualization of the 5-mc levels was determined via measured absorbance at 450 nm on a Victor 2 plate reader. Percentage levels of 5-mc were calculated via a standard curve.

Behavioral Assays

Due to higher than zero BEC levels in LEE pups at wean, behavioral assays were only performed at P30. Therefore, the 10-day post-wean period was considered a "washout" period in the paradigm. All behavioral analyses and scoring were performed and analyzed by trained researchers blind to the experimental condition. Mice were acclimated to the dimly lit behavioral testing room for a minimum of one hour before testing.

Suok Bar Test

The Suok bar test was used to assess anxiety-like behaviors and sensorimotor integration (Kalueff et al., 2008). Mice were subjected to a single, 5-minute, trial on an elevated (to a height of 20 cm) three-meter-long aluminum rod (diameter 3cm). The rod

is separated into 10 cm segments by line markings and is flanked by two clear acrylic wall ends, where the mouse cannot traverse further. To start the test, the mouse was placed in the center zone of the rod with their snouts facing one of the acrylic wall ends. The 5-minutes began once the mouse contacted the rod with all four paws. If the mouse fell from the apparatus the timer was paused, the mouse was repositioned on to the same location on the rod, then the timer was resumed. Trained researchers blind to experimental conditions documented events: (1) total number of segments crossed, (2) total number of missteps while traversing the rod, (3) total number falls from the rod, (4) latency to leave the center zone of the rod in seconds, and (5) horizontal/vertical exploratory movements indicated by head movements off the bar, by the number of rears, and acrylic wall leanings. Following the completion of each trial, the apparatus was cleaned with Virkon to remove olfactory cues. Trials were recorded for backup. Further detail provided in El Shawa et al., 2013.

Three-Chambered Sociability Test

The three-chambered sociability (SOC) test was used to examine social interaction behavior or sociability (Yang et al., 2011). Mice were subjected to three ten-minute trials in the three-chambered apparatus. The first trial is considered the acclimation trial; mice were restricted to the center chamber for ten minutes. In the second trial, the mice had unrestricted exploration of the entire apparatus. During this trial, an empty inverted metal wire cup is placed in the center of the flanking chambers. Lastly, the third trial introduces a novel, age-matched, same-sex mouse to the flanking chambers. This novel mouse is restricted under the inverted metal wire cup and the

amount of time the experimental mouse spends with the novel mouse is recorded. The second and third trials were video recorded for later analysis and confirmation.

Statistical Analyses

All statistical analyses were completed using Jamovi (v 2.3.28.0, The Jamovi project). A statistical software based on R (R Core Team, 2021) Between groups tests were carried out using ANOVA with Type III sums of squares. When appropriate, multiple comparisons were made using Tukey HSD (Fox and Weisberg, 2020; Lenth, 2020).

RESULTS

P20 Offspring Metrics

Our paradigm explored and confirmed its ability to produce gross alterations in P20 offspring's brain and body development. To accomplish this, we evaluated brain weights, body weights, brain-body ratio, and cortical length measurements (Figure 3.2) at wean by sex.

A two-way, ANOVA, condition (control vs. LEE) × sex identified a main effect of the condition on offspring brain weight, $F(1,31) = 41.76$, $p < .001$, $\eta^2 = .57$ (Figure 3.2A). There were no significant differences in offspring brain weight due to sex ($F(1,31) = .02$, $p = .90$, $\eta^2 = .00$) as well as no significant interaction (condition × sex; $F(1,31) = .03$, $p = .86$, $\eta^2 = .00$) (Figure 3.2A). Tukey HSD *post hoc* testing revealed significant differences between control ($M = 0.43$) and LEE ($M = 0.37$) offspring brain weight $t(31) = 6.46$, $p < .001$, $d = 2.19$..

Next, a two-way ANOVA, condition × sex, identified a main effect of condition on offspring body weight, $F(1,43) = 52.48$, $p < .001$, $\eta^2 = .54$ (Figure 3.2B). There were no effects of sex ($F(1,43) = .64$, $p = .43$,$\eta^2 = .01$) or their interaction (condition × sex; $F(1,43) = .39$, $p = .57$, $\eta^2 = .00$) on offspring bodyweight (Figure 3.2B). Tukey HSD *post hoc* testing revealed significant differences between control ($M = 9.68$) and LEE ($M = 5.40$) offspring body weight $t(43) = 7.24$, $p < .001$, $d = 2.14$.

Lastly, a two-way ANOVA, condition × sex, identified a main effect of condition on offspring ratio of pup's brain to body weight, $F(1,31) = 46.79$, $p < .001$, $\eta^2 = .60$ (Figure 3.2C). There were no effects of sex ($F(1,31) = .03$, $p = .86$, $\eta^2 = .00$) or their interaction (condition × sex; $F(1,31) = .25$, $p = .62$, $\eta^2 = .00$) on offspring ratio of pup's brain to body weight (Figure 3.2C). Tukey HSD *post hoc* testing revealed significant differences between control ($M = 0.05$) and LEE ($M = 0.08$) offspring ratio of pup's brain to body weight, $t(31) = -6.84$, $p < .001$, $d = -2.32$..

To examine cortical length (Figure 3.2D), a two-way, ANOVA, condition × sex, reported significant effects of condition on offspring cortical length, $F(1,14) = 6.95$, $p = .02$, $\eta^2 = .28$. Additionally, there were no effects of sex ($F(1,14) = 1.33$, $p = .27$,$\eta^2 = .05$) or their interaction (condition x sex; $F(1,14) = 2.78$, $p = .12$,$\eta^2 = .11$) on offspring cortical length (Figure 3.2D). Tukey HSD *post hoc* testing revealed significant differences between control ($M = 8.07$) and LEE ($M = 7.74$) offspring cortical length, $t(14) = 2.64$, $p = .02$, $d = 1.26$.

Overall, our data confirmed that mice exposed to ethanol via breast milk in infancy show signs of growth restriction, decreased brain weight and reduced cortical length.

Gene Expression in LEE neocortex

We examined the expression of two cortical patterning genes, *Id2* and *RZRβ,* in the neocortex of LEE and control mice. These two genes are important for neocortical patterning and arealization (Rubenstein et al., 1999, Dye et al., 2011A; 2011B) and are known to have altered expression patterns in a mouse model of FASD (El Shawa et al., 2013; Abbott et al., 2018). Here, we examine *Id2* and *RZRβ* expression patterns in LEE mice as compared to controls (Figure 3.3 Males; Figure 3.4 Females).

Id2 and *RZRβ* expression is highly complex, spans multiple cortical layers, and is expressed regionally within primary areas (Dye et al., 2011A; 2011B). *Id2* expression spans multiple cortical layers (II/III, V, and VI); however, expression levels are heavily reduced in layer IV of somatosensory cortex, notably absent where the barrel field is (*S1, denotes primary somatosensory cortex, Figure 3.3 A3,4; B3,4; Figure 3.4 A3,4; B3,4). Notably, high levels of *Id2* expression occur at the medial/caudomedial border of the motor and somatosensory cortices at wean and peri-pubescence (Dye et al., 2011B). Congruently, *RZRβ* is expressed primarily in layers IV and V (*S1, Figure 3.3 C2-4, D2-4; Figure 3.4 C2-4, D2-4) with high levels of expression occurring within the somatosensory cortex barrel field (S1BF) (Dye et al., 2011B). Interestingly, this creates a border of expression in S1BF between *Id2* and *RZRβ* that is easily identified and seems to

be unaltered by our LEE paradigm. However, the medial/caudomedial border of motor and somatosensory, visualized through *Id2* expression, appeared to be altered due to LEE (yellow triangle, Figure 3.3 A3; B3; Figure 3.4 A3; B). In addition, our previous work in LEE has identified a reduction in cortical thickness in the frontal cortex (Perez et al., 2023); therefore, we quantified expression of *Id2* and *RZRβ* at the frontal cortex. Specifically, we targeted the medial wall expression of both genes through the conversion of expression patterns into binary using ImageJ and reported altered *Id2* expression due to LEE.

Gene expression Quantification via Measurement of Id2 Medial-Lateral Expression and Transcript Analysis

Patterns of *Id2* and *RZRβ expression* shift developmentally from P0 to P30, and boundaries between *Id2* and *RZRβ* correlate with cortical area borders, specifically in the frontal-parietal lobes, where the motor cortex abuts somatosensory cortex (Dye et al., 2011A; 2011B). In our PrEE model, we see consistent shifts in the putative border between motor and somatosensory cortex, as determined through intraneocortical connectivity of primary areas and the expression boundary of *Id2* and *RZRβ* in layers 2/3 (El Shawa et al., 2013). Indeed, the edge of the strong, layer 2/3 dorsal Id2 expression that begins in the cingulate cortex and extends through motor cortex, correlates with the rostral-medial border of motor and somatosensory cortex (El Shawa et al., 2013). As with PrEE mice, we observed a visible shift in *Id2* expression pattern at the presumed border of motor and somatosensory cortex due to LEE (arrows in Fig. 3.5 A1-B2). Using a

digital micrometer, we measured the distance from the midline to the cessation of strong *Id2* expression (a method used in Dye et al., 2012). A two-way, ANOVA, condition × sex, reported significant effects of condition on distance from midline for the edge of *Id2* expression, $F(1,19) = 16.41$, $p < .001$, $\eta^2 = .45$, Fig. 3.5C. Notably, there were no effects of sex ($F(1,19) = .10$, $p = .75$, $\eta^2 = .00$), as LEE mice in both males and females show a shift in expression when compared to controls. There was no significant interaction (condition x sex; $F(1,19) = 1.20$, $p = .29$,$\eta^2 = .03$) on distance from midline. Tukey HSD *post hoc* testing revealed significant differences between control ($M = .98$) and LEE ($M = .76$) on distance from midline, $t(19) = 4.05$, $p < .001$, $d = 1.71$. Our results suggest that LEE results in medial shifts in *Id2* expression, compared to controls, at the motor and somatosensory cortex boundary, with the effect present in both males and females. Additionally, we quantified gene expression profiles for *Id2* and. *RZRβ* in the frontal cortex using transcript density analysis, as described in Dye et al., 2012, images shown in Fig. 3.6A1-D3. This cortical area was selected based on previous literature indicating an altered cortical thickness in the frontal lobe for male LEE mice at P20 (Perez et al., 2023). To accomplish this, we employed a three-way ANOVA, condition × sex × gene (*Id2* vs. *RZRβ*), to investigate transcript density percentage in the medial wall of the frontal cortex. This analysis identified significant main effects of condition ($F(1,27) = 6.42$, $p = .01$, $\eta^2 = .15$), sex ($F(1,27) = 4.34$, $p = .05$, $\eta^2 = .07$), and gene ($F(1,27) = 19.69$, $p < .001$, $\eta^2 = .31$) on transcript density in frontal cortex. However, no two-way (condition × sex; condition × gene; or sex × gene) or three-way (condition × sex × gene) interactions were significant (Figure 3.6 E). Tukey HSD *post hoc* testing revealed a

significant difference in LEE (M = 46.30) compared to control (M = 39.25) in overall transcript density in the frontal cortex, $t(27) = -3.07$, $p = .01$, $d = -1.07$. Tukey HSD *post hoc* testing revealed a significant difference in females (M = 45.17) compared to males (M = 40.38) in overall transcript density in the frontal cortex, $t(27) = -2.08$, $p = .05$, $d = -0.73$. Tukey HSD *post hoc* testing revealed a significant difference in *Id2* (M = 47.87) compared to *RZRβ* (M = 37.68) in overall transcript density in the frontal cortex, $t(27) = -4.44$, $p < .001$, $d = -1.55$. These results indicate that LEE may induce a change in *Id2* expression that is sex-specific with varying levels of transcript in discrete regions of cortex.

P20 DNA 5mc Methylation

To assess the effects of the exposure paradigm on the epigenetic marker, 5mc, we measured methylation percentage from caudal and rostral portions of the cortex from control and LEE mice. We carried out a three-way ANOVA, condition× sex) × cortical area (caudal vs. rostral), on the methylation percentage in mouse cortex. In this analysis, none of the main effects or interactions were significant (Figure 3.7). These results suggest that our methods did not detect alterations to DNA 5mc methylation in our subjects.

P30 Behavioral Analyses

To assess the impact of the exposure paradigm on behavioral development, we use behavioral testing to investigate potential differences. Previously, we employed a

battery of behavioral tests to examine behavioral phenotypes in LEE mice (Perez et al., 2023); here, we test LEE mice on two additional assays: the Suok Bar Test and the Three-Chambered Sociability Test.

The Suok bar test assesses anxiety-like behaviors and sensorimotor integration. We investigated the sensorimotor integration behaviors by recording segments crossed, falls, and missteps during testing (Figure 3.8 A-C). Congruently, the presence or absence of anxiety-like behaviors was investigated through latency to leave the center of the rod and exploration during testing (Figure 3.8 D, E). First, a two-way, ANOVA, condition × sex, failed to identify a significant effect of condition ($F(1,21) = .45$, $p = .51$, $\eta^2 = .02$) or sex ($F(1,21) = .60$, $p = .45$, $\eta^2 = .02$) on the segments crossed by offspring. However, we report a significant interaction (condition × sex) on segments crossed, $F(1,23) = 5.14$, $p = .03$, $\eta^2 = .19$. Subsequent *post hoc* Tukey HSD reports no significant comparisons between groups (Figure 3.8 A). A two-way condition ANOVA, condition × sex, failed to identify a significant effect of sex ($F(1,21) = .02$, $p = .88$, $\eta^2 = .00$) or interaction (condition × sex, $F(1,21) = .00$, $p = .96$, $\eta^2 = .00$) on the number of offspring falls. However, LEE offspring fell from the bar at a significantly higher rate than controls, $F(1,21) = 4.65$, $p = .04$, $\eta^2 = .18$. (Figure 3.8 B). Next, a two-way ANOVA, condition × sex, failed to identify a significant effect of condition ($F(1,21) = 1.69$, $p = .21$, $\eta^2 = .07$), sex ($F(1,21) = .37$, $p = .55$, $\eta^2 = .02$), or their interaction (condition × sex, $F(1,21) = .34$, $p = .57$, $\eta^2 = .02$) on the missteps made by offspring (Figure 3.8 C). A two-way ANOVA, condition × sex, failed to identify a significant effect of condition ($F(1,21) = .15$, $p = .70$, $\eta^2 = .01$), sex ($F(1,21) = .51$, $p = .49$,$\eta^2 = .02$), or their interaction (condition × sex,

$F(1,21) = .20$, $p = .67$, $\eta^2 = .01$) on the latency to leave the center of the rod by offspring (Figure 3.8D). A two-way ANOVA condition × sex, provided evidence that LEE offspring explored more than controls, $F(1,21) = 6.67$, $p = .02$, $\eta^2 = .22$. Additionally, there was a trend toward more exploration by females than males, $F(1, 21) = 3.29$, $p = .08$, $\eta^2 = .11$, The interaction between the two factors, however, failed to reach significance, $F(1,21) = .04$, $p = .84$,$\eta^2 = .00$. Tukey HSD *post hoc* testing revealed a significant increase in LEE ($M = 107.48$) compared to control ($M = 75.25$) on total exploratory behaviors, $t(23) = -2.71$, $p = .01$, $d = -1.20$ (Figure 3.8 E). Lastly, a two-way ANOVA, condition × sex, failed to identify a significant effect of condition ($F(1,35) = .09$, $p = .77$, $\eta^2 = .00$), sex ($F(1,35) = .14$, $p = .71$, $\eta^2 = .00$), or their interaction (condition × sex, $F(1,35) = .01$, $p = .92$, $\eta^2 = .00$) on the offspring sociability (Fig. 3.8 F). Overall, these data suggest that our exposure paradigm generates behavioral aberrations at P30 which include altered sensorimotor integration and anxiety in LEE mice regardless of sex.

DISCUSSION

Our study explored the developmental and behavioral impacts of LEE on offspring development, focusing on physical growth metrics, neocortical gene expression, epigenetic alterations, and behavioral phenotypes. The findings demonstrate clear effects of LEE on gross physical development, cortical gene expression, and behavior. These results contribute to understanding how maternal consumption of alcohol during lactation impacts offspring development at multiple biological levels.

Physical Development

LEE was associated with significantly reduced brain and body weight at P20. Despite these reductions, the brain-to-body weight ratio was higher in LEE offspring, suggesting a disproportionate impact of ethanol exposure on somatic growth relative to neural development. Our results are consistent with our laboratory's prior studies in LEE (Perez et al., 2023). Additionally, LEE significantly reduces cortical length, potentially indicating that gross cortical morphology is not resistant to ethanol exposure. These findings align with prior research from our laboratory demonstrating a reduction in cortical length in prenatal ethanol exposed mice (El Shawa et al., 2013; Abbott et al., 2018); additionally, our reductions in cortical length align with our prior studies in LEE (Perez et al., 2023). Gross development seems to be highly altered due to LEE; therefore, we posit that these changes may arise from increased cell-death, interference with the activity of growth factors, and changes in the regulation of gene activity (Goodlett & Horn, 2001).

Gene Expression Alterations in Cortical Development

Our study identified two significant alterations in gene expression patterns. First, we report a medial shift of *Id2* expression at the somatosensory and motor cortex border due to LEE. Second, we report increased levels of *Id2* in the frontal cortex of LEE mice compared to controls. These genes are critical for cortical arealization and patterning, suggesting that ethanol exposure during lactation may disrupt cortical development

through specific molecular pathways. *Id2* is a member of the helix-loop-helix (HLH) proteins that regulate cell differentiation and promote axonal growth (Ling et al., 2014). Specifically, *Id2* expression has been shown to enhance axon growth through increased microtubule polymerization when overexpressed in a mouse model (Yun et al., 2021). Typical *Id2* expression is high during prenatal and early postnatal periods in the brain and there is a reduction of expression as the brain ages (Yun et al., 2021) which indicates a natural reduction in axonal growth. Pairing this with the cortex-wide synaptic pruning that peaks around 14-21 days postnatal in mice (Lewis, 2011), our results suggest that altered neuronal development is present in LEE mice at P20 and is mediated by increased *Id2* expression. This overexpression of *Id2* in the frontal cortex and a medial shift in the somatosensory and motor cortices due to LEE, may alter typical synaptic pruning and may lead to altered connectivity within the rostral regions of cortex in LEE mice. Our previous work in LEE characterized dendritic spine density within the frontal cortex and identified a trend toward significance due to LEE, indicating a decrease in dendritic spine density compared to controls (Perez et al., 2023). Together, our findings suggest that the altered *Id2* expression along with a trend towards significance in dendritic spine density, may drive the altered behavior observed due to LEE. These alterations may manifest in cortex-wide connectivity issues, mediated by increased *Id2* expression during pruning. Further studies will explore intraneocortical connectivity between the frontal and motor cortex to further characterize the LEE phenotype.

Epigenetic Characterization of LEE

Despite hypothesized changes in DNA methylation as a potential mechanism underlying the observed phenotypes, no significant alterations in global 5mc levels were detected in the cortex of LEE mice. Our findings are in stark contrast to those observed in prenatal ethanol exposure models (Abbott et al., 2018). This may reflect the need for more targeted epigenetic assays, such as locus-specific methylation profiling of histone modification analyses, to capture subtle or region-specific changes. In addition, alcohol exposure may lead to alterations in DNA methyltransferases (DNMTs) which are responsible for the transfer of a methyl group onto the fifth carbon position of cytosine to form 5mc (Goll & Bester, 2005). Previous prenatal ethanol exposure studies have identified a significant reduction in DNMT mRNA levels in the murine cortex (Abbott et al., 2018). Our future research will characterize the impacts of LEE on DNMTs and other epigenetic markers.

Behavioral Consequences of Lactational Ethanol Exposure

We employed behavioral assessments that targeted sensorimotor integration, anxiety-like behaviors, and sociability. Our assessments revealed subtle but potentially meaningful disruptions in sensorimotor integration and anxiety-like behaviors. The implications of these findings are important for understanding the functional consequences of molecular and structural brain changes due to LEE. Notably, we did not observe any alterations in the social behavior of our LEE mice compared to controls; therefore, LEE might produce more subtle disruptions in neural circuits governing social

behavior. Future studies will further target mice's social behaviors to develop a comprehensive understanding of social development and LEE.

Our results from the Suok bar test indicated that sensorimotor integration and anxiety-like responses were altered due to LEE. Specifically, LEE mice expressed increased falls during testing, increased exploratory behaviors, and a sex-specific alteration of segments crossed. Specifically, our results indicated that male LEE mice decreased their movement compared to controls and female LEE mice increased their movement compared to controls. This may indicate a sex-specific impact on activity levels of LEE mice with the movement reduction in males attributed to increased anxiety-like behaviors and the movement increase in females attributed to hyperactivity. Previous research has established that paternal (Conner et al., 2020) or prenatal (El Shawa et al., 2013) ethanol exposure increases segments crossed in addition to an increase in falls. Previous LEE research has identified that increased risk-taking/exploratory behaviors are present at P30 using the Elevated Plus Maze (Perez et al., 2023) and our current study bolsters the behavioral phenotype of LEE to include increased exploratory behaviors via the Suok bar test. Together, our results are consistent with these findings and contribute to the characterization of alcohol exposure during lactation and the alteration of sensorimotor and exploratory behaviors. Congruently, ethanol exposure in 8-week-old mice has been previously shown to induce anxiety-like behaviors in various behavioral assays (Xu et al., 2022). This effect was primarily driven by the activation of iron-dependent cell death (ferroptosis) in neurons within the hippocampus and frontal cortex (Xu et al., 2022). Ferroptosis initiates iron-mediated lipid peroxidation, which induces

cell death (Li et al., 2020). Recently, ferroptosis has emerged as a potential mechanism of damage in many diseases including alcohol use disorders. Notably, a sulfated polysaccharide (fucoidan) has been identified as a possible protective agent against ferroptosis in the liver (Xue et al., 2022). In our laboratories previous studies, we have identified choline as a potential ameliorating agent for the negative effects of alcohol exposure during prenatal development (Bottom et al., 2020). Therefore, our future research will focus on the role of alcohol induced ferroptosis in prenatal- and early postnatal- alcohol exposure models. The research on ferroptosis and LEE is scarce; therefore, we will focus on the potential ameliorating effects of fucoidan, with and without choline supplementation, on LEE mice. Our future studies aim to identify a protective factor that may assist in reducing LEE phenotypes.

Implications and Future Directions

Our results emphasize the adverse effects of alcohol consumption during lactation on offspring development, particularly concerning brain and body growth, cortical gene expression, and behavior. These findings elucidate the need for public health interventions targeting maternal alcohol consumption during the postnatal period. Furthermore, the observed sex-specific effects on behavior suggest that future studies should examine the differences between sex, developmental timing, and ethanol exposure in more detail. For example, male and female mice begin puberty at slightly different ages, with males at P27 and females at P25 (Zhou et al., 2007; Ismail et al., 2011; Molenhuis et al., 2014). Notably, alcohol may disrupt typical hormonal release associated

with the onset of puberty (Srivastava et al., 2014; Srivastava et al., 2018) which may contribute to the sex-specific behavioral effects of LEE. Future research will expand on these findings by employing advanced epigenomic and transcriptomic techniques to uncover the mechanisms driving LEE's disruptions in cortical development. Additionally, studies examining behavioral outcomes beyond P30 are critical for understanding the long-term impact of LEE.

In summary, our study provides novel insights into the developmental consequences of lactational ethanol exposure, integrating findings across biological scales to elucidate the risks posed by maternal alcohol consumption during lactation. These data highlight the necessity for informed guidelines to protect maternal and infant health in this vulnerable period.

FIGURES

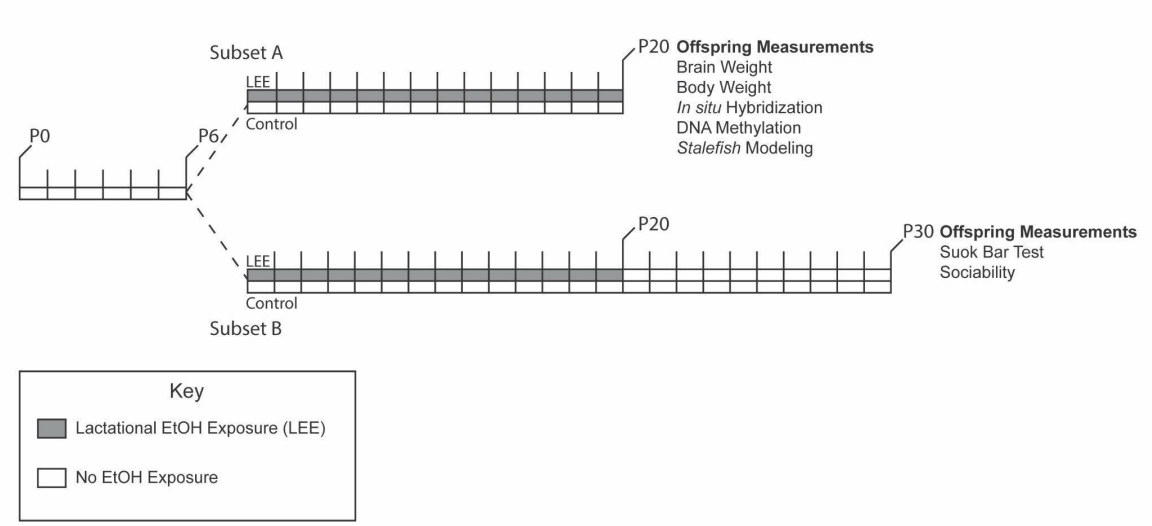

Figure 3.1 LEE Paradigm
Visualization of our exposure model. In sum, ethanol naive offspring and dams are split into two subsets. Subset A, with experimental endpoint of P20 and Subset B, with an experimental endpoint of P30. Within each subset, mice were pseudo-randomly selected to be in the "No EtOH Exposure" (control) group or "LEE" group. For behavior analysis, the LEE group was removed from ethanol exposure at P20 and a ten day cessation period allowed blood ethanol levels to reach zero.

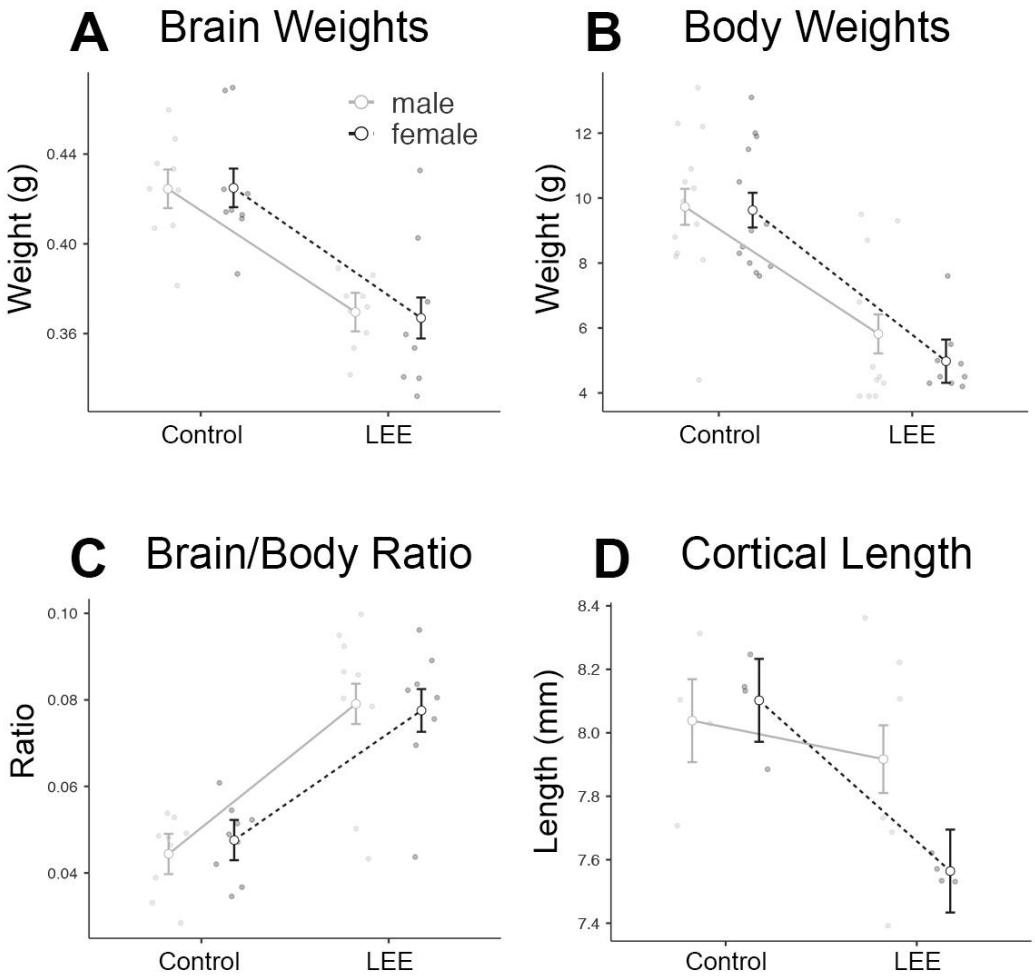

Figure 3.2 Offspring metrics
We report reduced brain weights (A) and body weights (B) due to LEE for male and female mice. In addition, we report an increased Brain/Body ratio (C) for LEE which is indicative of altered growth patterns, compared to controls. Lastly, we report reductions in brain cortical length due to LEE compared to controls, regardless of sex. (D). Data represented as Mean ± S.E.M. Data is jittered on the X axis to show individual data points.

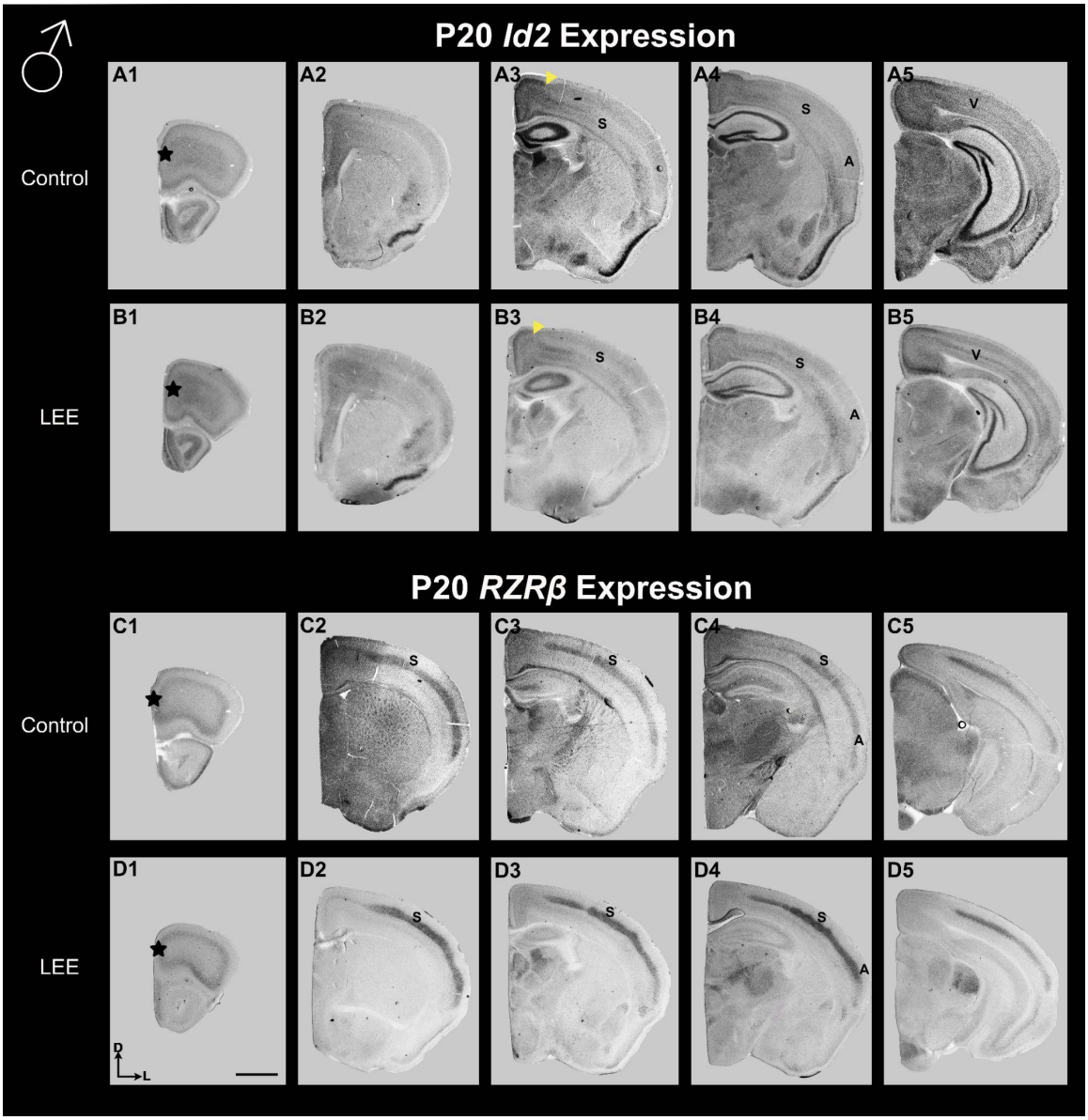

Figure 3.3 *in situ* Hybridization of *Id2* and *RZRβ* of male mice at P20
Representative coronal sections of *Id2* (Control, A1-5; LEE, B1-5) and *RZRβ* (Control, C1-5; LEE, D1-5) in male mice. Notable areas are identified within the figure. Black five pointed star represents the Prelimbic cortex. Yellow triangle represents the border of somatosensory and motor cortices, note LEE expression is shifted medially. *S1 = primary somatosensory cortex (*Id2*: A3,4; B3,4; *RZRβ*: C2-4; D2-4), *A1 = primary auditory cortex (*Id2*: A4; B4; *RZRβ*: C4; D4), *V1 = primary visual cortex (*Id2*: A5; B5) Sections oriented from rostral (1) to caudal (5). Sections orientated dorsal (D) up and lateral (L) to the right. Scale bar, 100 microns.

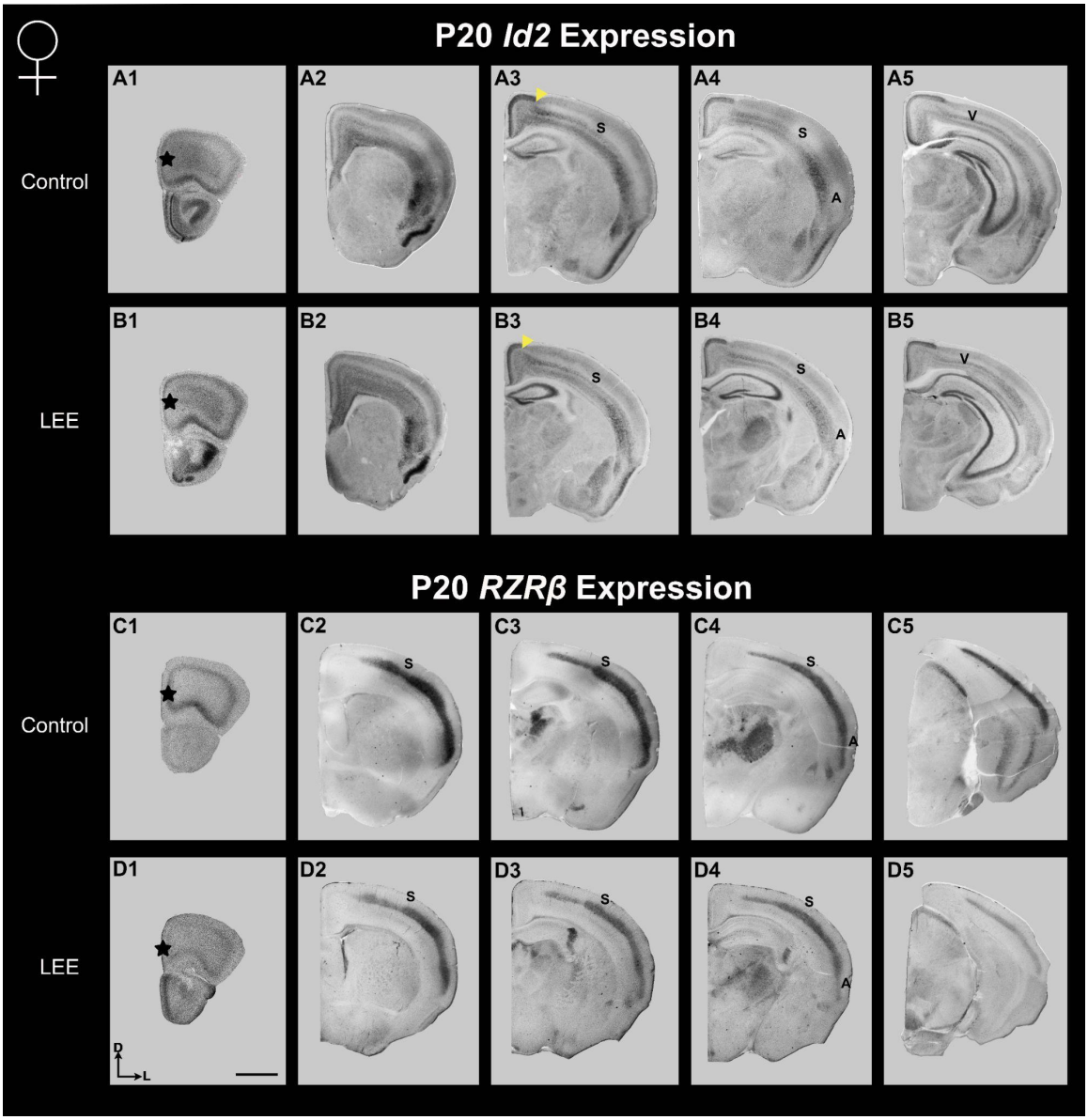

Figure 3.4 *in situ* Hybridization of *Id2* and *RZRβ* of female mice at P20
Representative coronal sections of *Id2* (Control, A1-5; LEE, B1-5) and *RZRβ* (Control, C1-5; LEE, D1-5) in female mice. Notable areas are identified within the figure. Black five pointed star represents the Prelimbic cortex. Yellow triangle represents the border of somatosensory and motor cortices, note LEE expression is shifted medially. *S1 = primary somatosensory cortex (*Id2*: A3,4; B3,4; *RZRβ*: C2-4; D2-4), *A1 = primary auditory cortex (*Id2*: A4; B4; *RZRβ*: C4; D4), *V1 = primary visual cortex (*Id2*: A5; B5). Sections oriented from rostral (1) to caudal (5). Sections orientated dorsal (D) up and lateral (L) to the right. Scale bar, 100 microns.

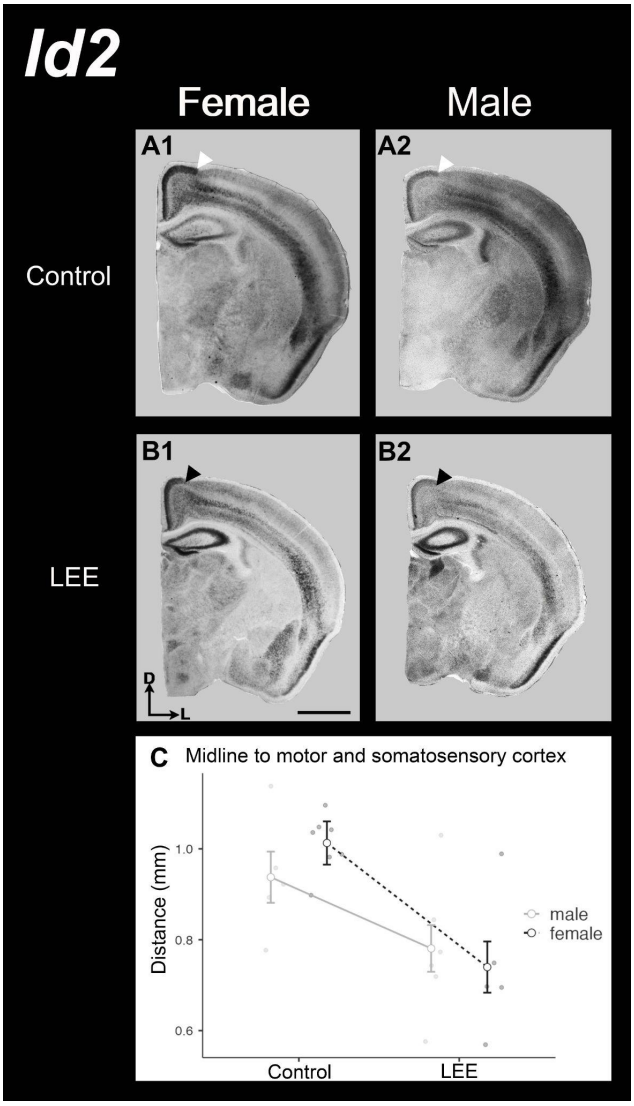

Figure 3.5 Analysis of motor and somatosensory cortex expression position between control and LEE mice at P20

Representative coronal sections of *Id2* expression patterns. LEE female (B1) and male (B2) expression is truncated and is shifted medially (black arrows) compared to control (white arrows) female (A1) and male (A2). Indicating that LEE may alter gene expression patterns in the border between motor and somatosensory cortex (C). Sections orientated dorsal (D) up and lateral (L) to the right. Scale bar, 100 microns.

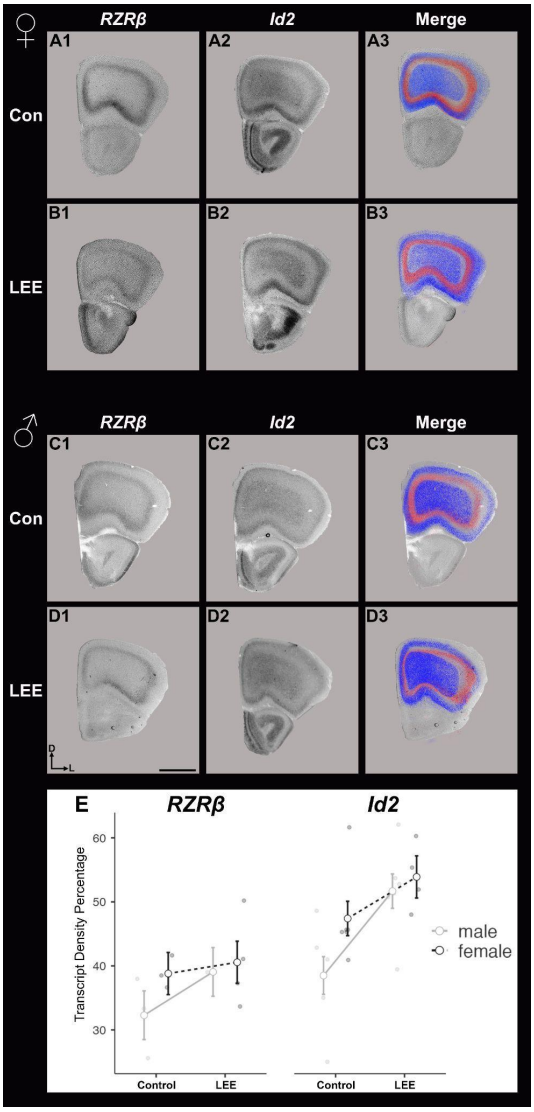

Figure 3.6 Analysis of Id2 and RZRβ expression in frontal cortex at P20
P20 coronal sections of offspring hybridized to *RZRβ* (A1, B1, C1, D1) and *Id2* (A2, B2, C2, C3) at the medial frontal cortex were analyzed by pseudo coloring of expression patterns. Merging the patterns together (A3, B3, C3, D3) reveals complementary patterning of *RZRβ* and *Id2* gene expression. Subsequent analysis of transcript density (E) was employed to determine altered expression strength of *RZRβ* and *Id2* at the medial frontal cortex. A static electronically drawn region of interest (ROI) was placed on sections of binary-converted in situ hybridization (ISH) to quantify levels of mRNA expression. LEE mice (both male and female) have increased levels of *Id2* gene expression in the medial frontal cortex. Sections orientated dorsal (D) up and lateral (L) to the right. Scale bar, 100 microns. Data represented as Mean ± S.E.M. Data is jittered on the X axis to show individual data points.

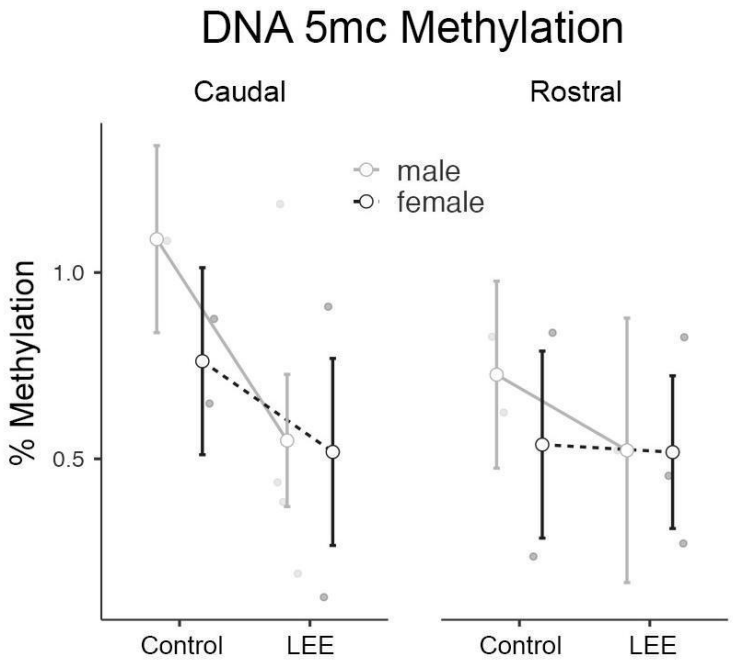

Figure 3.7 Epigenetic evaluation of global 5mC levels in cortex at P20
Global 5mC levels in rostral and caudal cortical tissue were analyzed for LEE and control mice. Our evaluation reports no significant differences in 5mC levels due to LEE. Data represented as Mean ± S.E.M. Data is jittered on the X axis to show individual data points.

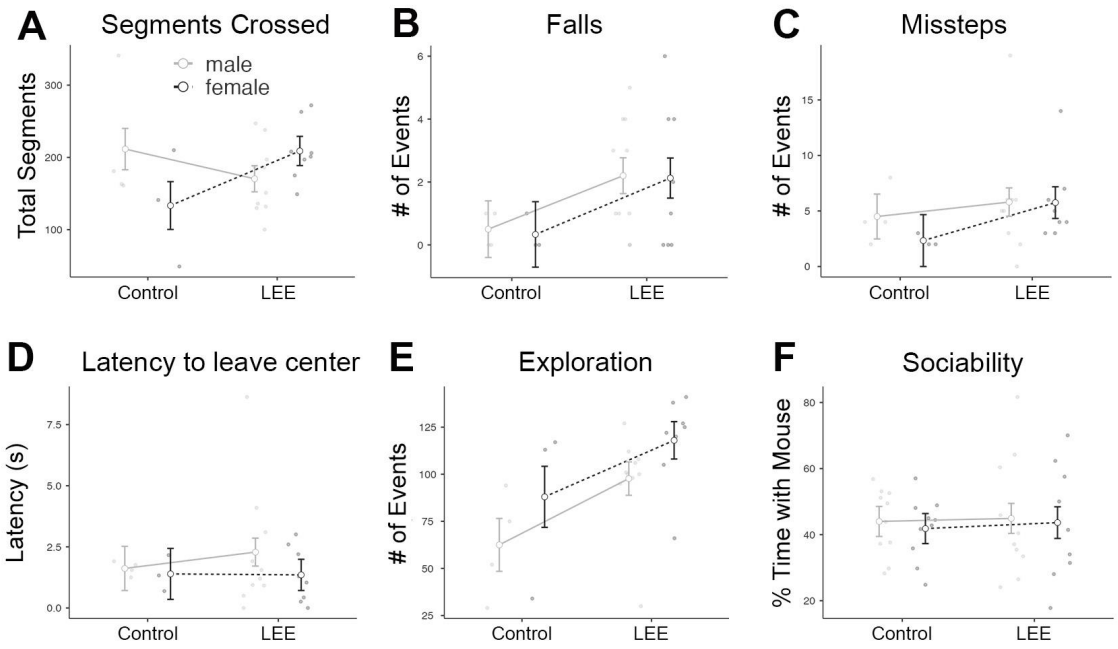

Figure 3.8 Behavioral analysis using the Suok Bar Test and Three-Chambered Sociability Test.
Behavioral analysis was performed on the Suok Bar Test (A-E) and the Three-Chambered Sociability Test (F). Measures of sensorimotor integration include segments crossed (A), falls (B), and Missteps (C). Measures of anxiety on the Suok include latency to leave center (D) of the bar and exploration (E) while on the bar. Our analyses revealed increases of exploratory behaviors in LEE mice compared to controls. In addition, we identified a trend towards significance in increased falls for LEE mice compared to controls. Lastly, we report a significant interaction effect of condition and sex for segments crossed. Indicating that male LEE mice cross fewer segments compared to controls and that female LEE mice cross more segments compared to controls. No differences present in mouse sociability metrics. Data represented as Mean ± S.E.M. Data is jittered on the X axis to show individual data points.

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General Conclusion

Our current research provides a comprehensive characterization of the behavioral, physiological, and genetic impacts of ethanol exposure during early development. In our murine model, we have demonstrated the deleterious and transgenerational effects of Prenatal Ethanol Exposure (PrEE). In addition, our results from the Lactational Ethanol Exposure (LEE) mouse model elucidates the importance of abstaining from alcohol use throughout the perinatal period, specifically during breastfeeding.

PrEE data from our laboratory over the past decade suggests that deficits in brain and body development, altered gene expression and neural connectivity are heritable, and persist well into the third filial generation (F3), even in the absence of subsequent ethanol exposures. The PrEE study reported in Chapter 1 identified how prenatal ethanol exposure can impact developmental programs that control cortical thickness, brain size, and social behavior. As a whole, our data in our PrEE FASD mouse model strongly suggest that maternal drinking during pregnancy can have detrimental effects that can be long lasting, pronounced, and most importantly, heritable.

To extend our PrEE model, we established and validated a novel alcohol exposure model where young postnatal mice consume ethanol via breastmilk during maternal lactation and characterized some behavioral, physiological, and genetic phenotypes of LEE. We reported reductions in brain weights, body weights, and cortical lengths that appear to persist from peri- and pre-pubescent periods. We identified frontal cortex thinning in LEE male mice, with reductions in dendritic spine density within the prelimbic cortex for LEE mice regardless of sex. In conjunction, we reported shifts in

typical gene expression patterns in the rostral portions of the neocortex due to LEE. Specifically, we observed a shift in the expression of *Id2* at the dorsal motor-somatosensory cortex boundary. Additionally, we quantified and reported an increased level of *Id2* expression in the frontal cortex of LEE mice. These alterations in body and brain development may alter typical behavioral patterns in LEE. Collectively, our results suggested that LEE may lead to increased risk-taking, increased hyperactivity, increased active stress-coping responses to environmental stressors, transient deficits in motor coordination, impaired sensorimotor integration, and altered anxiety-like behaviors. Notably, some of the LEE-induced phenotypes are sex-specific, highlighting the complexity and nuances of LEE that remain unexplored and emphasizing the need for further investigation.

Overall, our research unravels some of the complexities associated with ethanol exposure during the pre- and postnatal period. Much like Hercules' battle with the Hydra, where innovation and collaboration were key to overcoming complexity, our approach aims to emulate this approach. We aim to unravel the multifaceted mechanisms underlying PrEE and LEE, equipped with modern tools and a collaborative mindset. We are committed to advancing the understanding and communication of these issues to improve the well-being of affected individuals.

Childhood experiences are among the most significant determinants of a child's developmental trajectories. Our research aims to bolster the recommendations made for safe maternal practices during and after pregnancy. Alcohol, a well-established teratogen, poses a substantial public health concern during these critical developmental periods. Our

research reinforces the need for public educational programs and advocacy for those considering pregnancy, are pregnant, and/or have young children. By de-stigmatizing diagnoses and emphasizing the importance of support and treatment, we can create pathways towards autonomy and self-fulfillment for affected individuals. This responsibility lies with society to ensure that all children, and the adults they become, have the opportunity to thrive.