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## Behavioural And Molecular Consequences Of Postnatal Stress In A Mouse Model Of Fetal Alcohol Spectrum Disorder

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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## Abstract

Fetal alcohol spectrum disorders (FASD) are caused by prenatal alcohol exposure (PAE) and affect 1-5% of the North American population. Children born with FASD often face maternal separation throughout childhood. How this early life stress (ELS) affects the severity of FASD-related deficits is poorly understood. Using a mouse model, this dissertation establishes that behavioural deficits accumulate following prenatal alcohol exposure and early life stress, assessed using tests for activity, anxiety-like behaviour as well as learning and memory. Hippocampal gene expression was evaluated using RNA-seq followed by clustering of expression profiles through weighted gene co-expression network analysis (WGCNA). A set of transcripts are associated with anxiety-like behaviour ( $r = 0.79$ ,  $p = 0.002$ ) and treatment ( $r = 0.68$ ,  $p = 0.015$ ). Genes in this module are overrepresented by transcriptional regulation and neurodevelopment genes. One member of this module, *Polr2a*, is downregulated by the combination of treatments. Hippocampal promoter DNA methylation was assessed using methylated DNA immunoprecipitation sequencing (MeDIP-Seq). Methylation at different genes is affected by each treatment independently, and a unique set of genes are affected by the combination of treatments. PAE leads to altered promoter DNA methylation at genes important for transcriptional regulation and ELS leads to changes at genes important for histone methylation and immune response. The combination of treatments results in DNA methylation changes at genes important for neuronal migration and immune response. The results from the same samples show that genes with altered expression and promoter methylation are critical in brain development and function. Also, there is minimal complementarity between changes in promoter DNA methylation and gene expression. Mechanisms beyond promoter DNA methylation are likely involved in lasting gene expression changes leading to behavioural deficits seen in FASD. Although further research is required to elucidate the mechanism, the results included may be valuable towards early and reliable diagnosis, together with the development of novel strategies for the amelioration of FASD-related deficits.

## Keywords

Fetal alcohol spectrum disorder, prenatal alcohol, DNA methylation, gene expression, hippocampus, mouse, brain, learning and memory, early life stress, maternal separation

## Summary for Lay Audience

Fetal alcohol spectrum disorders (FASD) result from maternal alcohol consumption during pregnancy. Children born with FASD have a range of behavioural problems that include anxiety and hyperactivity alongside learning and memory deficits. Unfortunately, children born with FASD often face early life stress through maternal separation, various home placements and other adversity. To investigate molecular changes that may explain behavioural problems in children born with FASD, a mouse model was developed. Pregnant mice were exposed to alcohol, then the offspring were subjected to maternal separation as early life stress. Behavioural tests demonstrated FASD-related problems, including anxiety-like behaviour and hyperactivity, as well as learning and memory deficits resulting from each treatment. Behaviours are a result of protein interactions in the brain that are the product of gene expression. Changes in gene expression and promoter DNA methylation (involved in turning genes on or off) were assessed in the hippocampus region of the brain that is critical for behaviour. Gene expression results show that genes involved in immune response and brain maturation are affected by prenatal alcohol exposure and early life stress. Promoter DNA methylation is also affected by prenatal alcohol exposure and early life stress. Interestingly, not all changes in gene expression were directly related to promoter DNA methylation, suggesting the involvement of other regulatory mechanisms in FASD. This research is important for pushing the field of FASD forward by refining an animal model of FASD to include the developmental period after birth. It demonstrates that brain development continues after birth and provides a postnatal window for further negative or positive intervention. Understanding how the still-developing brain responds to the postnatal environment at the molecular level is valuable for the development of potential treatments.

## Co-Authorship Statement

The contents of this thesis contain modified portions of published manuscripts, for which I (BA) was the primary author. I performed or assisted with all aspects of manuscript preparation including experimental design, sample collection and preparation, experimental execution, data analysis, and writing.

Chapter 1 contains material from a published book chapter entitled “Maternal Separation Stress in Fetal Alcohol Spectrum Disorders: A Case of Double Whammy”. This book chapter was published in *Neuroscience of Alcohol* on March 22, 2019. It is co-authored by Shiva M. Singh (SMS). Both BA and SMS made substantial contributions to the concept and design of the work. The authors wish to acknowledge previous Singh lab members working on the FASD mouse project that contributed to the work discussed in this book chapter. BA created the first draft, with both authors substantially revising and approving the published version. This book chapter was published by Academic Press, an imprint of Elsevier © 2019. All rights reserved. Authors are permitted to include contributions in full or in part in a thesis or dissertation for non-commercial purposes.

Chapter 2 contains material from a published manuscript entitled “Developmental and behavioral consequences of early life maternal separation stress in a mouse model of fetal alcohol spectrum disorder”. This manuscript was published in *Behavioural Brain Research* on April 19, 2016. It is co-authored by SMS. Both BA and SMS made substantial contributions to the conception and design of the work. BA raised the mice, performed behaviour assays, and data analysis. The authors wish to acknowledge Yuchen Li, Shruthi Rethi, Ali Pensamiento, and David Seok for contributions in data collection, along with Eric Chater-Diehl and Ben Laufer for contributions to experimental design. BA was responsible for data analysis. Both authors completed data interpretation. BA created the first manuscript draft. Both authors substantially revised it and have approved the published version. This manuscript was published by Elsevier © 2016. Licensed under the Creative Commons CC-BY-NC-ND license.

Chapter 3 contains material from a published manuscript entitled “Hippocampal transcriptome analysis following maternal separation implicates altered RNA processing in a mouse model of fetal alcohol spectrum disorder”. This manuscript was published in the

Journal of Neurodevelopmental Disorders on May 16, 2020. It is co-authored by Christina A. Castellani (CAC) and SMS. BA was responsible for data acquisition. The authors continue to acknowledge Yuchen Li, Shruthi Rethi, Ali Pensamiento, and David Seok for contributions in data collection, together with Eric Chater-Diehl and Ben Laufer for contributions to experimental design. BA and CAC were responsible for data analysis. All authors completed data interpretation. BA created the first manuscript draft. All authors substantially revised it and have approved the published version. The manuscript was published by Springer Nature as open access, Alberry, Castellani, and Singh © 2020. Licensed under a Creative Commons Attribution 4.0 International License (CC BY).

Chapter 4 contains material from a published manuscript entitled “Hippocampal DNA methylation in a mouse model of fetal alcohol spectrum disorder that includes maternal separation stress only partially explains changes in gene expression”. This manuscript was published in *Frontiers in Genetics* on February 27, 2020. It is co-authored by SMS. Both BA and SMS made substantial contributions to the conception and design of the work. BA was responsible for data acquisition and analysis. The authors continue to acknowledge Yuchen Li, Shruthi Rethi, Ali Pensamiento, and David Seok for contributions in data collection, as well as Eric Chater-Diehl and Ben Laufer for contributions to experimental design. Both authors completed data interpretation. BA wrote the first draft of the manuscript. Both authors substantially revised it and have approved the published version. The manuscript was published by Frontiers as open access, Alberry and Singh © 2020. Licensed under the Creative Commons Attribution License (CC BY).

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## List of Abbreviations

ADHD – attention deficit hyperactivity disorder

AMPA – stimulus-dependent  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor

ANOVA – analysis of variance

B6 – C57BL/6 mouse

BDNF – brain-derived neurotrophic factor

BED – browser extensible data format

BLI – binge-Like Injection model of alcohol exposure

C57BL/6 – inbred laboratory mouse strain

CaMKII – Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

CC – control group

cDNA – complementary DNA

ChIP-Seq – chromatin immunoprecipitation sequencing

CPD – continuous preference drinking model of alcohol exposure

CS – control-stress group

CTCF – CCCTC-binding factor

CYP2E1 – cytochrome P450 2E1

DMR – differentially methylated region

DNA – deoxyribonucleic acid

DNAm – DNA methylation

DNMT – DNA methyltransferase

DV – dependent variable

EC – ethanol-control group

ECM – extracellular matrix

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ELS – early life stress

ES – ethanol-stress group

F – females

FASD – fetal alcohol spectrum disorder

FDR – false discovery rate

G0 – gestational day 0

G15 – gestational day 15

GABA – gamma-aminobutyric acid

GE – gene expression

GEO – gene expression omnibus

GFAP  $\alpha$ -glial fibrillary acidic protein

GO – gene ontology

H3K27 – histone 3 lysine 27

H3K27ac – histone 3 lysine 27 acetylation

H3K36me3 – histone 3 lysine 36 trimethylation

H3K4 – histone 3 lysine 4

H3K4me3 – histone 3 lysine 4 trimethylation

H3K9ac – histone 3 lysine 9 acetylation

H4K16ac – histone 4 lysine 16 acetylation

HC – home cage activity test

HDAC – histone deacetylase

IGF – insulin-like growth factor

IV – independent variable

KEGG – Kyoto encyclopedia of genes and genomes

LncRNA – long non-coding RNA

M – males

MACS – model-based analysis for ChIP-Seq

MAPK – mitogen-activated protein kinase

ME – module eigengene

MeDIP-Seq – methylated DNA immunoprecipitation sequencing

miRNA – microRNA

mRNA – messenger RNA

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

OFT – open field test

P10 – postnatal day 10

P14 – postnatal day 14

P2 – postnatal day 2

P21 – postnatal day 21

P25 – postnatal day 25

P3 – postnatal day 3

P34 – postnatal day 34

P50 – postnatal day 50

P70 – postnatal day 70

PAE – prenatal alcohol exposure

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

piRNA – Piwi-interacting RNA

POMC – pro-opiomelanocortin

PPAR – peroxisome proliferator-activated receptor

PRC1 – polycomb repressive complex 1

PTSD – post-traumatic stress disorder

qPCR – real-time (quantitative) polymerase chain reaction

RIN – RNA integrity number

RNA – ribonucleic acid

RNA-seq – RNA sequencing

rRNA – ribosomal RNA

SEM – standard error of the mean

siRNA – short interfering RNA

SRY – sex determining region Y

TCF – T-cell factor

TPM – transcripts per million

WGCNA – weighted gene co-expression network analysis

## Chapter 1. Introduction

### 1.1 Fetal alcohol spectrum disorders are a major societal concern

Maternal consumption of alcohol during pregnancy is associated with a spectrum of adverse clinical outcomes, including stillbirth (Cornman-Homonoff et al., 2012), spontaneous abortion (Kesmodel et al., 2002), premature birth (Sokol et al., 2007), birth defects (O’Leary et al., 2010), and growth restriction (Sabra et al., 2018). Prenatal alcohol exposure (PAE) also causes fetal alcohol spectrum disorders (FASD), a common, heterogeneous set of neurodevelopmental disorders that begin *in utero*, manifest during childhood, and last a lifetime. FASD is characterized by a collection of disorders, including developmental delays, growth restrictions, physical abnormalities, and behavioural deficits such as intellectual impairments (Chudley et al., 2005; Sokol et al., 2003; Streissguth and O’Malley, 2000).

FASD is a serious societal concern, and its prevalence remains high. In Canada, an estimated 10% of pregnant women consume alcohol (Popova et al., 2017), and the prevalence of an FASD diagnosis in Canadian 7- to 9-year-olds is estimated between 2-3% (Popova et al., 2018). In Canada, the prevalence of FASD is 5 to 67 times higher than global estimates (Lange et al., 2017). Similarly, in a cross-sectional study of four communities in the United States, the estimated prevalence of FASD is 1.1-5% (May et al., 2018). Despite societal efforts to raise awareness of the risks, gestational alcohol use persists in North America. This incidence has increased in recent years given that alcohol-related emergency visits have risen 4.4 times more than overall emergency visits in Ontario, Canada between 2003 and 2016 (Myran et al., 2019). Specifically, this increase is greater for women (86.5%) than men (53.2%), with 25- to 29-year-old women showing the highest increase (240%). Of the medical harms identified, suspected fetal damage including fetal alcohol syndrome rose 2133.3% between 2003 and 2016. Given that this concerning trend may extend to populations beyond Ontario, it does not bode well for a focus on FASD prevention via alcohol avoidance.

FASD remains a costly societal burden throughout an affected individual’s lifetime. Individuals with FASD often suffer from poor judgement, are easily distracted, and have difficulty perceiving social cues (Streissguth et al., 1991). Additionally, they often have

poor academic performance, social deficiencies, intellectual impairments, as well as early and repeated delinquency (Fast et al., 1999; Fast and Conry, 2004). Beyond personal consequences, the economic burden of FASD in Canada in 2013, including costs due to productivity losses, the correctional system, and health care, was approximately \$1.8 billion (Popova et al., 2016). FASD diagnosis is challenging as there are no reliable biomarkers nor a cure. Additionally, many mental disorders are comorbid with FASD, with attention-deficit/hyperactivity disorder occurring in 50% of people with FASD (Weyrauch et al., 2017). It will require a concerted effort to prevent or improve the manifestation and work towards amelioration of the detrimental effect of PAE on the developing brain.

## 1.2 Brain development continues postnatally and is influenced by the environment

Mammalian neurodevelopment involves the orchestration of cellular processes that are sensitive to prenatal and postnatal environmental stresses over time (Tau and Peterson, 2010), and may last for over two decades in humans. This period of neurodevelopment is also sensitive to the environment. For example, exposure to early life stress (ELS) via neglect or abuse increases the risk of psychiatric disorders later in life (Kisely et al., 2018). Child maltreatment, including physical abuse, emotional abuse, and neglect is associated with depressive disorders, drug use, suicide attempts, together with sexually transmitted infections and risky sexual behaviour (Norman et al., 2012). More specifically, childhood emotional abuse and neglect are associated with depression, anxiety, and post-traumatic stress disorder (PTSD) in adults (Kisely et al., 2018).

## 1.3 Children with FASD often face unfavourable environments

Children born with FASD are often born in an unstable home environment that may include a variety of stresses, specifically maternal separation. Children with FASD often enter childcare systems such as foster care or orphanages (Lange et al., 2013). Unfortunately, children with PAE often have comorbid prenatal and postnatal risk factors, including drug exposures and nutritional deficiencies. In a Canadian report of children with confirmed PAE, 95% had other prenatal substance exposure, with 61% experiencing deprivation as a failure to meet basic needs before two years old (Lebel et al., 2019). In a study from Washington State, 74% of children with confirmed PAE were not living with their birth

parents, with over 4 out-of-home placements on average per child (Astley Hemingway et al., 2020). In a recent study from one national medical adoption unit in Israel, 20.2% of children had a known history of PAE, and of these just 22.2% had no discernable abnormalities (Tenenbaum et al., 2020). Furthermore, many children that did not have a known PAE history otherwise fit the FASD diagnostic criteria, supporting the notion that FASD is underdiagnosed.

#### 1.4 PAE followed by postnatal stress is most detrimental

Little is known about how early life stress (ELS) negatively impacts children born with FASD (Price et al., 2017). Following PAE and abuse or neglect during early development, children are more likely to have impaired speech (Coggins et al., 2007) as well as behavioural deficits including impaired memory and attention (Henry et al., 2007; Koponen et al., 2009, 2013). More recently, research has highlighted how common mental health problems are in youth born with PAE. While an FASD diagnosis is associated with increased risk for the development of comorbid neurodevelopmental disorders, more adverse childhood events are associated with increased rates of neurodevelopmental disorders particularly for people with FASD, as compared to unaffected controls (Kambeitz et al., 2019). PAE alone does not sufficiently predict the rate of later disorders, while youth with adverse childhood experiences, specifically out-of-home care, have the highest risk of mental health problems (Koponen et al., 2020). Additionally, children with PAE and subsequent postnatal adversity have different brain structures and symptom profiles than children without additional postnatal adversity (Andre et al., 2020). Together, these reports highlight the importance of the postnatal environment in the manifestation of FASD. The postnatal environment may improve or worsen the outcome of a child born with PAE but remains to be comprehensively researched and established. Given the extensive heterogeneity and variability in time and dose of PAE in humans, most FASD research must rely on model organisms. These models have been instrumental in assessing the impact of PAE at the molecular and behavioural levels.

#### 1.5 Molecular models of FASD

No single model captures the human FASD etiology and experience. Yet, given that human FASD experiments are not ethically possible, the research community must make use of

animal models to gain a more mechanistic understanding of what happens following PAE and how it impacts neurodevelopment. Animal models allow researchers to manipulate and control the environment to better assess molecular alterations associated with the clinical manifestations. Experiments designed to evaluate the effect of PAE and ELS during neurodevelopment are described below.

Stem cells are appropriate to investigate molecular mechanisms associated with the effect of alcohol during the early embryonic period. Research using ethanol treatment of stem cells has found increased apoptosis and decreased  $\alpha$ -glial fibrillary acidic protein (GFAP) expression suggesting reduced astrocyte differentiation (Nash et al., 2012), along with changes in gene expression (Vangipuram and Lyman, 2012; Veazey et al., 2013), histone modifications (Veazey et al., 2013), and DNA methylation (Khalid et al., 2014) that also prevent appropriate differentiation (Roitbak et al., 2011; Zhou et al., 2011a).

Nonmammalian systems have been leveraged for their ease of investigating embryonic development. Zebrafish seem ideal to investigate the effect of environmental stressors on embryogenesis since adults are small and easy to maintain, generation time is short, and hundreds of translucent embryos are produced at once. These embryos develop rapidly outside the mother, making them easier to target directly and subsequently observe. Of course, there are numerous differences between zebrafish and humans, but many developmental genes and pathways are conserved between the two species (Kalinka and Tomancak, 2012). In practice, zebrafish have been used to model embryonic alcohol exposure by adding ethanol directly to the water. Embryonic ethanol treatment results in craniofacial, cardiac, and neural defects that resemble those seen in children with FASD (Sarmah et al., 2020). Chicken embryos have also been used to explore embryonic PAE, including the identification of apoptotic neural crest cell death (Cartwright et al., 1998) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) signalling for ethanol-induced apoptosis (Garic et al., 2011).

Rodents have been frequently used as a model for FASD for several key reasons. The wealth of databases and genetic tools available for use makes murine studies accessible and comparable. Particularly for alcohol research, there are strain differences in ethanol preference, with strains like the C57BL/6 willfully consuming up to 70% of their liquid



diet as 10% ethanol by choice during gestation (Kleiber et al., 2011). Following PAE in mice, many physical and behavioural characteristics associated with FASD are observed, including postnatal growth restriction and craniofacial dysmorphism (Kaminen-Ahola et al., 2010b; Kleiber et al., 2011; Marjonen et al., 2015; Schambra et al., 2015). Additionally, researchers have been able to focus on specific brain regions that may be partially responsible for behavioural deficits observed in the models but also in FASD.

## 1.6 Behavioural models of FASD

Hyperactivity, anxiety-like behaviours, alongside learning and memory deficits are the hallmarks of FASD and are regularly assessed in various animal models of FASD. In rodents, increased activity is observed following continued moderate exposure during trimester one-equivalent period (Fish et al., 2016; Mantha et al., 2013; Sanchez Vega et al., 2013), trimester two-equivalent period (Mantha et al., 2013; Muñoz-Villegas et al., 2017), and trimester three-equivalent period (Ieraci and Herrera, 2020; Xu et al., 2018). Interestingly, it seems the hyperactivity phenotype that is prominent in adolescence may diminish or reverse with age (Cantacorps et al., 2018).

While it is challenging to know how anxiety manifests in a non-human animal, several tests have been developed to evaluate deviations from typical behaviours. Zebrafish are social animals that practice shoaling behaviour. Embryonic ethanol exposure results in dose-dependent impairments to this social behaviour in adults (Buske and Gerlai, 2011; Fernandes and Gerlai, 2009; Parker et al., 2014) even after two years (Fernandes et al., 2015b). Similarly, a two-hour embryonic exposure is sufficient to reduce social behaviour assessed four months later (Baggio et al., 2018). In rats, PAE is associated with reduced social preference in females, but not males (Diaz et al., 2020). Similarly, an anxiety phenotype was observed only in young females following early PAE (Lucia et al., 2019), although the reverse has also been shown, with increased anxiety-like behaviour in males, but not females (Wieczorek et al., 2015). Continuous PAE throughout gestation results in an anxiety phenotype in adult mice (Cantacorps et al., 2018; Kleiber et al., 2011).

Deficits in learning and memory are modelled in different species with varied results depending on the type of test used or exposure paradigm. In zebrafish, a two-hour ethanol exposure at 16-hours post-fertilization is enough to impair associative learning (Fernandes

et al., 2014), although the severity of spatial learning deficits increases with dosage (Carvan et al., 2004). In rodents, early PAE results in spatial learning and memory deficits (Fish et al., 2016; Houlé et al., 2017; Mantha et al., 2013), with sex differences depending on the test used (Lucia et al., 2019; Schambra et al., 2017). A binge-like exposure during the trimester three-equivalent period is sufficient for learning and memory impairments in various behaviour tests (Hamilton et al., 2016; Ieraci and Herrera, 2020; Lee et al., 2016; MacIlvane et al., 2016; Mantha et al., 2013). Similarly, continuous PAE in mice results in impaired cognitive control and lower sensitivity to different stimuli in learning and memory tests (Olguin et al., 2020), as well as spatial learning and memory deficits (An and Zhang, 2015; Kleiber et al., 2011). In nonhuman primates, PAE is associated with poor performance on a memory task (Fedorchak and Miller, 2019).

## 1.7 Cellular and molecular perturbations in FASD

Ethanol is a teratogen that crosses the placenta and the blood-brain barrier, disrupting development. Alcohol is metabolized in the brain and liver by two main enzymes, alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) (Koop, 2006). During human gestation, CYP2E1 is first detected in the brain as early as seven weeks (Brzezinski et al., 1999). Metabolism by CYP2E1 generates free radicals, which may target polyunsaturated fatty acid side chains in brain tissue membranes, ultimately resulting in dysfunction (Gemma et al., 2007). This dysfunction regularly manifests in perturbations in apoptosis, brain development and function.

### 1.7.1 Apoptosis

Free radicals produced during alcohol metabolism may lead to uncontrolled apoptosis following DNA damage (Ramachandran et al., 2001). In the brains of ethanol-exposed fish, there is reduced cell proliferation and increased apoptosis (Joya et al., 2014). Furthermore, binge-like trimester three-equivalent PAE in mice is associated with increased expression (Chater-Diehl et al., 2016) along with activation of apoptotic signal protein caspase-3, CC3, *Casp3* (Bird et al., 2018; Shivakumar et al., 2020), expression of caspase 6, *Casp6* (Schaffner et al., 2020), and increased apoptosis in the somatosensory cortex and several regions of the hippocampus (Camargo Moreno et al., 2017). In human brain organoids, ethanol exposure results in premature differentiation and apoptosis (Zhu et al., 2017). Thus,

it is not surprising that research has directly linked p53 signalling following ethanol exposure to the resulting apoptosis (Camargo Moreno et al., 2017; Yuan et al., 2017). Finally, following brief alcohol exposure in non-human primates, there is widespread neuro-apoptosis (Farber et al., 2010), and oligodendrocyte apoptosis (Creeley et al., 2013).

### 1.7.2 Brain development

Many studies have investigated structural alterations alongside changes in messenger RNA (mRNA) and protein expression following PAE. There are frequent alterations to genes important for brain development including cell proliferation, neurogenesis, cell migration, adhesion, and synaptogenesis.

Beginning with zebrafish models, embryonic ethanol exposure leads to a reduction in the number of differentiated neurons, sensory neurons, and the length of motoneuron axons (Joya et al., 2014). Ethanol also inhibits cell-cell adhesion through altered expression and function of cell adhesion molecules (Charness et al., 1994; Miñana et al., 2000; Wilkemeyer et al., 1999). Impaired cell adhesion and migration following embryonic ethanol exposure are associated with reduced expression of the cell adhesion gene protocadherin-18a, *pcdh18a* (Sarmah et al., 2013). Embryonic ethanol exposure leading to gastrulation defects is associated with reduced expression of the pluripotency factor sex determining region Y (SRY)-box 2, *sox2* (Sarmah et al., 2020).

In mouse models, PAE is associated with reduced expression of homeobox A1, *Hoxa1* (Wang et al., 2009), as well as cortical thinning, impaired neuroepithelial proliferation, neuronal migration, and neuron maturity (Öztürk et al., 2017). Binge-like trimester three-equivalent PAE is associated with decreased expression of synaptic plasticity genes including Finkel-Biskis-Jenkins (FBJ) osteosarcoma oncogene, *Fos*, early growth response 1, *Egr1*, and activity regulated cytoskeletal-associated protein, *Arc* (Shivakumar et al., 2020), alongside fewer hippocampal interneurons (Bird et al., 2018). Ten days of PAE in mice is enough for the observation of fewer immature neurons in the male hippocampus (Olateju et al., 2018). Impaired hippocampal neurogenesis is associated with PAE in a nonhuman primate model (Fedorchak and Miller, 2019). Further, Wnt signalling, important for brain development and adult brain function (Oliva et al., 2013), is often dysregulated following PAE. Specifically, dysregulation of the Wnt transcription factor 7

like 2, *Tcf7l2*, has been reported in four different FASD models (Berres et al., 2017; Chater-Diehl et al., 2016; Kleiber et al., 2014; Zhou et al., 2011b).

### 1.7.3 Brain function

FASD-related phenotypes observed across animal models are often associated with changes in corresponding gene expression in the brain or specific regions. Interestingly, while thousands of genes are differentially expressed immediately following ethanol exposure, few of these genes remain differentially expressed just one day later (Boschen et al., 2020), indicating that lasting changes may be in response to molecular changes initiated by alcohol exposure.

In zebrafish, changes in social behaviour are accompanied by changes in serotonin and oxytocin receptor expression (Parker et al., 2014), together with reduced dopamine production and metabolism with a social stimulus (Fernandes et al., 2015a). Additionally, brain glutamate uptake is reduced following embryonic ethanol exposure (Baggio et al., 2017).

In rodent models, PAE consistently leads to changes in brain gene expression. In the rat hypothalamus, PAE is associated with increased inflammatory gene expression in microglia and more microglia present (Chastain et al., 2019). In the orbitofrontal cortex, frequently associated with executive control, there are sex differences in the number and function of gamma-aminobutyric acid (GABA)-ergic interneurons in response to moderate PAE (Kenton et al., 2020). In the pituitary of females following PAE, there is decreased dopamine D2 receptor mRNA and protein levels (Gangisetty et al., 2015). Increased risk-taking behaviour is associated with increased dopamine in the nucleus accumbens following PAE (Muñoz-Villegas et al., 2017). In the hippocampus, hyperactivity and learning and memory impairments following PAE are associated with reduced N-methyl-D-aspartate (NMDA)-R2B and increased brain-derived neurotrophic factor (BDNF) protein levels in the hippocampus (Ieraci and Herrera, 2020). Conversely, early PAE results in increased mRNA expression of *Bdnf*, *Grin2a*, and *Grin2b* (Lucia et al., 2019). Also, binge-like trimester three-equivalent exposure leads to decreased dendritic complexity (Boschen et al., 2017).

Beyond typical brain functions, immune response in the brain has been implicated in several studies modelling FASD. Continuous PAE in mice results in increased serum, hippocampal, and prefrontal cortex cytokines (Bodnar et al., 2016), as well as increased levels of innate immune signal proteins and receptors – TLR4, TLR2, NFkB/p65, NLRP3, Caspase 1, IL-1B – depending on duration and brain region assayed (Cantacorps et al., 2017). In the hypothalamus, there is increased inflammatory gene expression and more microglia present following trimester one-equivalent PAE in rats (Chastain et al., 2019). Early life adversity reduces serum cytokine levels in controls, but not following PAE in rats (Rainekei et al., 2017). Also, serum levels of C-reactive protein, a marker of inflammation, are higher following PAE and higher still with the combination of PAE and ELS. In the amygdala, PAE reduced chemokine (C-X-C motif) ligand 1, CXCL1, and interleukin 10, IL-10, further emphasizing the potential role of the immune response in FASD (Rainekei et al., 2017). In a rat model that includes postnatal environmental stress, only the combination of PAE and maternal separation results in decreased allopregnanolone, a neuroactive steroid (Biggio et al., 2018). While foot shock stress increases plasma allopregnanolone and corticosterone, this response is exaggerated in animals following PAE and ELS (Biggio et al., 2018).

## 1.8 Epigenetics and FASD

The epigenome regulates gene expression, and its tight control is essential for typical development. The epigenome is one way the internal or external environment, either prenatally or postnatally, can interact with the genome, affect gene expression, and ultimately lead to changes in neurodevelopment and behaviour. Importantly, many changes in gene expression following PAE involve genes with crucial epigenetic roles, including proteins important for reading and writing epigenetic marks. Included in the scope of the epigenome are noncoding RNAs that interfere with the translation of mRNA into proteins, histone modifications and DNA methylation that alter chromatin structure to regulate transcription.

### 1.8.1 Noncoding RNA

Cellular phenotypic outcomes are often the result of inter-related epigenetic mechanisms. For example, noncoding RNAs are important for gene expression regulation at the

transcriptional and post-transcriptional levels. They include long non-coding RNAs (lncRNAs) (Lee, 2012), microRNAs (miRNAs) (Chuang and Jones, 2007), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) (Holoch and Moazed, 2015). In FASD research, the noncoding RNA research has been focused on the relationship between microRNAs and their mRNA gene targets. Following PAE, several miRNAs and their predicted mRNA targets are reciprocally differentially expressed in mice (Laufer et al., 2013; Wang et al., 2009). Similarly, in a trimester one-equivalent exposure model, microRNAs important for cell state and dendritic spines in the hippocampus are differentially expressed following PAE (Marjonen et al., 2015). In a rat model, third trimester-equivalent exposure results in increased variance in miRNA expression and increased expression of *miR-200c*, a miRNA important for neurogenesis (Balaraman et al., 2017).

### 1.8.2 Histone modifications

Histone modifications are another strong candidate for the mechanism of action following PAE. Modifications to histone tails at specific genomic loci are the foundation of epigenetic mechanisms, with a wide variety of known potential modifications (Allis and Jenuwein, 2016). While modifications can include methylation that is dependent on the same methyl source as DNA methylation (discussed below), histone acetylation is another common modification. In pregnant mice, exposure to isotope-labelled ethanol results in labelled acetyl groups being incorporated as histone acetylation in the gestating fetal brains (Mews et al., 2019).

PAE is often associated with altered expression of histone modifiers, including deacetylases (HDACs) and methyltransferases, and genome-wide changes in the abundance of modifications. Early embryonic PAE leads to increased *Hdac2* expression in rat hippocampus (Lucia et al., 2019), and continuous PAE results in its increase in female pituitary along with *Hdac4* and *G9a* (Gangisetty et al., 2015). Binge-like trimester three-equivalent PAE in rats leads to increased H3 lysine 9 acetylation (H3K9ac) and decreased protein levels of HDAC1 and SIRT1 in the hypothalamus (Chastain et al., 2019), alongside changes in other modifications that may correlate with the phenotype (Veazey et al., 2015). In mice, PAE is associated with increased H3 lysine 4 trimethylation (H3K4me3) levels

and increased expression of the chromatin modification gene lysine (K)-specific methyltransferase 2E, *Kmt2e* (Schaffner et al., 2020). It also leads to increased levels of chromatin modifiers, including histone deacetylases (HDAC1-3), and reduced histone 3 (H3) and 4 (H4) acetylation in the hippocampus and neocortex (Shivakumar et al., 2020). Further, decreased hippocampal and temporal lobe ependyma H3K36me3 has been observed in a nonhuman primate model of FASD (Jarmasz et al., 2019). In human post-mortem brain samples, PAE is associated with region-specific decreases in H3K4me3, H3K9ac, H3K27ac, H4K12ac, and H4K16ac (Jarmasz et al., 2019).

Additionally, altered histone modifications at specific genes are regularly reported in the literature. In a neurosphere cell culture model, Homeobox genes important for neurogenesis have altered histone H3K4 or H3K27 trimethylation following ethanol treatment (Veazey et al., 2013). While there are often changes in histone modifications at specific genes, these do not regularly translate into altered expression (Veazey et al., 2017). Following binge-like trimester three-equivalent exposure, many genes have been implicated by regional differences in H3K4 and H3K27 methylation, particularly increased H3K4 methylation and decreased H3K27 methylation (Chater-Diehl et al., 2016).

### 1.8.3 DNA methylation

DNA methylation is a strong candidate for a biological mechanism of action for PAE because ethanol impairs folate transport to the developing fetus (Hutson et al., 2012), a key methyl donor important for DNA methylation. PAE in a mouse model results in changes in expression of the DNA methylation-dependent metastable epiallele, *Agouti viable yellow* ( $A^{vy}$ ), indicating that DNA methylation changes occur after PAE (Kaminen-Ahola et al., 2010b).

In various studies, PAE alters the expression of DNA methyltransferases. In a rat model, PAE results in increased gene expression of DNA methyltransferases (DNMTs), *Dnmt1* and *Dnmt3a*, alongside increased DNMT activity (Perkins et al., 2013). In a murine cell culture model, there is a dose-dependent increase in *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* expression, with increased DNMT3A protein abundance (Miozzo et al., 2018). While others have found dose-dependent decreased expression of *Dnmt1* (Veazey et al., 2017). Further, increased gene expression of *Dnmt1* and *Dnmt3a* in male rat hippocampus

following early PAE (Lucia et al., 2019) or continuous PAE (Gangisetty et al., 2015) has been observed.

Other DNA methylation genes have been implicated in FASD. In rodent models, PAE results in increased methyl CpG binding protein 2, *Mecp2*, gene expression (Gangisetty et al., 2015; Perkins et al., 2013), and protein level (Chastain et al., 2019; Öztürk et al., 2017). In a murine cell culture model, increased expression of DNA demethylation genes *Tet1* and *Tet2* has been observed following ethanol treatment (Veazey et al., 2017).

Several studies regarding DNA methylation have focused attention on a specific imprinted locus in the genome, insulin-like growth factor 2, IGF2/H19. H19 is a long noncoding RNA only transcribed from the maternal allele, while the paternal H19 is normally methylated at the promoter and not expressed. Paternal H19 allele methylation allows for the expression of paternal IGF2, which is not typically maternally expressed (DeChiara et al., 1991). Interestingly, DNA methylation at this locus following PAE in mice has been reported to be both increased (Laufer et al., 2013) and decreased (Haycock and Ramsay, 2009), along with decreased in human buccal swabs (Portales-Casamar et al., 2016). Furthermore, a polymorphism at this locus is associated with placental DNA methylation and head circumference following PAE (Marjonen et al., 2017).

Gene promoter DNA methylation changes following PAE have been reported in several models. Following ethanol treatment of rat neural stem cells, there is promoter hypermethylation of the chromatin remodelling complex member SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2, *Smarca2*, together with adhesion and polarity genes DiGeorge syndrome critical region gene 2, *Dgcr2*, and Par-6 family cell polarity regulator alpha, *Pard6a* (Zhou et al., 2011a). Conversely, the transcription factors cut like homeobox 2, *Cux2*, POU class 4 homeobox 3, *Pou4f3*, and SRY-box transcription factor 7, *Sox7*, are each hypomethylated following ethanol treatment (Zhou et al., 2011a). In a trimester one-equivalent model, there are corresponding changes in DNA methylation status at candidate genes with altered gene expression in the hippocampus, but also in peripheral tissue (Marjonen et al., 2015). Similarly, decreased hypothalamic *Slc6a4* mRNA following PAE was accompanied by increased promoter methylation (Ngai et al., 2015), and increased *Slc17a6* alongside



decreased promoter methylation (Zhang et al., 2015). Binge-like trimester three-equivalent exposure results in altered promoter DNA methylation, particularly for oxidative stress and peroxisome biogenesis genes (Chater-Diehl et al., 2016). Interestingly, the Wnt transcription factor *Tcf7l2* mentioned previously as differentially expressed also has complementary changes in DNA methylation and histone modifications in this model (Chater-Diehl et al., 2016, 2018). These results suggest changes in gene-specific expression in response to PAE may be the result of a variety of epigenetic mechanisms. Although DNA methylation, non-coding RNAs, and histone modifications have all been implicated, their inter-relationship is not known.

Global changes in DNA methylation following PAE have repeatedly been reported in rodents (Laufer et al., 2013; Liu et al., 2009; Lussier et al., 2018a; Öztürk et al., 2017), human embryonic stem cells (Khalid et al., 2014), and nonhuman primates (Jarmasz et al., 2019). In human post-mortem brain, reduced DNA methylation in the CA1 region of the hippocampus is associated with PAE (Jarmasz et al., 2019). The research aimed at finding a specific DNA methylation signature in peripheral tissue for FASD has had limited success (Cobben et al., 2019; Laufer et al., 2015; Lussier et al., 2018b; Portales-Casamar et al., 2016). While not yet specific or accurate enough for diagnosis, it suggests the potential for biomarker discovery. As detection technology and knowledge of DNA methylation states and cofactors improve, there will be improvements in biomarker detections and precision.

## 1.9 Refining a mouse model of FASD

To further elaborate on these processes, research from the Singh laboratory has used two different neurodevelopmental alcohol exposure paradigms in C57BL/6 (B6) mice. First, in the Continuous Preference Drinking (CPD) model pregnant mice consume moderate amounts of 10% alcohol (Kleiber et al., 2011). Consumption is monitored daily with two accessible bottles containing either water or 10% alcohol solution for each pregnant female. The resulting pups have neurodevelopmental and behavioural deficits that persist into adulthood (**Table 1.1**) (Kleiber et al., 2011). To gain further insight, an assessment of gene expression in the adult whole brain found many alterations (Kleiber et al., 2012), which allowed for modelling of gene networks that may contribute to the manifestation of FASD-associated aberrations (**Table 1.2**).

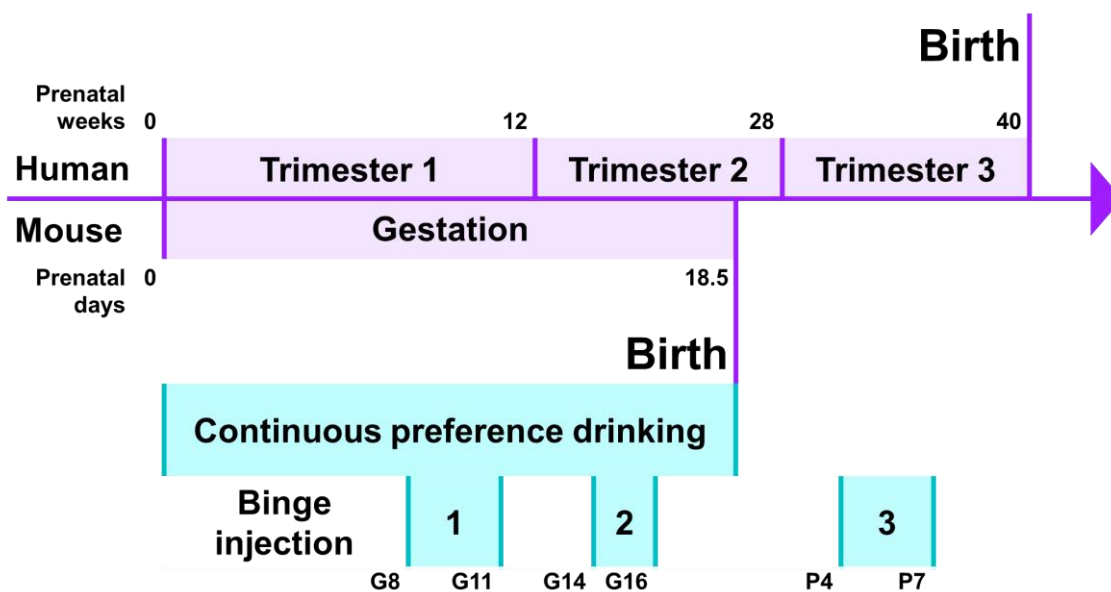
**Table 1.1.** Phenotypic characterization following neurodevelopmental alcohol exposure in mice.

Exposure Model	Phenotype	Reference
Continuous Preference Drinking (CPD)	<ul style="list-style-type: none"><li>• Delayed achievement of developmental milestones</li><li>• Reduced activity in a novel open field and a home environment</li><li>• Impaired learning of target in Barnes maze test for learning &amp; memory</li></ul>	(Kleiber et al., 2011)
Binge-like injection (Trimester 1 equivalent)	<ul style="list-style-type: none"><li>• Delayed achievement of developmental milestones</li><li>• Increased activity in a novel open field and a home environment</li><li>• Impaired learning of target in Barnes maze test for learning &amp; memory</li></ul>	(Mantha et al., 2013)
Binge-like injection (Trimester 2 equivalent)	<ul style="list-style-type: none"><li>• Delayed achievement of developmental milestones</li><li>• Increased activity in a novel open field environment</li><li>• Increased time spent in the centre of open field</li><li>• Increased time spent in the light area of light/dark box</li><li>• Impaired learning and memory of target in Barnes maze test for learning &amp; memory</li></ul>	(Mantha et al., 2013)
Binge-like injection (Trimester 3 equivalent)	<ul style="list-style-type: none"><li>• Delayed achievement of developmental milestones</li><li>• Increased activity in a home environment</li><li>• Reduced time spent in the centre of the open field (anxiety-like behaviour)</li><li>• Impaired learning and memory of target in Barnes maze test for learning &amp; memory</li></ul>	(Mantha et al., 2013)

**Table 1.2.** Gene expression characterization following neurodevelopmental alcohol exposure in mice.

Model (tissue)	Top affected pathways	Reference
Continuous preference drinking (whole brain)	Cellular development, tissue development, embryonic development; Free radical scavenging, cellular growth & proliferation; Lipid metabolism, small molecule biochemistry, vitamin & mineral metabolism	(Kleiber et al., 2012)
Binge-like injection trimester 1 (whole brain)	Endoplasmic reticulum stress pathway; Xenobiotic metabolism signalling; Glucocorticoid receptor signalling; Phospholipase C signalling; Antiproliferative role of somatostatin receptor 2	(Kleiber et al., 2013)
Binge-like injection trimester 2 (whole brain)	Fatty acid biosynthesis; Serotonin receptor signalling; Regulation of actin-based motility by Rho	(Kleiber et al., 2013)
Binge-like injection trimester 3 (whole brain)	Glutamate receptor signalling; Retinoic acid-mediated apoptosis signalling; Ephrin receptor signalling; Circadian rhythm signalling; One carbon pool by folate	(Kleiber et al., 2013)
Binge-like injection trimester 3 (hippocampus)	Olfactory transduction; Colorectal cancer; Free radical scavenging, gene expression, dermatological diseases & conditions; Cellular development, developmental disorder, hereditary disorder	(Chater-Diehl et al., 2016)

Alternatively, the Binge-Like Injection (BLI) model uses alcohol injections during different developmental stages, matching the first, second, and third trimesters of human pregnancy (**Figure 1.1**). Using this model, the nature and extent of behavioural deficits observed were determined to be dependent on timing (**Table 1.1**) (Mantha et al., 2013). Further, adult gene expression differences are also dependent on timing (Kleiber et al., 2013, 2014; Mantha et al., 2014; Stringer et al., 2013). These results show subtle, reliable, and relevant changes in genome-wide gene expression. Genes affected in each study have been implicated in cognitive dysfunction, anxiety, hyperactivity, and mood disorders – phenotypes shared by individuals diagnosed with FASD. Predominantly, early alcohol exposure during the human trimester one equivalent in mice affects cell proliferation genes; cell migration and differentiation by exposure in the human trimester two equivalent; and cellular communication and neurotransmission by exposure during the human trimester three equivalent (Kleiber et al., 2013). Hub genes connect networks and pathways identified for each model, providing commonality in FASD gene expression features, and establishing a lifelong gene expression footprint left by alcohol exposure (**Table 1.2**).



**Figure 1.1.** Methods and timing of neurodevelopmental alcohol exposure used in the mouse model of FASD. G indicates gestational days and P indicates postnatal days, aligned to human trimesters one, two, and three based on hallmarks of neurodevelopment.

In both models, profound changes in whole-brain DNA methylation and non-coding RNA (ncRNA) expression may underlie lasting changes in gene expression and behaviour (Laufer et al., 2013). Epigenetic changes were assessed in the BLI model in the adult hippocampus, revealing altered gene expression, DNA methylation, and histone methylation (Chater-Diehl et al., 2016). These results argue that FASD is an epigenetic disorder. Prenatal alcohol exposure leaves long-lasting alterations to DNA methylation as a footprint on select gene promoters, often within 50 of approximately 100 imprinted regions of the genome (Laufer et al., 2013). This effect is not random and repeated under different treatment protocols. It affects imprinted regions harbouring sequences interacting with regulatory proteins and ncRNAs. It also affects promoters of major network nodes, such as Pten signalling, that contain transcription repressor CTCF-binding sites and affect cytosine methylation. We conclude that alcohol serves as a potent methylation modifier during prenatal development. This change involves specific sites, genes, and pathways connecting prenatal alcohol exposure to FASD phenotypes.

**Table 1.3.** Observed epigenetic features following neurodevelopmental alcohol exposure in mice.

Molecular feature	Model (tissue)	Top affected pathways	Reference
DNA methylation	Continuous preference drinking (whole brain)	Cdk5 signalling; Pten signalling; Behaviour, neurological disease, & psychological disorders	(Laufer et al., 2013)
	Binge-like injection trimester 3 (hippocampus)	Cellular Movement, Cell Death & Survival, Cellular Development; Cell Cycle, Cellular Development, Cellular Growth & Proliferation; Peroxisome; Lysosome	(Chater-Diehl et al., 2016)
Histone modification: H3K27me3	Binge-like injection trimester 3 (hippocampus)	Endocrine System Development & Function, Molecular Transport, Protein Synthesis; Cancer, Skeletal & Muscular Disorders, Tissue Morphology; Cellular Function & Maintenance, Inflammatory Response, Hematological System Development; MTOR signalling pathway	(Chater-Diehl et al., 2016)
Histone modification: H3K4me3	Binge-like injection trimester 3 (hippocampus)	Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry; Regulation of cellular mechanics by calpain protease; Fatty acid $\beta$ -oxidation; Pathways in cancer; Fatty acid metabolism	(Chater-Diehl et al., 2016)

The literature summarized above argues that the selection of the model used must be based on the specific research objective. The research presented in this thesis is unique in that it combines the impact of PAE and ELS to assess resulting behavioural and molecular changes. Specifically, this study is aimed at assessing brain gene expression changes and the promoter DNA methylation changes that may account for them. To meet the goals outlined below, the continuous preference drinking (CPD) B6 mouse model of FASD was selected (Kleiber et al., 2011).

### 1.10 Hypothesis

Early life stress via maternal separation augments negative behavioural impacts of prenatal alcohol exposure in a mouse model of FASD that are reflected in lasting alterations in hippocampal gene expression and altered promoter DNA methylation.

### 1.11 Objectives

1. Characterize phenotypic deficits resulting from PAE and ELS, including:
  - a. Pup weight and survival
  - b. Activity in a novel environment
  - c. Anxiety-like behaviour in a novel environment
  - d. Activity in a familiar, home environment
  - e. Spatial learning and memory
2. Assess underlying hippocampal transcriptomic changes that may account for phenotypic deficits following PAE and ELS
  - a. Use a genome-wide method (RNA-Seq) for the examination of alterations to the full transcriptome
    - i. Characterize co-expression networks of transcripts in association with treatments and phenotypic information (weighted gene co-expression network analysis)



- b. Use a gene-specific method (reverse transcription qPCR) to validate specific changes identified by the genome-wide method
3. Investigate potential DNA methylation changes that may underly transcriptomic alterations following PAE and ELS
  - a. Use a genome-wide promoter DNA methylation method (MeDIP-Seq) to examine alterations to the promoter methylome
  - b. Investigate the overlap between genes implicated by promoter methylation and transcriptomic changes that may explain phenotypic deficits

## 1.12 Thesis Organization

Following this introductory chapter that outlines the problem, hypothesis and objectives, the results of this thesis are organized into three published data chapters. Chapter 2 details developmental and behavioural alterations following PAE and ELS (Alberry and Singh, 2016). Chapter 3 presents the analysis of the consequences of PAE and ELS on the adult hippocampal transcriptome (Alberry et al., 2020). Chapter 4 describes changes to promoter DNA methylation in the adult hippocampus following each treatment and relates the results to those reported in previous chapters (Alberry and Singh, 2020). Finally, the results are interpreted and discussed in the context of published literature and implications in Chapter 5, followed by concluding remarks.

## Chapter 2. Phenotypic consequences of PAE and ELS in a mouse model of FASD

### 2.1 Abstract

FASD is characterized by various behavioural deficits that are highly heterogeneous in their presentation. The postnatal environment may contribute to this heterogeneity as children with FASD are often raised in suboptimal conditions, including maternal separation stress. In this chapter, the phenotypic results are presented following maternal separation in a mouse model of FASD. The model uses the combination of PAE via maternal preference consumption of 10% ethanol in water with ELS via daily 3-hour maternal separation and isolation. The results presented in this chapter focus on development and behavioural features, including activity, anxiety-like behaviour, as well as learning and memory. PAE influenced the number of pups surviving to postnatal day 2 and 70, with fewer surviving pups associated with the severity of ethanol exposure. PAE and ELS both had effects on pup weight at postnatal day 21, with the amount of ethanol exposure positively correlating with pup weight. Females were more active than males in a novel open field environment, but not following PAE. Also, PAE results in overall increased exploratory behaviour in the open field. Further, PAE and ELS both result in overnight hypoactivity in a home cage environment, alongside learning deficits that were influenced by sex in the Barnes maze for learning and memory. These results are attributed to environmental interactions involving PAE and ELS.

## 2.2 Background

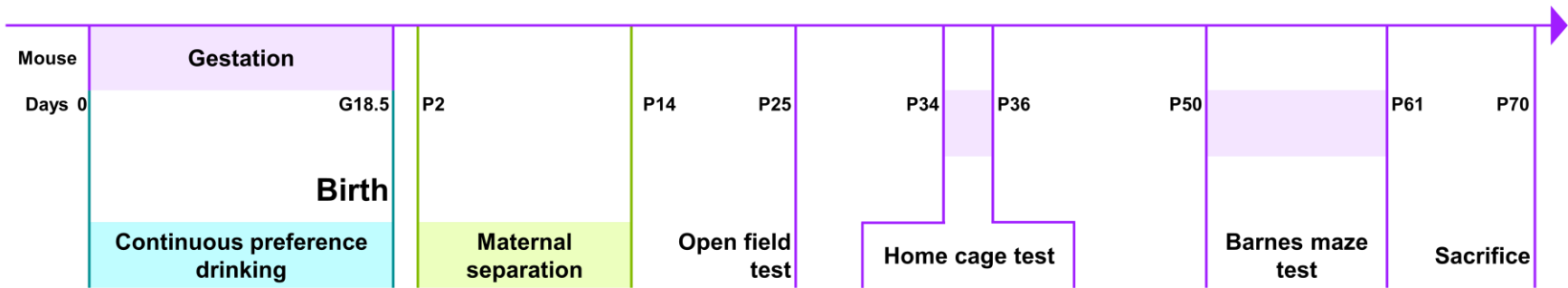
The role of early postnatal stresses on the manifestation of FASD-associated behavioural deficits has not been adequately explored. This chapter hypothesizes that postnatal stress may compound the deficits in individuals born with FASD. Further, comprehensive studies on this hypothesis are now feasible with suitable animal models.

C57BL/6J (B6) mice voluntarily consume high, biologically relevant volumes of ethanol when given free access to 10% ethanol in water, even while pregnant (Kleiber et al., 2011; Middaugh et al., 1999). The pups prenatally exposed to ethanol develop learning deficits, anxiety-like behaviours, and changes in activity (Allan et al., 2003; Kaminen-Ahola et al., 2010a; Kleiber et al., 2011; Marjonen et al., 2015). The degree of these deficits is variable and may depend on additional factors. One such factor is early life stress during development that also affects adult behaviours (Anisman et al., 2008). In particular, ELS introduced by maternal separation and isolation in mice may lead to increased anxiety-like behaviours in adults (Romeo et al., 2003). Mouse models adapted from rat models of maternal separation have found the first 10 postnatal days to be the most critical period for stress, likely due to the hippocampal plasticity at this time (Fenoglio et al., 2006). From this, mouse models of various forms of separation have focused on postnatal days 1-14 (Franklin et al., 2010; Veenema et al., 2008). While this time corresponds to the prenatal brain growth spurt in humans that occurs during the third trimester (Dobbing and Sands, 1979), this model best mimics the early life deprivation that may be mirrored in humans. Mice beyond postnatal day 14 are independently mobile, and increasingly less dependent on maternal care, having reached many developmental milestones. This research sought to model the period of early life when humans are most dependent on an external source of care. The postnatal environment has the potential to affect adult behaviour in cases of fetal alcohol exposures, supporting a multifactorial model for FASD that must include postnatal stresses. The most realistic mouse model for behavioural outcomes in FASD should include the additional postnatal environment, particularly maternal separation stress often encountered during the development of children born with FASD. Such results are not available in the literature and form the focus of this research. Specifically, the effect of early-life maternal separation stress on the development and behavioural outcome in adults resulting from maternal ethanol consumption during pregnancy in mice is presented here.

## 2.3 Methods & materials

### 2.3.1 Animals

Male and female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and subsequently maintained and bred in the Animal Care Facility at Western University. Before breeding, mice were housed in same-sex colonies of up to four individuals per colony, with *ad libitum* access to food and water. Cage, bedding, and nest material were standard between cages. Colony rooms were maintained on a 14/10-hour light/dark cycle, humidity between 40-60%, and ambient temperature 21-24°C. All protocols complied with ethical standards established by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee at Western University (London, Ontario, Canada)(**Appendix A**). A visual timeline summary of treatments and tests is represented in **Figure 2.1**.



**Figure 2.1.** Developmental timeline of treatments and testing procedures in the FASD mouse model.

### 2.3.2 Continuous preference drinking

Female mice approximately ten weeks of age were individually housed and randomly assigned to one of two groups: control dams with free access to water only, or ethanol consumption dams with free access to both water plus a 10% ethanol in water solution in a model of exposure referred to as the Continuous Preference Drinking Model (CPD) (Kleiber et al., 2011). The ethanol group females were introduced to ethanol in a stepwise manner with increasing concentrations available from 2%, 5%, and finally 10%, each introduced after 48 hours at the previous concentration. Briefly, each mouse had free access to either water alone, or 10% ethanol in water solution and water in modified serological pipettes with fitted 2.5-inch stainless steel sipper tubes (**Figure 2.2**). Daily liquid consumption was monitored as previously described (Kleiber et al., 2011; Treadwell et al., 2004; Weng et al., 2009). The volume of liquid consumed from each tube was recorded daily, and tube position was alternated each second day to minimize position effects.

After 10 days of 10% ethanol availability, 12-week-old female mice were mated overnight with 15-week-old B6 males. During mating, only water was available to the breeding pair to avoid male ethanol consumption. Removal of males after 24 hours represented gestational day 0 (G0). Voluntary ethanol consumption by pregnant females was recorded from G0 to postnatal day 10 (P10) when ethanol was removed and only water was provided to all mice. The weight of each pregnant female was monitored every other day. While blood alcohol levels were not taken to minimize maternal stress, voluntary consumption of 10% ethanol at 14g ethanol/kg body weight per day has been shown to produce peak blood alcohol levels of 120mg/dl (Allan et al., 2003). Experimental mice consumed an average of 8g ethanol/kg body weight per day, representing a modest level of ethanol exposure.

### 2.3.3 Early life stress via maternal separation & isolation

Postnatal maternal separation was introduced as previously described (Benner et al., 2014; Savignac et al., 2011). Briefly, half of the pups (2-4 pups/litter) were randomly selected for maternal separation on postnatal day 2 (P2). They were removed from the nest and isolated for 3 hours/day during the light phase from 10:00 – 13:00 each day up to and including postnatal day 14 (P14). Tail colouring with permanent markers was used to distinguish pups undergoing separation. During separation, pups were isolated in 8 oz. Dixie cups with

bedding and nest material in individual cages. The pups not selected for separation remained with the dam and other littermates during this time. Following the separation period, pups were weaned at postnatal day 21 (P21) and housed with same-sex littermates, males (M) or females (F), in cages of 2-4 individuals. Following this, experimental mice belonged to one of eight groups based on sex, PAE, and ELS: control males and females, ethanol males and females, stress males and females, and finally ethanol + stress males and females.



**Figure 2.2.** Animal treatment apparatuses for (A) prenatal alcohol exposure and (B) early life stress.

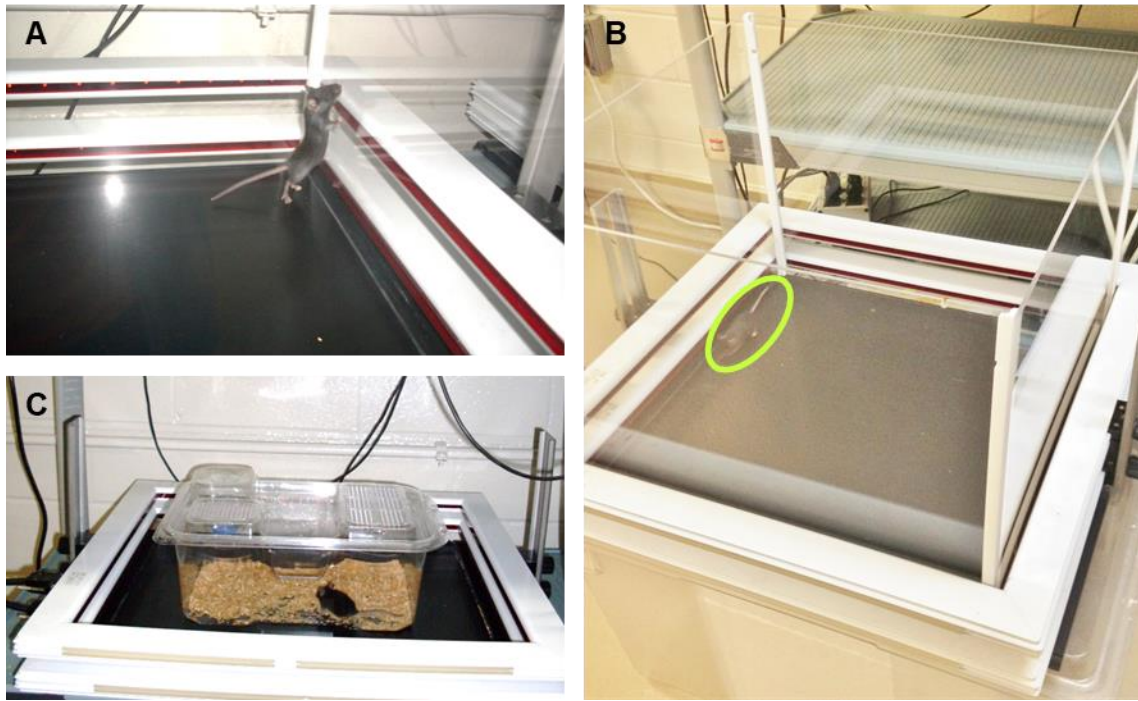


#### 2.3.4 Open-field locomotor test for activity & anxiety

Activity in a novel open-field environment was assessed on postnatal day 25 (P25) mice using the infrared Actimeter system, measured using Acti-Track software (Panlab, Barcelona, Spain) (Kleiber et al., 2011). This age was selected as it corresponds to the prepubescent period of development in mice (Clarkson and Herbison, 2006), which is a developmental time in humans often examined in hyperactive behaviour disorders (Langleben et al., 2002). Also, previous reports using the CPD model of PAE at this time found differences in activity between ethanol and control mice (Kleiber et al., 2011). The open-field arena consists of a black plexiglass surface (45 x 45 cm) enclosed by four clear acrylic walls (35 cm high), surrounded by two infrared frames that each produces a 16x16 grid of intersecting beams to track mouse movement in two parallel planes (**Figure 2.3 A, B**). Locomotor activity was calculated from infrared beam break data, including the number of rears onto hind limbs using the dual planes. This test also allows for the assessment of anxiety-like behaviour by considering the centre zone of the open field independently. Mice that are less active in the centre zone, spend less time in the centre zone, enter the centre zone less often, or take longer to initially explore the centre zone display anxiety-like traits. Anxiety-like behaviour was measured as the proportion of time spent within the inner central zone (22.5 x 22.5 cm) as compared to the outer periphery zone (11.25 cm from walls). Testing was completed within 3-5 hours after the light cycle began. For each test, a mouse was placed in the same corner of the area and allowed to freely explore for 15 minutes, during which time the experimenter left the room and returned after the test period was completed and the mouse was returned to its home cage. Between each test, the surface and walls of the arena were cleaned with 30% isopropanol.

### 2.3.5 Home cage activity test

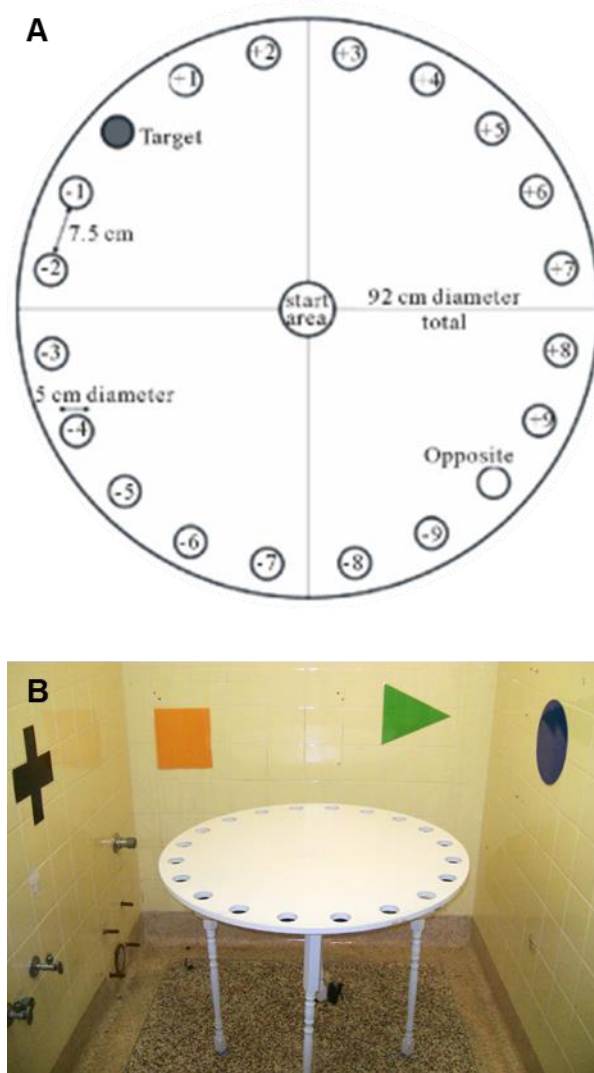
Activity in a familiar environment beginning on postnatal day 34 (P34) was evaluated using the infrared Actimeter system and the Acti-Track Software (Panlab, Barcelona, Spain). Each mouse was individually housed in a transparent plastic cage (34 cm (L) x 24 cm (W) x 14 cm (H), Innovive, San Diego, CA, USA) with standard bedding and *ad libitum* access to food and water (**Figure 2.3 C**). Following a 24-hour acclimation period, cages were placed on a black plexiglass surface (45 x 45 cm) within an infrared frame 8 hours before testing began. Infrared beam breaks were recorded over 12 hours, spanning an entire dark phase and including one hour of light at the beginning and end. Recordings were taken over two consecutive nights and averaged to obtain data for locomotor activity, distance travelled, speed, and the number of rears. Following the second night of testing, mice were returned to their original cages.



**Figure 2.3.** Infrared Actimeter testing apparatus for the open field test (A, B) and the home cage activity test (C). The test subject is circled in green in panel B.

### 2.3.6 Barnes maze test for learning & memory

A modified version of the Barnes Maze (Sunyer et al., 2007) for mice was constructed and conducted as previously described (Kleiber et al., 2011). Briefly, the testing apparatus consisted of a white circular platform (92 cm diameter, raised 75 cm from the floor) with 20 holes (5 cm in diameter) each spaced evenly around the periphery (2 cm from the edge of the platform). Each hole was blocked from the bottom except one serving as the target hole, under which a black escape box (13.5 cm (L) x 13.5 cm (W) x 5 cm (H)) was placed, allowing mice to escape the bright light of the testing platform. Images of various shapes and colours were placed around the walls of the testing room to act as additional visual cues for orientation. A light (220 lx) and camera were ceiling-mounted directly above the centre of the platform. The test consists of four 3-minute trials spaced 15 minutes apart per day over four consecutive days (acquisition days) beginning at postnatal day 50 (P50) (day 1). For each trial, a mouse was placed in a cylinder (14 cm (H), 10 cm (D)) at the centre of the platform. After 10 seconds, a computer-generated white noise (85 dB) began and the cylinder was lifted, allowing the mouse to freely explore the maze and find the escape hole. Trials were concluded when the mouse entered the escape box, at which point the white noise was turned off. If the mouse did not enter the escape box after 3 minutes, the mouse was guided towards the target hole by the experimenter. At the end of each trial, the mouse was left undisturbed in the escape box for 1 minute before being returned to its home cage. Between trials, the maze and escape box were cleaned with 30% isopropanol. Probe testing days occurred on days 5 and 12 after initial testing began. For probe trials, the escape box was removed, and the target hole was covered from the bottom to mimic all other holes. The mouse was placed in the cylinder, then after 10 seconds white noise began, and the cylinder was removed. The mouse could explore the platform for 1 minute before being returned to its home cage. All trials were analyzed using AnyMaze digital tracking software (Stoelting, Wood Dale, IL, USA) for latency to reach the target hole, distance travelled, and time spent in the target quadrant. Also, probe days were scored manually by observers.



**Figure 2.4.** Barnes maze test for learning and memory schematic (A) and apparatus in the testing room (B).

### 2.3.7 Statistical analysis

All data are reported as mean  $\pm$  standard error of the mean. Data were analyzed in R using analysis of variance methods where appropriate, depending on the independent variables (IVs) and dependent variable (DV) being measured. Tukey's honest significant difference (HSD) *post hoc* test was conducted where applicable.

Total maternal liquid consumption from 10 days before mating, during gestation, and 10 days after birth was compared by multi-factor analysis of variance (ANOVA), using total liquid consumption (DV) and treatment as well as day as IVs. Additionally, each phase (before mating, gestation, and after birth) were each analyzed by multi-factor ANOVA independently, as they each presented different patterns of liquid consumption. For ethanol exposed dams, total ethanol consumption was assessed in a one-way ANOVA as the DV, with the day as the IV. Maternal weight gain throughout gestation was assessed by multi-factor ANOVA with weight as the DV, and day together with treatment as IVs. For each subsequent metric, simple linear regressions were calculated to determine the relationship between the outcomes (DVs) and mean daily ethanol consumption as a percentage of total liquid consumption (IV).

Gestational day of parturition, number of live pups, number of dead pups at birth, number of pups surviving to P2, and pup weight at P2 were all independently assessed by Student's t-test as the DV, with treatment as the IV. Pup weight at P21 was assessed by multi-factor ANOVA as the DV, with prenatal treatment and postnatal treatment as IVs. Pup survival to postnatal day 70 (P70) was assessed by Pearson's chi-square test for independence using PAE, ELS, and sex as independent, categorical variables.

From the Open Field Test, total activity, distance travelled, number of rears, latency to the centre zone, total activity in the centre zone, time spent in the centre zone, and number of entries to the centre zone were each assessed independently as DVs in a multi-factor ANOVA using sex, PAE, and ELS as IVs. Data from the Home Cage Activity Test were assessed by multi-factor ANOVA using the total hourly activity as the DV, and hour of testing, sex, PAE, and ELS as IVs.

Following Barnes Maze testing, data were log-transformed for normalization of latency to reach the target. A multi-factor ANOVA was performed using latency to reach the target (DV) and sex, prenatal treatment, postnatal treatment, and day of testing as IVs. Subsequent *post hoc* analyses using Tukey's HSD were performed using each IV alone with the DV and in combination with each other. For memory probe trials, the student's t-test was used to compare the mean number of visits to the target hole and the mean number of visits to non-target holes for each group. The t-test was two-tailed, with unequal variance assumed. A multi-factor ANOVA was subsequently performed using the number of visits to the target (DV) and sex, prenatal treatment, and postnatal treatment (IVs).

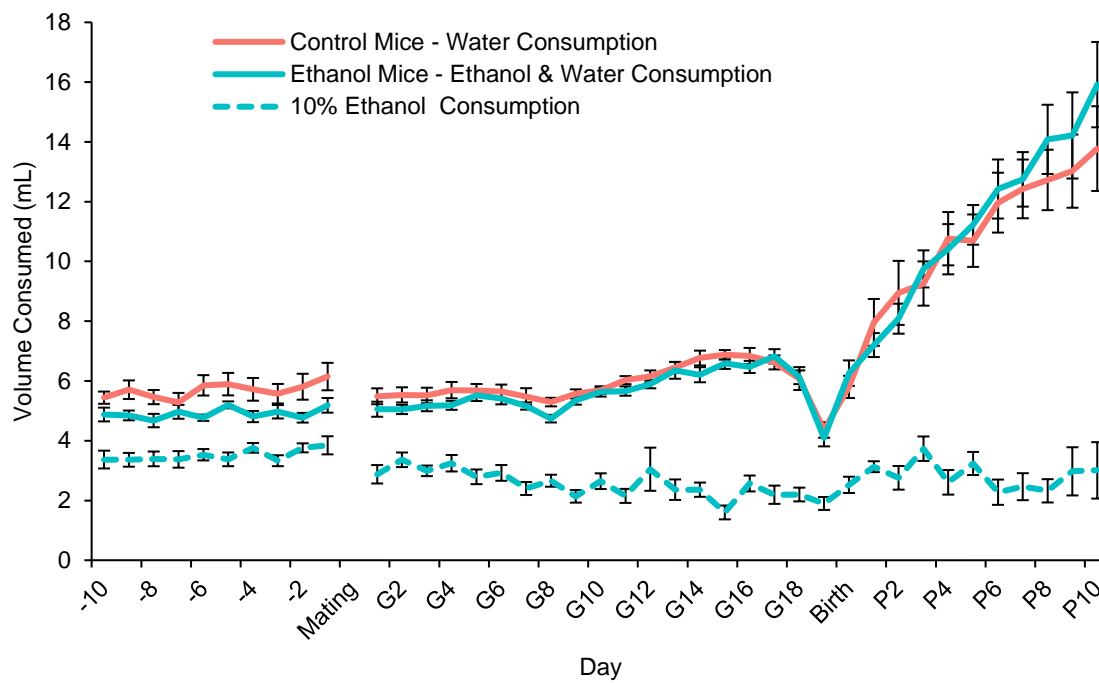
## 2.4 Results

### 2.4.1 Maternal alcohol consumption

When the phases of exposure (before mating, during gestation, after birth) are assessed together, there is a significant main effect of day on total liquid consumption,  $F(42,1021) = 49.79$ ,  $p < 0.001$ . However, there is no associated effect of ethanol exposure on total liquid consumption,  $F(1,1021) = 1.44$ ,  $p > 0.05$ . As each phase of exposure presents a unique pattern of consumption (**Figure 2.5**), they were also assessed independently. Before mating, ethanol mice had 10 days of exposure to 10% ethanol solution. As expected, during this time there is no main effect of day on total liquid consumption,  $F(9,258) = 0.81$ ,  $p > 0.05$ . Interestingly, there is a main effect of treatment, in that control mice regularly consume more total liquid volume than ethanol mice before mating,  $F(1,258) = 35.13$ ,  $p < 0.001$ . During gestation, there are main effects of both day,  $F(19,483) = 15.92$ ,  $p < 0.001$ , and treatment,  $F(1,483) = 14.64$ ,  $p < 0.001$ , again with control dams consuming more total liquid volume than ethanol mice. Finally, in the days following birth, there is only a main effect of day on total liquid consumption,  $F(10,280) = 16.7$ ,  $p < 0.001$ , not of treatment,  $F(1,280) = 1.96$ ,  $p > 0.05$ .

Mean daily ethanol consumption for ethanol mice ranges from 25% to 67% of total daily liquid consumption throughout gestation, with a gestational mean of 46%. This level of exposure corresponds to approximately 8 g/kg of ethanol daily. There is a significant effect of day on ethanol consumption,  $F(41,472) = 2.38$ ,  $p < 0.001$ . *Post hoc* analysis reveals the only days on which ethanol consumption significantly differs are G15 when compared to 1, 2 and 4 days before mating ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.05$ , respectively), as well as P3 ( $p < 0.05$ ).





**Figure 2.5.** Maternal daily liquid consumption.

Mean ( $\pm$  SEM) volume liquid consumption of ethanol-exposed females and control females 10 days before mating, during gestation, and 10 postnatal days following birth,  $N = 13-15$ .

### 2.4.2 Maternal weight gain

While there is a significant effect of day on maternal weight,  $F(16,439) = 136.71, p < 0.001$ , alcohol consumption during gestation has no significant effect on maternal weight gain,  $F(1,439) = 3.36, p > 0.05$ . *Post hoc* analysis reveals no significant differences in weight on any day during gestation between ethanol and control dams.

### 2.4.3 Litter size & survival

No significant differences exist between PAE and control litters for the gestational day of parturition,  $t(28) = 0.59, p > 0.05$ , number live pups,  $t(28) = 0.69, p > 0.05$ , and number of dead pups at birth  $t(28) = 0.29, p > 0.05$ . In addition, PAE litters do not differ from control litters in the number of pups surviving to P2,  $t(28) = 0.85, p > 0.05$ , nor in pup weight at P2  $t(147) = 0.81, p > 0.05$ . However, following a simple linear regression, the number of live pups at P2 is negatively related to mean daily ethanol consumption as a percentage of total liquid diet for ethanol-exposed litters,  $F(1,13) = 8.62, p < 0.05$ , with an  $R^2$  of 0.40. The effects of PAE and ELS on pup weight and survival are shown in **Table 2.1**. There is a significant effect of PAE,  $F(1,131) = 5.16, p < 0.05$ , and ELS,  $F(1,131) = 4.28, p < 0.05$ , on pup weight at P21. However, there is no significant interaction between PAE and ELS for pup weight at P21,  $F(1,131) = 3.10, p > 0.05$ . In addition, a simple linear regression shows pup weight at P21 is positively related to mean daily ethanol consumption as a percentage of total liquid diet for E mice,  $F(1,71) = 5.91, p < 0.05$ , with an  $R^2$  of 0.09. The number of pups surviving to P70 is dependent on PAE,  $X^2(1, N = 149) = 4.75, p < 0.05$ . ELS does not affect the number of pups surviving to P70,  $X^2(1, N = 149) = 2.76, p > 0.05$ . Both pup weight at P21,  $F(1,131) = 3.05, p > 0.05$ , and number of pups surviving to P70,  $X^2(1, N = 149) = 1.21, p > 0.05$ , are not influenced by sex. A simple linear regression shows number of live pups at P70 is negatively related to mean daily ethanol consumption as a percentage of total liquid diet for ethanol-exposed litters,  $F(1,13) = 6.07, p < 0.05$ , with an  $R^2$  of 0.32.

**Table 2.1.** Effect of PAE and ELS on pup survival.

	Ethanol + stress	Ethanol	Stress	Control
P2 pups surviving	32	41	33	43
P21 pups surviving	30	35	33	42
P21 weight (g)	$8.22 \pm 0.20^{i,ii}$	$8.38 \pm 0.21^i$	$7.29 \pm 0.23^{ii}$	$8.06 \pm 0.26$
P70 pups surviving	30	33	33	41
P70 percent surviving	94% <sup>iii</sup>	80% <sup>iii</sup>	100%	95%

<sup>i</sup> Ethanol exposed pups weigh more at P21,  $F(1,131) = 5.16, p < 0.05$

<sup>ii</sup> Separated pups weigh less at P21  $F(1,131) = 4.28, p < 0.05$

<sup>iii</sup> Fewer ethanol exposed pups survive to P70,  $X^2(1, N = 149) = 4.75, p < 0.05$

#### 2.4.4 Open field & home cage activity

From the open field test in a novel environment, multiple activity parameters were assessed including total activity during a 15-minute trial, total distance travelled, and the number of rears (**Table 2.2**). This test also allows for the assessment of anxiety-like behaviour by considering the centre zone of the open field independently. Mice that are less active in the centre zone, spend less time in the centre zone, enter the centre zone less often, or take longer to initially explore the centre zone are considered to have displayed anxiety-like traits. The significance of each effect or interaction is outlined in **Table 2.3**. Concerning total activity, the effects of PAE or ELS alone are insignificant,  $F(1,94) = 0.56, p > 0.05$ , and  $F(1,94) = 0.04, p > 0.05$ , respectively. While there is no significant interaction between PAE and ELS,  $F(1,94) = 0.76, p > 0.05$ , there is a significant effect of sex,  $F(1,94) = 5.08, p < 0.05$ , and a significant interaction between sex and PAE,  $F(1,94) = 4.52, p < 0.05$ , in total activity. *Post hoc* analysis reveals control females are significantly more active than control males ( $p < 0.05$ ), and this difference is not replicated between PAE mice. In terms of total distance travelled, the effects of PAE or ELS are not significant,  $F(1,94) = 1.28, p > 0.05$ ,  $F(1,94) = 0.01, p > 0.05$ , respectively. While there is no significant interaction between PAE and ELS,  $F(1,94) = 0.04, p > 0.05$ , there is between sex and PAE  $F(1,94) = 4.82, p < 0.05$ . While PAE males tend to travel further than controls, *post hoc* analysis reveals no significant differences between groups. By considering the number of rears throughout the 15-minute trial, no significant relationships emerge. The effect of PAE,  $F(1,94) = 2.25, p > 0.05$ , ELS,  $F(1,94) = 0.45, p > 0.05$ , or the interaction between PAE and ELS,  $F(1,94) = 1.37, p > 0.05$ , are all non-significant. However, a simple linear regression finds the number of rears is positively related to mean daily ethanol consumption as a percentage of total liquid diet,  $F(1,53) = 5.39, p < 0.05$ , with an  $R^2$  of 0.09.

When considering anxiety-like behaviours, there are no significant effects on activity in the centre zone by PAE,  $F(1,94) = 0.39, p > 0.05$ , nor ELS,  $F(1,94) = 1.32, p > 0.05$ , and no interaction between PAE and ELS,  $F(1,94) = 0.00, p > 0.05$ . Additionally, there are no significant effects on time spent in the centre zone by PAE,  $F(1,94) = 0.07, p > 0.05$ , nor ELS,  $F(1,94) = 0.07, p > 0.05$ , and no interaction between PAE and ELS,  $F(1,94) = 0.26, p > 0.05$ . There is a significant effect of PAE on latency to enter the centre zone,  $F(1,94) = 5.75, p < 0.05$ , in that PAE mice are quicker to enter the centre zone ( $p < 0.05$ ).

No significant effect of ELS on latency to enter the centre zone emerges,  $F(1,94) = 0.02$ ,  $p > 0.05$ , nor significant interaction between PAE and ELS,  $F(1,94) = 1.79$ ,  $p > 0.05$ . There are no significant effects of PAE,  $F(1,94) = 0.37$ ,  $p > 0.05$ , nor ELS,  $F(1,94) = 3.50$ ,  $p > 0.05$ , nor significant interaction between PAE and ELS,  $F(1,94) = 0.05$ ,  $p > 0.05$ , on the number of entries into the centre zone. However, there is a significant interaction between sex and PAE in the number of entries to the centre zone,  $F(1,94) = 4.276$ ,  $p < 0.05$ . Despite a trend of PAE males to make more entries to the centre zone than controls, *post hoc* analysis reveals no significant differences between groups. There are no additional significant effects or interactions on any of the other parameters measured.

**Table 2.2.** Effect of PAE and ELS on activity and anxiety-like behaviours in the open field test (Mean  $\pm$  SEM).

Sex	Group	Total Activity (beam breaks)	Total Distance (cm)	Number of Rears	Latency to Centre (s)	Centre Activity (beam breaks)	Centre Time (s)	Entries to Centre
Female	Ethanol + stress	2896.56 $\pm$	2444.53 $\pm$	60.11 $\pm$	63.60 $\pm$ 25.38	387.89 $\pm$ 53.55	84.33 $\pm$	39.00 $\pm$ 5.75
		227.99	306.36	13.31			11.21	
	Ethanol	2795.86 $\pm$	2225.39 $\pm$	42.57 $\pm$	76.91 $\pm$ 21.37	313.86 $\pm$ 25.65	74.51 $\pm$ 7.87	32.43 $\pm$ 4.40
		132.38	202.40	9.47				
	Stress	3209.33 $\pm$	3056.62 $\pm$	43.00 $\pm$	99.00 $\pm$ 18.41	407.17 $\pm$ 99.76	75.40 $\pm$	42.67 $\pm$ 9.23
		307.62	483.20	6.58				
	Control	2996.13 $\pm$	2632.75 $\pm$	50.67 $\pm$	74.53 $\pm$ 10.33	379.40 $\pm$ 39.19	88.71 $\pm$	40.53 $\pm$ 4.15
		132.22	206.32	4.43				
Male	Ethanol + stress	2748.76 $\pm$	2682.78 $\pm$	54.88 $\pm$	43.21 $\pm$ 9.04	369.18 $\pm$ 36.57	91.58 $\pm$ 9.65	41.12 $\pm$ 4.53
		102.55	373.48	5.57				
	Ethanol	2858.13 $\pm$	2728.30 $\pm$	59.74 $\pm$	59.00 $\pm$ 9.06	352.13 $\pm$ 35.90	86.40 $\pm$	34.30 $\pm$ 2.81
		108.38	358.08	4.89				
	Stress	2652.77 $\pm$	2080.19 $\pm$	44.00 $\pm$	93.58 $\pm$ 28.12	343.08 $\pm$ 40.14	85.37 $\pm$	35.92 $\pm$ 4.06
		142.23	175.00	5.67				
	Control	2509.23 $\pm$	2042.22 $\pm$	53.15 $\pm$	84.57 $\pm$ 20.59	286.31 $\pm$ 22.36	80.08 $\pm$	27.54 $\pm$ 2.63
		132.96	164.03	7.02				

**Table 2.3.** F Statistic for each main effect or interaction for activity and anxiety-like behaviours in the open field test.

		Total Activity	Total Distance	Number of Rears	Latency to Centre	Activity in Centre	Time in Centre	Entries to Centre
Main Effects	Sex	5.076*	0.223	0.618	0.73	0.896	0.185	1.369
	Ethanol	0.558	1.276	2.252	5.749*	0.39	0.069	0.374
	Stress	0.04	0.013	0.447	0.016	1.317	0.066	3.499
Interactions	Sex-Ethanol	4.521*	4.817*	0.167	1.228	2.238	0.479	4.276*
	Sex-Stress	0.449	0.359	1.142	0.362	0.025	0.208	0.365
	Ethanol-Stress	0.759	0.037	1.369	1.789	0.002	0.26	0.048
	Sex-Ethanol-Stress	0.064	0.026	1.016	0.018	0.33	0.357	0.125

\*  $p < .05$

The home cage activity test allows for assessment of the most active 12-hour period during the dark phase of the light-dark cycle, parameters measured included activity, mean speed, distance travelled, and number of rears (**Table 2.4**) with the significance of each effect shown in **Table 2.5**. Concerning total activity throughout the 12-hour testing period, the effect of PAE is insignificant,  $F(1,105) = 0.28$ ,  $p > 0.05$ . ELS has a significant effect on total activity,  $F(1,105) = 9.68$ ,  $p < 0.01$ , as does sex,  $F(1,105) = 32.816$ ,  $p < 0.001$ . There are no significant interactions between PAE and ELS, sex and PAE, sex and ELS, nor sex, PAE, and ELS ( $p$ 's  $> 0.05$ ). *Post hoc* analysis reveals females are more active than males ( $p < 0.001$ ), and separated mice are significantly less active than controls ( $p < 0.01$ ). Mean speed (cm/s) analysis reveals a slightly different pattern – PAE and ELS do not have any significant effect  $F(1,105) = 0.53$ ,  $p > 0.05$ , and  $F(1,105) = 2.78$ ,  $p > 0.05$ , respectively. There is a main effect of sex on mean speed,  $F(1,105) = 5.92$ ,  $p < 0.05$ , and a significant interaction between sex and ELS,  $F(1,105) = 5.84$ ,  $p < 0.05$ . There are no significant interactions between PAE and ELS, sex and PAE, nor sex, PAE, and ELS. *Post hoc* analysis reveals females have a significantly higher mean speed than males ( $p < 0.05$ ). This relationship is true for control males and females ( $p < 0.01$ ), however not for separated males and females ( $p > 0.05$ ). In addition, control females have higher mean speeds than separated females ( $p < 0.05$ ), a relationship not duplicated in males ( $p > 0.05$ ). When considering total distance travelled, no significant effects of PAE or ELS are observed,  $F(1,105) = 0.54$ ,  $p > 0.05$ , and  $F(1,105) = 2.84$ ,  $p > 0.05$ , respectively. A main effect of sex remains,  $F(1,105) = 5.82$ ,  $p < 0.05$ , and a significant interaction between sex and ELS  $F(1,105) = 5.81$ ,  $p < 0.05$ . No significant interactions between PAE and ELS, sex and PAE, nor sex, PAE, and ELS emerge. *Post hoc* analysis indicates females travel significantly more than males ( $p < 0.05$ ). Control females travel more than control males ( $p < 0.01$ ), and control females travel more than separated females ( $p < 0.05$ ), again a relationship not seen in males ( $p > 0.05$ ). The mean total number of rears in the overnight home cage activity test is not influenced by PAE,  $F(1,100) = 0.009$ ,  $p > 0.05$ . There is a main effect of ELS on number of rears,  $F(1,100) = 8.84$ ,  $p < 0.01$ , and a main effect of sex,  $F(1,100) = 19.542$ ,  $p < 0.001$ . No significant interactions emerge. *Post hoc* analysis reveals females rear more than males ( $p < 0.001$ ) and control mice rear more than separated mice ( $p < 0.01$ ).



**Table 2.4.** Effect of PAE and ELS on total activity, mean speed, distance travelled, and the number of rears in the overnight home cage activity test (Mean  $\pm$  SEM).

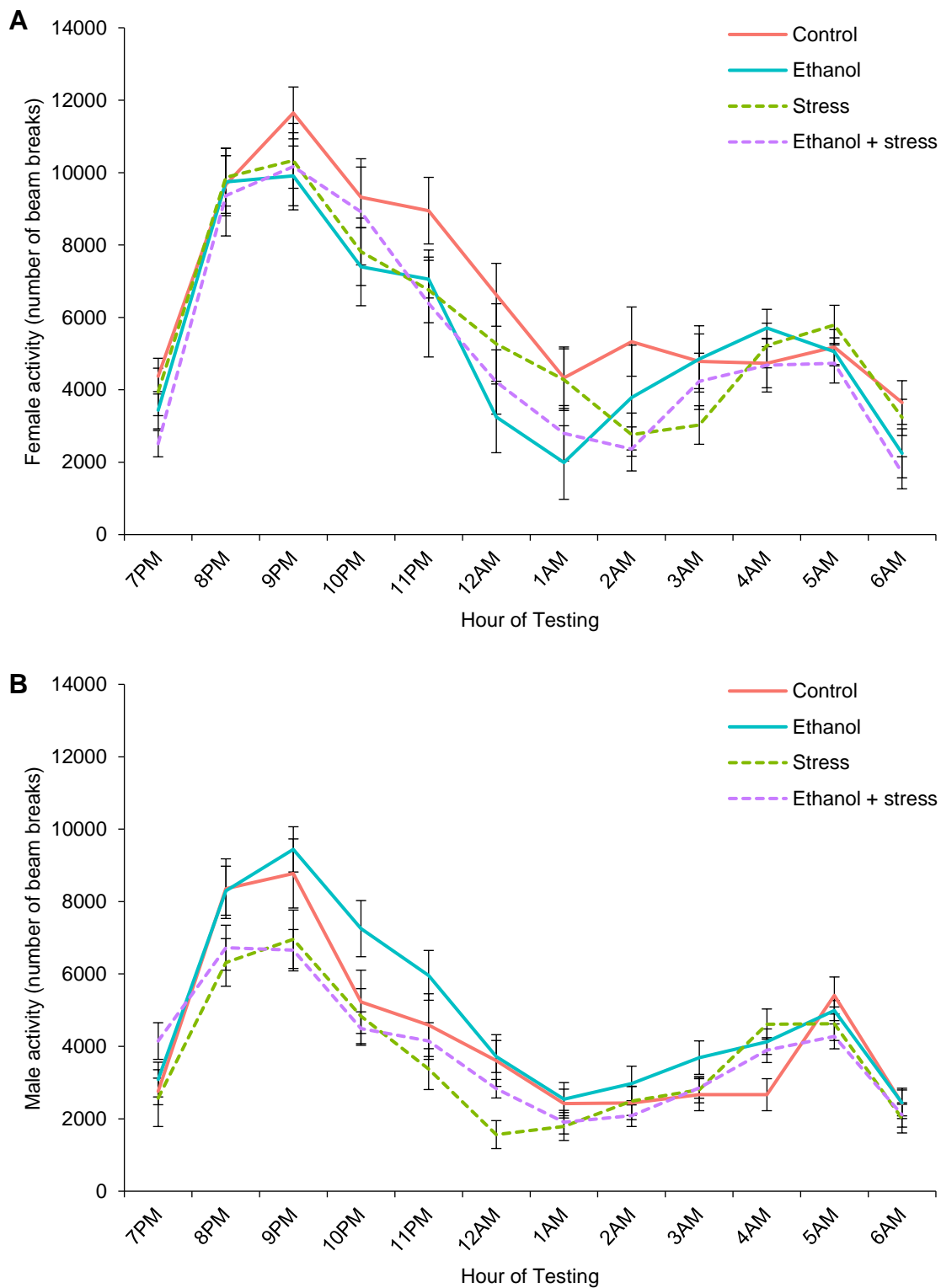
Sex	Group	Total Activity (beam breaks)	Mean Speed (cm/s)	Total Distance (cm)	Number of Rears
Female	Ethanol + stress	62074.19 $\pm$ 6755.45	0.96 $\pm$ 0.18	41551.17 $\pm$ 7697.70	2044.00 $\pm$ 471.21
	Ethanol	64423.93 $\pm$ 6283.68	1.71 $\pm$ 0.92	73027.92 $\pm$ 39745.15	2231.00 $\pm$ 313.17
	Stress	68308.08 $\pm$ 4384.90	0.92 $\pm$ 0.05	39763.11 $\pm$ 2396.05	2295.15 $\pm$ 202.41
	Control	78599.05 $\pm$ 6218.12	2.58 $\pm$ 0.68	111544.19 $\pm$ 29505.0	2410.26 $\pm$ 245.56
Male	Ethanol + stress	44012.68 $\pm$ 3066.80	0.93 $\pm$ 0.29	39822.89 $\pm$ 12331.93	1303.91 $\pm$ 115.09
	Ethanol	58528.95 $\pm$ 3782.14	1.03 $\pm$ 0.11	44465.67 $\pm$ 4852.34	2016.76 $\pm$ 145.50
	Stress	43941.71 $\pm$ 4404.80	1.19 $\pm$ 0.48	51541.61 $\pm$ 20798.76	1233.46 $\pm$ 165.04
	Control	52623.92 $\pm$ 4419.79	0.72 $\pm$ 0.08	31537.36 $\pm$ 3354.52	1801.62 $\pm$ 205.94

**Table 2.5.** F Statistic for each main effect or interaction for total activity, mean speed, distance travelled, and the number of rears in the overnight home cage activity test.

		Total Activity	Mean Speed	Total Distance	Number of Rears
Main Effects	Sex	32.816***	5.921*	5.823*	19.542***
	Ethanol	0.275	0.532	0.535	0.009
	Stress	9.682**	2.777	2.835	8.840**
Interactions	Sex-Ethanol	3.103	0.657	0.642	1.117
	Sex-Stress	0.379	5.840*	5.810*	2.755
	Ethanol-Stress	0.004	0.000	0.000	0.138
	Sex-Ethanol-Stress	0.847	1.260	1.360	0.013

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

By examining mean activity by hour of testing in the overnight home cage activity test (**Figure 2.6**), no significant effect of PAE is observed,  $F(1,1260) = 1.25$ ,  $p > 0.05$ . However, there is a significant effect of ELS on activity,  $F(1,1260) = 44.08$ ,  $p < 0.0001$ , and a complex interaction between sex, PAE, and ELS,  $F(1,1260) = 3.86$ ,  $p < 0.05$ . In addition, significant effects of hour,  $F(11,1260) = 88.70$ ,  $p < 0.0001$ , and sex,  $F(1,1260) = 149.39$ ,  $p < 0.0001$ , on activity are revealed. There are also significant interactions between hour and sex,  $F(11,1260) = 3.43$ ,  $p < 0.001$ , as well as between sex and PAE,  $F(1,1260) = 14.12$ ,  $p < 0.001$ . *Post hoc* analysis reveals control females are significantly more active than control males ( $p < 0.0001$ ), similarly PAE females are significantly more active than PAE males ( $p < 0.0001$ ). Further, PAE females are less active than their control counterparts ( $p < 0.01$ ), however this relationship is not replicated in males ( $p > 0.05$ ). When considering sex, PAE, and ELS together, many significant relationships are revealed. Female ethanol mice are significantly less active than female control mice ( $p < 0.01$ ) (**Figure 2.6A**), a relationship not duplicated in males ( $p > 0.05$ ) (**Figure 2.6B**). Female ethanol + stress mice are also significantly less active than control females ( $p < 0.001$ ) (**Figure 2.6A**), again not replicated in males ( $p > 0.05$ ) (**Figure 2.6B**). In the PAE condition, female ethanol + stress mice are not significantly less active than ethanol females ( $p > 0.05$ ) (**Figure 2.6A**), while ethanol + stress males are significantly less active than ethanol males ( $p < 0.0001$ ) (**Figure 2.6B**).

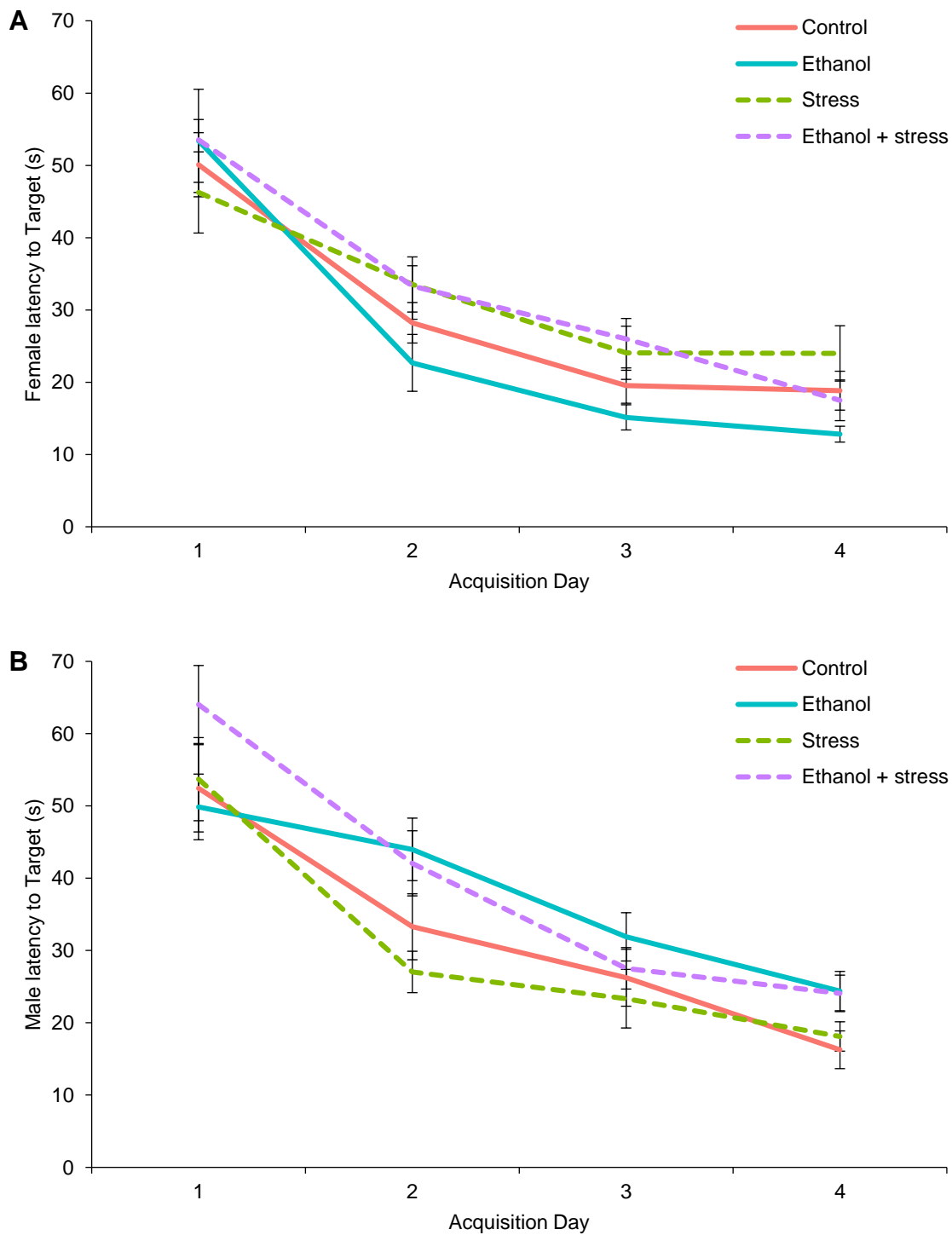


**Figure 2.6.** Overnight activity by hour.

The mean ( $\pm$  SEM) number of beam breaks by the hour of testing for females (A) and males (B) following PAE with and without ELS, N = 6-22.

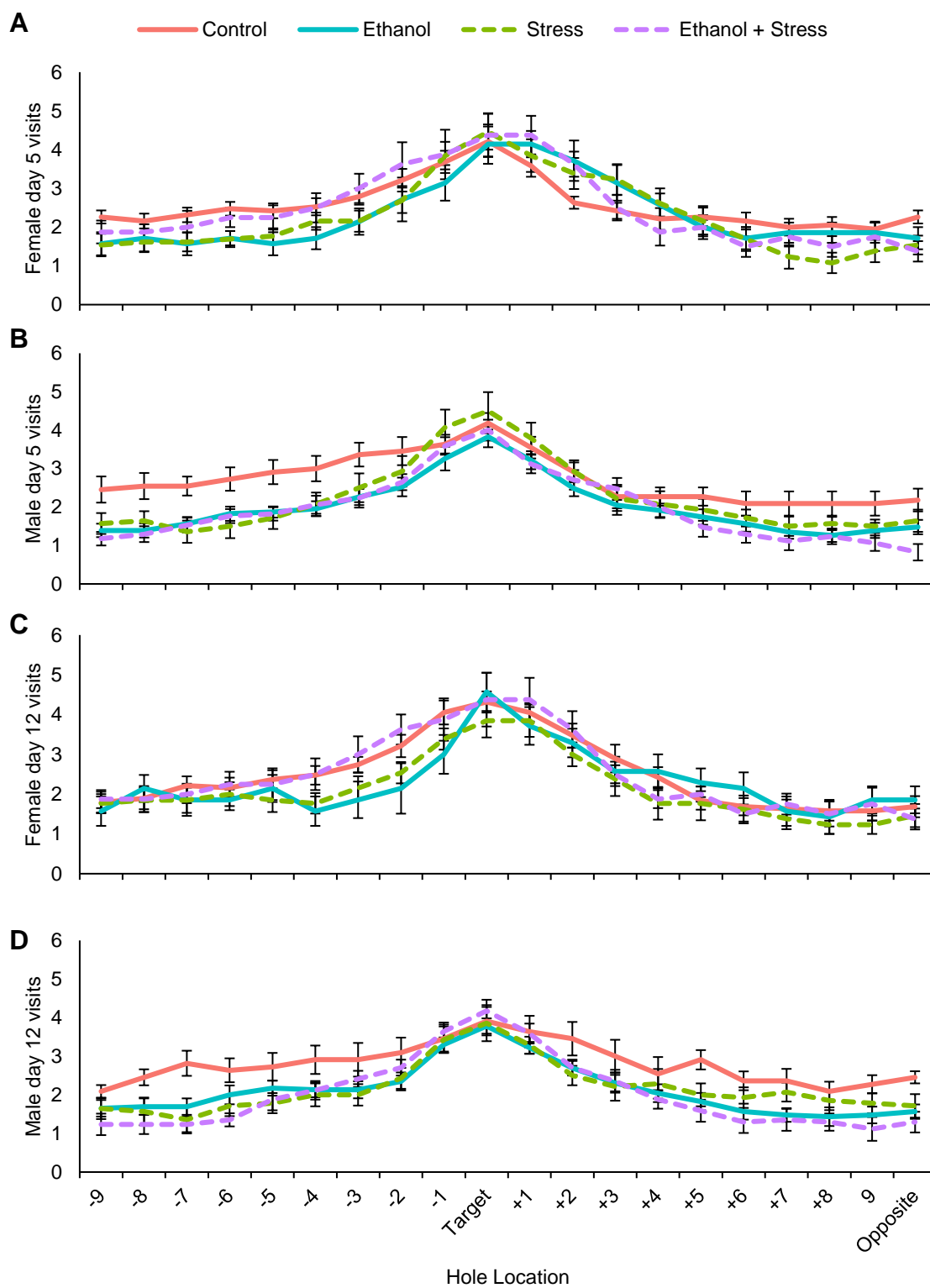
#### 2.4.5 Barnes maze test for learning & memory

Learning acquisition testing in the Barnes maze occurs over four consecutive days, consisting of four 15-minute trials spaced 15 minutes apart on each day. There is a significant main effect of day,  $F(3,1786) = 130.24$ ,  $p < 0.001$ , with all mice showing decreased latency to find the target hole across the 4 trial days. Sex,  $F(1,1786) = 10.339$ ,  $p < 0.01$ , PAE,  $F(1,1786) = 9.645$ ,  $p < 0.01$ , and ELS,  $F(1,1786) = 3.878$ ,  $p < 0.05$ , also have main effects on latency. Interestingly, there are significant interactions between sex and ethanol,  $F(1,1786) = 9.578$ ,  $p < 0.01$ , as well as between day, sex, and ELS,  $F(3,1786) = 3.82$ ,  $p < 0.01$ , on latency to target. These relationships are evident in **Figure 2.7** – the effect of separation on latency is particularly clear in females on the 3<sup>rd</sup> and 4<sup>th</sup> acquisition day. Conversely, the effect of ethanol on latency is particularly evident in males on acquisition days 2-4. *Post hoc* analyses of latency (DV) and day of testing (IV) reveal that mean latency on each day is significantly different than every other day ( $p$ 's  $< 0.001$ ). *Post hoc* analysis using latency (DV) and sex (IV) finds females have significantly faster trials than males ( $p < 0.01$ ). When considering latency (DV) and ELS (IV), *post hoc* analysis finds ELS mice have significantly slower times than control counterparts ( $p < 0.05$ ). *Post hoc* analysis using latency (DV) and PAE (IV) reveals PAE subjects are significantly slower than their control counterparts ( $p < 0.01$ ). When sex is included as an independent variable, only PAE males are significantly slower than controls ( $p < 0.001$ ), an effect not seen in females ( $p > 0.05$ ). No additional significant relationships are revealed by *post hoc* analysis.



**Figure 2.7.** Learning acquisition in the Barnes maze test for learning and memory. Mean latency ( $\pm$  SEM) to enter the target hole across four trials on acquisition days 1-4 for females (A) and males (B) following PAE with and without ELS, N = 7-23.

Memory for the learned location of the target hole did not appear to be affected by sex or treatment on Probe Day 5 and 12 as all groups showed significant differences between number of explorations of target compared to non-target holes, suggesting all groups visited the target hole significantly more than an average non-target hole (**Figure 2.8**). There were no significant main effects on number of visits to the target hole on day 5 or 12, respectively by sex,  $F(1,104) = 0.63, p > 0.05$ ;  $F(1,104) = 1.29, p > 0.05$ , PAE,  $F(1,104) = 1.04, p > 0.05$ ;  $F(1,104) = 0.33, p > 0.05$ , nor ELS,  $F(1,104) = 0.86, p > 0.05$ ;  $F(1,104) = 0.02, p > 0.05$ , and no significant interaction between PAE and ELS,  $F(1,104) = 0.03, p > 0.05$ ,  $F(1,104) = 0.38, p > 0.05$ . No other significant relationships were found.



**Figure 2.8.** Mean number of hole visits on probe days in the Barnes Maze. The mean number of visits to each hole ( $\pm$  SEM) on probe days 5 (A, B) and 12 (C, D) for females (A, C) and males (B, D),  $N = 7$  23.

## 2.5 Discussion

Animal models for human disorders have been widely used to understand disease etiology. Such models are not often realistic, particularly regarding neuropsychiatric disorders as causation is not typically known. FASD is a notable exception where animal models, particularly rodents, have been used to investigate the developmental and behavioural deficits associated with PAE (Patten et al., 2014). Such experiments in rodents have established a relationship between behavioural abnormalities and timing (Mantha et al., 2013) or dosage of ethanol (Brys et al., 2014; Cullen et al., 2013, 2014). These experiments introduced alcohol as an injection at a given time or by choice of consumption during gestation. Control mice regularly consumed more daily liquid volume than ethanol mice both before and during gestation (**Figure 2.5**). As food intake was not monitored throughout this study, the potential nutritional differences that may arise as a result of chronic alcohol exposure cannot be eliminated as a potential confound (Forsander, 1988). Without any attempts to measure or mitigate the potential effect of dehydration as a side effect of chronic alcohol use, the effects of PAE must include the additional potential of maternal dehydration, and any other changes in the dam because of alcohol exposure. Alcohol consumption continued until P10 rather than terminating at birth to reduce potential changes in maternal care resulting from withdrawal that may have additionally affected the postnatal development of the pups. At P10, mice begin opening their eyes and become quite active, relying less on the dam for nutrition and care. The potential for direct ethanol exposure by consumption by pups is a risk mitigated by selecting P10 as the end of availability. Dams in this study consumed less ethanol on average daily than in a previous study (8 g/kg body weight compared to 16 g/kg body weight) (Kleiber et al., 2011). Furthermore, litter effects were not measured or controlled for, as groups contain littermates. The influence of a litter coincides with the level of ethanol exposure and cannot be further separated in this study design. As such, maternal influence may contribute potential confounds to this research. The results included in this report are unique in that they add early postnatal maternal separation stress on pups resulting from PAE that is often experienced by children who develop FASD. The interaction between PAE and postnatal stress has been explored at different age equivalents including adolescence (Comeau et al., 2014) and adulthood (Gangisetty et al., 2014; Hellemans et al., 2008; Lan et al., 2015; Uban



et al., 2013). The results included in this chapter show that the postnatal environment is important in the outcome, discussed below.

### 2.5.1 Pup growth & survival

Growth and bodyweight of pups following PAE have been reported in many treatment paradigms in rodents, and the results are conflicting. In this experiment, there are two significant effects on pup size. First, there was a main effect of PAE on pup weight at P21, where PAE pups tended to weigh more than control pups (**Table 2.1**). This effect coincided with a weak, positive correlation between mean gestational ethanol exposure and weight at P21 for E mice, a result that needs validation through further study. While defects in body growth are more typical of FASD (Kleiber et al., 2011), heavier pups have been seen in rat models of PAE (Kim et al., 2013; Lilliquist et al., 1999). Second, pups that had undergone ELS tended to weigh less than the control counterparts (**Table 2.1**), consistent with previous research (Savignac et al., 2011). More importantly, some reports of slow growth in PAE or ELS may be attributed to nutritional differences, such as reduced access to the maternal source of nutrition, suggesting that body weight may not represent a reliable reflection of the underlying effects of treatment. While no interaction between PAE and ELS on pup weight was observed, this may be a result of rather mild forms of exposure paradigms used.

While at P2 there was no significant difference in the number of live pups between ethanol and control dams, there was a strong, negative correlation between the number of pups surviving to P2 and mean gestational ethanol exposure for ethanol dams. By P70, fewer PAE mice survived than control (**Table 2.1**). There remained a strong, negative correlation between the number of pups surviving to P70 and mean gestational ethanol exposure. Many studies have examined the initial effects of PAE on the developing brain, finding increased apoptosis consistently in various brain regions, during various developmental stages (Dikranian et al., 2005; Ikonomidou et al., 2000; Olney et al., 2002; Sulik, 2005; Zhou et al., 2011b). PAE during early gestation has also resulted in congenital defects, preterm birth, and spontaneous abortion, particularly at higher doses (Harlap and Shiono, 1980). Although non-surviving pups were not autopsied, their deaths were likely a result of the teratogenic effect of alcohol. The results also suggest the severity of the effect of PAE

varies greatly, even within a litter. This individual variation, even within a litter, may reflect the uterine position during gestation (Lister et al., 2013).

### 2.5.2 Activity & anxiety-like behaviour

Females in this experiment were more active than males in a novel environment, and PAE eliminates such sex differences (**Table 2.2, Table 2.3**). These results are consistent with previous literature (Boehm et al., 2008), and highlight the sex-dependent effect of PAE exploratory activity. The finding that the amount of gestational ethanol exposure correlated with the number of rears is very modest (only 9% of variability accounted for) and will need further support through validation. The absence of an effect of ELS on anxiety-like behaviours is common in mice (Savignac et al., 2011), and may result from compensation in maternal care by mothers when pups are returned to the litter (Millstein and Holmes, 2007). ELS models alone present unique difficulties in mice (Millstein and Holmes, 2007). Perhaps lengthening the duration of daily separation or the number of days may induce more behavioural changes (Parfitt et al., 2004). Furthermore, making ELS unpredictable may increase the severity of deficits, as it may be more difficult for mice to habituate to the stress (Franklin et al., 2010, 2011; Weiss et al., 2011).

From the overnight home cage activity test, activity, mean speed, distance, and the number of rears were assessed (**Table 2.4, Table 2.5**). All measures showed significant differences between sexes, with females scoring higher in each parameter. ELS had a main effect on both activity and the number of rears, with stressed mice obtaining lower scores than controls. Mean speed and distance had interactions between sex and ELS, such that the effect of stress was most prominent in females. These sex differences in response to stress are common in rodent literature, with implications extending to differences in brain development. In particular, females tend to have decreased hippocampal neurogenesis following maternal separation while males do not, and in some cases have increased neurogenesis (Loi et al., 2014; Oomen et al., 2009). Overnight activity in a home cage environment followed an hourly trend, with all mice peaking in activity in the first 3 hours of testing, which appeared to also be influenced by sex (**Figure 2.6**). Females were more active than males at most time points. Interestingly, female activity was particularly affected by PAE (**Figure 2.6A**). These findings that PAE led to hypoactivity has been found

before using the same exposure paradigm (Kleiber et al., 2011), and reduced locomotor activity following PAE has been reported elsewhere (Wang et al., 2009). Hypoactivity may be a result of the major apoptosis events that occur during ethanol exposure, also associated with reduced cognitive processing (Jacobson et al., 1994). While males did not show any significant differences following PAE or ELS (**Figure 2.6B**), separation following PAE led to a reduction in activity (**Figure 2.6B**), an emphasis not seen in females (**Figure 2.6A**). In a rat model, only the combination of PAE and postnatal stress were sufficient to alter anxiety-like behaviour (Biggio et al., 2018). These findings highlight the nuances of the effect of ethanol in consideration of the time of testing or exposure and the importance of sex differences.

### 2.5.3 Learning & memory

PAE influenced latency to reach the target hole in the Barnes Maze Test (**Figure 2.7**). This suggests that learning the location of the target is negatively impacted by PAE. This deficit was evident in only males (**Figure 2.7B**). This is relevant, as at higher ethanol exposure levels (16 g/kg as compared to the current 8 g/kg) previously modelled, there was no significant main effect of sex or interaction with treatment observed (Kleiber et al., 2011). The delay in learning is characteristic of many FASD mouse models, independent of the exposure paradigm (Allan et al., 2014; Berman and Hannigan, 2000; Chokroborty-Hoque et al., 2014; Hamilton et al., 2014; Lilliquist et al., 1999; Livy et al., 2003; Murawski et al., 2013; Wagner et al., 2014). In humans, learning takes longer in individuals with FASD and is greatly impacted by repetition (Engle and Kerns, 2011). The repetition necessary to learn the target hole location in the Barnes Maze supports this fact – PAE mice were still able to learn the location, albeit at a reduced rate when compared to controls. While these deficits may seem subtle, the accumulation of subtle learning deficits over time may greatly impact a child, particularly when also faced with a poor and unstimulating early environment. Interestingly, although PAE and ELS result in varied learning impairments in this experiment, they have no measured effect on memory. Although such results follow previous work in ELS rats (Oomen et al., 2010), they remain unexplained. The Barnes Maze is not considered a particularly stressful test when compared to the Morris water maze (Harrison et al., 2009), however, it relies on the mouse's instinct to escape the bright, open platform for a more secure, dark enclosed space.

## 2.6 Conclusions

This research revealed several important phenotypic and behavioural outcomes following PAE and ELS in a mouse model of FASD. PAE may lead to increased pup weight, decreased survival, increased activity, learning deficits in males, as well as hypoactivity in females. Further, ELS may lead to suppressed pup weight, hypoactivity, and learning deficits. Both PAE and ELS represent negative environmental assaults on a continuum of neurodevelopment and maturation. Some features negatively affected by PAE are made worse by ELS (hypoactivity and learning deficits), while others are independent (exploratory behaviour). It is worth noting that while PAE did not protect from, nor make more severe, the later effects of ELS on any behavioural traits measured as a significant interaction, the added negative impact of ELS on individuals following PAE should continue to be explored. Sex contributes still more complexity to the interactions between prenatal and postnatal stresses. Sex differences arise during brain development because of epigenetic programming. While these differences exist, how each sex perceives and responds to different environmental stresses remains an area of great research interest. The results argue that the development of an animal model for FASD should include the postnatal environment. More emphasis must be placed on understanding how each of these behavioural alterations arises. While this research contributes to preliminary knowledge regarding behavioural outcomes following PAE and ELS, the underlying mechanisms remain unknown. The ethanol exposure paradigm used in this study uses continuous voluntary consumption, offering a mild, yet chronic exposure as opposed to binge-like injection models. The difficulties in establishing a reliable model of FASD that encompasses ELS cannot be understated when behavioural results are so variable across different exposures. This research has contributed additional knowledge to the field of FASD research in animal models, highlighting the necessity of considering sexes independently.

## Chapter 3. Adult hippocampal gene expression alterations following PAE and ELS

### 3.1 Abstract

PAE followed by ELS via maternal separation was used to refine the mouse model of FASD. It incorporates maternal separation, an early life stress often faced by children diagnosed with FASD. Transcriptomic changes that result from these treatments were analyzed using RNA-seq followed by clustering of expression profiles through weighted gene co-expression network analysis (WGCNA). Gene expression changes in the mouse hippocampus were validated using reverse transcription qPCR. This chapter details the results of an association between adult hippocampal gene expression and PAE followed by ELS that is related to behavioural changes presented in Chapter 2. Expression profile clustering using WGCNA identifies a set of transcripts, module 19, associated with anxiety-like behaviour ( $r = 0.79$ ,  $p = 0.002$ ) as well as treatment group ( $r = 0.68$ ,  $p = 0.015$ ). Genes in this module are overrepresented by genes involved in transcriptional regulation and other pathways related to neurodevelopment. Interestingly, one member of this module, *Polr2a*, polymerase (RNA) II (DNA directed) polypeptide A, is downregulated by the combination of PAE and ELS in an RNA-Seq experiment and qPCR validation ( $q = 2 \times 10^{-12}$ ,  $p = 0.004$ , respectively). Together, transcriptional control in the hippocampus is implicated as a potential underlying mechanism leading to anxiety-like behaviour via environmental insults.

## 3.2 Background

In rodents, ELS has a notable effect on hippocampus-specific learning and memory processes (Oomen et al., 2010; Pillai et al., 2018; Rice et al., 2008). Stress activates hippocampal neurons, ultimately leading to increased glucocorticoid receptor signalling (Hatalski et al., 2000). The behaviours affected by PAE and ELS include anxiety-like behaviour as well as learning and memory. While many brain regions may be relevant, the focus of this study centres on the hippocampus due to its essential role in spatial learning (Jarrard, 1993). The hippocampus is critical for spatial learning and memory, through synaptic plasticity it is susceptible to the environment in ways that are often adaptive, although also make it vulnerable to chronic stress. The molecular mechanisms involved in this interaction, however, have not been investigated.

Performing detailed molecular research on these topics in humans is challenging, so animal models must be used. Given that children with PAE often face postnatal chronic stress, an animal model of FASD using C57BL/6J (B6) mice was developed (Kleiber et al., 2011). Further, this model has been used to explore how postnatal stresses associated with maternal separation may compound behavioural and developmental deficits in PAE mice (Chapter 2) (Alberry and Singh, 2016). The results follow the literature and show that PAE results in learning deficits, anxiety-like behaviours, and changes in activity (Allan et al., 2003; Kaminen-Ahola et al., 2010a; Kleiber et al., 2011; Marjonen et al., 2015). Specifically, following PAE and ELS, mice were slower to reach the location of a learned target in the Barnes maze test for learning and memory (Chapter 2) (Alberry and Singh, 2016). In the open field test (OFT), mice are placed in a novel environment where the amount of exploration of the centre zone is indicative of anxiety-like behaviour. In this test, ethanol mice prenatally exposed to alcohol are quicker to enter the centre zone than controls. Finally, the home cage activity test (HC) is used to assess activity in a familiar environment. In the HC test, mice that had faced postnatal maternal separation were less active than controls (Chapter 2) (Alberry and Singh, 2016). Also, ELS introduced by maternal separation and isolation during early development in mice may lead to increased anxiety-like behaviours in adults (Romeo et al., 2003). The results of rodent models of maternal separation have found the first 10 postnatal days represent the most critical period

(Fenoglio et al., 2006), as such, separation models have focused on this time (Franklin et al., 2010; Veenema et al., 2008).

Neurodevelopment continues into adulthood and can be affected by PAE and/or ELS at any time during this period, suggesting that early postnatal environment can alter adult behaviour in progeny following PAE (Chokroborty-Hoque et al., 2014). This influence may involve changes in gene expression as the foundation for alterations in neurodevelopment and brain function. In this chapter, the mouse model is used to identify changes in hippocampal gene expression following PAE and ELS in mice, which may represent a more realistic model for FASD.

### 3.3 Methods & materials

#### 3.3.1 Hippocampal dissection & RNA isolation

On postnatal day 70, male mice belonging to either control, ethanol, stress, or ethanol + stress groups were sacrificed via carbon dioxide asphyxiation and cervical dislocation. Hippocampus was dissected from the whole-brain as previously described (Spijker, 2011). Hippocampus samples were transferred to individual tubes containing phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen, and stored at -80°C. Using a pestle, samples were ground over liquid nitrogen to create a powder. While on ice, stages of buffer RLT (Qiagen, Valencia, CA) were added and mixed by pipetting. Following 10 min incubation, samples were centrifuged. The supernatant was loaded onto AllPrep DNA spin columns and the AllPrep DNA/RNA Mini Kit Protocol (Qiagen, Valencia, CA) was followed to isolate DNA and RNA from the same tissue sample. Total RNA was suspended in 100 µL of RNase-free water. RNA quantification was determined by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

#### 3.3.2 RNA-Seq

RNA samples were sent on dry ice to The Centre for Applied Genomics (TCAG) (The Hospital for Sick Children, Toronto, Ontario, Canada). The RNA quality was analyzed by Agilent Bioanalyzer 2100 RNA Nano (Agilent Technologies, Santa Clara, CA) by assessing 28S/18S ratios of ribosomal RNA bands. All samples used had RNA integrity numbers (RINs) greater than 8, indicating they were of high quality and not degraded. RNA Library preparation followed Illumina TruSeq Stranded Total RNA Library Preparation protocol to include poly(A) messenger RNA (mRNA) and long noncoding RNA (lncRNA) using 400 ng total RNA as starting material, with ribosomal RNA (rRNA) depletion using RiboZero Gold. Following RNA fragmenting at 94°C for 4 min, transcripts were converted to double-stranded complementary DNA (cDNA), end-repaired, and 3' adenylated for ligation of TruSeq adapters. Sample fragments were amplified with different barcode adapters for multiplex sequencing under the following PCR conditions: 10 s denaturation at 98°C, 13 cycles of 10 s at 98°C, 30 s at 60°C, 30 s at 72°C, and final extension of 5 min at 72°C. To confirm fragment size, 1 µL of the final RNA library was loaded on a Bioanalyzer 2100 DNA High Sensitivity chip (Agilent Technologies, Santa Clara, CA). Kapa Library Quantification Illumina/ABI Prism Kit protocol (KAPA Biosystems) was



used to quantify RNA libraries by qPCR. After pooling in equimolar amounts, libraries were paired-end sequenced on an Illumina HiSeq 2500 platform using a High Throughput Run Mode flowcell and V4 sequencing chemistry according to recommended Illumina protocol to generate 126-bp long paired-end reads. Sequence data is available at the Gene Expression Omnibus (GEO), accession number GSE133369. Read quality was assessed using FastQC, with all reads passing the per-base sequence quality and per sequence quality score analysis modules, indicating minimal degradation throughout runs and an error rate of less than 0.2%, respectively. Similarly, all reads passed the per base N content and sequence length distribution analysis modules, indicating consistency in quality and sequence length, as expected. The per base sequence content analysis module results for each read have non-uniform base composition and the sequence duplication levels are higher than FastQC predicts, consistent with RNA libraries. There are overrepresented sequences in each read, although none represent hits in the FastQC database of common contaminants, indicating these are naturally present sequences. None of the reads fail the adapter content analysis module, suggesting that no sequence is present in more than 10% of all reads. Finally, as typical with RNA-Seq libraries, there are some highly represented kmers in each read, likely derived from highly expressed sequences.

### 3.3.3 Pseudoalignment and differential expression analysis

Paired-end reads for each sample were quantified via pseudoalignment to version 38 of the Ensembl annotation of the mouse transcriptome using kallisto (Bray et al., 2016). To estimate the inferential variance of transcript abundance, 100 bootstrap samples were taken. Differential analysis of gene expression was determined using sleuth (Pimentel et al., 2017) via transcript *p*-value aggregation (Yi et al., 2018). The kallisto-sleuth pipeline for differential expression of transcripts via *p*-value aggregation was chosen over other methods for its improved sensitivity and conservative false discovery rate (Pimentel et al., 2017). For comparison, HISAT2 (Kim et al., 2019) and featureCounts (Liao et al., 2014) were also used to align reads and assign features to version 38 of the Ensembl annotation of the mouse transcriptome. The sleuth object model was defined based on the treatment group, with Wald Tests performed to compare each experimental treatment group (ethanol, stress, and ethanol + stress) to the control group. Similarly, for comparison DESeq2 was used for differential analysis of gene expression (Love et al., 2014) using aligned reads

from the HISAT2-featureCounts pipeline, also defining the model based on the treatment group as when using sleuth. Generalized hypergeometric tests for enrichment of gene ontology (GO) terms and Kyoto encyclopedia of genes and genomes (KEGG) pathways were used for genes represented by transcripts differentially expressed in each treatment group following the kallisto-sleuth pipeline using the goana and kegg functions in the *limma* software package (Ritchie et al., 2015) in R, filtered by significance ( $p < 0.05$ ). The background gene set used was created using biomaRt (Smedley et al., 2015) in R to obtain gene IDs from the “mmusculus\_gene\_ensembl” dataset (mm10) with transcript variants detected in any sample from the RNA-Seq experiment.

### 3.3.4 Weighted gene co-expression network analysis

Transcript abundance in transcripts per million (tpm) was used for weighted gene co-expression network analysis (WGCNA) for transcripts detected in all samples (92 187) (Langfelder and Horvath, 2008). As less than 20 samples were used, the soft power threshold was set at 9 to produce adjacency matrices from correlation values for each combination of transcripts. A topological overlap matrix and topological overlap dissimilarity matrix were produced and used for agglomerative hierarchical clustering by the average linkage method. Transcripts were clustered based on the topological overlap between them. Modules were defined using blockwise network analysis with a maximum block size of 15 000, minimum module size of 30, merge cut height of 0.35, with deepSplit at the default 2 for medium sensitivity. Transcripts that did not cluster in a specific module were placed in module 0 and not considered for further analysis. Modules were numerically labelled by module size, with module 1 being the largest module. Each module was represented by the module eigengene (ME), the first principle component of the module.

Co-expression modules were related to 11 traits based on treatment as well as phenotypic results described in Chapter 2 (Alberly and Singh, 2016). Briefly, this includes prenatal ethanol treatment, postnatal stress treatment, experimental group, Barnes maze learning score, weight at postnatal day 21, activity, distance travelled, latency to enter the centre zone, and the number of entries into the centre zone of the open field test, along with activity, and the number of rears in the home cage activity test. Each of these traits was experimentally assigned as a treatment or a measured outcome significantly different

following at least one of the treatments. Module-trait correlations were filtered by significance ( $p < 0.05$ ). Modules of interest were selected as significantly correlated modules to each trait, resulting in 20 modules of interest. Generalized hypergeometric tests for enrichment of GO terms were used for genes represented by transcripts present in each module using Enrichr (Kuleshov et al., 2016), filtered by significance ( $p < 0.05$ ). Similarly, KEGG pathways were determined using the *kegga* function in the *limma* software package (Ritchie et al., 2015) in R, filtered by significance ( $p < 0.05$ ).

### 3.3.5 qPCR for gene expression

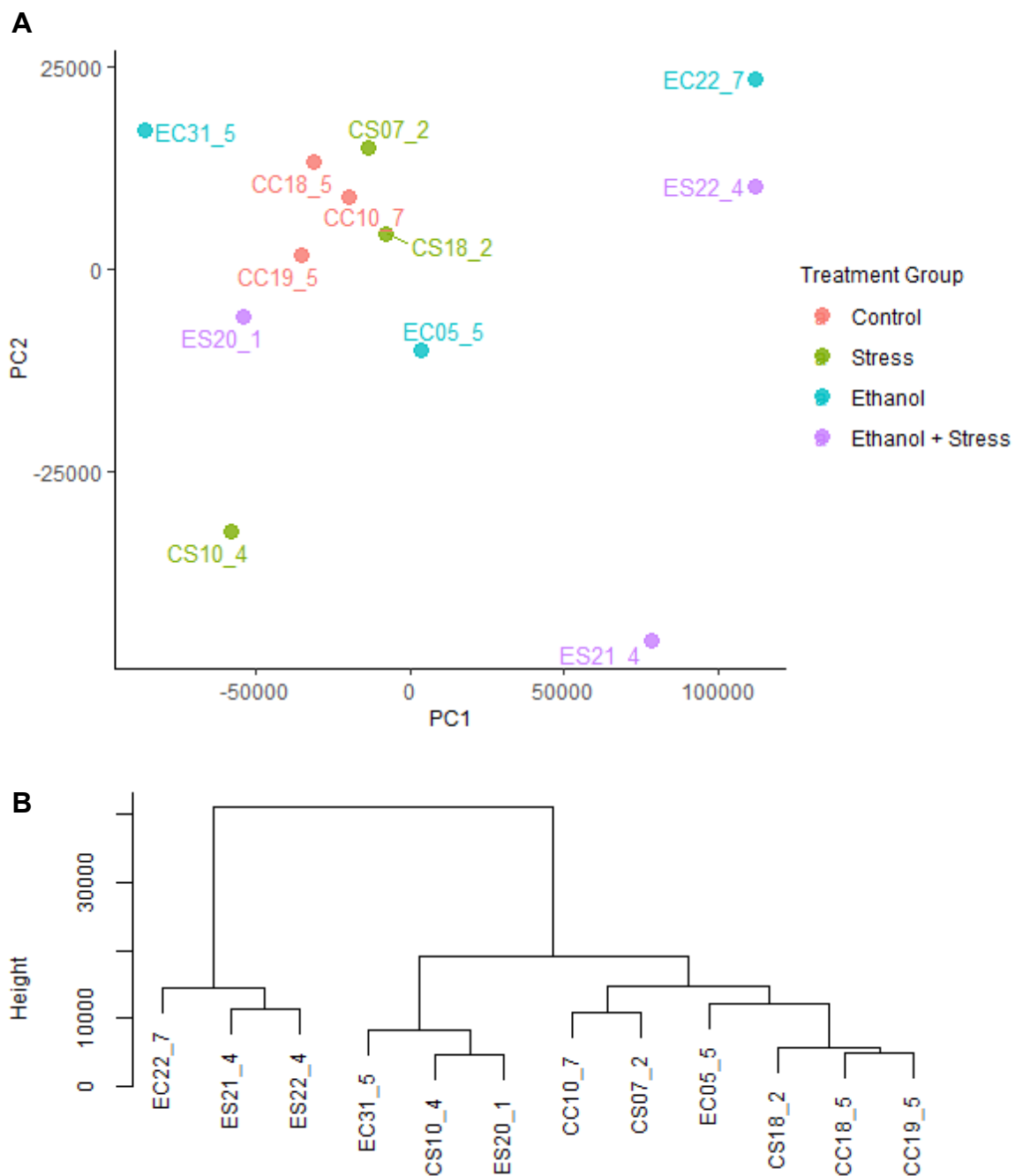
Purified hippocampal RNA was converted to cDNA using the SuperScript IV VILO Master Mix with ezDNase Enzyme following the manufacturer's instructions (Thermo Fisher Scientific). TaqMan Assays were used to investigate the gene of interest, *Polr2a* (ID Mm00839502\_m1, FAM labelled) in a multiplex reaction with TATA box binding protein (*Tbp*) as an endogenous reference gene (ID Mm01277042\_m1, VIC labelled) with the TaqMan Fast Advanced Master Mix according to the manufacturer's instructions (Applied Biosystems). The  $2^{-\Delta\Delta C_t}$  method was used to assess relative quantity.

### 3.4 Results

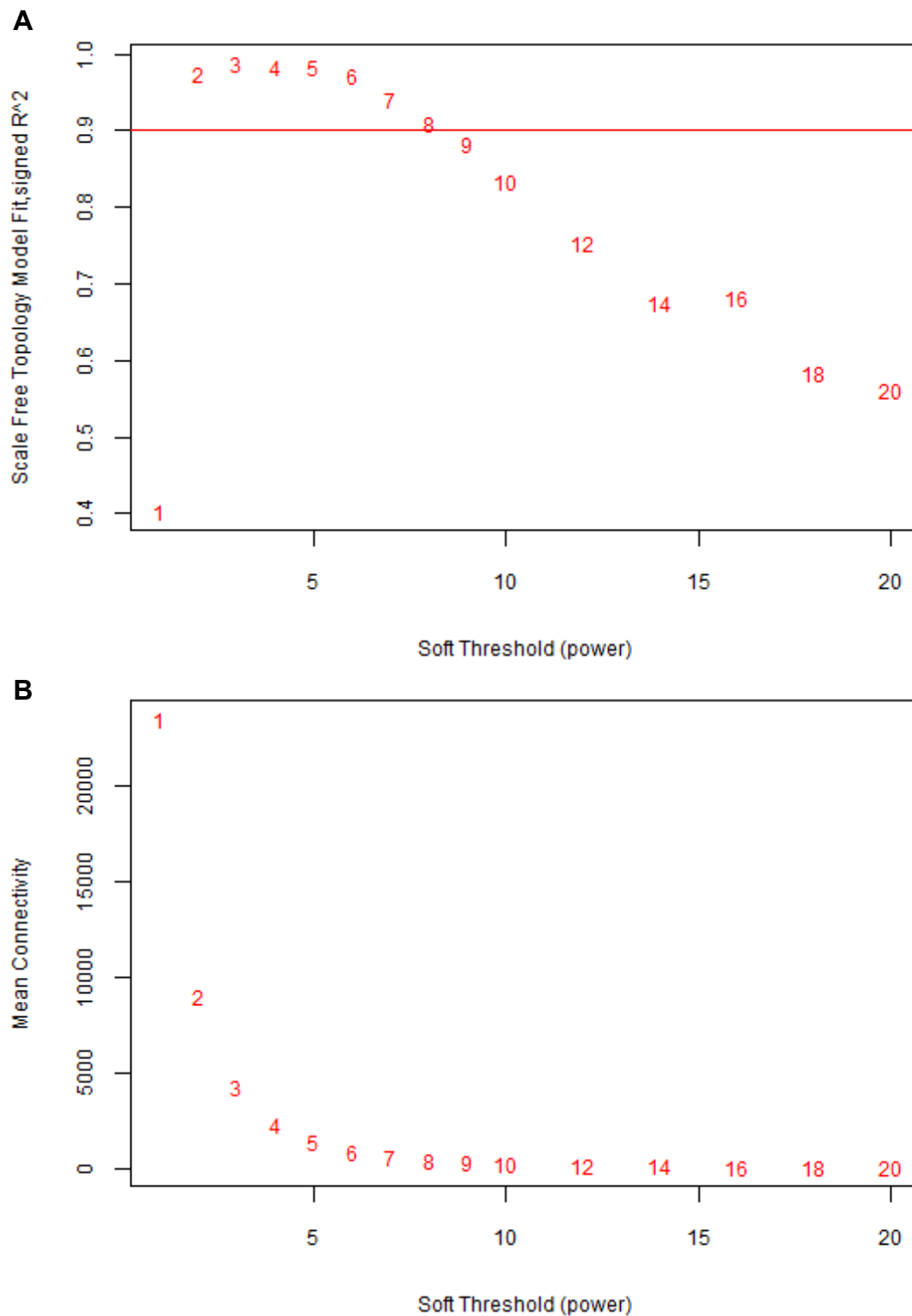
RNA-Seq was performed on hippocampal RNA samples from three individuals for each of four groups of mice representing a control group with no experimental exposure, an ethanol group of mice prenatally exposed to ethanol, a stress group with mice subjected to postnatal maternal separation stress, and an ethanol + stress group with mice prenatally exposed to ethanol followed by postnatal maternal separation stress. Transcriptomes from these 12 samples were assessed via RNA-Seq to determine how they differ between treatment groups and control. Reads were pseudoaligned via kallisto and compared to alignment by HISAT2 and featureCounts for feature assignment, with kallisto resulting in between 3.09% fewer to 0.91% more successfully assigned reads of the total number of reads processed for each sample than HISAT2-featureCounts (**Table 3.1**). Additionally, sample hierarchical clustering indicates no obvious outliers (**Figure 3.1**). Using a soft-thresholding power of nine (**Figure 3.2**), WGCNA was used to cluster transcripts into modules based on correlated expression across samples that can be assessed with other known traits (**Figure 3.3**). For optimal correlations between modules and traits of interest, a cutHeight of 0.35 was employed. A lower cutHeight results in more modules with fewer genes per module, with adjacent modules sharing trait associations, while increasing cutHeight results in fewer, larger modules. Here, it results in 44 modules, represented by 43 to 11 946 transcripts in each module (**Figure 3.4**).

**Table 3.1.** Read mapping statistics following RNA-Seq and pseudoalignment of reads via kallisto, alignment via HISAT2 and feature association using featureCounts.

Group	ID	Total reads	Pseudo-aligned reads (kallisto)	Aligned reads (HISAT2)	Assigned fragments (feature Counts)	Difference (kallisto – feature Counts)	Uniquely mapped reads (kallisto)	Multi-mapped reads (kallisto)	Non-mapped reads (kallisto)	Uniquely mapped reads (HISAT2)	Multi-mapped reads (HISAT2)	Non-mapped reads (HISAT2)
Control	CC 19-5	18059636	13053473 (72.28%)	15461256 (85.61%)	13097397 (72.52%)	-43924 (-0.24%)	4812139 (36.86%)	8241334 (63.14%)	5006163 (27.72%)	13458552 (74.52%)	2002704 (11.09%)	2598380 (14.39%)
Control	CC 18-5	16858034	12148157 (72.06%)	14532030 (86.2%)	12142944 (72.03%)	5213 (0.03%)	4456289 (36.68%)	7691868 (63.32%)	4709877 (27.94%)	12644297 (75%)	1887733 (11.2%)	2326004 (13.8%)
Control	CC 10-7	19901561	13416225 (67.41%)	16594331 (83.38%)	13419437 (67.43%)	-3212 (-0.02%)	4917401 (36.65%)	8498824 (63.35%)	6485336 (32.59%)	14085477 (70.78%)	2508854 (12.61%)	3307230 (16.62%)
Stress	CS 18-2	20961646	15060196 (71.85%)	18093057 (86.32%)	14869470 (70.94%)	190726 (0.91%)	5551751 (36.86%)	9508445 (63.14%)	5901450 (28.15%)	15511180 (74%)	2581877 (12.32%)	2868589 (13.68%)
Stress	CS 10-4	17233196	12417524 (72.06%)	14707852 (85.35%)	12291539 (71.32%)	125985 (0.73%)	4545698 (36.61%)	7871826 (63.39%)	4815672 (27.94%)	12866052 (74.66%)	1841800 (10.69%)	2525344 (14.65%)
Stress	CS 07-2	21927501	14521973 (66.23%)	18465720 (84.21%)	14758654 (67.31%)	-236681 (-1.08%)	5322094 (36.65%)	9199879 (63.35%)	7405528 (33.77%)	15369516 (70.09%)	3096204 (14.12%)	3461781 (15.79%)
Ethanol	EC 31-5	20157771	15038239 (74.6%)	17642991 (87.52%)	14803303 (73.44%)	234936 (1.17%)	5486156 (36.48%)	9552083 (63.52%)	5119532 (25.4%)	15541694 (77.1%)	2101297 (10.42%)	2514780 (12.48%)
Ethanol	EC 22-7	19884564	12311281 (61.91%)	16940669 (85.2%)	12926150 (65.01%)	-614869 (-3.09%)	4632403 (37.63%)	7678878 (62.37%)	7573283 (38.09%)	13830187 (69.55%)	3110482 (15.64%)	2943895 (14.8%)
Ethanol	EC 05-5	20687853	14348817 (69.36%)	18057745 (87.29%)	14478319 (69.98%)	-129502 (-0.63%)	5347074 (37.26%)	9001743 (62.74%)	6339036 (30.64%)	15383176 (74.36%)	2674569 (12.93%)	2630108 (12.71%)
Ethanol + Stress	ES 22-4	19626221	13041690 (66.45%)	16963386 (86.43%)	13464230 (68.6%)	-422540 (-2.15%)	4926144 (37.77%)	8115546 (62.23%)	6584531 (33.55%)	14283450 (72.78%)	2679936 (13.65%)	2665835 (13.58%)
Ethanol + Stress	ES 21-4	21851098	14321259 (65.54%)	18774161 (85.92%)	14823963 (67.84%)	-502704 (-2.3%)	5409882 (37.78%)	8911377 (62.22%)	7529839 (34.46%)	15827225 (72.43%)	2946936 (13.49%)	3076937 (14.08%)
Ethanol + Stress	ES 20-1	19088967	14475675 (75.83%)	16801322 (88.02%)	14352874 (75.19%)	122801 (0.64%)	5296289 (36.59%)	9179386 (63.41%)	4613292 (24.17%)	14893906 (78.02%)	1907416 (9.99%)	2287645 (11.98%)

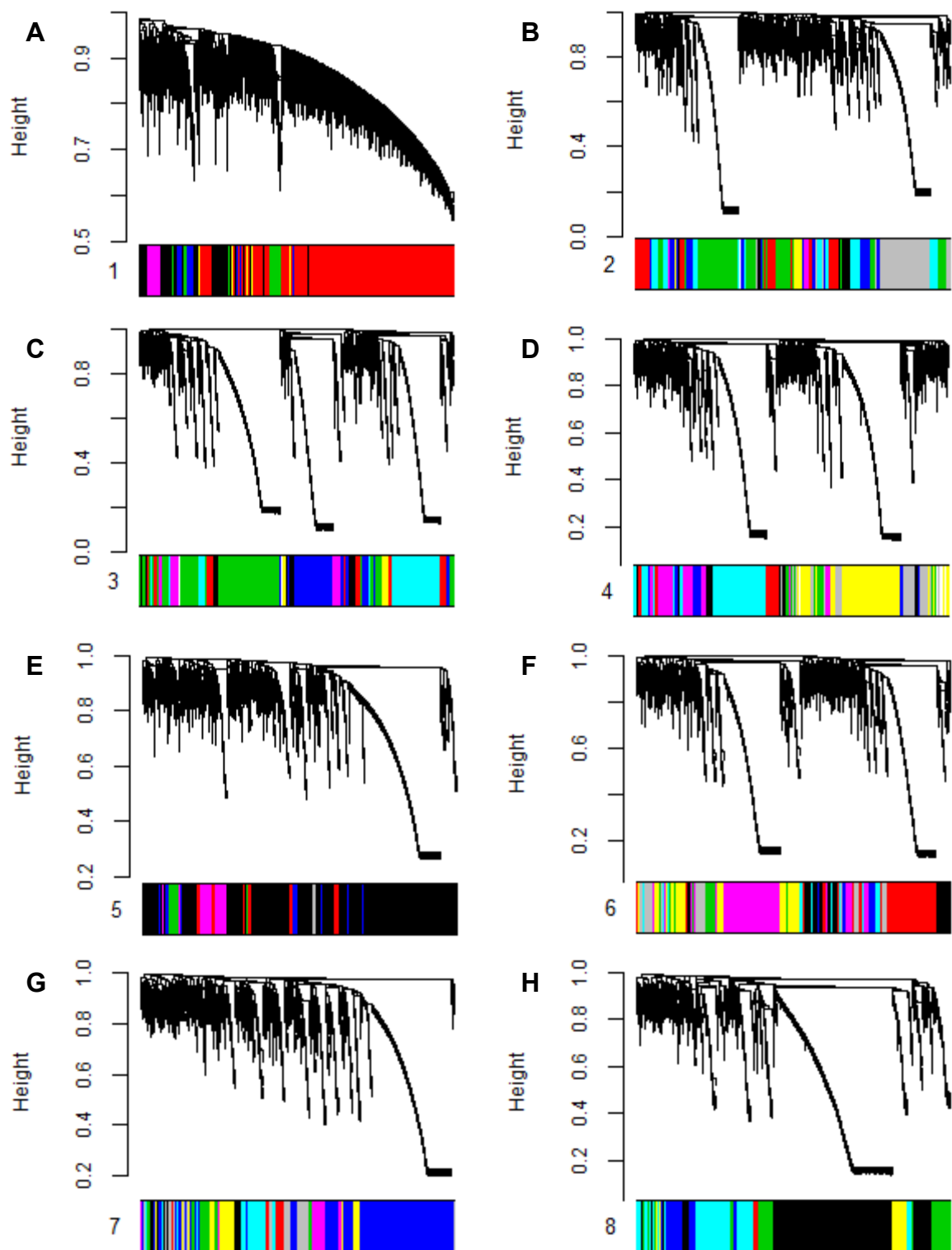


**Figure 3.1.** Transcriptome-based sample clustering. Principal component analysis plot for principal components one and two (A), as well as sample hierarchical clustering for outlier detection (B).



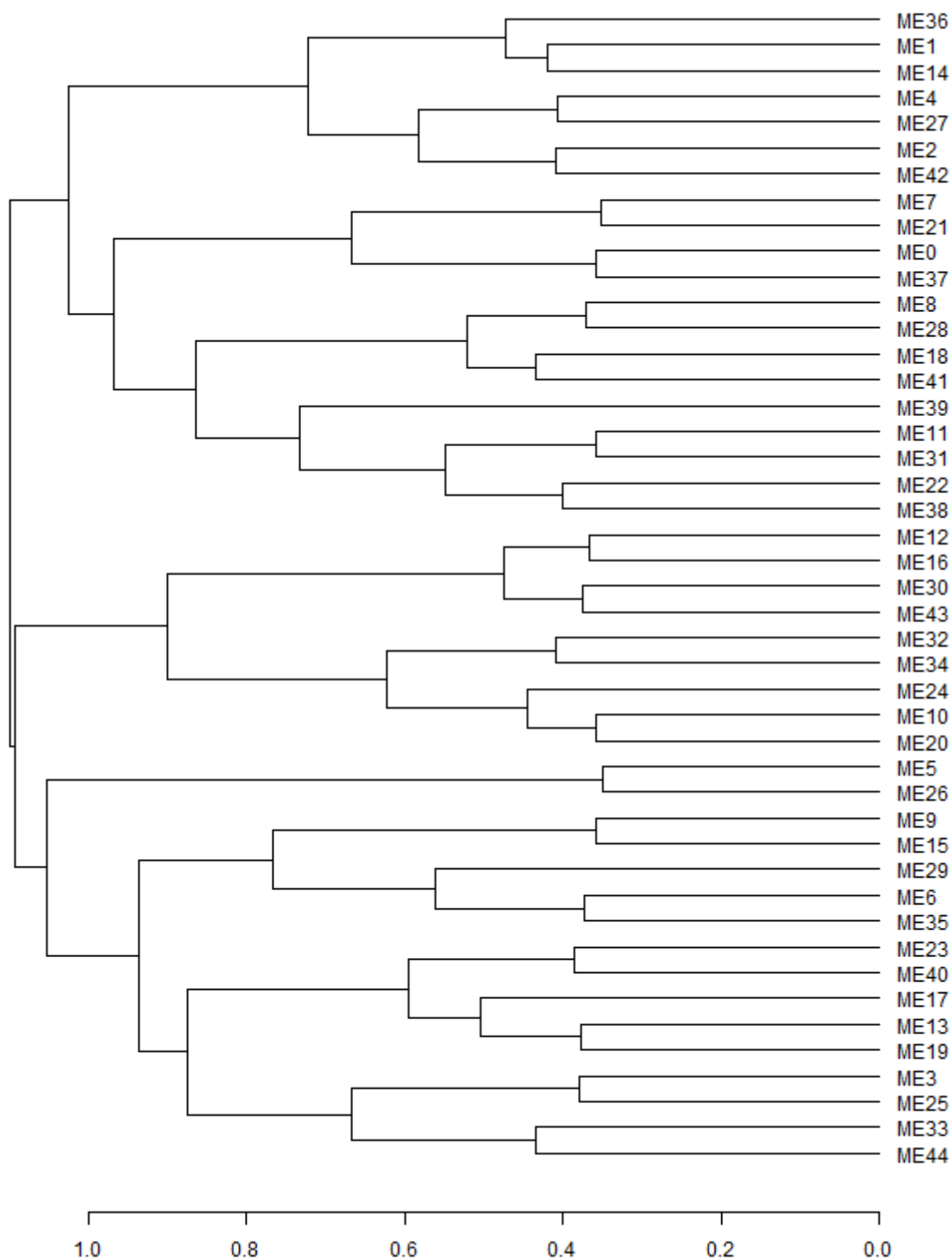
**Figure 3.2.** Connectivity analysis for soft thresholding power.

Connectivity analysis of scale-free topology fit for soft-thresholding powers where numbers indicate soft-thresholding power and the red line is a fit index of 0.9 (A), alongside mean connectivity of the network for different soft-thresholding powers (B).



**Figure 3.3.** Transcript similarity clustering dendrograms for blockwise analysis. Dendrograms for blocks one through eight (A to H, respectively), created by clustering transcripts based on topological overlap with different modules indicated by colour below and block number on the left.

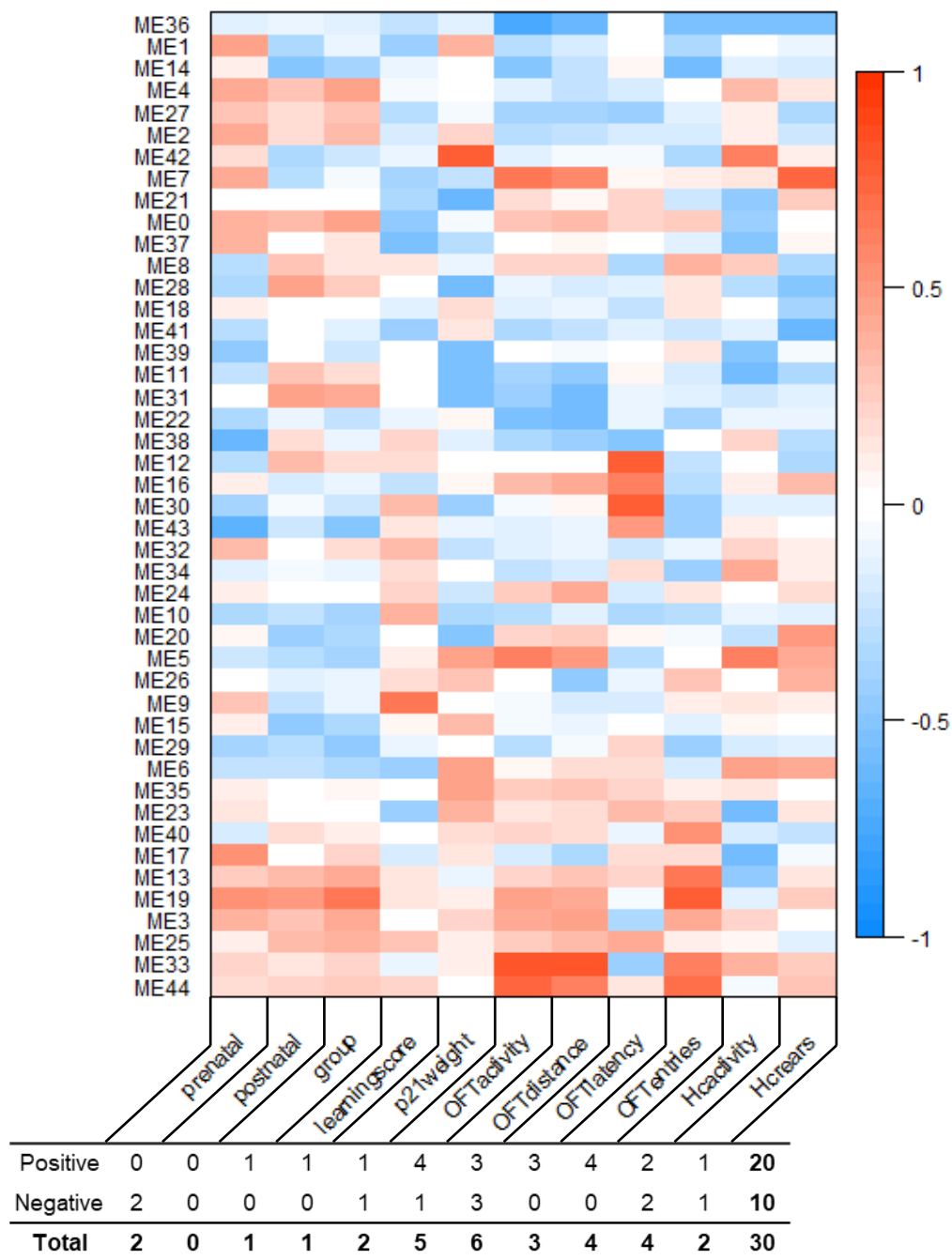




**Figure 3.4.** Module formation through clustering of module eigengenes. Hierarchical clustering dendrogram of module eigengenes with the dissimilarity of eigengenes given by  $1 - (\text{eigengene correlation})$ .

### 3.4.1 WGCNA reveals associations between gene modules, treatment, and outcomes

The correlation was determined between each module produced using WGCNA and 11 traits specified either by assignment or previous measurement, including prenatal treatment, postnatal treatment, treatment group, Barnes Maze learning score (learningscore), weight on postnatal day 21 (p21weight), open field test measures of activity (OFTactivity), distance (OFTdistance), latency to enter the centre (OFTlatency), and the number of entries to the centre (OFTentries), as well as home cage measures of activity (HCactivity) and the number of rears (HCrears) (Chapter 2) (Alberry and Singh, 2016) (**Appendix B**). These traits have unique correlations with modules with visibly shared patterns (**Figure 3.5**). Although some modules share similar correlations, no two traits have the same correlation status with each module. Modules clustering next to one another share similar patterns of correlation between some traits, but no two modules have the same profile at this level. Using a nominally significant cut-off ( $p < 0.05$ ), 30 module-trait relationships emerge, with a range from zero to six significantly correlated modules per trait. Further, most of these relationships (20/30) are positive correlations, while others (10/30) are negative. Also, 20 of the 44 modules (45.56%) are significantly correlated to at least one trait, four correlated to two traits, and three modules correlated to three traits. It is worth noting, however, that these traits are not independent. While there are several overlapping correlations between traits, only one module, module 19 (ME19) is correlated with both experimental treatment and measured behavioural outcome.

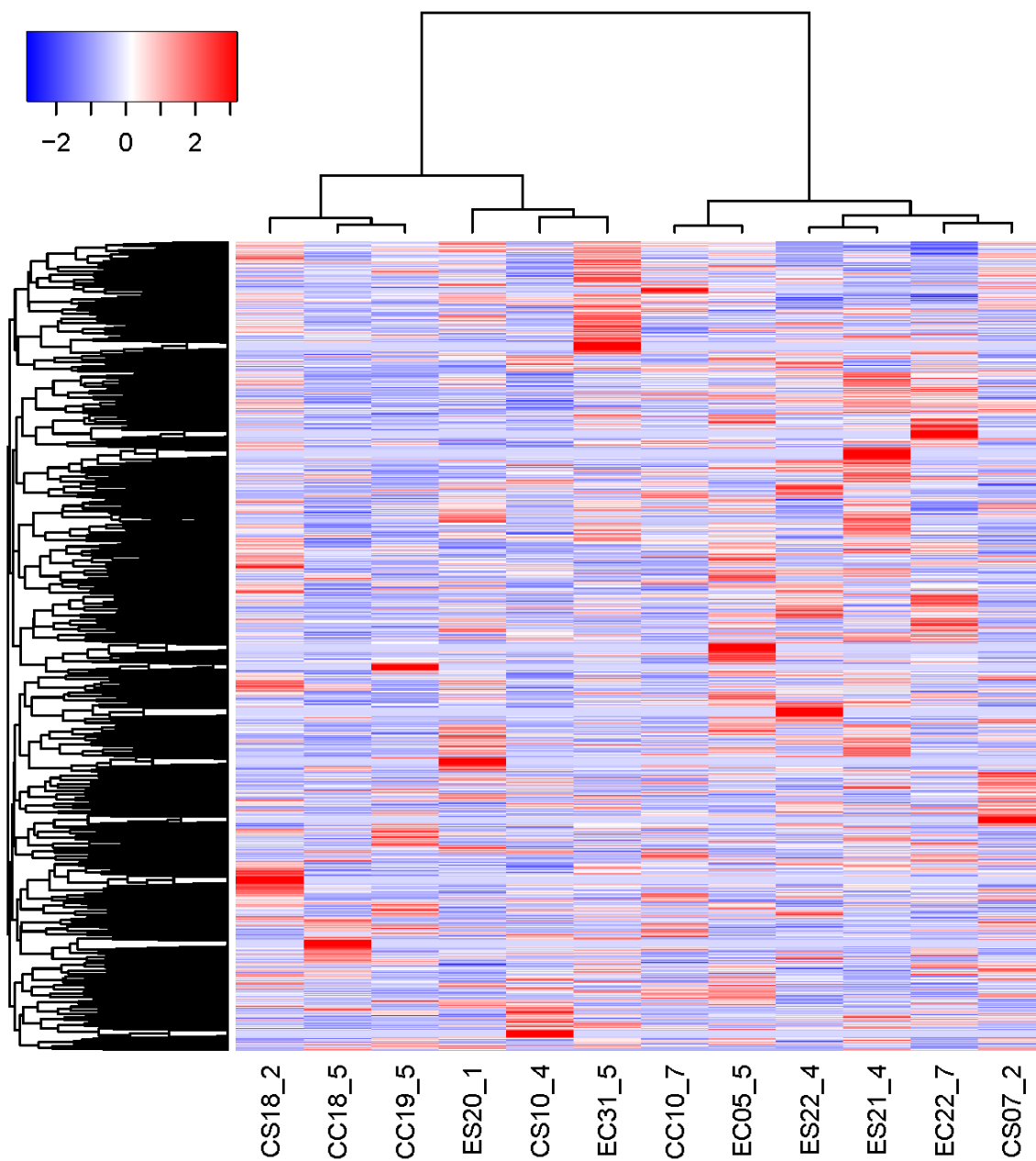


**Figure 3.5.** Module-trait correlation heatmap.

Heatmap of correlations with 11 traits: prenatal or postnatal treatment, experimental group (group), Barnes maze learning score (learningscore), weight at postnatal day 21 (p21weight), open field test activity (OFTactivity), distance (OFTdistance), latency to enter the centre (OFTlatency), number of centre entries (OFTentries), home cage activity (HCactivity), and number of rears (HCrears), with positive (red) and negative (blue) correlations, with the number of significantly ( $p < 0.05$ ) positively or negatively correlated modules with each trait indicated below.

### 3.4.2 Module 19: RNA polymerase II-associated functions are correlated to the experimental group and anxiety-like behaviour

One module, ME19, is correlated with experimental group (group) ( $r = 0.68$ ,  $p = 0.015$ ), alongside number of entries into the centre during the open field test (OFTentries) ( $r = 0.79$ ,  $p = 0.002$ ). This module is composed of 895 transcripts that align to 739 annotated genes, including two complete protein complexes (**Appendix C**). Epidermal growth factor and its receptor (EGF:EGFR) are represented in this module by *Egf* and *Egfr* transcripts. Also, the NMDA glutamate receptor (NMDAR) is represented by *Grin2b*, glutamate receptor, ionotropic, NMDA2B (epsilon 2), and *Grin1*, glutamate receptor, ionotropic, NMDA1 (zeta 1). Expression patterns of module 19 gene transcripts are not driven by individual samples, as indicated by heatmap and hierarchical clustering (**Figure 3.6**). Using a modest threshold for inclusion ( $p < 0.05$ ), module 19 genes are members of KEGG pathways important in transcription such as RNA degradation ( $p = 0.006$ ), as well as neurodevelopment and neurodegeneration, including adherens junction ( $p = 0.015$ ) (**Table 3.2, Appendix C**). Major gene ontologies implicated by genes in this module using the same threshold are important for neurodevelopment and neurodegeneration, such as beta-catenin-TCF complex ( $p = 6 \times 10^{-4}$ ), Notch signalling ( $p = 7 \times 10^{-4}$ ), and mitogen-activated protein kinase (MAPK) cascade ( $p = 9 \times 10^{-4}$ ), together with transcription, including RNA polymerase II functions ( $p = 0.001$ ) (**Table 3.2, Appendix C**).



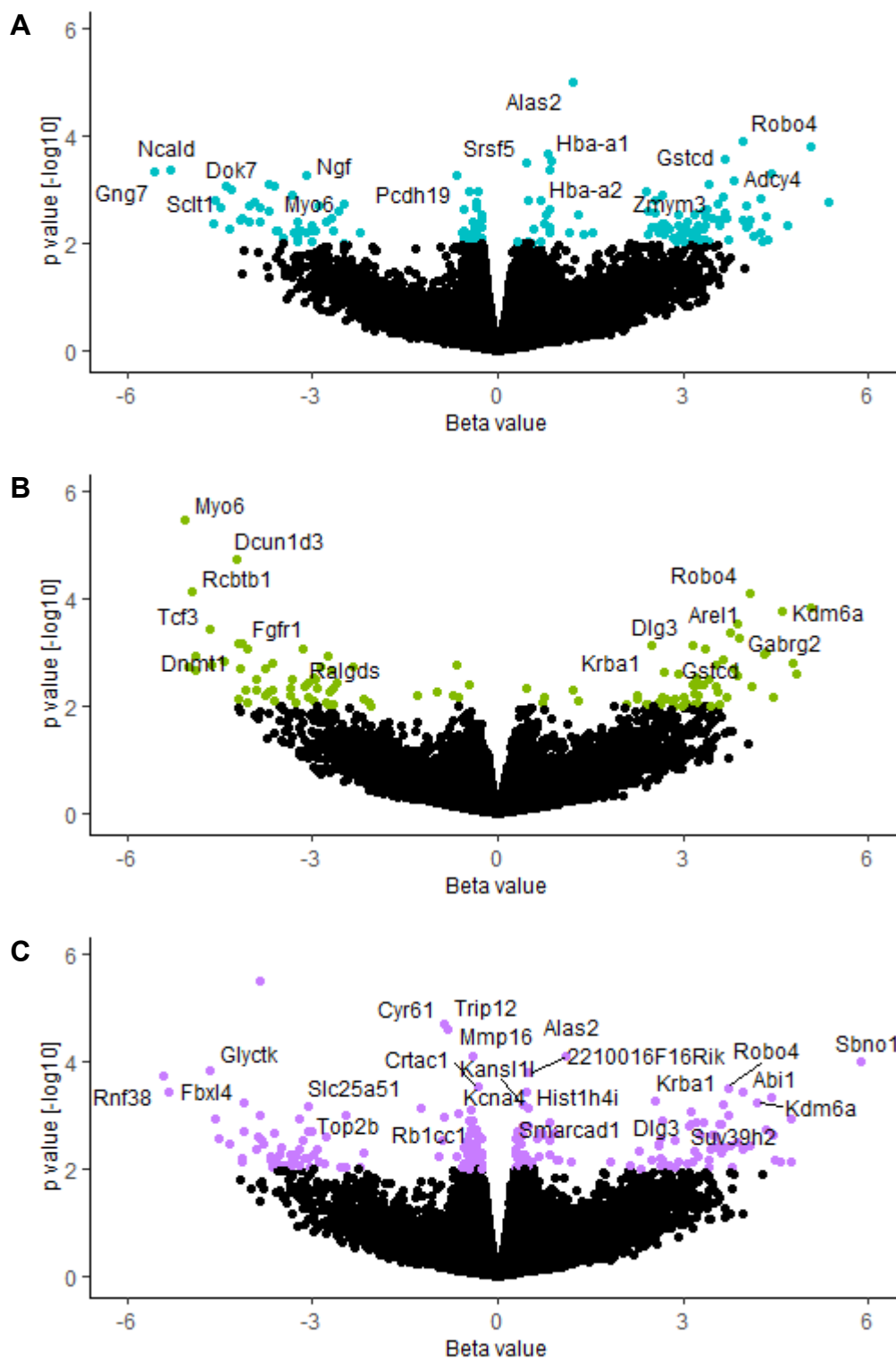
**Figure 3.6.** Hierarchical clustering and heatmap of transcripts representing module 19 gene expression for each sample.

**Table 3.2.** Top 5 most significantly over-represented KEGG pathways and gene ontology (GO) terms represented by genes in module 19.

Term	p-value
<b>Molecular function</b>	
RNA polymerase II regulatory region sequence-specific DNA binding	0.001
Chromo shadow domain binding	0.001
RNA polymerase II distal enhancer sequence-specific DNA binding	0.003
Ubiquitin-like protein ligase binding	0.005
RNA polymerase II core promoter proximal region sequence-specific DNA binding	0.006
<b>Biological process</b>	
Positive regulation of binding	0.001
Notch signaling pathway	0.001
MAPK cascade	0.001
Regulation of protein targeting to mitochondrion	0.001
Regulation of protein metabolic process	0.001
<b>Cellular component</b>	
Beta-catenin-TCF complex	0.001
Melanosome	0.001
Pigment granule	0.001
Azuophil granule lumen	0.005
Microbody	0.006
<b>KEGG pathway</b>	
RNA degradation	0.005
Rap1 signaling	0.008
Prostate cancer	0.009
Arginine and proline metabolism	0.010
Adherens junction	0.011

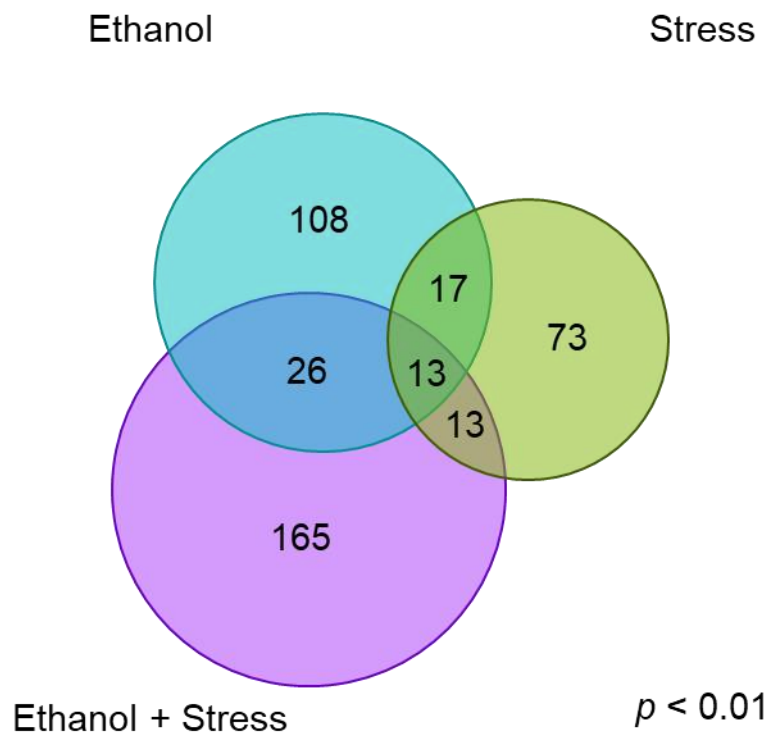
### 3.4.3 Prenatal ethanol exposure and early life maternal separation stress are associated with changes in gene expression

Differentially expressed genes were determined at the transcript level for each experimental treatment group compared to the control group via sleuth (**Appendix D**). Genes with larger effect size (beta values) tend to also reach greater significance (**Figure 3.7 A, B, C**). Filtering for existing transcripts aligned to the mouse genome (mm10), 164 unique transcripts were altered by ethanol, 116 by stress, and 217 by the combination of two treatments ( $p < 0.01$ ) (**Figure 3.8**). There was some overlap between lists, with 13 transcripts shared by all three lists. Differentially expressed genes were analyzed for enrichment of gene ontology and KEGG pathways using annotated genes from these transcripts for ethanol (**Table 3.3, Appendix D**), stress (**Table 3.4, Appendix D**), and ethanol + stress (**Table 3.5, Appendix D**). Transcripts differentially expressed following PAE are important for mRNA processing ( $p < 1.01 \times 10^{-5}$ ) and synapse localization ( $p < 0.001$ ). Following ELS, transcripts important for cell polarity ( $p = 8.64 \times 10^{-5}$ ) and several neurological pathways ( $p < 0.001$ ) are differentially expressed. For mice exposed to both treatments, altered transcripts are important for stimulus-response ( $p < 2.39 \times 10^{-4}$ ). Taken together, some ontologies and pathways altered by either PAE or ELS are shared, particularly with synaptic functions (synapse, synapse part, postsynapse, GABAergic synapse). Also, genes related to hemoglobin binding have altered expression in the ethanol as well as ethanol + stress groups ( $p < 1.69 \times 10^{-5}$ ).



**Figure 3.7.** Differential gene expression between groups detected by sleuth. Volcano plots indicating effect size (Beta value) and significance ( $p < 0.01$  by colour) for each transcript in ethanol (A), stress (B), and ethanol + stress (C) groups compared to control treatment with nominally significant ( $p < 0.001$ ), annotated genes labelled.





**Figure 3.8.** Venn diagram of overlapping transcripts differentially expressed in each treatment as compared to controls as detected by sleuth ( $p < 0.01$ ).

**Table 3.3.** Top 5 most significantly over-represented GO terms and KEGG pathways represented by annotated genes of transcripts differentially expressed in the ethanol group compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Haptoglobin binding	1.64E-09
Oxygen binding	5.48E-07
Hemoglobin alpha binding	1.97E-06
Hemoglobin binding	1.69E-05
Protein binding	1.84E-05
<b>Biological process</b>	
Regulation of alternative mRNA splicing, via spliceosome	3.75E-07
Regulation of mRNA splicing, via spliceosome	8.29E-07
Alternative mRNA splicing, via spliceosome	1.25E-06
Regulation of RNA splicing	8.49E-06
Regulation of mRNA processing	1.01E-05
<b>Cellular component</b>	
Hemoglobin complex	1.64E-09
Haptoglobin-hemoglobin complex	3.67E-09
Synapse	2.09E-05
Synapse part	2.86E-05
Postsynapse	0.001
<b>KEGG pathway</b>	
African trypanosomiasis	6.90E-06
Malaria	3.50E-05
Steroid biosynthesis	0.010
Porphyrin and chlorophyll metabolism	0.028
ECM-receptor interaction	0.029

**Table 3.4.** Top 5 most significantly over-represented GO terms and KEGG pathways represented by annotated genes of transcripts differentially expressed in the stress group compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Protein binding	8.30E-05
Binding	1.28E-04
Protein serine/threonine/tyrosine kinase activity	0.001
Transferase activity	0.002
Catalytic activity, acting on a protein	0.002
<b>Biological process</b>	
Microtubule cytoskeleton organization involved in establishment of planar polarity	8.64E-05
Golgi organization	4.85E-04
Endomembrane system organization	0.001
Embryo development	0.001
Organonitrogen compound metabolic process	0.001
<b>Cellular component</b>	
Intracellular part	7.05E-05
Intracellular	9.42E-05
Organelle part	1.43E-04
Intracellular organelle part	3.46E-04
Basal cortex	0.001
<b>KEGG pathway</b>	
Circadian entrainment	0.002
Retrograde endocannabinoid signalling	0.009
Glycerophospholipid metabolism	0.012
GABAergic synapse	0.012
Morphine addiction	0.012

**Table 3.5.** Top 5 most significantly over-represented GO terms and KEGG pathways represented by annotated genes of transcripts differentially expressed in the ethanol + stress group compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Haptoglobin binding	6.68E-09
Oxygen binding	2.17E-06
Hemoglobin alpha binding	4.56E-06
Hemoglobin binding	3.89E-05
Oxygen carrier activity	9.20E-05
<b>Biological process</b>	
Macromolecule modification	2.34E-04
Response to chemical	2.37E-04
Response to temperature stimulus	2.39E-04
Cellular protein modification process	2.55E-04
Protein modification process	2.55E-04
<b>Cellular component</b>	
Hemoglobin complex	6.68E-09
Haptoglobin-hemoglobin complex	1.49E-08
Intracellular part	1.39E-04
Intracellular	2.07E-04
Cell part	0.001
<b>KEGG pathway</b>	
African trypanosomiasis	2.66E-05
Malaria	1.31E-04
Mucin type O-glycan biosynthesis	0.002
Glycine, serine and threonine metabolism	0.008
Systemic lupus erythematosus	0.008

Some overlap exists between these three lists, specifically when examining the top 25 most significant annotated transcripts in each list (Table 3.6). Most notably, *Robo4*, Roundabout guidance receptor 4, and *Krba1*, KRAB-A domain containing 1, are upregulated in each treatment group, with effect sizes ranging from 2.40 to 4.07. The most significantly differentially expressed gene following PAE is aminolevulinic acid synthase 2, erythroid, *Alas2* (beta = 1.21,  $p = 1.04 \times 10^{-5}$ ), and it is also upregulated in the ethanol + stress group (beta = 1.08,  $p = 7.71 \times 10^{-5}$ ). Similarly, *Suv39h2*, suppressor of variegation 3-9 2, is upregulated following PAE (beta = 3.64,  $p = 1.34 \times 10^{-3}$ ) along with the combined ethanol + stress treatments (beta = 3.74,  $p = 9.76 \times 10^{-4}$ ). *Kdm6a*, lysine (K)-specific demethylase 6a, *Sbno1*, strawberry notch 1, and *Dlg3*, discs large MAGUK scaffold protein 3, are shared between the stress and ethanol + stress groups as upregulated when compared to controls.

**Table 3.6.** Top 25 annotated gene transcripts identified in each treatment group, where beta value represents the effect size for each transcript detected.

Ethanol			Stress			Ethanol + Stress		
Gene	p-value	Beta	Gene	p-value	Beta	Gene	p-value	Beta
Alas2	1.04E-05	1.21	Myo6	3.34E-06	-5.06	Polr2a	4.71E-17	-0.96
Robo4	1.27E-04	3.95	Dcun1d3	1.80E-05	-4.24	Esrrb	2.88E-07	-3.66
Gstcd	2.83E-04	3.66	Rcbtb1	7.47E-05	-4.94	Cyr61	2.03E-05	-0.86
Hba-a1	2.91E-04	0.86	Robo4	8.00E-05	4.07	Trip12	2.56E-05	-0.82
Srsf5	3.17E-04	0.46	Kdm6a	1.66E-04	4.59	Alas2	7.71E-05	1.08
Hba-a2	4.24E-04	0.84	Tcf3	3.60E-04	-4.65	Mmp16	7.72E-05	-0.41
Ncald	4.43E-04	-5.29	Are1l	4.23E-04	3.77	Sbno1	9.86E-05	5.88
Gng7	4.83E-04	-5.57	Gabrg2	5.55E-04	3.89	Glyctk	1.47E-04	-4.66
Pcdh19	5.28E-04	-0.66	Dnmt1	6.76E-04	-4.19	2210016F16Rik	1.56E-04	0.49
Ngf	5.62E-04	-3.09	Fgfr1	6.79E-04	-4.13	Rnf38	1.90E-04	-5.42
Adcy4	7.04E-04	3.82	Krba1	7.19E-04	2.49	Crtac1	2.97E-04	-0.33
Zmym3	7.77E-04	3.40	Dlg3	7.68E-04	3.16	Robo4	3.13E-04	3.72
Dok7	7.88E-04	-3.70	Ralgds	8.60E-04	-3.16	Abi1	3.72E-04	3.96
Myo6	8.78E-04	-3.62	Gstcd	8.61E-04	3.36	Hist1h4i	3.76E-04	0.45
Sclt1	8.91E-04	-4.40	Stx5a	1.05E-03	4.30	Krba1	5.56E-04	2.54
Slitrk2	1.08E-03	-0.32	Tnpo3	1.15E-03	-4.89	Kdm6a	5.79E-04	4.20
Krba1	1.09E-03	2.40	Haghl	1.20E-03	-2.76	Fbx14	6.02E-04	-4.11
Arpp21	1.25E-03	2.66	Per3	1.38E-03	3.64	Kansl1l	6.41E-04	0.41
Slc25a40	1.29E-03	-3.34	0610010F05Rik	1.46E-03	-4.43	Top2b	6.87E-04	-3.07
Suv39h2	1.34E-03	3.64	Sbno1	1.58E-03	4.77	Slc25a51	7.24E-04	-1.25
Bcor1l	1.50E-03	4.26	Tmem194b	1.59E-03	-3.66	Smarcad1	7.47E-04	0.48
Itgb5	1.55E-03	-4.57	Tpp2	1.77E-03	-4.64	Kcna4	8.24E-04	-0.44
Msi1	1.55E-03	0.48	Pnpla6	1.88E-03	-5.01	Dlg3	8.87E-04	3.12
A230050P20Rik	1.61E-03	0.70	Mettl5	1.89E-03	-2.34	Rblcc1	9.35E-04	-0.64
Slc17a5	1.71E-03	-0.34	Gtf2a2	1.90E-03	-2.87	Suv39h2	9.76E-04	3.74

For comparison, following HISAT2 alignment and featureCounts for feature assignment, DESeq2 was used to determine if the top 25 most significantly differentially expressed genes determined by sleuth were replicable using a different analysis pipeline. For the ethanol group, three genes among the top 25, *Alas2*, *Hba-a1*, hemoglobin alpha, adult chain 1, and *Hba-a2*, hemoglobin alpha, adult chain 2, also have significantly increased expression ( $q < 0.01$ ) in the DESeq2 analysis (**Table 3.7**). For the stress group, none of the top 25 genes determined by sleuth were significantly differentially expressed using DESeq2 (**Table 3.8**). For the ethanol + stress group, five of the top 25 genes determined by sleuth, *Alas2*, *Mmp16*, matrix metalloproteinase 16, *2210016F16Rik*, RIKEN cDNA 2210016F16 gene, *Crtac1*, cartilage acidic protein 1, and *Kcna4*, potassium voltage-gated channel, shaker-related subfamily, member 4, were also significantly ( $q < 0.01$ ) differentially expressed in the same direction of change using DESeq2 (**Table 3.9**). For global differential expression in each treatment comparison using DESeq2, no genes reach significance using a Benjamini-Hochberg corrected  $p$ -value ( $q$ -value) cut-off of 0.05. By employing a more liberal threshold ( $p < 0.01$ ), 59, 12, and 103 genes are differentially expressed as detected by DESeq2 following ethanol, stress, or ethanol + stress treatments, respectively (**Appendix E**). Differentially expressed genes ( $p < 0.01$ ) as detected by DESeq2 following each treatment show minimal overlap, with just two genes shared between all three treatments, *Alas2* and *Cpne7*, copine VII (**Figure 3.9**). The overlap between the genes detected by DESeq2 and sleuth is also minimal at this level of significance (**Figure 3.10**). All detected genes for each treatment group were  $p$ -value-ranked following each analysis pipeline and compared. There are moderate correlations in gene rank between the kallisto-sleuth pipeline and the HISAT2-featureCounts-DESeq2 pipeline, with significant ( $p < 2.2 \times 10^{-16}$ ) correlation coefficients of 0.434, 0.428, and 0.573 for ethanol, stress and ethanol + stress, respectively (**Figure 3.11**).

**Table 3.7.** Top 25 genes for ethanol as compared to controls via the kallisto-sleuth pipeline found using the HISAT2-featureCounts-DESeq2 pipeline.

Gene	kallisto-sleuth		HISAT2-featureCounts-DESeq2		
	p-value	Beta	p-value	q-value	Log2 Fold Change
Hba-a1	0.00029	0.86384	0.00001	0.00015	1.28488
Alas2	0.00001	1.20885	0.00001	0.00015	1.19906
Hba-a2	0.00042	0.83562	0.00020	0.00161	1.15508
Slitrk2	0.00108	-0.32270	0.02289	0.11972	-0.26801
Slc17a5	0.00171	-0.33624	0.02494	0.11972	-0.30286
Msi1	0.00155	0.48386	0.03448	0.13792	0.44246
Pcdh19	0.00053	-0.65955	0.05288	0.18130	-0.33378
Bcorl1	0.00150	4.25901	0.17626	0.52879	0.20871
Gng7	0.00048	-5.56601	0.21736	0.57963	0.20130
Zmym3	0.00078	3.40203	0.39920	0.84866	-0.11510
Myo6	0.00088	-3.62465	0.40500	0.84866	-0.12976
Krba1	0.00109	2.40217	0.42433	0.84866	0.08831
Arpp21	0.00125	2.66427	0.47841	0.86388	0.12818
Dok7	0.00079	-3.70025	0.50393	0.86388	0.08809
Slc25a40	0.00129	-3.33901	0.70404	0.98264	-0.06511
Adcy4	0.00070	3.81995	0.77946	0.98264	-0.08270
Srsf5	0.00032	0.46260	0.81493	0.98264	0.03386
Robo4	0.00013	3.95223	0.83522	0.98264	-0.05641
Gsted	0.00028	3.65831	0.86505	0.98264	0.03952
Ngf	0.00056	-3.09032	0.87249	0.98264	-0.03963
Suv39h2	0.00134	3.63797	0.89379	0.98264	-0.02773
Ncald	0.00044	-5.28714	0.93289	0.98264	0.00700
Itgb5	0.00155	-4.56688	0.94720	0.98264	0.00796
Sclt1	0.00089	-4.39975	0.98264	0.98264	-0.00381
A230050P20Rik	0.00161	0.69778	-	-	-

Note: - indicates gene not present in this analysis.



**Table 3.8.** Top 25 genes for stress as compared to controls via the kallisto-sleuth pipeline found using the HISAT2-featureCounts-DESeq2 pipeline.

Gene	kallisto-sleuth		HISAT2-featureCounts-DESeq2		
	p-value	Beta	p-value	q-value	Log2 Fold Change
Kdm6a	0.00017	4.59443	0.10729	0.98825	-0.17518
Krba1	0.00072	2.48690	0.24661	0.98825	-0.12964
Gstcd	0.00086	3.35821	0.39286	0.98825	0.19742
Tpp2	0.00177	-4.64336	0.49359	0.98825	-0.10023
0610010F05Rik	0.00146	-4.42984	0.55443	0.98825	0.05924
Myo6	0.00000	-5.06472	0.57301	0.98825	-0.08782
Tnpo3	0.00115	-4.88651	0.66298	0.98825	-0.05107
Tcf3	0.00036	-4.64774	0.74259	0.98825	0.04453
Dcun1d3	0.00002	-4.23587	0.75899	0.98825	0.04612
Per3	0.00138	3.63989	0.76666	0.98825	0.03564
Rcbtb1	0.00007	-4.94358	0.77695	0.98825	0.02487
Stx5a	0.00105	4.29944	0.77997	0.98825	0.02794
Ralgds	0.00086	-3.16036	0.79517	0.98825	0.02882
Pnpla6	0.00188	-5.00831	0.82999	0.98825	0.03902
Gtf2a2	0.00190	-2.87306	0.83043	0.98825	-0.04313
Sbno1	0.00158	4.77340	0.85293	0.98825	-0.02699
Arell1	0.00042	3.76911	0.85343	0.98825	0.03688
Dnmt1	0.00068	-4.18863	0.86082	0.98825	-0.03495
Fgfr1	0.00068	-4.12772	0.87447	0.98825	0.02914
Dlg3	0.00077	3.16031	0.87568	0.98825	-0.03454
Gabrg2	0.00055	3.89300	0.89824	0.98825	-0.01214
Mettl5	0.00189	-2.34197	0.92357	0.98825	-0.01603
Haghl	0.00120	-2.75506	0.94707	0.98825	0.00822
Robo4	0.00008	4.06756	0.99773	0.99773	-0.00077
Tmem194b	0.00159	-3.66217	-	-	-

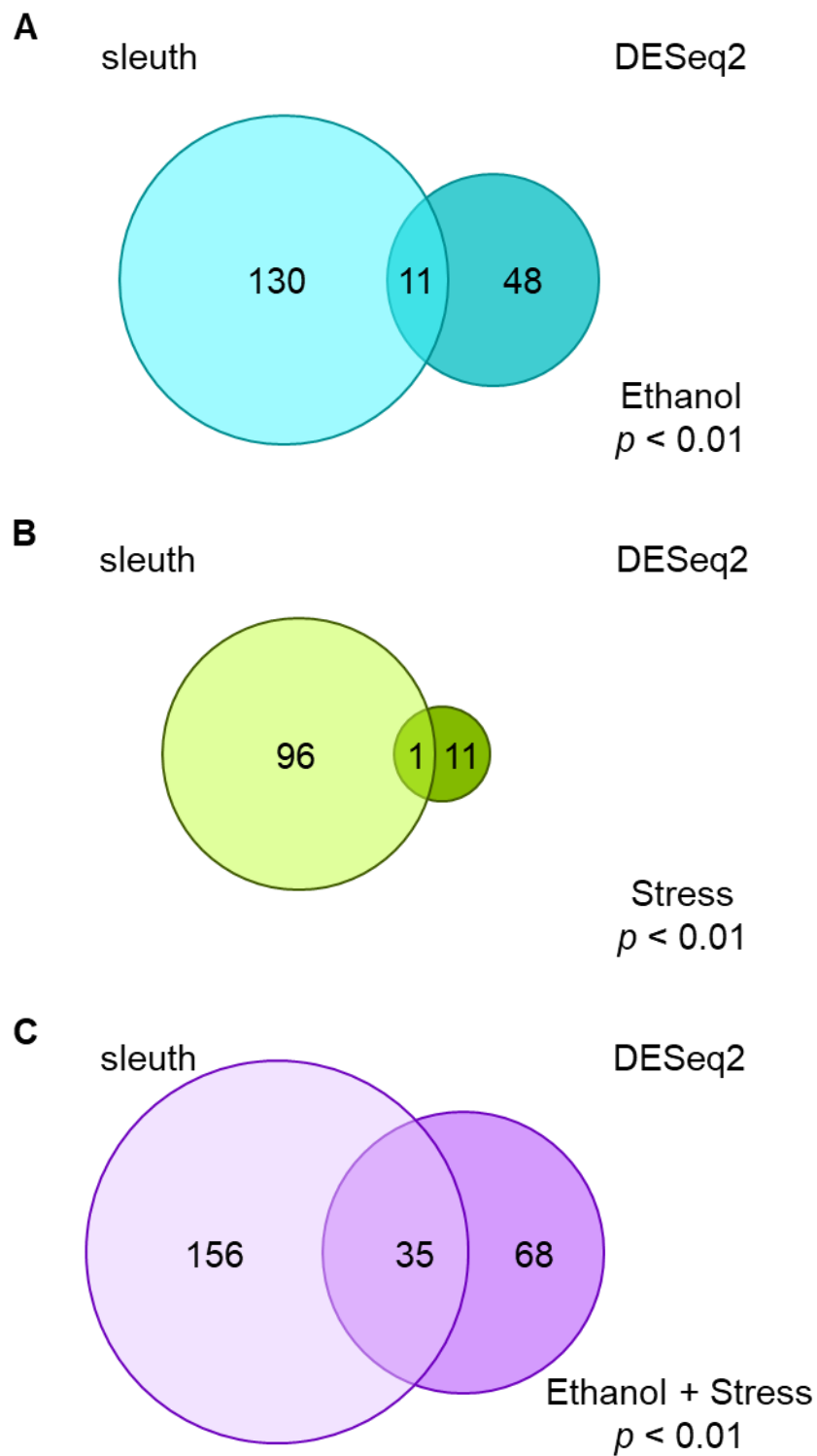
Note: - indicates gene not present in this analysis.

**Table 3.9.** Top 25 genes for ethanol + stress as compared to controls via the kallisto-sleuth pipeline found using the HISAT2-featureCounts-DESeq2 pipeline.

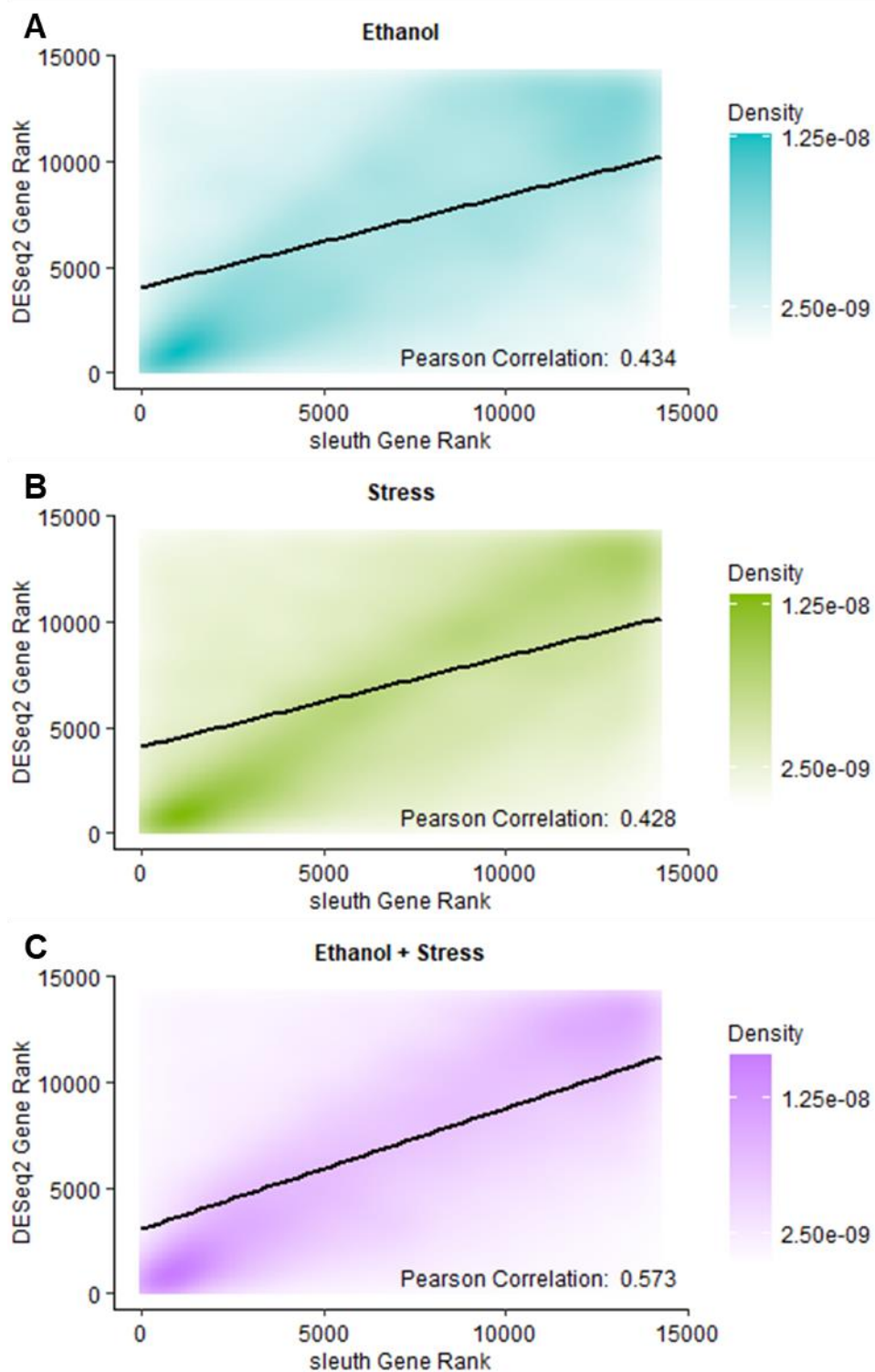
Gene	kallisto-sleuth		HISAT2-featureCounts-DESeq2		
	p-value	Beta	p-value	q-value	Log2 Fold Change
Alas2	0.00008	1.08411	0.00004	0.00097	1.12445
Mmp16	0.00008	-0.40807	0.00020	0.00202	-0.47048
2210016F16Rik	0.00016	0.49154	0.00026	0.00202	0.72152
Crtac1	0.00030	-0.33206	0.00037	0.00215	-0.49230
Kcna4	0.00082	-0.44395	0.00064	0.00295	-0.59650
Smarcad1	0.00075	0.47626	0.03789	0.13491	0.21456
Polr2a	0.00000	-0.95671	0.04106	0.13491	-0.33345
Esrrb	0.00000	-3.66468	0.05409	0.15551	-0.84118
Top2b	0.00069	-3.06706	0.09044	0.21094	-0.26385
Kansl1l	0.00064	0.41337	0.09356	0.21094	0.30696
Trip12	0.00003	-0.82176	0.10089	0.21094	-0.27312
Kdm6a	0.00058	4.19874	0.12358	0.23687	-0.16732
Rb1cc1	0.00093	-0.64383	0.17212	0.30451	-0.15819
Dlg3	0.00089	3.12257	0.19092	0.31365	-0.28884
Glyctk	0.00015	-4.66467	0.22754	0.34889	0.45365
Krba1	0.00056	2.53848	0.26212	0.37679	-0.12534
Sbno1	0.00010	5.88303	0.36510	0.46620	-0.13192
Fbxl4	0.00060	-4.11002	0.37457	0.46620	0.15410
Suv39h2	0.00098	3.74023	0.38512	0.46620	-0.18252
Robo4	0.00031	3.71660	0.52361	0.60215	0.17060
Rnf38	0.00019	-5.41755	0.86762	0.95026	-0.01959
Slc25a51	0.00072	-1.25375	0.92423	0.95465	0.00896
Abi1	0.00037	3.95501	0.95465	0.95465	0.00472
Cyr61	0.00002	-0.85992	-	-	-
Hist1h4i	0.00038	0.45037	-	-	-

Note: - indicates gene not present in this analysis.



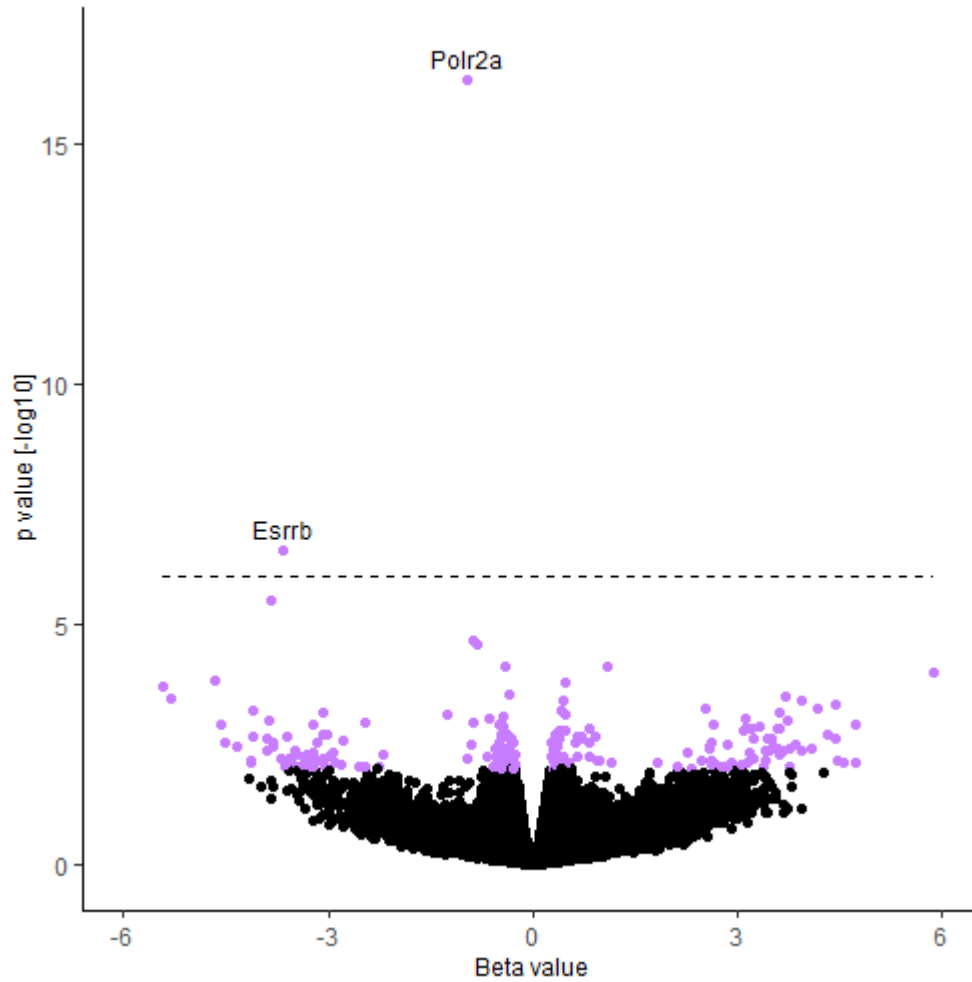


**Figure 3.10.** Venn diagrams of overlapping differentially expressed genes as detected by sleuth and DESeq2 ( $p < 0.01$ ). (A) ethanol treatment, (B) stress treatment, (C) ethanol + stress treatments.



**Figure 3.11.** Gene rank by  $p$ -value density plots between sleuth (x-axis) and DESeq2 (y-axis) analysis pipelines for each treatment. (A) ethanol ( $r = 0.434$ ,  $p < 2.2 \times 10^{-16}$ ), (B) stress ( $r = 0.428$ ,  $p < 2.2 \times 10^{-16}$ ), and (C) ethanol + stress ( $r = 0.573$ ,  $p < 2.2 \times 10^{-16}$ ).

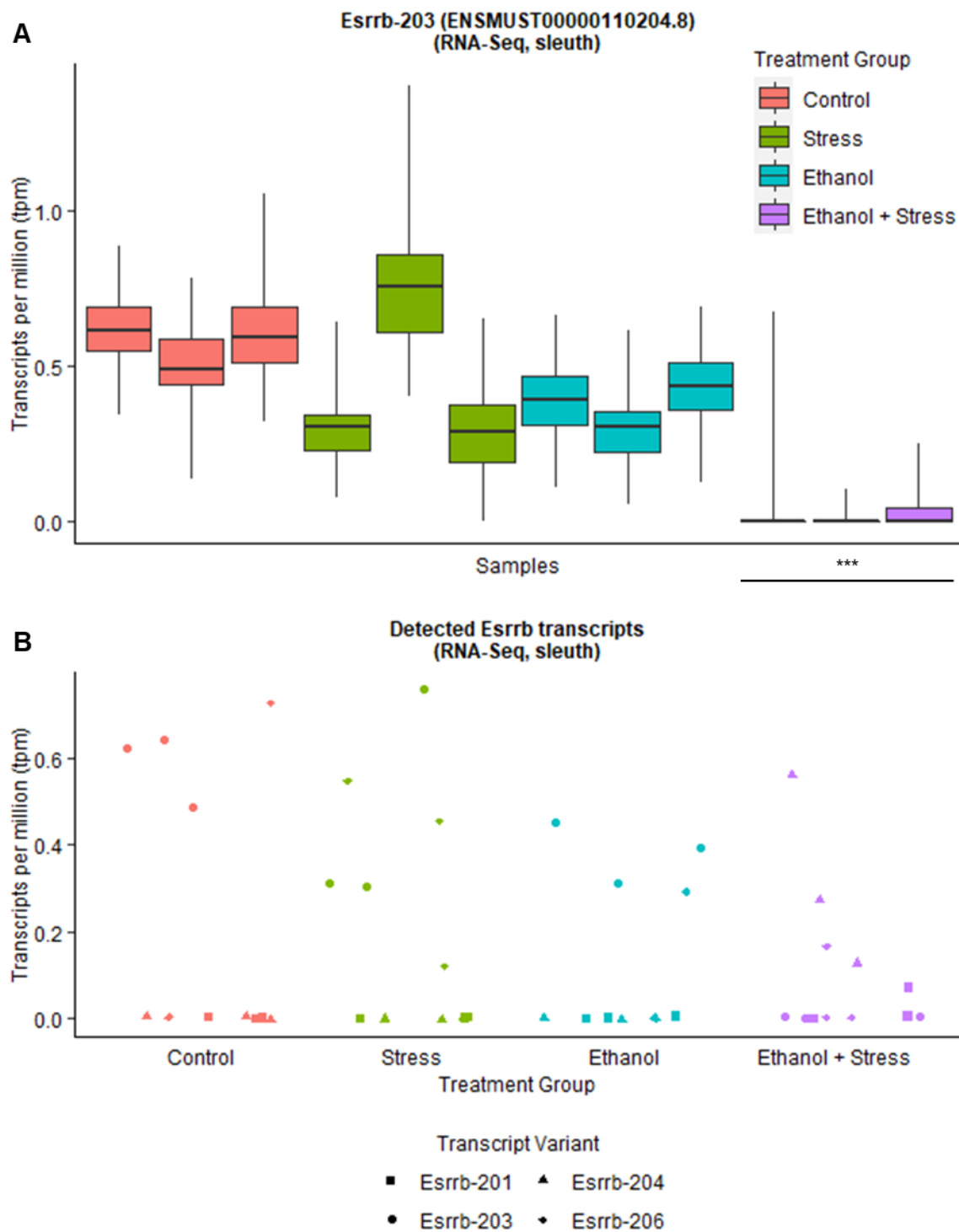
Using a  $p$ -value cut-off adjusted for the false discovery rate (FDR) ( $q < 0.01$ ), PAE or ELS alone does not result in any transcripts with robust changes in differential expression. However, two genes show robust changes in differential expression ( $q < 0.01$ ) between the ethanol + stress group compared to the control group, *Esrrb*, estrogen related receptor, beta and *Polr2a*, polymerase (RNA) II (DNA directed) polypeptide A (**Figure 3.12**). Interestingly, these two genes are also members of module 19 from WGCNA. Each of these is the result of a single transcript downregulated following the combination of ethanol + stress.



**Figure 3.12.** Differential gene expression between ethanol + stress and control groups as detected by sleuth. Volcano plot indicating effect size (Beta value) and significance for transcripts with an expanded scale and significant ( $q < 0.05$ ) annotated genes labelled for ethanol + stress compared to control. The dashed line indicates the cut point used in **Figure 3.7**.

For *Esrrb*, there are six known expressed transcript variants, five of which are protein-coding. The largest, *Esrrb-203* (ENSMUST00000110204.8) (4196bp) is the transcript differentially expressed between the ethanol + stress group and controls (**Figure 3.13A**). Other variants are detected in different groups (**Figure 3.13B**), including the expression of *Esrrb-206* in samples within each group. Interestingly, *Esrrb-204* was only detected in the ethanol + stress group. While there is limited evidence of isoform switching in mice, humans have three forms of *ESRRB*, each likely with a distinct function. The short form is most homologous to mouse *Esrrb*, and there is evidence that these variants may regulate cell cycle progression differently (Heckler and Riggins, 2015). The low level of *Esrrb* expression in the RNA-Seq experiment excluded it from further validation.

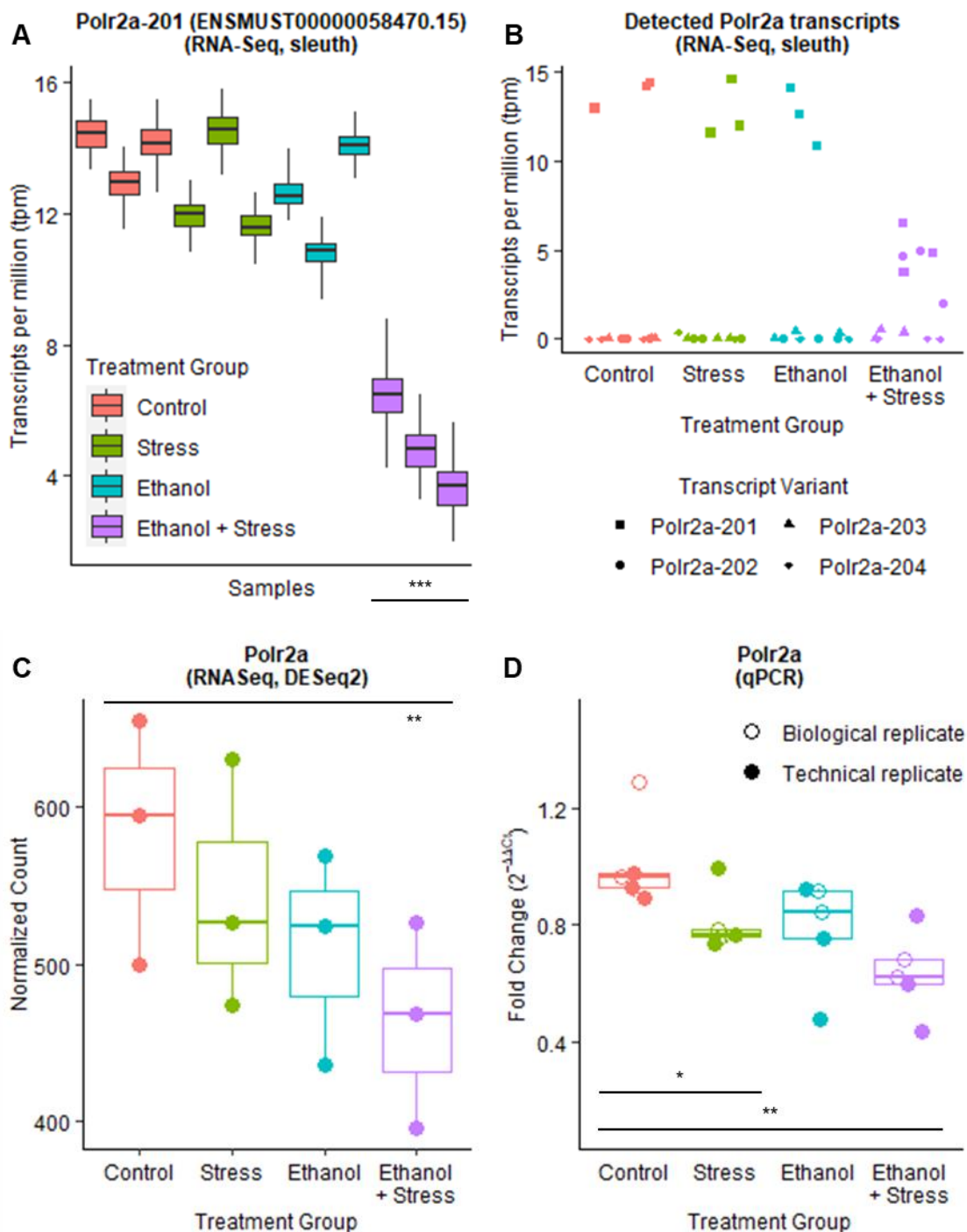




**Figure 3.13.** *Esrrb* RNA-Seq transcript abundance in transcripts per million (tpm) as detected using the kallisto-sleuth analysis pipeline.

(A) *Esrrb*-203 abundance (tpm  $\pm$  inferential variance), \*\*\* $q < 0.01$ . (B) Transcript abundance (tpm) for all detected *Esrrb* transcript variants.

Regarding *Polr2a*, in mice, there are four known expressed transcripts, two of which are protein-coding. The larger, *Polr2a-201* (ENSMUST00000058470.15) (6740bp) is the transcript differentially expressed between the ethanol + stress group and controls (**Figure 3.14A**). In humans, there is only one protein-coding variant homologous to this gene. Interestingly, most of the non-coding isoforms are minimally detected in all groups, however, the other protein-coding variant, *Polr2a-202*, is expressed in each sample following ethanol + stress treatment (**Figure 3.14B**). As a comparison measure, the expression patterns of *Polr2a* was also determined using the DESeq2 pipeline and are remarkably similar to the sleuth pipeline using the same RNA-Seq data (**Figure 3.14C**). Specifically, using the HISAT2-featureCounts-DESeq2 pipeline, *Polr2a* has a fold change of -0.80 ( $p = 0.041$ ) following ethanol + stress as compared to controls. Downregulation of *Polr2a* was validated by qPCR, with a 1.28-fold decrease in expression following stress ( $p = 0.048$ ), and a 1.59-fold decrease following the combination of ethanol + stress ( $p = 0.004$ ) (**Figure 3.14D**).



**Figure 3.14.** *Polr2a* transcript abundance.

(A) *Polr2a-201* abundance in transcripts per million (tpm  $\pm$  inferential variance) detected by sleuth for RNA-Seq, \*\*\* $q < 0.01$ . (B) Abundance (tpm) for all detected *Polr2a* transcript variants by sleuth for RNA-Seq. (C) Normalized counts determined using DESeq2 for RNA-Seq, \*\* $p < 0.01$ . (D) Relative quantity (fold change via the  $2^{-\Delta\Delta C_t}$  method) detected by reverse transcription qPCR. Biological replicates (open circles) were not used in the RNA-Seq, technical replicates (closed circles) are in the RNA-Seq, \* $p < 0.05$ , \*\*  $p < 0.01$ .

### 3.5 Discussion

FASD is a complex societal burden that while preventable, remains common. Besides PAE, children born with FASD are often also exposed to a stressful postnatal upbringing that invariably includes early maternal separation. The main objective of this study is to understand how ELS may complicate molecular changes and behavioural deficits following PAE. The experimental design used has assessed the behavioural changes that result from PAE and ELS (Chapter 2) (Alberry and Singh, 2016). It is followed by the transcriptomic changes reported here, with a focus on the adult hippocampus. The results reported here indicate that the manifestation of FASD-related deficits may be compounded by additional maternal separation stress via alterations in hippocampal gene expression. Specifically, the analysis of this multifaceted data by WGCNA has led to the identification of modules of genes similarly expressed between samples that correlate with treatment and behavioural outcomes. One module of genes, module 19, correlates with treatment and anxiety-like behaviour in the progeny (number of centre entries in the open field test). Genes in this module are related to transcription and neurodevelopment through various gene ontologies and KEGG pathways. The transcript-level differential expression analysis indicates that ELS, as well as the combination of PAE and ELS, results in the downregulation of a transcript responsible for an RNA polymerase II subunit (*Polr2a*) in the hippocampus. Together, these results suggest that the long-term changes in behaviour following PAE and ELS likely result from altered hippocampal gene expression.

#### 3.5.1 WGCNA reveals FASD-relevant module of co-expressed transcripts

The behavioural changes in these mice significantly different between treatment groups are described in Chapter 2 (Alberry and Singh, 2016). This chapter adds to that data through the analysis of RNA-Seq gene expression results from the adult hippocampus of the same mice together with the novel use of WGCNA in an *in vivo* FASD model. WGCNA is a valuable systems biology tool for the determination of modules of correlated gene expression and the relation of these modules to sample traits (Langfelder and Horvath, 2008). WGCNA identifies a single module of hippocampal gene expression, module 19, that correlates with experimental treatment as well as with a reliably measured phenotypic outcome. In this case, the number of entries into the centre zone of the open field test by mice is analogous to an anxiety-like behaviour (Choleris et al., 2001; Prut and Belzung,

2003). Interestingly, several FASD-related deficits, including learning, memory, stress response, and anxiety-like behaviours are under hippocampal control. Specifically, increased adult hippocampal neurogenesis has been associated with reduced anxiety-like behaviour in mice (Hill et al., 2015). Additionally, stress-induced alterations in hippocampal microglia have also been associated with anxiety-like behaviour (Kreisel et al., 2014). Moreover, exposure to chronic stress is associated with activated hippocampal microglia in rats (Tynan et al., 2010). Such results argue that the WGCNA identified a set of genes, represented by module 19, that is related to the interplay between ethanol exposure, stress, and anxiety-like behaviour. Hence, these genes may be relevant to the etiology of FASD.

Next, the focus centres on the set of genes represented in module 19. The genes affected represent pathways that may be disturbed in FASD. Genes responsible for the MAPK cascade (21 genes) and notch signalling (8 genes) are included in this module. MAPK signalling has been identified as a strong candidate pathway in FASD and is fundamental to fetal development (Lombard et al., 2007). The MAPK signalling cascade has also been implicated in ethanol-induced apoptosis through the activation of p53 signalling in neural crest cells (Yuan et al., 2017). Additionally, human neural precursors display impaired neurogenesis following alcohol exposure via the downregulation of MAPK genes (Louis et al., 2018). The MAPK pathway is also involved in chronic stress, with increased negative regulation via MAPK phosphatase-1 in the hippocampus of chronically stressed rodents (Duric et al., 2010). Taken together, it is unsurprising to find MAPK cascade members in module 19, with expression correlated to the treatment group along with anxiety-like behaviour in the resulting progeny. Further, genes involved in Notch signalling are also overrepresented in module 19. Notch signalling is important for neurogenesis and embryonic development through its influence on the expression of associated transcription factors, even in the adult brain (Ables et al., 2011). Notch signalling dysregulation also occurs in PAE models involving mice (Ninh et al., 2019) and zebrafish (Muralidharan et al., 2018). These results further support the notion that MAPK and notch signalling, alongside other signalling cascades, implicate a role for altered transcriptional control in the adult hippocampus following PAE and ELS.

### 3.5.2 Gene expression patterns implicate neurodevelopmental dysregulation

This is the first report of genome-wide changes in hippocampal gene expression following a continuous preference exposure of alcohol in a mouse model of FASD. These results argue that PAE and ELS lead to alterations in specific hippocampal genes depending on the type and combination of exposures. Of interest to this discussion are two transcripts, *Robo4* and *Krba1*, that are among the most significantly altered transcripts in each treatment group, both upregulated in every group compared to control ( $p < 0.001$ ). The protein product for *Robo4* is the roundabout (Robo) receptor, which interacts with the astrocyte-secreted slit guidance ligand 2 (Slit2) during central nervous system development, specifically as an axon repellent during axon guidance (Brose et al., 1999). *Krba1* is predicted to regulate transcription via DNA template binding as a zinc-finger protein that represses RNA polymerase promoters (Urrutia, 2003), and is associated with gestational long-term exposure to air pollution (Winckelmans et al., 2017). If the upregulation of these two genes following each treatment results in decreased viability, abnormal synapse formation, or improper transcriptional regulation during neurodevelopment, they may partially explain the observed behavioural deficits in FASD. While the function of *Robo4* and *Krba1* in the adult hippocampus is unclear, they are both constitutively expressed in the hippocampus, as reported in the Allen Mouse Brain Atlas (Lein et al., 2007). The dysregulation of these genes may occur during neurodevelopment and persist into adulthood and may also be relevant in the adult stage.

Two transcripts, *Alas2* and *Suv39h2*, are among the most significantly altered transcripts in the two PAE treatment groups (ethanol, ethanol + stress) as detected using the kallisto-sleuth pipeline. Additionally, *Alas2* is consistently altered in every treatment group using the second, independent analytical pipeline, HISAT2-featureCounts-DESeq2. There have been mixed reports regarding environmental exposures and *Alas2* expression, including downregulation following voluntary maternal ethanol consumption (Rosenberg et al., 2010) or stress exposure in heavy drinkers (Beech et al., 2014), and upregulation following lead exposure (Schneider et al., 2012) or repeated stress (Stankiewicz et al., 2014). The protein product of *Suv39h2* is a histone methyltransferase, specifically for H3K9, often resulting in trimethylation that inhibits gene expression (Peters et al., 2001). Further,

differential histone methylation in the brain has also been implicated in FASD (Chater-Diehl et al., 2016, 2017).

Finally, three transcripts, *Kdm6a*, *Sbno1*, and *Dlg3*, were among the most significantly altered transcripts in response to ELS (stress and ethanol + stress groups). *Kdm6a* encodes a histone demethylase that removes suppressive chromatin marks and controls gene expression in microglia for clearance of dying neurons and non-functional synapses (Ayata et al., 2018). *Sbno1* is a nuclear-localized transcriptional regulator important in Notch and Hippo signalling (Watanabe et al., 2017), which has been associated with schizophrenia (Fromer et al., 2016; Radulescu et al., 2018), and is likely important for brain function. *Dlg3* encodes synapse-associated protein 102 and is important for synapse formation in the brain, whereby knockout mice have synaptic plasticity impairments, including impaired spatial learning (Cuthbert et al., 2007). In humans, *DLG3* mutations cause non-syndromic X-linked intellectual disability (Tarpey et al., 2004). There is upregulation of a *Dlg3* transcript in the stress and ethanol + stress groups compared to control, in agreement with other findings that overexpression inhibits proliferation and induces apoptosis (Liu et al., 2014). Taken together, the upregulation of these three genes in the stress and ethanol + stress groups may lead to altered brain transcriptional regulation and synaptic plasticity required for normal learning processes and brain function.

### 3.5.3 Gene expression dysregulation complements WGCNA results

The molecular functions most significantly enriched by module 19 genes involve RNA polymerase II DNA binding. These are genes correlated with the experimental treatment group and anxiety-like behaviour. When considering differentially expressed genes, the most significantly differentially expressed transcript coding for an RNA polymerase II subunit (*Polr2a*) is downregulated following stress and ethanol + stress as compared to control. *Polr2a*, polymerase (RNA) II (DNA directed) polypeptide A, encodes for the largest subunit of RNA polymerase II, an enzyme responsible for mRNA synthesis. Like the combination of ethanol + stress presented here, a mouse model of stress + morphine from postnatal days 5 to 9 found decreased *Polr2a* expression following stress alone (Juil et al., 2011). Conversely, when combined with morphine treatment, there was an associated upregulation of *Polr2a*, suggesting expression of this gene is particularly sensitive to

environmental insults. This report is not the first evidence of *Polr2a* alterations in FASD research – in a mouse model of binge-like PAE, increased promoter DNA methylation of *Polr2a* was found in the hippocampus (Chater-Diehl et al., 2016). Additionally, *Polr2a* expression is decreased in the dentate gyrus of the hippocampus alongside increased anxiety-like behaviour following induced glucocorticoid receptor overexpression (Wei et al., 2012). WGCNA has also been used to create gene co-expression modules related to ethanol exposure in human embryonic stem cells to help understand molecular mechanisms underlying FASD. Despite the vastly different experimental designs, one module associated with ethanol treatment in the stem cells was also most significantly enriched with RNA polymerase II activity (Khalid et al., 2014).

### 3.6 Conclusions

This research used WGCNA to identify a module of genes potentially implicated in FASD. In this analysis, module 19 was associated with anxiety-like behaviour, an association potentially driven by microglia. Additionally, the *Kdm6a* histone demethylase found differentially expressed in stress and ethanol + stress groups is important for microglia function in the clearance of dying neurons. Developmental alcohol exposure leads to lasting disruption of microglia in rodents (Chastain et al., 2019). RNA isolated from the adult hippocampus was examined in this study. This is a more focused tissue type than whole brain homogenate, representing RNA from a mixed cell population. However, multiple lines of evidence suggest microglia and neurons respond differently and need to be assessed separately. Further work in this area should focus on cell type differences that underlie the observations described here. Also, alterations in gene expression in this study were assessed at postnatal day 70, following PAE and/or ELS up to postnatal day 14. It seems unlikely that the direct effects of the treatments used persist 8 weeks later. It is logical to argue that these exposures cause long-lasting transcriptomic effects. In this context, epigenetic mechanisms are known to be involved in the programming of gene expression throughout development, and as mediators of experience to finely control expression. As in these results, alterations to epigenetic marks such as DNA methylation, histone modifications, and microRNA expression have been implicated following PAE and ELS. As such, future experiments should aim to classify alterations in epigenetic terms that may be responsible for the persistent changes in gene expression seen over a long period. Finally, transcriptome



changes persist in the hippocampus of adult mice prenatally exposed to alcohol and/or postnatally exposed to early life stress. Some transcripts are co-expressed in a way that correlates with experimental treatment and behavioural outcomes. These transcripts code for genes important in RNA processing and management throughout neurodevelopment and beyond. Also, some transcripts are significantly differentially expressed between treatment groups. Specifically, the largest protein-coding variant of *Polr2a* (*Polr2a-201*) is downregulated following ELS as well as the combination of PAE and ELS. These lasting alterations in transcripts responsible for RNA processing likely underlie the behavioural deficits observed. They suggest that postnatal stresses further complicate the effects caused by PAE in FASD. Further understanding of how gene expression changes occur and persist may result in earlier detection, prognosis, and amelioration in humans faced with FASD.

## Chapter 4. Adult hippocampal promoter DNA methylation alterations following PAE and ELS

### 4.1 Abstract

The molecular impact of PAE and ELS often associated with FASD is not understood and forms the focus of this research. What initiates and maintains the behavioural changes established in Chapter 2 (Alberry and Singh, 2016) and the associated hippocampal gene expression changes discussed in Chapter 3 (Alberry et al., 2020) remains to be established and forms the focus of this chapter. Specifically, the changes in promoter DNA methylation that are known to affect gene expression were assessed using MeDIP-Seq following PAE and ELS. The novel results show that different sets of genes with altered promoter DNA methylation are affected by both treatments independently, and a unique set of genes are affected by the combination of the two treatments. PAE leads to altered promoter DNA methylation at genes important for transcriptional regulation. Maternal separation leads to changes in genes important for histone methylation and immune response, and the combination of two treatments results in DNA methylation changes at genes important for neuronal migration and immune response. Interestingly, there is minimal complementarity between changes in promoter DNA methylation and gene expression, although genes involved tend to be critical for brain development and function. While remaining to be validated, such results argue that mechanisms beyond promoter DNA methylation must be involved in lasting gene expression alterations leading to behavioural deficits in FASD.

## 4.2 Background

Given that PAE is the cause of FASD, and neurodevelopment is long-lasting, spanning decades, research must also focus on postnatal factors that may determine the manifestation of this disorder. A mouse model of FASD using C57BL/6J (B6) mice has been developed (Kleiber et al., 2011), establishing that FASD is like an iceberg. The perceptible phenotypic deficits resulting from PAE are just the tip – learning deficits, anxiety-like behaviours, and altered activity patterns (Allan et al., 2003; Kaminen-Ahola et al., 2010a; Kleiber et al., 2011; Marjonen et al., 2015). Most underlying biological defects are not visible and are reflected in molecular changes.

Since children with FASD often face additional early life stress, the model has been extended to explore how postnatal maternal separation may impact developmental and behavioural deficits following PAE described in Chapter 2 (Alberry and Singh, 2016). There are changes in hippocampal gene expression that persist into adulthood following PAE combined with ELS in B6 mice described in Chapter 3 (Alberry et al., 2020). Given that the initiation and maintenance of aberrant gene expression in response to PAE includes epigenetic mechanisms, primarily DNA methylation, non-coding RNA, and histone modifications (Chater-Diehl et al., 2016; Chen et al., 2013; Laufer et al., 2013), these processes are expected to be critical in most models of FASD. In this research, the focus is on DNA methylation for two key reasons. First, while many epigenetic mechanisms may alter transcription, promoter DNA methylation may repress transcription via blocked transcription factor binding, the canonical relationship between gene expression and promoter DNA methylation (Jaenisch and Bird, 2003). Second, recent FASD studies in humans have been generating promising results with the involvement of DNA methylation (Laufer et al., 2015; Portales-Casamar et al., 2016).

This research is novel in that it combines the effect of PAE and ELS on the FASD iceberg analogy at three levels – behaviour, hippocampal gene expression and hippocampal DNA methylation on the same samples. The hippocampus is implicated in the manifestation of FASD-related behaviours. This chapter details the assessment of promoter DNA methylation changes in the mouse model of FASD that includes PAE and ELS. The results show that although the behavioural changes are compatible with molecular changes,

alterations in gene expression are only partly reflected in promoter-specific DNA methylation. It may represent a feature of the experimental design or reflect a general feature of the development of FASD. Although one is tempted to suggest that epigenetic changes other than DNA methylation must represent underlying mechanisms involved in altering gene expression and the development of FASD, the issue deserves further experimentation and comprehensive evaluation.

## 4.3 Methods & materials

### 4.3.1 Hippocampal dissection & DNA isolation

Male mice were sacrificed via carbon dioxide asphyxiation and cervical dislocation on postnatal day 70, with hippocampus dissected and samples kept at  $-80^{\circ}\text{C}$ , as mentioned previously (Chapter 3.3.1). The AllPrep DNA/RNA Mini Kit Protocol (Qiagen, Valencia, CA) was followed to isolate DNA and RNA from the same sample. DNA quantification was determined by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

### 4.3.2 Methylated DNA immunoprecipitation sequencing (MeDIP-Seq)

Three DNA samples were randomly selected from each treatment group (12 total DNA samples from individual mice processed separately) and sent on dry ice to Arraystar Inc. (Rockville, MD), with one sample per group as an input sample for sequence control. DNA was quantified by NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE), then fragmented to a range of 200-1500 bp using a Diagenode Bioruptor, end-repaired, and 3' adenylated for ligation of genomic adapters. Fragments were immunoprecipitated by anti-5-methylcytosine antibody and PCR amplified. AMPure XP beads were used to select fragments from 300-800 bp, then fragments were quantified by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Following denaturation with 0.1 M NaOH, single-stranded DNA molecules were captured and amplified *in situ* on an Illumina flow cell. Libraries were sequenced on the Illumina HiSeq 4000 platform using the HiSeq 3000/4000 SBS Kit (300 cycles) protocol. Off-Line Basecaller (OLB V1.8) was used for base calling. Reads were aligned to the mouse genome (UCSC MM10) using HISAT2 (V2.1.0) after passing the Solexa CHASTITY quality filter and resulting BED files were used for differential methylation analysis. Sequences have been made available at GEO accession number GSE137984.

### 4.3.3 Differential methylation analysis

Aligned reads from HISAT2 were used for peak calling using model-based analysis for ChIP-Seq (MACS) (version 1.4.2) (Zhang et al., 2008), where enriched regions (peaks) for each sample were identified by comparison to input background samples for each group using a dynamic Poisson distribution ( $q < 10^{-5}$ ). MeDIP enriched regions were annotated

to the nearest gene using UCSC RefSeq, with differentially methylated regions (DMRs) in the promoter of known genes for ethanol vs control, stress vs control, as well as ethanol + stress vs control, identified by diffReps (cut-off:  $\log_2\text{FC} = 1.0$ ,  $p = 10^{-4}$ ) (Shen et al., 2013). Promoter regions are defined here as 2000 bp upstream and downstream from the transcription start site of a gene. Generalized hypergeometric tests for enrichment of gene ontology (GO) terms and Kyoto encyclopedia of genes and genomes (KEGG) pathways were used for genes represented by promoter DMRs for each treatment group using `goana` and `kegga` functions in the `limma` software package (Ritchie et al., 2015) in R, filtered by significance ( $p < 0.05$ ).

#### 4.3.4 Comparison to transcriptome analysis

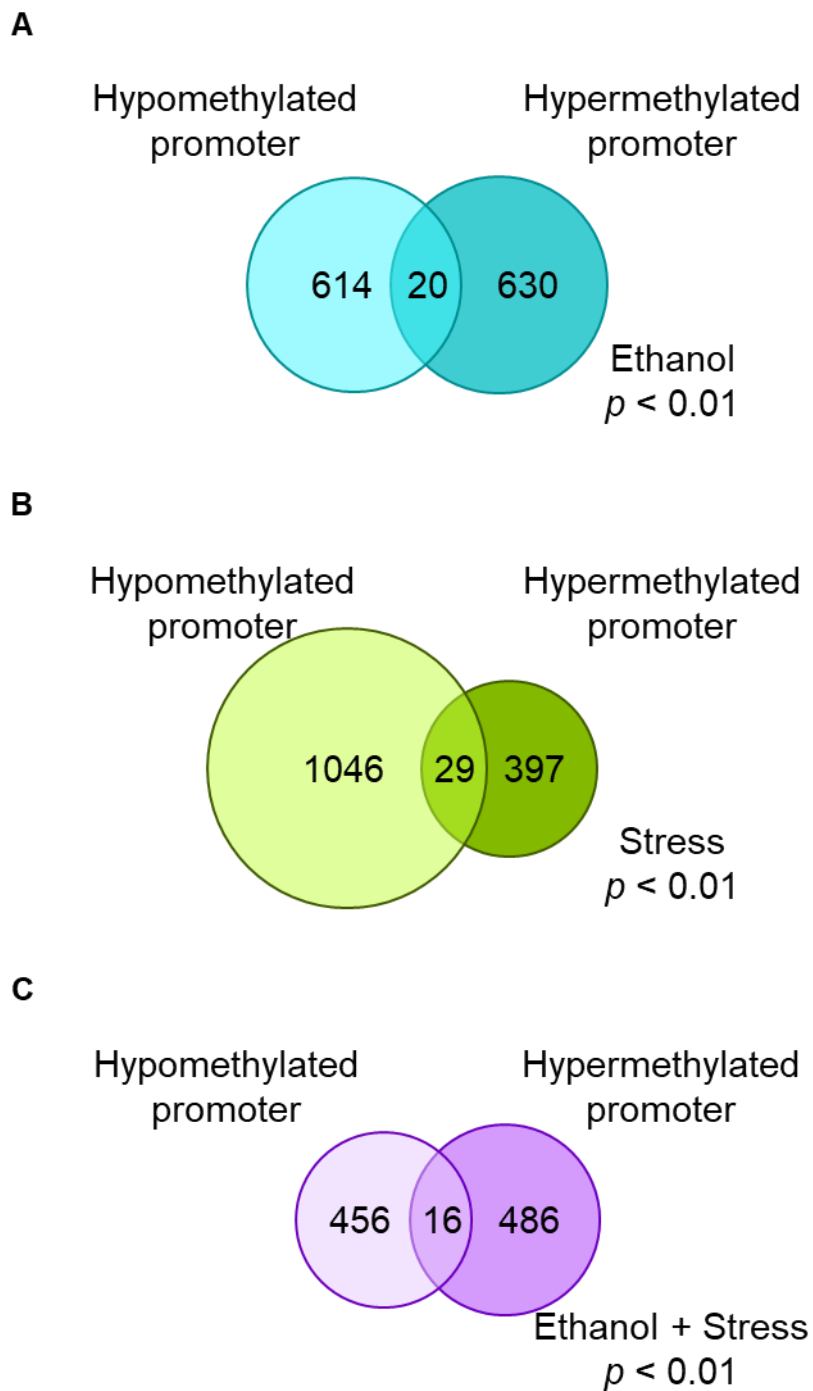
Genes represented by promoter DMRs for each treatment group were compared to transcript-level expression analysis from the RNA-Seq experiment using RNA isolated from the same samples described in Chapter 3 (Alberry et al., 2020). Gene lists used here were filtered by significance ( $p < 0.01$ ), resulting in a list of 164 unique transcripts altered in the ethanol group, 116 for stress, and 217 for the combination of ethanol + stress (genes included in **Appendix D**).

## 4.4 Results

Hippocampal DNA samples from the same samples used for RNA-Seq analysis for each of four groups of mice representing a control group with no experimental interventions, an ethanol group that faced PAE, a stress group that faced ELS, and an ethanol + stress group subject to both treatments were used for these results (see methods for specific details). DNA methylation profiles were assessed via MeDIP-Seq to determine how treatment groups differ from each other and the controls.

### 4.4.1 DNA methylation changes following different treatments

Differentially methylated regions (DMRs) were determined for each treatment group compared to controls, then associated with annotated genes via proximity to transcription start sites. Promoter regions are defined here as 2 kb upstream or downstream from the transcription start site of a gene. Genes with promoter DMRs were considered for this analysis. In this way, several DMRs considered spatially distinct can be present in the promoter region of single or multiple genes. A gene implicated by more than one DMR is considered separately when the DMRs are in opposition, but only once if the DMRs are in the same direction. 1264 genes have promoter DMRs altered by PAE as compared to controls (**Figure 4.1A**, details in **Appendix F**). Following ELS, 1472 genes are represented by promoter DMRs (**Figure 4.1B**, details in **Appendix F**). When mice faced both ethanol + stress, 958 genes have promoter DMRs (**Figure 4.1C**, details in **Appendix F**). Interestingly, while the ethanol group has a similar number of genes with hypo- and hypermethylated promoter DMRs, the stress group genes are represented by 73% hypomethylated promoter DMRs. Genes with promoter DMRs in the ethanol + stress group are also more evenly distributed between hypo- and hypermethylated. While a single DMR is either hypo- or hypermethylated, a single gene may be represented by multiple DMRs with different states. There are between 16 and 29 genes in each comparison that are represented by both hypo- and hypermethylation of promoter DMRs.



**Figure 4.1.** The number of genes with significant ( $p < 0.01$ ) promoter DNA methylation changes in each treatment group.

(A) prenatal ethanol treatment, (B) early life stress treatment, or (C) the combination of prenatal ethanol treatment and early life stress as compared to controls.



Hypo- and hypermethylated DMR-associated genes for each treatment group were analyzed separately for gene ontology (GO) and KEGG pathway enrichment. In the ethanol group, genes related to voltage-gated calcium channel activity tended to be hypomethylated at their promoter regions ( $p < 0.007$ ) (**Table 4.1**). Also, brain-related terms including NMDA receptor activity ( $p = 0.001$ ), positive regulation of behaviour ( $p = 0.002$ ), circadian rhythm ( $p = 0.003$ ), and apical dendrite ( $p = 0.03$ ) are all biological processes over-represented by genes with hypomethylated promoters following PAE. Interestingly, components responsible for transcriptional regulation are also implicated by genes with hypomethylated promoters, including polycomb repressive complex 1 (PRC1) complex ( $p = 4.04 \times 10^{-4}$ ), transcription factor TFIID complex ( $p = 0.03$ ), and RNA polymerase II transcription factor complex ( $p = 0.046$ ). Genes related to phosphoinositide kinase ( $p < 0.02$ ) and cytoskeleton regulation ( $p = 0.005$ ) are hypermethylated at their promoter regions following PAE (**Table 4.2**). In the stress group, genes important for histone modifications ( $p < 0.01$ ) and neuronal organization ( $p = 0.007$ ) are overrepresented by genes with hypomethylated promoters (**Table 4.3**). Conversely, genes with hypermethylated promoters following ELS are important for cytokine interactions ( $p < 0.006$ ) (**Table 4.4**). When mice face both ethanol + stress, genes important for neuron migration ( $p < 0.005$ ) are hypomethylated at their promoters (**Table 4.5**). As with stress alone, the combination of ethanol + stress results in promoter hypermethylation for genes responsible for cytokine interactions ( $p < 0.01$ ) (**Table 4.6**).

**Table 4.1.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypomethylated promoter DMR-associated genes implicated by ethanol treatment compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Hydrolase activity, hydrolyzing O-glycosyl compounds	0.001
Voltage-gated cation channel activity	0.005
Voltage-gated calcium channel activity	0.007
High voltage-gated calcium channel activity	0.007
Protein tyrosine kinase activity	0.008
<b>Biological process</b>	
Regulation of cation channel activity	8.71E-05
Regulation of NMDA receptor activity	0.001
Positive regulation of behaviour	0.002
Regulation of skeletal muscle cell differentiation	0.002
Positive regulation of circadian rhythm	0.003
<b>Cellular component</b>	
PRC1 complex	4.04E-04
Apical dendrite	0.025
Transcription factor TFIID complex	0.026
Nuclear ubiquitin ligase complex	0.043
RNA polymerase II transcription factor complex	0.046
<b>KEGG pathway</b>	
PPAR signaling pathway	0.018
Hypertrophic cardiomyopathy (HCM)	0.019
Fructose and mannose metabolism	0.024
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.027
Glycosaminoglycan degradation	0.028

**Table 4.2.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypermethylated promoter DMR-associated genes implicated by ethanol treatment compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Phosphatidylinositol bisphosphate kinase activity	0.001
Phosphatidylinositol-4,5-bisphosphate 3-kinase activity	0.002
Phosphatidylinositol 3-kinase activity	0.003
1-phosphatidylinositol-3-kinase activity	0.014
C3HC4-type RING finger domain binding	0.015
<b>Biological process</b>	
Regulation of transport	0.004
Photoreceptor cell development	0.005
Eye photoreceptor cell development	0.006
Lymphocyte differentiation	0.010
Anion transport	0.010
<b>Cellular component</b>	
Unconventional myosin complex	0.026
Proton-transporting V-type ATPase complex	0.048
<b>KEGG pathway</b>	
Carbohydrate digestion and absorption	0.003
Regulation of actin cytoskeleton	0.005
Pyrimidine metabolism	0.011
Focal adhesion	0.013
Glyoxylate and dicarboxylate metabolism	0.017

**Table 4.3.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypomethylated promoter DMR-associated genes implicated by stress treatment compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
SUMO-specific protease activity	0.005
Protein-arginine omega-N asymmetric methyltransferase activity	0.007
Non-membrane spanning protein tyrosine kinase activity	0.009
Histone-arginine N-methyltransferase activity	0.010
Arginine N-methyltransferase activity	0.014
<b>Biological process</b>	
Histone methylation	0.003
Smooth muscle contraction	0.005
Apoptotic nuclear changes	0.007
Regulation of microtubule cytoskeleton organization	0.007
Neuron projection extension in neuron projection guidance	0.007
<b>Cellular component</b>	
Multivesicular body membrane	0.005
T cell receptor complex	0.009
Actin cytoskeleton	0.017
Multivesicular body	0.021
Cytoplasmic stress granule	0.024
<b>KEGG pathway</b>	
Chemokine signaling pathway	0.020

**Table 4.4.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypermethylated promoter DMR-associated genes implicated by stress treatment compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Voltage-gated potassium channel activity in ventricular cardiac muscle cell action potential repolarization	4.96E-04
Voltage-gated potassium channel activity involved in cardiac muscle cell action potential repolarization	0.002
Oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	0.004
Cytokine activity	0.006
Peptidyl-proline 4-dioxygenase activity	0.006
<b>Biological process</b>	
Positive regulation of protein secretion	3.88E-04
Regulation of macrophage cytokine production	0.001
Ventricular cardiac muscle cell membrane repolarization	0.001
Positive regulation of cytokine secretion	0.001
Cardiac muscle cell membrane repolarization	0.001
<b>Cellular component</b>	
Multivesicular body	0.025
T cell receptor complex	0.045
Platelet alpha granule	0.045
<b>KEGG pathway</b>	
Cytokine-cytokine receptor interaction	1.34E-06
Neuroactive ligand-receptor interaction	0.008
Antigen processing and presentation	0.013
TGF-beta signaling pathway	0.013
Hematopoietic cell lineage	0.015

**Table 4.5.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypomethylated promoter DMR-associated genes implicated by combined ethanol + stress treatments compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Acetylglucosaminyltransferase activity	0.001
Rho guanyl-nucleotide exchange factor activity	0.014
Ether hydrolase activity	0.014
Phosphofructokinase activity	0.014
Transmembrane receptor protein tyrosine kinase activity	0.015
<b>Biological process</b>	
mRNA polyadenylation	4.50E-04
RNA polyadenylation	0.001
Regulation of neuron migration	0.001
Positive regulation of neuron migration	0.002
Neuron projection extension	0.005
<b>Cellular component</b>	
L-type voltage-gated calcium channel complex	0.011
Azurophil granule membrane	0.013

*Note:* no KEGG pathways reached  $p < 0.05$ .

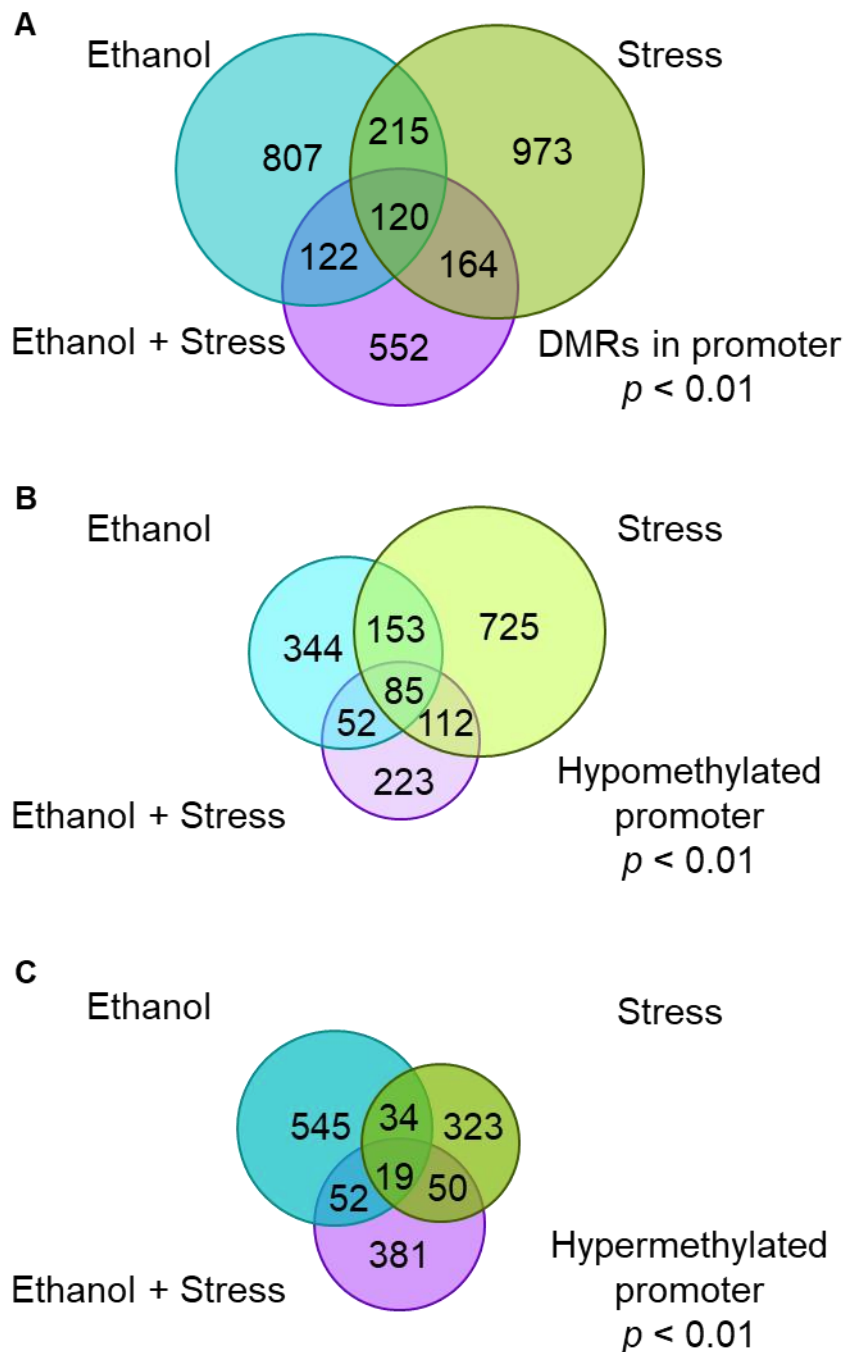
**Table 4.6.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypermethylated promoter DMR-associated genes implicated by combined ethanol + stress treatments compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Anion transmembrane transporter activity	0.005
Inorganic anion transmembrane transporter activity	0.008
Organic anion transmembrane transporter activity	0.009
Cytokine receptor activity	0.011
Extracellular ATP-gated cation channel activity	0.012
<b>Biological process</b>	
Positive regulation of calcium-mediated signalling	0.006
Monocarboxylic acid metabolic process	0.006
Sphingosine-1-phosphate signaling pathway	0.009
Amylin receptor signaling pathway	0.009
Response to insulin	0.009
<b>Cellular component</b>	
Integral component of nuclear inner membrane	0.003
Extrinsic component of external side of plasma membrane	0.012
G-protein coupled receptor dimeric complex	0.020
Brush border membrane	0.035
<b>KEGG pathway</b>	
Cytokine-cytokine receptor interaction	1.68E-05
Hematopoietic cell lineage	0.001
Mucin type O-glycan biosynthesis	0.001
JAK-STAT signaling pathway	0.003
Dilated cardiomyopathy (DCM)	0.026

#### 4.4.2 Genes with promoter DNA methylation changes are shared by different treatments

While most promoter DMRs are specific to each treatment, there are regions of overlap between treatments (**Figure 4.2**). 120 genes are implicated by promoter DMRs following ethanol, stress, as well as ethanol + stress (**Figure 4.2A**). When directionality is considered, there are 85 genes represented by hypomethylated promoter DMRs in all three treatment groups (**Figure 4.2B**). Finally, there are 19 genes with hypermethylated promoter DMRs in all three treatment groups (**Figure 4.2C**). Genes consistently implicated by promoter DMR hypomethylation tend to be important for histone methylation ( $p < 0.04$ ) and cyclic adenosine monophosphate (cAMP) protein kinase activity ( $p < 0.04$ ) (**Table 4.7**). Conversely, genes with promoter DMR hypermethylation are important for ion channel activity ( $p < 0.007$ ) together with neuroactive ligand-receptor interactions ( $p = 0.04$ ) and chemokine signalling ( $p < 0.003$ ) (**Table 4.8**).





**Figure 4.2.** The number of genes with significant promoter DNA methylation changes shared following prenatal ethanol treatment, early life stress, or the combination of ethanol + stress.

(A) all genes with altered promoter DNA methylation together, (B) gene with hypomethylated promoters, and (C) genes with hypermethylated promoters.

**Table 4.7.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypomethylated promoter DMR-associated genes implicated by all three treatment categories – ethanol, stress and combined ethanol + stress compared to control ( $p < 0.01$ ).

Term	p-value
<b>Molecular function</b>	
Camp-dependent protein kinase regulator activity	0.029
Protein-arginine omega-N asymmetric methyltransferase activity	0.034
Histone-arginine N-methyltransferase activity	0.038
Camp-dependent protein kinase inhibitor activity	0.038
Arginine N-methyltransferase activity	0.042
<b>Biological process</b>	
Endosome to lysosome transport via multivesicular body sorting pathway	0.025
Phagosome-lysosome fusion	0.025
Choline catabolic process	0.025
Regulation of protein metabolic process	0.027
Regulation of high voltage-gated calcium channel activity	0.029
<b>Cellular component</b>	
L-type voltage-gated calcium channel complex	0.029
Perinuclear endoplasmic reticulum	0.034

*Note:* no KEGG pathways reached  $p < 0.05$ .

**Table 4.8.** Top 5 most significantly ( $p < 0.05$ ) overrepresented GO terms and KEGG pathways represented by hypermethylated promoter DMR-associated genes implicated by all three treatment categories – ethanol, stress and combined ethanol + stress compared to control ( $p < 0.01$ ).

Term	p-value
Molecular function	
ATP-gated ion channel activity	0.007
Extracellular ATP-gated cation channel activity	0.007
Nucleotide receptor activity	0.007
Purinergic receptor activity	0.008
Mannose binding	0.010
Biological process	
Mesodermal cell fate commitment	0.006
Bone resorption	0.008
Neuromuscular process controlling balance	0.013
Neuromuscular process	0.015
Response to interferon-alpha	0.016
Cellular component	
Integral component of nuclear inner membrane	0.011
Multivesicular body	0.028
KEGG pathway	
Chemokine signaling pathway	0.001
Cytokine-cytokine receptor interaction	0.003
Neuroactive ligand-receptor interaction	0.042

#### 4.4.3 DNA methylation alterations in relation to gene expression

Since hippocampal transcriptome profiles exist for these mice (Chapter 3), genes with altered promoter DNA methylation could be compared to genes with altered expression. The number of genes represented by altered promoter methylation following each treatment is represented by the direction of gene expression change following the RNA-Seq study previously reported are presented here (**Table 4.9**). While many genes are included for comparison, the overlap between genes with altered DNA methylation and gene expression is minimal. For ethanol mice, 1264 genes with promoter DMRs were compared to the previously reported 164 genes altered in these samples. There are two genes with hypomethylated promoters and increased expression, *Slc2a9*, solute carrier family 2 (facilitated glucose transporter), member 9, and *Blvrb*, biliverdin reductase B (flavin reductase (NADPH)). Conversely, for hypermethylated promoters, there are two transcripts with decreased expression following PAE, *Eif4g1*, eukaryotic translation initiation factor 4, gamma 1, and *Myo6*, myosin VI.

In the mice subjected to stress, 1472 genes with promoter DMRs were assessed in relation to the 116 genes altered in these samples. There are three genes with hypomethylated promoters and increased transcript expression, *Arpp21*, cyclic AMP-regulated phosphoprotein, 21, *Mark2*, microtubule affinity regulating kinase 2, and *Rab33b*, RAB33B, member RAS oncogene family. One gene, *Slc39a11*, solute carrier family 39 (metal ion transporter), member 11, is hypermethylated at its promoter and has reduced transcript expression following maternal separation.

For ethanol + stress mice, 958 genes with promoter DMRs were compared to the 217 genes altered in these samples. Interestingly, *Arpp21* is also hypomethylated at its promoter and overexpressed following ethanol + stress treatment alongside two additional genes, *Dlg3*, and *Pinx1*, PIN2/TERF1 interacting, telomerase inhibitor 1. Conversely, two genes share increased promoter methylation and decreased gene expression, *Slu7*, pre-mRNA-splicing factor SLU7, and *Unc119*, unc-119 lipid binding chaperone. While the number of genes with compatible altered promoter methylation and expression resulting from each treatment or combination of treatments is minimal, most of these genes have known brain function, ranging from neuronal migration to synapse formation and plasticity (**Table 4.10**).

**Table 4.9.** Relevant genes through the complementary direction of methylation (DNAm) and gene expression (GE) changes for genes differentially expressed with differential promoter methylation following ethanol, stress, or ethanol + stress.

Group	Direction		Number of genes	Gene name	DNA Methylation			Expression		
	DNAm	GE			DMR locus	fold change	p-value	beta	p-value	
Ethanol	↓	↑	2	<i>Slc2a9</i>	chr5:38481041-38481660	-1.80	0.006	2.576	0.005	
				<i>Blvrb</i>	chr7:27449261-27449600	-inf	0.007	3.366	0.005	
		↓	0	-	-	-	-	-	-	
		-	632	-	-	-	-	-	-	
	↑		↓	2	<i>Eif4g1</i>	chr16:20671221-20671520	inf	0.003	-3.487	0.008
					<i>Myo6</i>	chr9:80164241-80164600	3.03	0.009	-3.625	0.001
			↑	0	-	-	-	-	-	-
			-	648	-	-	-	-	-	-
Stress	↓			<i>Arpp21</i>	chr9:112234421-112234980	-3.79	0.002	2.253	0.006	
			↑	3	<i>Mark2</i>	chr19:7340121-7341000	-2.03	0.001	3.413	0.003
					<i>Rab33b</i>	chr3:51485781-51486080	-inf	0.003	2.830	0.009
		↓	2	-	-	-	-	-	-	
		-	1070	-	-	-	-	-	-	
	↑		↓	1	<i>Slc39a11</i>	chr11:113567341-113567720	3.33	0.010	-2.768	0.009
			↑	0	-	-	-	-	-	-
	-	425	-	-	-	-	-	-		

**Table 4.9.** Continued from the previous page.

Group	Direction		Number of genes	Gene name	DNA Methylation			Expression	
	DNAm	GE			DMR locus	fold change	p-value	beta	p-value
Ethanol + Stress				<i>Arpp21</i>	chr9:112184321-112185360	-1.05	0.006	2.663	0.001
		↑	3	<i>Dlg3</i>	chrX:100773301-100773560	-inf	0.009	3.123	0.001
		↓		<i>Pinx1</i>	chr14:63859301-63859580	-inf	0.007	0.439	0.005
		↓	2	-	-	-	-	-	-
		-	467	-	-	-	-	-	-
		↓	2	<i>Slu7</i>	chr11:43434381-43434620	inf	0.004	-0.459	0.007
		↑		<i>Unc119</i>	chr11:78341401-78341900	1.90	0.006	-3.900	0.004
		↑	1	-	-	-	-	-	-
	-	499	-	-	-	-	-	-	

**Table 4.10.** Genes of interest identified by altered promoter methylation and gene expression with literature-based evidence of central nervous system function.

Gene of interest	Central nervous system relevance
<i>Slc2a9</i>	Memory performance (Houlihan et al., 2010)
	Social phobia (Lyngdoh et al., 2013)
<i>Eif4g1</i>	Neurodegeneration (Dhungel et al., 2015)
<i>Myo6</i>	Dendritic spine length (Avraham et al., 1995)
	Postsynaptic receptor internalization (Osterweil et al., 2005)
	Presynaptic glutamate release potentiation (Yano et al., 2006)
<i>Arpp21</i>	Dendrite growth (Rehfeld et al., 2018)
<i>Mark2</i>	Neuron migration (Mejia-Gervacio et al., 2012; Sapir et al., 2008)
<i>Rab33b</i>	Axon growth (Huang et al., 2019)
<i>Dlg3</i>	Synapse formation, spatial learning (Cuthbert et al., 2007)
	Intellectual disability (Tarpey et al., 2004)
<i>Pinx1</i>	Late-onset Alzheimer disease (Tosto et al., 2019)
<i>Unc119</i>	Dendritic branching (May et al., 2014)

## 4.5 Discussion

The main objective of this research was to understand how ELS may complicate molecular changes underlying behavioural deficits that arise following PAE. Behavioural changes resulting from PAE and ELS have been characterized and described in Chapter 2 (Alberry and Singh, 2016), with lasting changes to the hippocampal transcriptome detailed in Chapter 3 (Alberry et al., 2020). In this chapter, the focus is on alterations to promoter DNA methylation in the same mice. The behavioural deficits originally reported are subtle, a direct result of the subtlety of the treatment paradigms used. Also, gene expression alterations are modest in fold change, detected at postnatal day 70, long after PAE and ELS. The resulting mice have provided the foundation for the assessment of long-term molecular changes that may explain observed changes in gene expression and observed behavioural deficits.

### 4.5.1 Ethanol-induced alterations in promoter DNA methylation

PAE results in gene-specific changes in promoter DNA methylation. Functionally, it includes promoter hypomethylation of genes important for brain function and formation as well as for transcriptional regulation. There is also promoter hypermethylation of genes important for phosphoinositide kinase activity and cytoskeleton regulation. These alterations may represent lasting evidence of altered gene expression during development that persists into adulthood. The ethanol exposure model employed here has been previously used to demonstrate lasting alterations to whole-brain DNA methylation profiles (Laufer et al., 2013). While this report includes comparable amounts of hypo- and hypermethylated gene promoters following continuous PAE, previous work using targeted binge exposure trimester three equivalent models have found alcohol-induced hypermethylation in the hippocampus in mice (Chater-Diehl et al., 2016) and rats (Otero et al., 2012). Conversely, a model of exposure more representative of trimester two equivalent exposure in mice found global hypomethylation in the neocortex, but this pattern is more complex when brain structures are independently assessed (Öztürk et al., 2017). The methylation results of this research using a continuous exposure model add that PAE irrespective of timing and dose is effective in altering hippocampal promoter DNA methylation in adults that had been exposed during gestation.



#### 4.5.2 Maternal separation-induced alterations in DNA methylation

This research shows that there are more genes with hypo- than hypermethylated promoters following ELS via maternal separation. The hypomethylated genes are important for histone methylation and chemokine activity. Meanwhile, hypermethylated promoters tend to be of genes important for cytokine interactions. The results align with a previous report that indicated ELS results in lasting changes to DNA methylation in rat hippocampus (Kosten et al., 2014). In humans, child abuse is associated with hypermethylation of several genomic loci in the hippocampus (McGowan et al., 2009). Meanwhile, maternal separation in rats induces an inflammatory immune response in the hippocampus (Roque et al., 2016). Interestingly, ELS is associated with reduced serum cytokine levels, suggesting that hypermethylation of related genes may not be hippocampal specific, rather playing a more ubiquitous role in the body

#### 4.5.3 Alterations in DNA methylation following PAE and ELS

The combination of PAE and ELS treatments results in the hypomethylation of genes important for neuronal migration and hypermethylation of genes related to cytokine interactions. Similarly, genes with hypermethylated promoters following any of the treatments are also important for chemokine and cytokine interactions. It is apparent from **Figure 4.1** that genes affected are treatment specific. Few genes are shared between ethanol only or stress only and the combination of treatments as implicated by promoter DMRs. PAE followed by ELS has been associated with altered immune system development, with early life adversity differentially affecting peripheral and central immune response depending on previous ethanol exposure (Rainecki et al., 2017).

#### 4.5.4 Overlaps between DNA methylation and gene expression changes

Changes in gene expression in association with behavioural deficits following PAE and ELS in these mice were reported in Chapter 3 (Alberry et al., 2020). Interestingly the module of genes most closely associated with treatment and anxiety-like behaviour includes genes important for transcriptional regulation and neurodevelopment. With these results in mind, the existing overlap between genes with altered expression and altered promoter DNA methylation in the adult hippocampus following PAE and ELS was characterized.

Following PAE, two genes have decreased promoter methylation and corresponding increased expression – *Slc2a9* and *Blvrb*. *Slc2a9* codes for a fructose transporter that acts as a uric acid transporter (Le et al., 2008). While not much is known regarding its role in the brain, SLC2A9 SNPs have been associated with better memory performance (Houlihan et al., 2010) and social phobia (Lyngdoh et al., 2013). *Blvrb* codes for an oxidoreductase important for heme degradation, with no clear link to brain function. PAE also results in promoter hypermethylation and decreased transcript expression for two genes, *Eif4g1* and *Myo6*. *Eif4g1* produces a translation initiation factor, eIF4G, with mutations present in familial Parkinson disease (Chartier-Harlin et al., 2011). This gene acts as a scaffold protein with others in the hippocampus in ways that are perturbed in neurodegeneration likely through protein misfolding (Dhungel et al., 2015). *Myo6* is an actin-based motor protein that has been previously linked to stress response in the mouse hippocampus (Tamaki et al., 2008). Mice lacking this gene have fewer synapses and shorter dendritic spines (Avraham et al., 1995). *Myo6* is important for postsynaptic stimulus-dependent  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor (AMPA) internalization (Osterweil et al., 2005), as well as for BDNF-mediated glutamate release from presynaptic terminals in long-term potentiation (Yano et al., 2006). Together, these results suggest the combined accumulation of DNA methylation, and subsequently reduced expression of these transcripts may be important for the altered brain-related phenotypes observed in PAE mice.

There are three genes with promoter hypomethylation and a corresponding increase in transcript expression following maternal separation stress, *Arpp21*, *Mark2*, and *Rab33b*. *Arpp21* is also implicated by the combination of ethanol + stress through promoter hypomethylation and increased expression. The ARPP21 protein product is composed of conserved domains for RNA-binding, with the ability to interact with eIF4G and preferentially bind to the 3' untranslated regions of mRNAs. Additionally, overexpression and knockdown experiments of ARPP21 show that it positively regulates dendrite growth (Rehfeld et al., 2018). While there is increased *Arpp21* expression following stress or ethanol + stress, a stress-induced decrease has also been reported (Constantinof et al., 2019). *Mark2* produces a serine/threonine-protein kinase involved in cell polarity and microtubule dynamics, along with neuron migration (Mejia-Gervacio et al., 2012; Sapir et

al., 2008). *Rab33b* is a member of Rab family proteins that regulate intracellular vesicular trafficking, particularly at the Golgi apparatus (Zheng et al., 1998). Recently, the zebrafish ortholog of *Rab33b*, *rab33ba*, has been shown to mediate axon outgrowth in the forebrain (Huang et al., 2019). *Slc39a11* is the only gene that has promoter hypermethylation and overexpression in the stress group. It codes for a Zrt-, Irt-like protein (ZIP) for zinc transport (Yu et al., 2013). Taken together, these results suggest that the loss of methylation and subsequent overexpression of these genes following postnatal maternal separation stress may lead to observed changes in behavioural phenotypes mediated by improper neuronal migration and growth.

The combination of ethanol + stress treatments results in three genes with increased expression and corresponding promoter hypomethylation, *Arpp21*, *Dlg3*, and *Pinx1*. *Arpp21* was introduced previously as it was also implicated by stress alone. Of these, *Dlg3* is one of the most significantly altered transcripts in response to stress and ethanol + stress treatments discussed in Chapter 3 (Alberry et al., 2020). It codes for synapse-associated protein 102, a protein important for synapse formation. Knockout mice have impaired synaptic plasticity and spatial learning (Cuthbert et al., 2007) while human *DLG3* mutations result in non-syndromic X-linked intellectual disability (Tarpey et al., 2004). *Pinx1* is a telomerase inhibitor with little known brain function, although variants have been associated with late-onset Alzheimer disease (Tosto et al., 2019). Two genes share decreased promoter methylation and increased expression following the combination of treatments, *Slu7* and *Unc119*. *Slu7* is a pre-mRNA splicing factor that forms part of the spliceosome and is important for genome stability (Jiménez et al., 2019). *Unc119* codes for a lipid binding chaperone required for G protein localization in sensory neurons (Zhang et al., 2011). Further, knockdown of *Unc119* results in neurotoxicity and decreased dendritic branching (May et al., 2014). These results suggest that PAE and ELS cause changes in the expression of many genes, few involving changes in promoter DNA methylation. Such results implicate mechanisms beyond promoter DNA methylation that may initiate and maintain alterations in gene expression important in the development of FASD and associated behavioural deficits.

#### 4.5.5 Beyond promoter DNA methylation

Promoter DNA methylation is an essential mechanism of epigenetic control for the expression of genes. It is affected by environmental factors, including ethanol and stress. Changes in promoter DNA methylation status can lead to the initiation and maintenance of gene expression over time. Promoter DNA methylation has been the research focus in FASD; however, DNA methylation does not act in isolation. While the focus of this chapter is on DMRs in gene promoters, methylation status at a single CpG site may be enough to influence gene expression. In this analysis, the influence of a single CpG that has this type of influence without resulting in a significantly altered region of DNA methylation may be missed. Similarly, the focus has been on promoter DNA methylation for its well-known influence on gene expression via altered transcription factor binding. Gene body or other intergenic changes in methylation were not captured in this research and may be important for gene expression through the work of enhancers or other contributors to three dimensional DNA structure (Greenberg and Bourc'his, 2019).

#### 4.6 Conclusions

The promoter DNA methylation results included in this chapter are novel in that they were obtained from the same mice used to assess behavioural changes described in Chapter 2 (Alberry and Singh, 2016) and hippocampal gene expression described in Chapter 3 (Alberry et al., 2020). This allows for an assessment of the role of promoter DNA methylation and gene expression in FASD, including any relationship between them in response to PAE, ELS, and the combination of both treatments. These results implicate many genes via altered expression or promoter DNA methylation, many of which are important for brain development and function. The results also argue that promoter methylation is only partly involved in the environmentally induced changes in gene expression in FASD.

## Chapter 5. Discussion and final conclusions

The present research has revealed several important insights in the manifestation of FASD following PAE and ELS in a mouse model. Chapter 2 concludes that PAE results in increased pup weight, activity, learning deficits in males, and hypoactivity in females, as well as decreased survival. Further, ELS leads to suppressed pup weight, hypoactivity, and learning deficits. Both PAE and ELS represent negative environmental incursions on a continuum of neurodevelopment, growth, and maturation. This model of PAE and maternal separation stress has also found sex-dependent changes in behaviour following the combination of two treatments, with both resulting in changes in activity and learning deficits. In a novel open field environment, females are more active than males, but not following PAE. There was also increased exploratory behaviour in the open field following PAE. Further, PAE and maternal separation stress each result in overnight hypoactivity in a home cage environment, together with sex-dependent learning deficits. It is worth noting that while PAE did not protect from nor exaggerate the later effects of ELS on any behavioural traits measured as a significant interaction, the added negative impact of ELS on individuals following PAE should continue to be explored. Sex contributes increased complexity to the interactions between prenatal and postnatal stresses. Sex differences arise during brain development because of epigenetic programming. While these differences exist, how each sex perceives and responds to different environmental stresses remains an area of great research interest.

In Chapter 3, experiments focused on hippocampal gene expression used WGCNA to identify module 19 as associated with changes in hippocampal gene expression and anxiety-like behaviour, an association potentially driven by microglia. Transcriptome changes persist in the hippocampus of adult mice prenatally exposed to alcohol and/or postnatally exposed to early life stress. Some transcripts are co-expressed in a way that correlates with experimental treatment and behavioural outcomes. These transcripts code for genes important in RNA processing and management throughout neurodevelopment and beyond. Also, some transcripts are significantly differentially expressed between treatment groups. Specifically, the largest protein-coding variant of *Polr2a* (*Polr2a-201*) is downregulated following ELS as well as the combination of PAE and ELS. These lasting

alterations in transcripts responsible for RNA processing likely underlie the behavioural deficits observed in these mice.

Chapter 4 details the experiments that identified genes with altered hippocampal promoter DNA methylation following PAE, ELS, and the combination of treatments found in this model (Alberly and Singh, 2020). The combination of PAE and ELS results in decreased promoter methylation of genes critical for neuronal migration. Conversely, the combination of PAE and ELS led to increased promoter methylation of genes important for immune response, including 21 members of the cytokine-cytokine receptor interaction KEGG pathway. While there is minimal overlap between promoter DNA methylation and gene expression, genes involved are critical for brain development and function. This work argues that while promoter methylation persists following PAE and ELS, other mechanisms may be responsible for the lasting changes in gene expression and ultimately the behaviour deficits that occur in FASD. Further, RNA expression is transient, acting at the moment measured, while DNA methylation alterations may represent past events, current status, and potential future expression.

## 5.1 Hope for the future

FASD is entirely biologically preventable. It requires the avoidance of alcohol before and during pregnancy. There is no safe time nor safe dose of alcohol during pregnancy known. This is a challenging proposition given that the use of alcohol in reproductive-age females is increasing, which is expected to lead to a corresponding increase in children born with FASD (Alberly and Singh, 2019). As of now, there is no known treatment or cure for FASD. Under the circumstances, there is a need to understand the molecular mechanisms involved and develop novel preventative and treatment modalities.

Research regarding the prevention of FASD has centred on increasing the availability of methyl donors during pregnancy and neurodevelopment through supplementation of known methyl donors such as folate and choline. In an embryonic cell culture model, co-administration of folic acid prevents the morphological delays and deficits, together with the downregulation of *Hoxa1* and its miRNA, *miR-10a* (Wang et al., 2009). Where PAE results in malformations, offspring of pregnant mice on a diet supplemented with choline, betaine, folic acid, vitamin B12, L-methionine, and zinc, followed by PAE have fewer

malformations than those with PAE alone (Downing et al., 2011). Gestational choline supplementation in a rat model reduces ethanol-associated alterations in gene expression and protein levels of DNA methylation genes *Dnmt1* and *Mecp2* in the hypothalamus (Bekdash et al., 2013). Similarly, choline supplementation prevents the ethanol-associated changes in gene expression of histone methylation genes *Set7/9*, *G9a*, and *Setdb1*, as well as the detected changes in histone modifications, H3K4me2,3 and H3K9me2 (Bekdash et al., 2013). Also, choline supplementation blocks an ethanol-associated increase in pro-opiomelanocortin, *POMC*, promoter methylation and the corresponding decrease in gene expression in the hypothalamus (Bekdash et al., 2013). Betaine supplementation during embryonic ethanol exposure in an avian embryo model reduces malformation defects observed following ethanol alone (Karunamuni et al., 2017). Choline supplementation as an intervention for women drinking during pregnancy has been minimally investigated. However, choline-supplemented infants have improved weight and head circumference at 6.5 and 12 months, in addition to better visual recognition memory compared to placebo infants (Jacobson et al., 2018). In a trial of choline administration in 2.5- to 5-year-olds with FASD, children who receive 500 mg of choline daily for nine months have higher non-verbal intelligence, visual-spatial skills, working memory, verbal memory, and fewer symptoms associated with attention deficit hyperactivity disorder (ADHD) than the placebo group when assessed four years later (Wozniak et al., 2020). While the methyl-donor supplementation hypothesis has gained support, the mechanism of action is still unknown. However, it remains an exciting avenue for potential prevention of FASD-related deficits following PAE. Further understanding towards how the postnatal environment may modulate FASD-related deficits will come from animal models. In one study, decreased dendritic complexity in the hippocampus associated with binge-like trimester three-equivalent exposure was avoided by exercise and environmental complexity (Boschen et al., 2017).

## 5.2 Concluding remarks

Although PAE and postnatal stress cause lasting brain damage, the interaction of these environmental assaults has been minimally explored in humans. In this thesis, I have assessed how behavioural changes result from the combination of PAE and ELS, corresponding hippocampal transcriptome and promoter DNA methylation alterations.

While there are some overlaps between the expression and corresponding promoter methylation of some genes, other epigenetic mechanisms beyond promoter DNA methylation are likely involved in the lasting gene expression alterations and behavioural abnormalities seen in pups subjected to PAE and ELS. Although logical, such conclusions must be assessed experimentally, as the hippocampus represents a heterogeneous cell population. Also, the treatments used are relatively mild, applied during neurodevelopment, with behavioural and molecular changes assessed in adulthood. Not surprisingly, this experimental paradigm has resulted in subtle changes in both gene expression and DNA methylation. Future research must focus on experimentally establishing mechanisms involved in altered gene expression and maintenance of these changes in different cell types within the brain. This will require modified experimental protocols and the use of single-cell sequencing and -omics integration. The results presented in this thesis argue that animal models for FASD must include the postnatal environment that may further complicate the effects caused by PAE. Postnatal neurodevelopment is malleable and responsive to the environment, both negative and positive. The malleability provides an opportunity to influence the development and alter outcomes. A greater understanding of how these changes occur and persist has the potential to lead to the development of novel strategies for the amelioration of FASD-related deficits for children born with this common and complex disorder. Most immediately, the results emphasize the need to avoid or minimize negative, stressful postnatal environments for children born with FASD. This strategy will require early and reliable diagnosis, which remains challenging. In this context, the development and characterization of a peripheral biomarker remain of utmost importance. Similarly, the potential to epigenetically manipulate brain gene expression should be the focus of future treatment research.



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## Appendices

### Appendix A – Ethics approval

2007-059-10::6:

**AUP Number:** 2007-059-10

**AUP Title:** Genetic Regulatory Mechanisms: Genes Determining Ethanol Preference in Mice

**Yearly Renewal Date:** 11/01/2013

**The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-059-10 has been approved, and will be approved for one year following the above review date.**

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

#### **REQUIREMENTS/COMMENTS**

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee

**Appendix B** – Correlation coefficients (*p*-values) of module-trait associations for each of the 44 modules produced by WGCNA and 11 traits

Module	prenatal	postnatal	group	learningscore	p21weight	OFTactivity	OFTdistance	OFTlatency	OFTentries	Hcactivity	Hcrears
ME0	0.38 (0.2)	0.33 (0.3)	0.46 (0.1)	-0.45 (0.1)	-0.059 (0.9)	0.29 (0.4)	0.33 (0.3)	0.23 (0.5)	0.25 (0.4)	-0.44 (0.2)	0.008 (1)
ME1	0.45 (0.1)	-0.34 (0.3)	-0.1 (0.7)	-0.43 (0.2)	0.4 (0.2)	-0.29 (0.4)	-0.18 (0.6)	-0.037 (0.9)	-0.33 (0.3)	-0.038 (0.9)	-0.11 (0.7)
ME2	0.42 (0.2)	0.17 (0.6)	0.34 (0.3)	-0.17 (0.6)	0.24 (0.5)	-0.32 (0.3)	-0.27 (0.4)	-0.18 (0.6)	-0.18 (0.6)	0.096 (0.8)	-0.23 (0.5)
ME3	0.38 (0.2)	0.29 (0.4)	0.43 (0.2)	0.037 (0.9)	0.23 (0.5)	0.42 (0.2)	0.48 (0.1)	-0.33 (0.3)	0.43 (0.2)	0.21 (0.5)	0.029 (0.9)
ME4	0.44 (0.2)	0.28 (0.4)	0.45 (0.1)	-0.06 (0.9)	-0.034 (0.9)	-0.14 (0.7)	-0.28 (0.4)	-0.2 (0.5)	0.009 (1)	0.36 (0.3)	0.13 (0.7)
ME5	-0.21 (0.5)	-0.3 (0.3)	-0.36 (0.2)	0.11 (0.7)	0.46 (0.1)	0.64 (0.03)	0.51 (0.09)	-0.32 (0.3)	-0.014 (1)	0.61 (0.03)	0.42 (0.2)
ME6	-0.25 (0.4)	-0.26 (0.4)	-0.34 (0.3)	-0.44 (0.2)	0.45 (0.1)	0.06 (0.9)	0.17 (0.6)	0.16 (0.6)	-0.19 (0.6)	0.45 (0.1)	0.41 (0.2)
ME7	0.41 (0.2)	-0.29 (0.4)	-0.077 (0.8)	-0.4 (0.2)	-0.25 (0.4)	0.66 (0.02)	0.6 (0.04)	0.076 (0.8)	0.11 (0.7)	0.14 (0.7)	0.73 (0.007)
ME8	-0.29 (0.4)	0.3 (0.3)	0.14 (0.7)	0.15 (0.6)	-0.12 (0.7)	0.23 (0.5)	0.21 (0.5)	-0.35 (0.3)	0.37 (0.2)	0.27 (0.4)	-0.34 (0.3)
ME9	0.28 (0.4)	-0.27 (0.4)	-0.11 (0.7)	0.64 (0.02)	-0.0057 (1)	-0.054 (0.9)	-0.19 (0.5)	-0.19 (0.6)	0.094 (0.8)	0.15 (0.6)	0.089 (0.8)

Module	prenatal	postnatal	group	learningscore	p21weight	OFTactivity	OFTdistance	OFTlatency	OFTentries	Heactivity	Hears
ME10	-0.33 (0.3)	-0.27 (0.4)	-0.39 (0.2)	0.36 (0.2)	-0.33 (0.3)	-0.32 (0.3)	-0.14 (0.7)	-0.34 (0.3)	-0.29 (0.4)	-0.086 (0.8)	-0.13 (0.7)
ME11	-0.25 (0.4)	0.31 (0.3)	0.17 (0.6)	-0.02 (1)	-0.54 (0.07)	-0.38 (0.2)	-0.48 (0.1)	0.061 (0.9)	-0.16 (0.6)	-0.59 (0.04)	-0.35 (0.3)
ME12	-0.29 (0.4)	0.36 (0.3)	0.19 (0.6)	0.17 (0.6)	0.029 (0.9)	-0.026 (0.9)	0.0033 (1)	0.8 (0.002)	-0.26 (0.4)	0.027 (0.9)	-0.32 (0.3)
ME13	0.26 (0.4)	0.33 (0.3)	0.41 (0.2)	0.14 (0.7)	-0.12 (0.7)	0.24 (0.5)	0.28 (0.4)	0.22 (0.5)	0.65 (0.02)	-0.46 (0.1)	0.15 (0.6)
ME14	0.093 (0.8)	-0.49 (0.1)	-0.39 (0.2)	-0.1 (0.8)	0.0031 (1)	-0.5 (0.1)	-0.26 (0.4)	0.045 (0.9)	-0.57 (0.05)	-0.12 (0.7)	-0.19 (0.6)
ME15	0.097 (0.8)	-0.45 (0.1)	-0.36 (0.3)	0.051 (0.9)	0.35 (0.3)	-0.073 (0.8)	-0.095 (0.8)	0.034 (0.9)	-0.13 (0.7)	0.066 (0.8)	0.036 (0.9)
ME16	0.1 (0.8)	-0.18 (0.6)	-0.11 (0.7)	-0.27 (0.4)	0.048 (0.9)	0.35 (0.3)	0.42 (0.2)	0.64 (0.03)	-0.29 (0.4)	0.09 (0.8)	0.32 (0.3)
ME17	0.53 (0.08)	0.0027 (1)	0.24 (0.5)	-0.18 (0.6)	0.12 (0.7)	-0.18 (0.6)	-0.32 (0.3)	0.16 (0.6)	0.2 (0.5)	-0.59 (0.04)	-0.048 (0.9)
ME18	0.089 (0.8)	-0.029 (0.9)	0.014 (1)	-0.12 (0.7)	0.19 (0.6)	-0.12 (0.7)	-0.083 (0.8)	-0.26 (0.4)	0.14 (0.7)	-0.021 (0.9)	-0.4 (0.2)
ME19	0.55 (0.06)	0.48 (0.1)	0.68 (0.02)	0.13 (0.7)	0.11 (0.7)	0.47 (0.1)	0.41 (0.2)	-0.075 (0.8)	0.79 (0.002)	-0.12 (0.7)	0.27 (0.4)
ME20	0.076 (0.8)	-0.41 (0.2)	-0.33 (0.3)	0.019 (1)	-0.49 (0.1)	0.22 (0.5)	0.27 (0.4)	0.067 (0.8)	-0.06 (0.9)	-0.26 (0.4)	0.49 (0.1)
ME21	0.023 (0.9)	-0.023 (0.9)	-0.011 (1)	-0.33 (0.3)	-0.62 (0.03)	0.17 (0.6)	0.076 (0.8)	0.21 (0.5)	-0.21 (0.5)	-0.47 (0.1)	0.28 (0.4)

Module	prenatal	postnatal	group	learningscore	p21weight	OFTactivity	OFTdistance	OFTlatency	OFTentries	Hcactivity	Hcrears
ME22	-0.34 (0.3)	-0.12 (0.7)	-0.26 (0.4)	-0.1 (0.7)	0.073 (0.8)	-0.54 (0.07)	-0.58 (0.05)	-0.084 (0.8)	-0.38 (0.2)	-0.1 (0.7)	-0.12 (0.7)
ME23	0.15 (0.6)	-0.037 (0.9)	0.035 (0.9)	-0.43 (0.2)	0.37 (0.2)	0.13 (0.7)	0.17 (0.6)	0.33 (0.3)	0.25 (0.4)	-0.57 (0.05)	0.12 (0.7)
ME24	0.081 (0.8)	-0.0032 (1)	0.034 (0.9)	0.21 (0.5)	-0.2 (0.5)	0.25 (0.4)	0.41 (0.2)	-0.17 (0.6)	0.13 (0.7)	0.00087 (1)	0.16 (0.6)
ME25	0.099 (0.8)	0.36 (0.3)	0.36 (0.2)	0.32 (0.3)	0.12 (0.7)	0.28 (0.4)	0.34 (0.3)	0.42 (0.2)	0.11 (0.7)	0.063 (0.8)	-0.14 (0.7)
ME26	0.023 (0.9)	-0.13 (0.7)	-0.11 (0.7)	0.19 (0.6)	0.32 (0.3)	0.028 (0.9)	-0.47 (0.1)	-0.12 (0.7)	0.32 (0.3)	0.021 (0.9)	0.38 (0.2)
ME27	0.28 (0.4)	0.17 (0.6)	0.28 (0.4)	-0.29 (0.4)	-0.052 (0.9)	-0.37 (0.2)	-0.4 (0.2)	-0.4 (0.2)	-0.14 (0.7)	0.12 (0.7)	-0.36 (0.3)
ME28	-0.33 (0.3)	0.46 (0.1)	0.27 (0.4)	0.0042 (1)	-0.57 (0.05)	-0.083 (0.8)	-0.17 (0.6)	-0.15 (0.6)	0.12 (0.7)	-0.3 (0.3)	-0.51 (0.09)
ME29	-0.36 (0.2)	-0.32 (0.3)	-0.45 (0.1)	-0.1 (0.8)	0.015 (1)	-0.3 (0.3)	-0.071 (0.8)	0.21 (0.5)	-0.41 (0.2)	-0.18 (0.6)	-0.15 (0.6)
ME30	-0.38 (0.2)	-0.05 (0.9)	-0.22 (0.5)	0.33 (0.3)	-0.41 (0.2)	-0.055 (0.9)	0.073 (0.8)	0.77 (0.004)	-0.42 (0.2)	-0.13 (0.7)	-0.14 (0.7)
ME31	-0.0066 (1)	0.45 (0.1)	0.4 (0.2)	-0.018 (1)	-0.56 (0.06)	-0.43 (0.2)	-0.58 (0.05)	-0.11 (0.7)	-0.15 (0.7)	-0.22 (0.5)	-0.16 (0.6)
ME32	0.34 (0.3)	0.029 (0.9)	0.18 (0.6)	0.35 (0.3)	-0.28 (0.4)	-0.14 (0.7)	-0.12 (0.7)	-0.22 (0.5)	-0.081 (0.8)	0.21 (0.5)	0.097 (0.8)
ME33	0.22 (0.5)	0.12 (0.7)	0.2 (0.5)	-0.092 (0.8)	0.11 (0.7)	0.83 (9e-04)	0.82 (0.001)	-0.44 (0.2)	0.64 (0.03)	0.37 (0.2)	0.27 (0.4)

Module	prenatal	postnatal	group	learningscore	p21weight	OFTactivity	OFTdistance	OFTlatency	OFTentries	Hcactivity	Hcrears
ME34	-0.15 (0.6)	-0.047 (0.9)	-0.11 (0.7)	0.17 (0.6)	0.029 (0.9)	-0.28 (0.4)	-0.19 (0.6)	0.16 (0.6)	-0.41 (0.2)	0.43 (0.2)	0.11 (0.7)
ME35	0.097 (0.8)	-0.0036 (1)	0.04 (0.9)	0.02 (1)	0.47 (0.1)	0.25 (0.4)	0.31 (0.3)	0.21 (0.5)	0.089 (0.8)	0.14 (0.7)	-0.019 (1)
ME36	-0.14 (0.7)	-0.11 (0.7)	-0.16 (0.6)	-0.26 (0.4)	-0.16 (0.6)	-0.73 (0.007)	-0.61 (0.03)	-0.027 (0.9)	-0.54 (0.07)	-0.52 (0.08)	-0.52 (0.08)
ME37	0.4 (0.2)	-0.029 (0.9)	0.15 (0.6)	-0.54 (0.07)	-0.28 (0.4)	0.037 (0.9)	0.059 (0.9)	-0.031 (0.9)	-0.14 (0.7)	-0.49 (0.1)	0.067 (0.8)
ME38	-0.61 (0.04)	0.17 (0.6)	-0.12 (0.7)	0.2 (0.5)	-0.13 (0.7)	-0.35 (0.3)	-0.41 (0.2)	-0.48 (0.1)	-0.034 (0.9)	0.2 (0.5)	-0.32 (0.3)
ME39	-0.46 (0.1)	0.0045 (1)	-0.2 (0.5)	0.035 (0.9)	-0.53 (0.07)	-0.014 (1)	-0.078 (0.8)	0.018 (1)	0.12 (0.7)	-0.5 (0.1)	-0.059 (0.9)
ME40	-0.17 (0.6)	0.19 (0.6)	0.094 (0.8)	0.032 (0.9)	0.16 (0.6)	0.2 (0.5)	0.18 (0.6)	-0.11 (0.7)	0.54 (0.07)	-0.18 (0.6)	-0.24 (0.5)
ME41	-0.28 (0.4)	0.0059 (1)	-0.12 (0.7)	-0.44 (0.2)	0.14 (0.7)	-0.34 (0.3)	-0.28 (0.4)	-0.14 (0.7)	-0.2 (0.5)	-0.14 (0.7)	-0.62 (0.03)
ME42	0.2 (0.5)	-0.33 (0.3)	-0.21 (0.5)	-0.12 (0.7)	0.77 (0.004)	-0.15 (0.6)	-0.06 (0.9)	-0.041 (0.9)	-0.34 (0.3)	0.62 (0.03)	0.12 (0.7)
ME43	-0.66 (0.02)	-0.21 (0.5)	-0.49 (0.1)	0.15 (0.6)	-0.098 (0.8)	-0.13 (0.7)	-0.1 (0.8)	0.5 (0.1)	-0.42 (0.2)	0.085 (0.8)	0.023 (0.9)
ME44	0.16 (0.6)	0.22 (0.5)	0.27 (0.4)	0.23 (0.5)	0.021 (0.9)	0.74 (0.006)	0.63 (0.03)	0.16 (0.6)	0.7 (0.01)	-0.047 (0.9)	0.31 (0.3)

**Appendix C** – Genes implicated by transcripts in module 19 associated with the experimental treatment group and the number of centre zone entries in the open field test; GO terms and KEGG pathways overrepresented ( $p < 0.05$ ) in module 19.

See additional digital files provided.

**Appendix D** – Differentially expressed gene lists for ethanol, stress, and ethanol + stress as compared to controls via the kallisto-sleuth pipeline, filtered by significance ( $p < 0.05$ ); GO terms and KEGG pathways overrepresented ( $p < 0.05$ ) by transcripts significantly differentially expressed ( $p < 0.01$ ) for each comparison.

See additional digital files provided.

**Appendix E** – Differentially expressed gene lists for ethanol, stress, and ethanol + stress as compared to controls via the HISAT2-featureCounts-DESeq2 pipeline, filtered by significance ( $p < 0.01$ ).

See additional digital files provided

**Appendix F** – Hypo- and hypermethylated promoter DMRs from the adult hippocampus for ethanol, stress, and ethanol + stress groups compared to untreated controls filtered by significance ( $p < 0.01$ ).

See additional digital files provided.



## Curriculum Vitae

### **Personal Information**

Bonnie Alberry, B.Sc.  
Ph.D. Candidate  
Molecular Genetics Unit  
Department of Biology  
Western University  
London, ON

### **Research Interests**

Exploring how the brain reacts and responds to the environment via epigenetics and gene expression in normal brain function and complex disease development

### **Education**

#### **Ph.D. Candidate, Biology, Cell & Molecular Stream (Current Position)**

Supervisor: Dr. Shiva M Singh, Department of Biology, Western University

Comprehensive Examination – September 3, 2015, Pass with Distinction

Topics: Developing and evaluating animal models; RNA-Seq for mutation detection;  
From environment to phenotype: how alcohol and stress affect brain and behaviour

Courses – Model Systems in Cell and Developmental Biology (A+), Advances in Epigenetics (A), The Biology of Aging (A-), and Functional Genomics & Systems Biology (A+)

#### **B.Sc. Neuroscience (Honours), Carleton University, 2012**

Undergraduate thesis entitled: The expression of microRNAs 103 and 107 in mouse liver and fat tissue is unaffected by a high-fat diet

## Publications

### Refereed Journal Articles:

1. **Alberry, B.**, Castellani, C.A., Singh, S.M. (2020). Hippocampal transcriptome analysis following maternal separation implicates altered RNA processing in a mouse model of fetal alcohol spectrum disorder. *Journal of Neurodevelopmental Disorders* **12**:15. doi: <https://doi.org/10.1186/s11689-020-09316-3>
2. **Alberry, B.**, & Singh, S.M. (2020). Hippocampal DNA methylation in a mouse model of fetal alcohol spectrum disorder that includes maternal separation stress only partially explains changes in gene expression. *Frontiers in Genetics* **11**:70. doi: <https://doi.org/10.3389/fgene.2020.00070>
3. Chater-Diehl, E., Sokolowski, D., **Alberry, B.**, & Singh, S.M. (2019). Coordinated Tcf7l2 regulation in a mouse model implicates Wnt signaling in fetal alcohol spectrum disorders. *Biochemistry and Cell Biology* **97**(4):375-379. doi: <https://doi.org/10.1139/bcb-2018-0215>
4. **Alberry, B.**, & Singh, S.M. (2016). Developmental and behavioral consequences of early life maternal separation stress in a mouse model of fetal alcohol spectrum disorder. *Behavioural Brain Research* **308**:94-103. <https://doi.org/10.1016/j.bbr.2016.04.031>
5. Chater-Diehl, E., Laufer, B.I., Castellani, C.A., **Alberry, B.**, & Singh, S.M. (2016). Alteration of gene expression, DNA methylation, and histone methylation in free radical scavenging networks in adult mouse hippocampus following fetal alcohol exposure. *PlosOne* **11**(5):e0154836. <https://doi.org/10.1371/journal.pone.0154836>
6. Chokroborty-Hoque, A., **Alberry, B.**, & Singh, S.M. (2014). Exploring the complexity of intellectual disability in fetal alcohol spectrum disorders. *Frontiers in Pediatrics* **2**:90; doi: <https://doi.org/10.3389/fped.2014.00090>
7. Kleiber, M.L., Diehl, E., Laufer, B.I., Mantha, K., Chokroborty-Hoque, A., **Alberry, B.**, & Singh, S.M. (2014). Long-term genomic and epigenomic dysregulation as a consequence of prenatal alcohol exposure: a model for fetal alcohol spectrum disorders. *Frontiers in Genetics* **5**:161; doi: <https://doi.org/10.3389/fgene.2014.00161>

### Book Chapters:

1. **Alberry, B.**, & Singh, S.M. (2019). Chapter 34 - Maternal separation stress in fetal alcohol spectrum disorders: a case of double whammy, in *Neuroscience of Alcohol: Mechanisms and Treatment*, Victor R. Preedy ed., Elsevier, 325-333; doi: <https://doi.org/10.1016/B978-0-12-813125-1.00034-9>

## Conference Proceedings:

1. Zai, G., **Alberry, B.**, Arloth, J., Bánlaki, Z., Bares, C., Boot, E., Camilo, C., Chadha, K., Chen, Q., Cole, C.B., Cost, K.T., Crow, M., Ekpor, I., Fischer, S.B., Flatau, L., Gagliano, S., Kirli, U., Kukshal, P., Labrie, V., Lang, M., Lett, T.A., Maffioletti, E., Maier, R., Mihaljevic, M., Mittal, K., Monson, E.T., O'Brien, N.L., Østergaard, S.D., Ovenden, E., Patel, S., Peterson, R.E., Pouget, J.G., Rovaris, D.L., Seaman, L., Shankarappa, B., Tsetsos, F., Vereczkei, A., Wang, C., Xulu, K., Yuen, R.K.C., Zhao, J., Zai, C.C., & Kennedy, J.L. (2016). Rapporteur summaries of plenary, symposia, and oral sessions from the XXIIIrd World Congress of Psychiatric Genetics Meeting in Toronto, Canada, 16-20 October 2015. *Psychiatric Genetics* **26**(6):229-257; doi: <https://dx.doi.org/10.1097%2FYPG.0000000000000148>
2. **Alberry, B.**, Laufer, B., Diehl, E., Kleiber, M., & Singh, S. (2017). Assessing fetal alcohol spectrum disorder etiology via hippocampal gene expression following continuous prenatal alcohol exposure and postnatal maternal separation in mice. *European Neuropsychopharmacology* **27**:S269-270. Abstract only. <https://doi.org/10.1016/j.euroneuro.2015.09.010>
3. Diehl, E., Laufer, B., Castellani, C., **Alberry, B.**, & Singh, S. (2017). Oxidative stress pathways implicated in comprehensive epigenetic and transcriptomic assessment of hippocampus in a model of fetal alcohol spectrum disorders. *European Neuropsychopharmacology* **27**:S271-272. Abstract only. <https://doi.org/10.1016/j.euroneuro.2015.09.010>

## Other Publications:

1. **Alberry, B.**, & Singh, S.M. (2019). Alarming Ontario drinking statistics obligates reappraisal of the impact of alcohol on newborns. *Canadian Medical Association Journal* **191**(46):E1283 doi: <https://doi.org/10.1503/cmaj.73305>
2. **Alberry, B.** (2017, November 26). 4<sup>th</sup> Canadian conference on epigenetics: mechanisms of disease [Blog post]. From <https://epigenie.com/conferences/4th-canadian-conference-on-epigenetics-mechanisms-of-disease/>

**Awards & Honours**

- 2019** Canadian Epigenetics Environment and Health Research Consortium Network Travel Award
- 2018** Children's Health Research Institute Travel Award for Trainees
- 2018** Graduate Student Teaching Award Nominee (Society of Graduate Students)
- 2017** Graduate Student Teaching Award Nominee (Society of Graduate Students)
- 2016** Graduate Teaching Award (Biology Undergraduate Program), Western University

- 2016** Graduate Student Teaching Award Nominee (Society of Graduate Students)
- 2015** World Congress of Psychiatric Genetics Oral Presentation Award Finalist
- 2015** World Congress of Psychiatric Genetics (WCPG) Early Career Investigator Award
- 2014** Graduate Student Teaching Award (Society of Graduate Students), Western University
- 2011** J. Lorne Gray Scholarship, Carleton University
- 2010** Clarence C. Gibson Scholarship, Carleton University
- 2009** E.W.R. Steacie Scholarship, Carleton University
- 2008** President's Scholarship, Carleton University

#### **Invited Talks**

- 2019** Research lecture: Early life stress in a mouse model of fetal alcohol spectrum disorder. Biology 4950 – Seminar in Genetics, Western University (January 18)
- 2018** Research lecture: Early life stress in a mouse model of fetal alcohol spectrum disorder. Biology 4950 – Seminar in Genetics, Western University (September 14)
- 2017** Research lecture: Prenatal alcohol exposure & early life stress in a mouse model of fetal alcohol spectrum disorders. Biology 4950 – Seminar in Genetics, Western University (September 15)
- 2017** Research lecture: Prenatal alcohol exposure & early life stress in a mouse model of fetal alcohol spectrum disorders. Biology 3596 – Genomics and Beyond: A Laboratory Course, Western University (April 3)
- 2016** Lecture: Developing & evaluating animal models. Biology 3598 – Behavioural Genetics. Western University (February 1)

## Conference Participation

### Invited Oral Presentations:

- 2020** Epigenetics of FASD – Part II: Impact of Postnatal Environment (with Morgan Kleiber). *London FASD Conference: Scientific Advances and Community Innovations*. London, ON, Canada (October 28)
- 2019** Exploring the molecular iceberg model for FASD: a hope for the future (with S.M. Singh). *FASD: Achieving New Heights Together!* Burlington, ON, Canada (March 22)
- 2018** FASD: an epigenetic neurodevelopmental disorder modelled in mice. *Biology Graduate Research Forum, Western University*. London, ON, Canada (October 19)
- 2015** Assessing fetal alcohol spectrum disorder etiology via hippocampal gene expression following continuous prenatal alcohol exposure and postnatal maternal separation in mice. *XXIII World Congress of Psychiatric Genetics (WCPG)*. Toronto, ON, Canada (October 18)

### Poster Presentations:

- 2019** Refining an epigenetic mouse model of fetal alcohol spectrum disorder that includes postnatal maternal separation stress. *The 6<sup>th</sup> Canadian Conference on Epigenetics*. Banff, AB, Canada (November 17-20)
- 2018** FASD: an epigenetic neurodevelopmental disorder modelled in mice. *The 5<sup>th</sup> Canadian Conference on Epigenetics*. Estérel, QC, Canada (September 30-October 3)
- 2016** Exploring the etiology of FASD: hippocampal gene expression after continuous prenatal alcohol exposure plus postnatal maternal separation in mice. *American Society of Human Genetics (ASHG) 2016 Annual Meeting*. Vancouver, BC, Canada (October 18-22)

**2015** Gene expression and regulation following prenatal alcohol exposure and early life stress in mice. *Neuroepigenetics*. Santa Fe, NM (February 23)

### **Teaching Experience**

Western University:

Seminar in Genetics (BIO 4950) (Teaching Assistant) (1 term, 2018)

Scientific Methods in Biology (BIO 2290) (Teaching Assistant) (1 term, 2018)

Molecular Genetics Laboratory (BIO 4583) (Teaching Assistant) (2 terms, 2016-2017)

Investigative Techniques in Genetics (BIO 4582) (Teaching Assistant) (6 terms, 2013-2016)

Lead Teaching Assistant, Faculty of Science (2 terms, 2014-2015)

Genomics and Beyond (BIO 3596) (Teaching Assistant) (2 terms, 2013, 2017)

Principles of Human Genetics (BIO 3592) (Teaching Assistant) (1 term, 2012)

### **Research Mentorship**

**2018** Larissa Peck – Undergraduate Independent Study

**2017** Wan-yu Elisa Chao – NSERC Undergraduate Research Project

**2016-2017** Ege Sarikaya – Undergraduate Honors Research Thesis Student. Thesis entitled: Altered hippocampal gene expression following prenatal alcohol exposure in mice: The Delta-Notch & Hippo signalling pathways

**2014-2015** Shruthi Rethi – Volunteer & Undergraduate Honors Research Thesis Student. Thesis entitled: The effect of prenatal alcohol exposure on learning and memory via hippocampal gene expression.

**2014-2015** Ali Pensamiento – Work Study Student

**2014** David Seok – NSERC Undergraduate Research Project

**2014** Cheryl Lin – Volunteer

**2014** Tianyi Yan – Volunteer

**2013-2014** Neethu Govindaraju – Scholar’s Elective Student

**2013-2014** Yuchen Li – Volunteer & NSERC Undergraduate Research Project

### **Service & Memberships**

**2016 – Present** Reviewer for *Epigenetics*, *Psychoneuroendocrinology*, *Brain Research*, and *Behavioural Brain Research*

**2015 – 2020** Member, Society of Graduate Students (SOGS) Bursaries and Subsidies Committee, Western University

**2012 – Present** Member, Society of Graduate Students, Western University

**2012 – Present** Member, Society of Biology Graduate Students, Western University

### **Media Coverage**

**2020** Research highlighted by EpiGenie (Blog). “Compound effects on DNA methylation: prenatal alcohol exposure and early life stress” (March 18)  
<https://epigenie.com/compound-effects-on-dna-methylation-prenatal-alcohol-exposure-and-early-life-stress/>

**2019** Book chapter highlighted by Alcohol News (Blog). “FASD News - 13/2019” (March 31) <http://alcoholweekly.blogspot.com/2019/03/fasd-news-132019.html>

**2016** Research highlighted by EpiGenie (Blog). “Trifecta of epigenetic methylation reveals a long-term profile of fetal alcohol exposure” (May 6)  
<https://epigenie.com/trifecta-of-epigenetic-methylation-reveals-a-long-term-profile-of-fetal-alcohol-exposure/>