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Ethanol exposure during synaptogenesis in a mouse model of fetal alcohol spectrum disorders: acute and long-term effects on gene expression and behaviour

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Ethanol exposure during synaptogenesis in a mouse model of fetal alcohol spectrum disorders: acute and long-term effects on gene expression and behaviour

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by

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Abstract

Alcohol is a neuroactive molecule that is able to exert variable and often detrimental effects on the developing brain, resulting in a broad range of physiological, behavioural, and cognitive phenotypes that characterize 'fetal alcohol spectrum disorders' (FASD). Factors affecting the manifestation of these phenotypes include alcohol dosage, timing of exposure, and pattern of maternal alcohol consumption; however, the biological processes that are vulnerable to ethanol at any given neurodevelopmental stage are unclear, as is how their disruption results in the emergence of specific pathological phenotypes later in life.

The research included in this thesis utilizes a C57BL/6J (B6) mouse model to examine the changes to gene expression and behaviour following a binge-like exposure to ethanol during synaptogenesis, a period of neurodevelopment characterized by the rapid formation and pruning of synaptic connectivity that correlates to brain development during the human third trimester. B6 pups were treated with a high dose (5 g/kg over 2 hours) of ethanol at postnatal day 4 (P4), P7, or on both days (P4+7). Mice were evaluated using a battery of behavioural tests designed to assess FASD-relevant phenotypes, and showed delayed achievement of neuromuscular coordination, hyperactivity, increased anxiety-related traits, and impaired spatial learning and memory.

Gene expression analysis identified 315 transcripts that were altered acutely (4 hours) following ethanol exposure. Up-regulated transcripts were associated with cellular stress response, including both pro- and anti-apoptotic molecules, as well as maintenance of cell structural integrity. Down-regulated transcripts were associated with energetically costly processes such as ribosome biogenesis and cell cycle progression. Genes critical to synapse formation were also affected, as well as genes important for the appropriate development of the hypothalamic-pituitary-adrenal axis.

Additionally, gene expression changes within the adult brain of mice treated with ethanol at P4+7 were examined to evaluate the long-term effects of neurodevelopmental alcohol exposure. Array analysis identified 376 altered mRNA transcripts with roles in synaptic function, plasticity, and stability, as well as epigenetic processes such as folate metabolism and chromatin remodeling. MicroRNA analyses identified changes in the levels of 33 microRNA species, suggesting that that long-term changes to gene expression following may be maintained (at least in part) via epigenetic mechanisms.

Taken together, these analyses illustrate the sensitivity of synaptogenesis to ethanol exposure, leading to a 'molecular footprint' of gene expression changes that persists into adulthood and may contribute to the emergence of long-term behavioural and cognitive phenotypes associated with FASD.

Keywords

fetal alcohol spectrum disorder, brain, development, gene expression, ethanol, mouse model, microarray, behaviour

Co-Authorship

Data contained within this thesis includes work from previously published manuscripts:

1. Some of the behavioural data included in this thesis can be found within a manuscript entitled "Neurodevelopmental timing of ethanol exposure may contribute to the observed heterogeneity of behavioural deficits in a mouse model of fetal alcohol spectrum disorders (FASD)" by Katarzyna Mantha, Morgan Kleiber, and Shiva M. Singh, published in the *Journal of Behavioral and Brain Science* (2013). Morgan Kleiber designed the behavioural battery of tests and performed all behavioural testing and statistical analysis for mice treated during the third trimester-equivalent. Testing and analysis of mice treated during the first and second trimester-equivalent were performed by Katarzyna Mantha, who also wrote the manuscript. All authors contributed to the editing of the final publication.

2. Data pertaining to acute gene expression changes in neonates and microRNA expression changes in adult mice following third trimester-equivalent ethanol exposure are published in the manuscript "Third trimester-equivalent ethanol exposure is characterized by an acute cellular stress response and an ontogenetic disruption of genes critical for synaptic establishment and function in mice" by Morgan L. Kleiber, Benjamin I. Laufer, Randa L. Stringer, and Shiva M. Singh, published in *Developmental Neuroscience* (2014). Morgan Kleiber performed all animal and mRNA work, including analysis of array data. Analysis of microRNA data was performed by Benjamin Laufer and Morgan Kleiber. Quantitative PCR confirmation of mRNA levels was conducted by Randa Stringer and Morgan Kleiber. Morgan Kleiber wrote the manuscript with contributions from Shiva M. Singh.

3. Array data and analyses related to the gene expression changes in adult mice are published in the manuscript "Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure" by Morgan L. Kleiber, Katarzyna Mantha, Randa L. Stinger, and Shiva M. Singh, within the *Journal of Neurodevelopmental Disorders* (2013). Morgan Kleiber performed all animal and mRNA experiments for mice treated during the third trimester-equivalent. Katarzyna Mantha performed animal and mRNA experiments for the first and second-trimester equivalent treatment groups. Randa Stringer performed PCR confirmation of gene expression for the first trimester-equivalent samples. Morgan Kleiber wrote the manuscript, with contributions from Katarzyna Mantha and Shiva M. Singh.

In all studies, Shiva M. Singh also provided intellectual contributions towards project development, manuscript revisions, and supervision.

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List of Abbreviations

ADH	alcohol dehydrogenase
ADHD	attention deficit hyperactivity disorder
ALDH	acetaldehyde dehydrogenase
ANOVA	analysis of variance
B6	C57BL/6 mouse strain
BAC	blood alcohol concentration
BCSN	gene network: behaviour, cell-to-cell signaling and interaction, nervous system development and function
BDNF	brain derived neurotrophic factor
CA1	cornu ammonis area 1
CA3	cornu ammonis area 3
CAM	cell adhesion molecule
CCN	gene network: cell morphology, cell-to-cell signaling and interaction, nervous system development and function
CDCG	gene network: cellular development, cellular growth and proliferation, tumor morphology
cDNA	complimentary deoxyribonucleic acid
CREB	cyclic-adenosine monophosphate (cAMP) response element binding protein
cRNA	complimentary ribonucleic acid
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual, Version 5
Dusp	dual specificity phosphatase
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
FAM	5’ 6-carboxyfluorescein
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
FDR	false discovery rate
G	gestational day
g	gram
GABA	γ-aminobutyric acid
GEO	Gene Expression Omnibus
GO	gene ontology
h	hour
HDAC	histone deacetylase
HPA	hypothalamic-pituitary-adrenal

IPA	Ingenuity Pathway analysis
IQ	intelligence quotient
LDB	light/dark box
MANOVA	multivariate analysis of variance
MAPK	mitogen-activated protein kinase
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
n	number
NCBI	National Center for Biotechnology Information
ncRNA	non-coding ribonucleic acid
ND/AE	neurodevelopmental disorder/alcohol exposed
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NPD	gene network: neurological disease, psychological disorders, behaviour
NT	neurotrophin
P	postnatal day
PAE	prenatal alcohol exposure
pFAS	partial fetal alcohol syndrome
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
GC-RMA	GeneChip Robust Multiarray Averaging
RM-ANOVA	repeated measures analysis of variance
ROS	reactive oxygen species
s	second
SAM	S-adenosylmethionine
SE/AE	static encelopathy/alcohol exposed
SEM	standard error of the mean
SPSS	Statistical Package for the Social Sciences
sscDNA	single-stranded complimentary deoxyribonucleic acid
TAMRA	3' tetramethylrhodamine
TGFβ	transforming growth factor beta
TMCS	gene network: tissue morphology, cell-to-cell signaling and interaction, cellular growth and proliferation
TRAIL	TNF-related apoptosis inducing ligand
µg	microgram
µl	microlitre

Chapter 1

INTRODUCTION

1.1 Fetal alcohol exposure and the developmental origins of health and disease

Development is guided by the interaction between the code inherited as one's genomic DNA and the dynamic systems that regulate its control. The development of the mammalian brain is acutely sensitive to this interplay: dependent on precise spatial and temporal gene regulation but highly responsive to the developmental environment, including the decisions a woman may make regarding her own health and her child's while pregnant.

Neurodevelopmental gene expression can be affected by maternal stress, diet, and the consumption of drugs or alcohol. How the prenatal environment influences the emergence of later-life physiological or psychological disorders forms the basis of the "developmental origins of health and disease" hypothesis (BARKER 2004, GILLMAN 2005), which has been applied to psychopathologies such as autism, schizophrenia, attention-deficit hyperactivity disorder, and anxiety and mood disorders (SCHLOTZ and PHILLIPS 2009, BALE et al. 2010, DUFOUR-RAINFRAY et al. 2011, KIRAN KUMAR et al. 2013, UHER 2014). Few disorders, however, have such a clear developmental etiology

as fetal alcohol spectrum disorders (FASD). Caused by prenatal alcohol exposure, FASD is one of the most prevalent examples of how disrupting the developmental environment can lead to a broad range of negative later-life physiological, behavioural, and cognitive outcomes.

1.2 Fetal alcohol spectrum disorders

Fetal alcohol spectrum disorders (FASD) is a non-diagnostic umbrella term that encompasses the physiological, neurological, behavioural, and cognitive features displayed by individuals exposed to alcohol by maternal consumption during pregnancy. Historically, very little attention was given to the relationship between alcohol and birth defects and much medical literature condoned moderate alcohol use during pregnancy until as recently as the middle of the 20th century (ABEL 2012). In 1968, French researchers described delayed growth and distinctive physiological characteristics in children of alcoholic mothers (LEMOINE et al. 1968), suggesting that prenatal alcohol exposure was harmful to the development of a fetus. This study remained largely ignored until 1973 when Jones and Smith reported similar findings (JONES et al. 1973), describing an array of physiological and intellectual abnormalities among children they defined as having 'fetal alcohol syndrome'.

It is remarkable that such a common substance, consumed for centuries, was so recently acknowledged as a health hazard and a cause of detrimental effects on a developing child. However, since the publication of these seminal studies, the effects of prenatal alcohol exposure have received increasing attention. Even so, the recognition of fetal alcohol-related disorders remains complicated, with variable diagnostic criteria and significant phenotypic heterogeneity. Recently, the term "neurodevelopmental disorder-prenatal alcohol exposed" achieved a code under the category "otherwise specified neurodevelopmental disorder" within the most recent American Psychiatric Association's Diagnostic and Statistical Manual (DSM-5) as a condition requiring further study (AMERICAN PSYCHIATRIC ASSOCIATION 2013). Some

classification terms have, however, been established within Canada and the United States to define the differences in the severity of physiological and behavioural features that are nonetheless consistent with prenatal alcohol exposure (BERTRAND et al. 2005, CHUDLEY et al. 2005) .

At its most severe, individuals exposed to heavy alcohol exposure during early gestation display prenatal and postnatal growth deficiencies, distinctive craniofacial features, physiological and central nervous system abnormalities, and significant cognitive impairment that define Fetal Alcohol Syndrome (FAS) (ASTLEY et al. 1999, ARCHIBALD et al. 2001, BERTRAND et al. 2005, LIPINSKI et al. 2012). More commonly, prenatal alcohol exposure results in less severe clinical phenotypes that include partial fetal alcohol syndrome (PFAS), in which individuals may present partial physiological features of FAS while remaining severely cognitively impaired. Other categories include static encephalopathy/alcohol exposed (SE/AE), meaning permanent or unchanging neurological damage that may or may not present physiologically as well as neurobehaviourally, and neurodevelopmental disorder/alcohol exposed (ND/AE) that may present solely as cognitive and behavioural changes in the absence of gross physiological abnormalities (ASTLEY and CLARREN 2000). Importantly, the latter categories most likely comprise the majority of FASD cases, making identification and diagnosis extremely difficult. Further, there is no necessary correlation between detectable neurophysiological abnormalities and severity of cognitive impairment (MATTSON et al. 1998).

1.2.1 Behavioural and cognitive phenotypes associated with FASD

Part of the difficulty with diagnosing FASD in the absence of confirmed prenatal alcohol exposure relates to the phenotypic heterogeneity associated with FASD. Also, the physical, cognitive, and behavioural impairments associated with prenatal alcohol exposure (PAE) may not be clearly “normal” or “abnormal”; indeed, individuals with PAE fall within a broad continuum that ranges from a normal spectrum of behaviours to distinctively impaired (MATTSON and RILEY 1998, ASTLEY et

al. 2009, KODITUWAKKU 2009). Many of these behaviours are also not specific to prenatal alcohol exposure and may manifest differently across the life of an affected individual.

The vulnerability of the developing brain to ethanol translates to risk for a broad range of later-life outcomes. Infants and young children with PAE often show impaired motor coordination, balance, and reflex responses that may persist into adulthood (COLES et al. 1985, CONNOR et al. 2006, LUCAS et al. 2014). Also, children with FAS and FASD typically have lower IQs than non-exposed children though there is considerable variability, with most individuals with (non-severe) prenatal alcohol exposure falling within a normal IQ range (MATTSON and RILEY 1998). Regardless, children with FASD exhibit increased rates of learning disabilities and, as a result, poor academic achievement (HOWELL et al. 2006). This may be due to impaired verbal and non-verbal learning, as well as deficits in verbal information encoding and visuospatial processing (MATTSON et al. 1996, ROEBUCK-SPENCER and MATTSON 2004, WILLFORD et al. 2004). Poor academic performance may also be attributable to inattention and activity levels (GREEN et al. 2009, ALVIK et al. 2013); indeed, attention-deficit/hyperactivity disorders (ADHD) is the most common psychiatric comorbidity of children with FASD, with three to nine times higher prevalence than the general population (BHATARA et al. 2006, HERMAN et al. 2008). Further compounding these disadvantages are associations with PAE and disruptive, impulsive, and delinquent behaviour, impaired social and adaptive functioning, and tendency to engage in dangerous or risk-taking behaviour (RASMUSSEN 2005, RASMUSSEN and WYPER 2007, JIRIKOWIC et al. 2008a, CROCKER et al. 2009, WARE et al. 2014). Additionally, recent associations have been made between prenatal alcohol exposure and circadian dysfunction, impacting adaptive function, mood, concentration and cognitive function (EARNEST et al. 2001, WENGEL et al. 2011, CHEN et al. 2012). Many of these phenotypes persist into adulthood and result in secondary psychiatric and psychosocial manifestations such as increased risk for depression, anxiety and oppositional defiance-related disorders, as well as an increased risk for alcohol and drug abuse, impairments in moral and social development, and failure to learn from experience

(STREISSGUTH and O'MALLEY 2000, STEINHAUSEN et al. 2003, BHATARA et al. 2006, NICCOLS 2007). This often leads to increased difficulty with the legal system, either as offenders or victims, and an increased prevalence within the correctional system (FAST and CONRY 2004, STREISSGUTH et al. 2004). Comprehensive medical and social programs are imperative to identify and improve the prognosis of individuals born with FASD; however, we still have little understanding of how alcohol exposure during neurodevelopment, including factors such as timing, dose, and pattern of exposure, induces such a broad and variable range of neurobehavioural phenotypes.

1.2.2 Epidemiology of fetal alcohol spectrum disorders

Given the inconsistencies with diagnostic criteria, variability of phenotypic presentation, and unlikelihood of accurately ascertaining the nature of gestational alcohol exposure, there are large discrepancies in estimates of FASD incidence and prevalence. It is, however, recognized that maternal alcohol consumption during pregnancy is the most common preventable cause of neurodevelopmental disabilities in Western cultures. The estimated prevalence of FAS is between 0.5 and 3 per 1,000 births; however, the estimated incidence of conditions falling within the FASD spectrum in Canada is approximately 9 in 1000 births (PUBLIC HEALTH AGENCY OF CANADA 2003). More recent studies have suggested that the prevalence is underestimated and may be as high as 2-5% (MAY et al. 2009, MAY et al. 2011). These numbers are considerably concentrated within low socio-economic communities, in children within the child care system, the Canadian correctional system, and in certain remote, rural or indigenous populations, where estimates of FAS and FASD rise to approximately 6.0% and 16.9%, respectively (WILLIAMS et al. 1999, MASOTTI et al. 2006, LANGE et al. 2013, POPOVA et al. 2015). In a comprehensive analysis by Stade *et al*. (2009) using a conservative estimate of 3 per 1000 births and including individuals from 0-53 years of age, the annual estimated cost in 2007 for health care, educational and social services, and other indirect costs associated with FASD was $5.3 billion (95% confidence interval: $4.12 - $6.4 billion) at the population level

(STADE et al. 2009). These numbers indicate that FASD is a pressing concern economically, medically, and sociologically, and research into the biological etiology of these disorders may aid in developing better diagnostic tools and improve the ultimate quality of life for individuals prenatally exposed to alcohol.

1.2.3 Maternal drinking patterns and associations with FASD

The number of individuals diagnosed with prenatal alcohol exposure-related disabilities has increased significantly over the last few decades. Part of this increase is due to improvements in social and medical awareness of these disorders (CHUDLEY et al. 2005, GOH et al. 2008, ASTLEY 2013). Regardless of this, in North America as many as 30% of women continue to drink during pregnancy, with approximately 5% engaging in "binge" drinking (ETHEN et al. 2009). This may occur for varied reasons: naïveté of potential consequences, addiction, or unawareness of pregnancy. Further, changes in social attitudes and drinking patterns among women of childbearing age also may contribute to these growing numbers.

Binge drinking is defined as the consumption of alcohol that raises one's blood alcohol concentration (BAC) to approximately 0.08% or above (NATIONAL INSTITUTE OF ALCOHOL ABUSE AND ALCOHOLISM 2004). For an average woman, this equates to approximately 4 standard drinks in two hours or less. According to a recent study conducted by the United States Center for Disease Control, binge drinking prevalence, frequency, and intensity was found to be highest in women aged 18-24 years of age (CENTER FOR DISEASE CONTROL 2012). This is troubling, given that binge drinking in the months prior to pregnancy considerably elevates the risk of drinking (and binge drinking) during pregnancy (ETHEN et al. 2009). Generally, mothers of individuals that fall with the SE/AE and ND/AE categories engage in drinking during the first trimester, but cut down substantially upon recognition of the pregnancy; however, when they do drink, they are likely to do so in a binge-type fashion. This is important given that a binge pattern of drinking, even infrequently, results in higher and prolonged BACs, which are strongly correlated with increased neurological and

cognitive damage, regardless of the trimester in which the binge episode occurred (MAY et al. 2013).

1.2.4 Effect of prenatal alcohol exposure on brain structure

Ethanol is a small molecule that easily crosses the placental and blood-brain barrier (IDANPAAN-HEIKKILA et al. 1972). One to two hours after maternal alcohol ingestion, blood alcohol levels of the fetus reaches an approximately equivalent level as maternal BAC; however, ethanol elimination by the fetus does not occur at the same level of metabolic efficiency, resulting in prolonged exposure time and reliance on maternal ethanol metabolism to eliminate alcohol from the bloodstream and the amniotic fluid (BURD et al. 2012). There is a dose-response relationship between maternal BAC levels, the duration of a high BAC, and the severity of neurological damage in the developing fetus (MAIER and WEST 2001). Consequently, binge drinking is more detrimental to the developing brain than drinking patterns that produce low or moderate BACs (MAIER and WEST 2001).

Much of the neuropathological data regarding how ethanol physiologically impacts the developing brain in humans has been obtained using neuroimaging techniques. Studies involving FAS and FASD patients have shown a wide array of morphological changes and a general reduction in brain volume. There seem to be few areas (if any) that are not susceptible to ethanol-induced damage, depending on timing of exposure. Fairly consistently, magnetic resonance imaging studies have found abnormal cortical thickness and diminution of white matter, the corpus callosum, the prefrontal cortex, the cerebellum, and the hippocampus (ARCHIBALD et al. 2001, BOOKSTEIN et al. 2002, SOWELL et al. 2002, RILEY et al. 2004, CORTESE et al. 2006). Given the critical roles in learning, memory, sensory information processing, and cognitive function played by these brain regions, these results are consistent with the kinds of deficits seen in FASD. At the same time, many individuals that have abnormalities in brain functioning show no gross anatomical abnormalities upon brain imaging (MATTSON and RILEY 1998, SPADONI et al. 2007). Further, alcohol-exposed children both with and

without physiological abnormalities are similarly impaired on tests of language, memory, fine-motor speed, and visual-motor integration, suggesting that the degree of behavioural and cognitive deficits may be independent of physical features (MATTSON et al. 1998). These findings suggest that reduced brain volume in particular regions cannot account for all the neurological phenotypes associated with FASD.

1.3 Animal models in FASD research

Understanding the biological mechanisms through which alcohol affects the developing brain and leads to behavioural and cognitive abnormalities is a complex and difficult challenge. The problem lies in the variability of phenotypes associated with FASD: there is no checklist of behaviours that can confirm prenatal alcohol exposure. Rarely, if ever, do researchers have information regarding the exact timing, dosage, or pattern of maternal alcohol consumption, nor can they determine the impact of potentially deleterious socioeconomic factors that may exacerbate FASD-associated traits. In order to better understand how prenatal alcohol exposure may result in FASD in human populations requires the development and use of effective animal models. Animal models are crucial to elucidating the biological mechanisms that initiate or underlie human psychiatric disorders in ways not possible in humans; additionally, these results can be effectively translated to improve human phenotypes (BALE et al. 2010, NESTLER and HYMAN 2010a). Animal models also allow for the control of genetic and environmental factors that confound the manifestation of FASD in humans, such as ethanol dosage, timing of exposure, pattern of exposure (acute or chronic), maternal nutrition, comorbid drug exposure, and genetic background. Creating a model for FASD in animals seems straight-forward given that these disorders have a very discrete etiology: prenatal alcohol exposure. However, to effectively model a human disorder, the model must provide a conserved biological basis that can replicate similar anatomical, molecular, and phenotypic features of the disorder.

1.3.1 A multi-species approach to understanding FASD

Animal models have played a critical role in FASD research since the late 1970s to establish that alcohol was indeed a teratogen and prenatal exposure was able to cause adverse physiological and behavioural effects (BOND and DI GIUSTO 1976, CHERNOFF 1977, HENDERSON and SCHENKER 1977, RANDALL and TAYLOR 1979). Also, these early studies determined that there was a relationship between dose of ethanol (ie, maternal BAC levels) and severity of offspring abnormalities (CHERNOFF 1977, RANDALL and TAYLOR 1979). In an important study conducted by Sulik *et al.* (1981), it was demonstrated that the distinctive facial features of FAS could be replicated in mice and that the appearance of these craniofacial abnormalities were dependent on the timing of exposure (SULIK et al. 1981). This study demonstrated that there were critical windows of vulnerability for certain physiological and neurological abnormalities. Since these pivotal studies, many different animal models have been developed and utilized over the last few decades, each lending itself more appropriately to certain research goals depending on the nature and complexity of the question. Fundamental biological questions regarding the effect of ethanol on evolutionarily-conserved developmental mechanisms have been addressed using organisms such as *Caenorhabditis elegans*, *Drosophila*, and zebrafish (DAVIS et al. 2008, MCCLURE et al. 2011, COLE et al. 2012). By far the most common model, however, are rodent species such as mice, rats, and guinea pigs. While each model may mimic one or several features of FASD, certainly none are entirely comprehensive. However, together they effectively contribute to our understanding of genetic and cellular mechanisms involved in prenatal alcohol exposure and how those mechanisms may lead to physiological changes, neuro-functional alterations, and behavioural and cognitive impairments in humans with FASD.

1.3.2 Modeling FASD in mice

Mouse models are extremely useful for evaluating the genetic and cellular impact of alcohol *in vivo* in a complex neurological system. In general, mice are often used for health-related research due to their short generation time, their ease of care, and basic genetic, developmental and physiological similarity to humans. There is considerable literature describing their physiology, behaviour, susceptibility to ethanol teratology, and developmental timeline with relation to the human timeline. They are also particularly amenable to genomic studies given the availability of tools such as DNA sequence and gene expression data (including spatial and temporal expression of genes during development), well-established behavioural assays, and availability of arrays and transgenic tools.

Mouse models, and in particular the C57BL/6 (B6) inbred strain, have been widely used in FASD research since the demonstration that they could be used to recapitulate strikingly similar physiological and craniofacial abnormalities to those observed in humans with FAS (SULIK et al. 1981). Since then, numerous mouse models have been developed using various ethanol dosages, treatments at different developmental stages, and multiple methods of ethanol administration. These models are used to examine the behavioural, physiological, cellular, and genetic consequences of ethanol exposure.

Mouse models of FASD generally fall within one of two categories: chronic moderate exposure throughout gestation or punctuated acute treatments at specific neurodevelopmental stages. The former often utilizes inbred strains with a high ethanol preference, such as B6, to model voluntary maternal ethanol consumption throughout gestation (ALLAN et al. 2003, BOEHM et al. 2008, KLEIBER et al. 2011, BRADY et al. 2012). Pregnant B6 females continue to consume approximately 70% of their total liquid intake in the form of a 10% ethanol solution when given a choice between the ethanol solution and water, exposing the developing fetus to low-to-moderate levels of ethanol (BACs reach approximately 80 – 200 mg/dl) throughout gestation. The resulting offspring show subtle but consistent behavioural phenotypes relevant

to FASD, such as delays in the development of neuromuscular reflexes and coordination, increases in novelty-induced anxiety, decreased sensitivity to ethanol during adolescence, and deficits in spatial learning and hippocampal-dependent learning tasks (BOEHM et al. 2008, KLEIBER et al. 2011, BRADY et al. 2012). Models using voluntary consumption throughout gestation have face validity in that they likely represent a common pattern of alcohol consumption in humans that choose to drink while pregnant; however, they can make it difficult to ascertain the effects of ethanol on processes that occur at specific neurodevelopmental stages. To address this, other models use an exposure paradigm where a punctuated high-dose or "binge"-like treatment is administered at a specific developmental time, either through oral intubation, injection, or inhalation (PARSON and SOJITRA 1995, IKONOMIDOU et al. 2000, HEATON et al. 2002b, BAKE et al. 2012, LIPINSKI et al. 2012, WAGNER et al. 2014). These "binge" paradigms results in higher BACs, usually above 200 mg/dl, and as is the case in humans, more severe neurological damage. In B6 mice, the threshold of 200 mg/dl is sufficient to induce significant apoptotic neurodegeneration in the developing brain, with the severity of damage proportional to the length of time BACs remain above this level (IKONOMIDOU et al. 2000). These binge models are also useful in making associations between the timing of exposure and specific physiological and behavioural phenotypes, as ethanol can be administered both during specific pregnancy trimester-equivalents, and in specific dosages (GOODLETT and JOHNSON 1997, MAIER et al. 1997, WOZNIAK et al. 2004, HUNT et al. 2009, LIPINSKI et al. 2012, MANTHA et al. 2013). The variability of these studies, however, in terms of the dosage administered and timing of exposure, make comparisons between different models difficult. A consistent ethanol administration paradigm across different developmental stages (and trimester-equivalents) would be useful to assess windows of vulnerability of developing brain regions, behavioural changes associated with these windows, and the effect of alcohol on molecular processes that may underlie these changes. Our laboratory has developed a treatment paradigm in B6 mice that represents a binge-like exposure, with a consistent dose, which we have administered via subcutaneous injection during the first, second, or third human trimester equivalent (KLEIBER et al. 2013, MANTHA et al. 2013). The results presented in this

thesis examine the effects of ethanol on developing brain during the third trimester-equivalent, an exceptionally sensitive period to the teratogenic effects of alcohol. However, results from multiple studies support that there is no safe time during pregnancy in which it is safe to consume alcohol and not risk the proper neurodevelopment of the fetus.

1.4 Biological mechanisms implicated in the neurodevelopmental effects of alcohol

Alcohol's adverse effects are likely due to a combination of direct and indirect interactions with biological processes occurring in the developing brain at the time of exposure. Part of the difficulty in assessing the etiology of FASD is the pleiotropic nature of ethanol's actions within the brain, as well as our incomplete understanding of the exact cellular and molecular processes involved in brain development. There are a number of studies, however, that have implicated specific biological mechanisms that consistently seem to be adversely affected by ethanol, all of which likely contribute to the heterogeneous physiological and behavioural phenotypes associated with prenatal alcohol exposure. Some of these processes are discussed below.

1.4.1 Alcohol metabolism

Genes involved in the metabolism of alcohol and its metabolic intermediates possess polymorphisms in human populations that affect gene functioning, making them natural targets for research examining susceptibility to the effects of alcohol (GOEDDE et al. 1983, CRABBE et al. 1999). The primary pathway for the metabolism of alcohol involves alcohol dehydrogenase (ADH), which converts alcohol into the toxic intermediate metabolite acetaldehyde, which is then metabolized by acetaldehyde dehydrogenase (ALDH) into acetate (ZAKHARI 2006). Both ethanol and acetaldehyde

easily cross the placental barrier and are readily accessible by the developing fetus very shortly after maternal consumption (BLAKLEY and SCOTT 1984). Studies have examined the association of different alleles of enzymes within the alcohol metabolism pathway, such as those within the alcohol dehydrogenase *ADH1B* gene, with the severity or frequency of FAS and FASD in various populations (STOLER et al. 2002, JACOBSON et al. 2006, GREEN and STOLER 2007). Inefficient ADH proteins limit the conversion of ethanol to the toxic metabolite acetaldehyde prolonging higher BACs; similarly, inefficient ALDH isozymes result in longer exposure to acetaldehyde. Further, ethanol may competitively inhibit the role of ADH in the catalyzation of retinol to retinoic acid, a potent mediator of embryogenesis and cellular differentiation, leading to retinoic acid deficiency (CHAMBON 1996, DUESTER 1996, ZACHMAN and GRUMMER 1998). While these studies point to genes that may mitigate the severity of ethanol neurotoxicity between individuals (WARREN and LI 2005), they do not explain the mechanisms that trigger ethanol's neurotoxic effects. More important, polymorphisms in genes related to alcohol metabolism are not sufficient to explain the full range of alcohol's effects on the developing brain.

1.4.2 Oxidative stress

Numerous studies have demonstrated that prenatal alcohol exposure results in increased reactive oxygen species (ROS) and oxidative stress in multiple brain regions (RAMACHANDRAN et al. 2001, RAMACHANDRAN et al. 2003, GREEN et al. 2006, CHU et al. 2007, HILL et al. 2014). This increase in ROS can cause damage to cell membranes, DNA, and proteins, and trigger the release of apoptosis-inducing factors from the mitochondria (REGO and OLIVEIRA 2003). Changes in ROS levels can also affect mitochondrial function, leading to altered intracellular and extracellular calcium concentrations. Given the need to tightly regulate calcium-mediated neuronal signaling when establishing appropriate synaptic connections, altered calcium concentrations could be detrimental during synaptogenesis.

The developing brain is vulnerable to ethanol-induced ROS damage for multiple reasons, including a high oxygen metabolic rate, the high concentration of fatty acids that can be used as substrates for ROS, and the relatively low concentration of endogenous antioxidant enzymes as compared to the adult brain (FLOYD and CARNEY 1992, HALLIWELL 1992, HENDERSON et al. 1999, SOKOLOFF 1999). Indeed, there is substantial evidence that supplementation with exogenous antioxidants can prevent certain ethanol-induced neurodevelopmental damage (HEATON et al. 2003, MARINO et al. 2004, KUMRAL et al. 2005). As with other mechanisms, certain regions show windows of vulnerability. Studies in rodents have demonstrated that the cerebellum, hippocampus, and hypothalamus, and striatum are all vulnerable to increases in ROS following alcohol exposure during the third trimester equivalent (HEATON et al. 2002b, HEATON et al. 2003, MARINO et al. 2004, DEMBELE et al. 2006). Further, oxidative stress has been shown to have a long-lasting impact on the function of these regions. For example, early neonatal ethanol exposure increases mitochondrial antioxidant manganese superoxide dismutase levels within the hypothalamus at P7 in rats (DEMBELE et al. 2006). This is associated with decreased expression of pro-opiomelanocortin that persists into adulthood, affecting hypothalamic-pituitary-adrenal signaling, endocrine function (DEMBELE et al. 2006) and anxiety and depressive-like behaviours (BROCARDO et al. 2012). While oxidative stress can certainly trigger cell death, these studies demonstrate that it can also affect long-term gene expression and cellular function, which then may contribute to FASD-associated phenotypes.

1.4.3 Growth factor and neurotrophic factor signaling

Growth factors are secreted, diffusible proteins essential for neuronal survival, proliferation, and maturation. Depending on the balance of mitogenic or anti-proliferative trophic signals, developing cells make decisions regarding progression or rate of mitosis, the initiation of programmed cell death, cell shape and size, and dendritic arborization (BIBEL and BARDE 2000). Many growth factors that are critical

to normal brain development such as the neurotrophins nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophins 3, 4, and 5 (NT-3, NT-4, and NT-5), as well as transforming growth factor-β (TGFβ) and insulin-like growth factors (IGFs), are disrupted by neurodevelopmental ethanol exposure(HEATON et al. 1995, HEATON et al. 2000b, MILLER 2003, PARKS et al. 2008, DE LA MONTE et al. 2011). This disruption may be direct, with ethanol acting as an agonist or antagonist on the growth factor receptor or within its signaling pathway, or indirect, inducing changes to the expression of genes involved in growth factor signaling or expression of the growth factor itself (RESNICOFF et al. 1993, ZHANG et al. 1998, MAIER et al. 1999, HEATON et al. 2000b, MOORE et al. 2004). By whatever mechanism, it seems that ethanol acts to reduce signaling of neurotrophic factors, encouraging the cell to undergo apoptosis. Supplementation of cells (*in vitro* and *in vivo*) with specific neurotrophins can reduce ethanol-induced neurotoxicity (LUO et al. 1997, JAURENA et al. 2011). Transgenic studies have demonstrated that the deletion of BDNF can increase ethanol-induced cell death in specific regions, while NGF overexpression can ameliorate ethanol neurotoxicity (HEATON et al. 2000a, HEATON et al. 2002a). This may be achieved via the up-regulation of anti-apoptotic and down-regulation of pro-apoptotic genes (ALOYZ et al. 1998, PEREZ-NAVARRO et al. 2005, CHIOU et al. 2007). Other growth factors, however, such as TGFβ, can promote cell death following ethanol exposure by up-regulating the expression of genes involved in apoptotic pathways (HICKS and MILLER 2011). Given that growth factors play such essential roles in brain development, altering their availability or signaling at critical neurodevelopmental times or in vulnerable regions likely contributes to both short and long-term ethanol effects.

1.4.4 Cell adhesion and interaction

During neurodevelopment, cell-cell recognition and adhesion are required for numerous processes including neuron survival, migration, and, during synaptogenesis, synaptic formation, maturation, and plasticity (RUTISHAUSER 1984,

YANG et al. 2014). Cell adhesion molecules (CAMs) establish synapse specificity by mediating protein interactions to properly align the pre- and post-synaptic neurons, recruit proteins integral to synaptic membranes such as neurotransmitter receptors and scaffolding proteins, facilitate vesicle reuptake and neurotransmitter release, and control the formation and balance of excitatory and inhibitory synapses (WASHBOURNE et al. 2004, WILLIAMS et al. 2010). Further, disruption of neural cell adhesion molecules leads to neurodevelopmental disorders resembling the physiological and behavioural phenotypes of FAS (CREMER et al. 1994, KAMIGUCHI et al. 1998). Ethanol has been shown to interact with neural cell adhesion molecule (NCAM) and L1-NCAM to affect neurodevelopmental cell adhesion (MINANA et al. 2000, AREVALO et al. 2008, LITTNER et al. 2013). The impact of this is likely substantial, but the effect of ethanol on other cell adhesion molecules remains relatively unexplored. However, given their pivotal role in synaptogenesis, any disruption of cell adhesion molecules have the potential to strongly contribute to the phenotypes associated with FASD that rely on synaptic maintenance and plasticity such as learning, memory, stress response, and motor function.

1.4.5 Neurotransmitter signaling

Synaptogenesis, the process by which cells establish synaptic connectivity, relies heavily on intercellular communication. Excitatory and inhibitory signals between synapses and dendrites are formed, maintained, and deleted by neurotransmission between pre- and post-synaptic neurons. John Olney and colleagues have demonstrated the profound effect of ethanol on neurotransmitter signaling during synaptogenesis, showing that ethanol interferes with NMDA and GABA receptors to trigger widespread neurodegeneration within the mouse brain (IKONOMIDOU et al. 2000, OLNEY et al. 2000, OLNEY et al. 2002c). Cell death, however, is not the singular mechanism by which ethanol acts through developing neurotransmitter systems. Ethanol is a unique drug in that it interferes with multiple neuronal signaling mechanisms, including ligand-gated ion channels such as those associated with γ-

aminobutyric acid (GABA), *N*-methyl-D-aspartate (NMDA), serotonin, dopamine, catecholamines, opioids, and steroids (HARRIS et al. 2008). Brain regions implicated in the phenotypic effects of prenatal alcohol exposure including the cerebellum, hippocampus, and neocortex show changes such as altered expression of GABA and NMDA receptor subunits, delayed developmental maturation of GABAergic and glutamatergic neurons, altered receptor functionality, or impaired plasticity (HSIAO et al. 2004, TOSO et al. 2006a, SERVAIS et al. 2007, INCERTI et al. 2010, PUGLIA and VALENZUELA 2010). Additionally, impairments in serotonergic and dopaminergic function result in reduced serotonin neuron innervation, alterations to hypothalamic-pituitary-adrenal axis function, circadian rhythm abnormalities, and altered reward pathway signaling (SHER 2004, ZHOU et al. 2005, HOFMANN et al. 2007, FERNANDES et al. 2015). The effects of neurodevelopmental disruption of neurotransmitter systems have been shown to persist into adulthood (IZUMI et al. 2005, TOSO et al. 2005, TOSO et al. 2006b), and as such have received much attention as contributors to the long-term phenotypic changes associated with fetal alcohol exposure. Certainly, even subtle changes to these major neurotransmitter systems during synaptogenesis likely has significant implications for the formation and refinement of neural circuits and contribute to learning and memory deficits, motor abnormalities, changes to stress responsiveness, and other FASD-associated phenotypes. However, while ethanol may act directly on neurotransmitter function during exposure, the persistent effects of neurodevelopmental alcohol exposure may be attributed to more long-lasting mechanisms, including changes to the expression of genes that facilitate and support interneuron communication.

1.5 Gene expression and epigenetic patterning as targets of neurodevelopmental alcohol exposure

Among the many biological mechanisms vulnerable to ethanol is the regulation of gene expression. As such, prenatal ethanol exposure is particularly troubling given that neurodevelopment is a carefully-orchestrated genetic process, involving precise

spatial and temporal coordination of the transcriptome. This includes the epigenetic patterning that is established during neurodevelopment and critical to appropriate gene expression in differentiated cells. The previously-mentioned biological mechanisms rely, at least in part, by ethanol-induced changes to the expression of genes involved in those processes. Alcohol acts pleiotropically in the adult brain, and so may interfere with the transcriptional regulation of a number of developmental pathways. Consequently, many of the studies examining ethanol-induced changes to neurodevelopmental gene expression have focused on candidate genes chosen based on evidence from these processes, including genes involved in cell survival, apoptosis, migration, oxidative stress, and neurotransmission (CLIMENT et al. 2002, TOSO et al. 2005, CHEN et al. 2006a, DEMBELE et al. 2006, TOSO et al. 2006b, CAMARILLO and MIRANDA 2008, BEKDASH et al. 2014, TYLER and ALLAN 2014). The genes examined by these studies include regulators of neurogenesis, NMDA and GABA receptor subunits, HPA and circadian-associated transcripts, and neurotrophic molecules and their receptors. However, a candidate approach does not fully account for the broad range of neurological phenotypes characteristic of a multifactorial spectrum disorder, nor does it capture the interactions between genes and biological pathways that support the involvement of specific developmental mechanisms.

1.5.1 The necessity of genome-wide strategies

Given that ethanol is a well-established instigator of gene expression changes in the adult brain (TREADWELL and SINGH 2004, MULLIGAN et al. 2006, CONTET 2012), it is surprising that few studies have examined the effect of alcohol exposure on genome-wide changes to neurodevelopmental mRNA expression. The studies that have examined the effect of ethanol on the developing brain using expression arrays typically focus on early neurodevelopmental exposure (ie, during the first trimester equivalent) (HARD et al. 2005, GREEN et al. 2007, DOWNING et al. 2012), providing insight into mechanisms that may produce physiological and neurological abnormalities associated with FAS but not the mechanisms that may underlie the

cognitive and behavioural changes in individuals as a consequence of later gestational exposure. Further, research has indicated that neurodevelopmental processes have windows of vulnerability to ethanol, suggesting that the genes affected during the first trimester equivalent are likely very different from those affected during later brain development. Given the amount of physiological and behavioural research that has focused on the third trimester equivalent as a critical period of brain development, the lack of data exploring the genetic mechanisms that may underlie these findings is surprising. FASD is a complex disorder, affecting multiple brain regions and associated with a broad range of phenotypic abnormalities. Therefore, a candidate gene approach may be insufficient to fully appreciate the range of effects that prenatal alcohol exposure induces on normal gene regulation and expression. Genome-wide expression studies also cannot focus on a single stage of development; understanding the effects of ethanol on late-gestation neurodevelopmental processes are essential to understanding why the third trimester-equivalent is so critically vulnerable to ethanol and why exposure during this period is sufficient to produce profound behavioural deficits.

Microarray data and subsequent gene set enrichment analyses can be useful when distilling the biological underpinnings of complex phenotypes and to identify major pathways involved in its cause or maintenance (BUNNEY et al. 2003, MIRNICS and PEVSNER 2004). This is particularly critical to FASD; ethanol exerts an initial cellular response within the developing brain, but it has also been shown to interfere with mechanisms, including epigenetic patterning, which may affect long-term gene expression and cellular function. While multiple studies have focused on the immediate effects of neurodevelopmental ethanol exposure, fewer have evaluated how exposure translates to mature brain dysfunction that contributes to the persistent phenotypes associated with FASD. Evaluation of the mechanisms that contribute to these long-term effects are perhaps more critical and beneficial to those prenatally exposed to alcohol, particularly if they may lead to potential diagnostic or therapeutic strategies. Further, studies examining the relationship between the acute effects of ethanol on gene expression at specific developmental stages, the persistent

changes it induces within the mature brain, and the manifestation of these neural changes as phenotypic characteristics would provide a more comprehensive picture of FASD as a neurodevelopmental disorder with life-long psychiatric consequences.

1.5.2 MicroRNA expression as a contributing mechanism

Alcohol-induced changes to developmental epigenetic patterning has recently been suggested as a mechanism of long-term maintenance of altered gene expression in the brain. Non-coding RNAs (ncRNAs) are abundantly expressed, some exclusively, in the mammalian brain (MEHLER and MATTICK 2007). They have demonstrated roles in mature brain function, including long and short-term memory formation, neuroprotective mechanisms, and in controlling the malleability and plasticity of neural networks (MEHLER and MATTICK 2007, MERCER et al. 2008, QURESHI and MEHLER 2012, JIMENEZ-MATEOS 2015). MicroRNAs (miRNAs) are able to fine-tune protein expression by interfering with mRNA translation (LAGOS-QUINTANA et al. 2001). Also, miRNAs have important roles in normal neurodevelopment, including mitigating the levels of gene expression that control cell proliferation, apoptosis, differentiation, and synapse formation and remodeling (MEHLER and MATTICK 2007, COOLEN et al. 2013, NOWAK and MICHLEWSKI 2013, HU et al. 2014).

In 2007, Sathyan et al. (2007) first explored the role of regulatory miRNAs in the teratogenic effects of ethanol on the developing brain. This study reported the potential interplay of miR-9, miR-21, miR-153, and miR-355 and their target mRNAs, illustrating the sensitive balance of antagonistic biological cues that may ultimately determine cellular death or survival and adaptation following ethanol insult (SATHYAN et al. 2007). Importantly, Sathyan and colleagues identified that miRNAs serve as an effective intermediary between an environmental teratogen and cellular response given their ability to rapidly affect the expression of multiple genes.

Not only are miRNAs involved in the immediate response of developing cells to ethanol, but PAE may alter the epigenetic patterning that regulates miRNA

expression, in turn changing the balance of mRNA regulation within the adult brain (MIRANDA 2007, BALARAMAN et al. 2013, LAUFER et al. 2013). Few studies have yet to explore the long-term dysregulation of miRNA expression as a potential mechanism underlying the phenotypic changes associated with FASD, but work in has identified a number of altered miRNAs in the adult brain of mice exposed to ethanol during neurodevelopment (BALARAMAN et al. 2013, LAUFER et al. 2013, MANTHA et al. 2014a). A number of the miRNAs identified in the adult brain of PAE mice have previously been implicated by studies exploring the acute effects of ethanol exposure. These include miR-335, which was found to be suppressed following high ethanol levels but upregulated following more moderate exposures, affecting neural stem cell differentiation (SATHYAN et al. 2007). Also found to be ethanol responsive during neurodevelopment was miR-10b (WANG et al. 2009), which was found to be down-regulated in the adult brain (MANTHA et al. 2014a). This miRNA is a regulator of the Hox gene family, which play key roles in neuronal migration (GEISEN et al. 2008). The relationship or significance between the alterations in miRNA expression acutely following ethanol exposure as well as in the adult brain of mice prenatally exposed to alcohol has yet to be determined. However, it is clear that miRNAs are powerful modulators of gene expression with the ability to affect numerous mRNA targets. Further, a given biological pathway or process may be affected from multiple vantages simultaneously in an antagonistic or synergistic manner. Given that recent results suggest a significant role for miRNAs in the etiology of FASD and that changes to their expression may persist long after alcohol exposure, it appears critical to evaluate their altered expression in the adult brain and their relationship to altered mRNAs and associated biological pathways to more fully understand the scope of changes that prenatal alcohol exposure may exert over the life of an affected individual.

1.6 Developmental timing and ethanol vulnerability

Mouse models have been invaluable in establishing that the structure of the developing brain, the extent of neurological damage, and the resulting phenotypic changes are highly dependent on the timing of alcohol exposure. The normal maturation of regions of the rodent brain follow a similar pattern as in humans, with caudal regions such as the hindbrain developing earlier than more rostral areas and a somewhat parallel sequence of processes in each region (RICE and BARONE 2000). There are important differences, however, in the timeline of mouse and human neurodevelopment that need to be acknowledged. In humans, neurodevelopmental processes occur at the scale of months, with stages defined by three trimesters of gestational development. In mice, these processes occur on a scale of days (BAYER et al. 1993a). Further, while the processes that occur during the first and second trimester in humans (the first to sixth month of pregnancy) also occur gestationally in mice, they equate to gestational day (G) 0 – 11 and G12-21, respectively. Given that parturition in mice occurs at 21-22 days post-conception, the neurodevelopmental processes associated with the third trimester in humans occur postnatally in mice (postnatal day 0 to 14, approximately). This means that third trimester-equivalent exposures must be modeled by neonate ethanol treatment, eliminating the kinetics of maternal ethanol metabolism and transfer through the bloodstream and placenta that certainly impact the effect of alcohol exposure during this period in humans. Regardless, mouse models have been instrumental in establishing that the brain is vulnerable to the teratogenic effects of alcohol throughout its development, as well as in determining specific neurodevelopmental processes and brain regions that may be vulnerable to ethanol exposure at various times.

1.6.1 The vulnerability of synaptogenesis to ethanol exposure

During the third trimester of pregnancy in humans and the first three postnatal weeks in mice, the brain undergoes a rapid period of growth and development during which

much of the physiological and molecular basis of neural connections are established (DOBBING and SANDS 1979, BAYER et al. 1993b, COHEN-CORY 2002). This process of synaptogenesis includes an overproduction of dendritic arborization and synapse formation, greatly increasing synaptic density and brain mass, lending this period the designation the "brain growth spurt" (CHANGEUX and DANCHIN 1976, DOBBING and SANDS 1979). This is followed by extensive apoptotic pruning and refinement of synaptic connectivity hypothesized to maximize transmission efficiency (PURVES and LICHTMAN 1980). This process is a fundamental developmental strategy and occurs in multiple brain regions across mammalian species (ANDERSEN 2003).

The formation, maintenance, and deletion of synapses requires the careful regulation of molecular cues such as neurotrophic factor expression, cell adhesion protein regulation, and neurotransmitter signaling (JUNG and BENNETT 1996, KNOLL and DRESCHER 2002, TAKASU et al. 2002, HERLENIUS and LAGERCRANTZ 2004, ZAGREBELSKY and KORTE 2014). These processes are essential to establish mature excitatory synaptic connectivity and required for the development of neuronal structure in an intact, adult brain (CLINE and HAAS 2008, WANG and KRIEGSTEIN 2008, WONDOLOWSKI and DICKMAN 2013). The importance of interneuron communication during this stage of development, however, also makes it attune to environmental cues including teratogens; indeed, synaptogenesis may be one of the most exquisitely ethanol-sensitive periods of brain development (IKONOMIDOU et al. 2000, OLNEY et al. 2002b). Even transient increases in blood alcohol at postnatal day 7 (P7), a peak period of synaptogenesis in mice, can lead to widespread cell death in multiple brain regions (WOZNIAK et al. 2004). This is associated with ethanol's ability to act as an *N*-methyl-D-aspartate (NMDA) receptor antagonist and a γ-aminobutyric acid (GABA) agonist, which can suppress the intensity of action potentials that are required to establish synaptic connectivity leading to the inappropriate apoptosis of developing neurons (IKONOMIDOU et al. 2000, OLNEY et al. 2002c). The regions that suffer a significant deletion of developing neurons following ethanol exposure during this 'brain growth spurt' are consistent with many of the behavioural abnormalities observed in FASD, including the hippocampus, cerebellum, cortex, amygdala, hypothalamus, and corpus

callosum (IKONOMIDOU et al. 2000, OLNEY et al. 2002a, DIKRANIAN et al. 2005). Further, early neonatal ethanol treatment (during synaptogenesis) is sufficient to produce multiple FASD-related behavioural traits in adolescent and adult mice, including spatial learning impairment, motor deficits, increased anxiety- and depression-like behaviours, and alterations to circadian regulation (SAKATA-HAGA et al. 2006, BROCARDO et al. 2012, VOLGIN and KUBIN 2012, DIAZ et al. 2014a, DIAZ et al. 2014b, WAGNER et al. 2014). However, not all cells exposed to ethanol during synaptogenesis undergo apoptosis. Many are exposed to ethanol – even within vulnerable regions – and survive to comprise the developmental basis of adult neural architecture. Also, it has been shown that alcohol-induced neurodegeneration is dependent on the transcription and translation of new molecules, including the caspase activator *Blc2-associated X protein* (*Bax*) (NOWOSLAWSKI et al. 2005). As such, the period of synaptogenesis becomes an intriguing period of development in terms of how ethanol may trigger a genetic or cellular response in developing neurons that initiates programmed cell death, how cells may balance pro- and anti-apoptotic signals, and what biological processes are affected in non-apoptotic cells by this neurodevelopmental disruption and in the mature brain, long after the cessation of alcohol, but while the phenotypes associated with FASD have manifested.

1.7 Summary and research rationale

It is apparent that FASD is an extremely socially and economically costly set of disorders, despite its preventability. While research examining the complex array of behavioural, physiological, and biological mechanisms that initiate or maintain the features of these disorders continues to progress, there appears to be a gap in our understanding of how disruptions to genetic processes may be involved. Further, the variability of ethanol dosage, timing of exposure, animal model, and analytic assay used prohibits any comparison between many FASD-associated studies, making the identification of underlying mechanisms extremely difficult.

Although our understanding of neurodevelopment is certainly incomplete, we are aware that the development of the brain is not uniform and that certain periods of vulnerability exist for specific regions. The work included in this thesis seeks to examine the effects of alcohol exposure on synaptogenesis, or the third trimester-equivalent in humans, as a known period of exceptional sensitivity to ethanol and a period important for the development of multiple behavioural and cognitive processes. This requires the development of an effective animal (mouse) model that can recapitulate a number of phenotypes associated with FASD across ontogeny. Also, evaluating the changes in gene expression only acutely following ethanol exposure, while important, will not be sufficient to predict how those changes will affect genetic processes in the adult brain. This work seeks to examine not only the acute genetic response, but those genes that exhibit altered expression in the adult brain that may contribute to the maintenance of FASD-related phenotypes.

Finally, this work is part of a more comprehensive, genome-wide analysis of transcripts disrupted at specific neurodevelopmental times, including all trimester equivalents, using a consistent dosage regimen and a consistent evaluation strategy. I feel this approach will provide a better understanding of the relationship between timing of exposure, vulnerable neurodevelopmental pathways, and phenotypes resulting from that treatment. This approach may facilitate a non-reductionist (systems) approach to understanding the etiology of FASD features and aid in the identification of molecular targets as diagnostic markers or for ameliorative therapeutics.

1.8 Hypothesis and research objectives

The primary goal of the research included in this thesis is to investigate, at a genome-wide level, the effect of alcohol on neurodevelopmental gene expression and how the disrupted genetic pathways may correlate with neurobehavioural changes consistent with human FASD.

I hypothesize that a binge-like exposure to ethanol during synaptogenesis induces alterations in behavioural and cognitive phenotypes relevant to FASD. These changes are associated with acute changes to gene expression that occur at the time of exposure as well as long-term changes that persist into adulthood. The genes affected by ethanol exposure during synaptogenesis will have relevance to molecular pathways important during the third trimester-equivalent as well as the behavioural changes observed in ethanol-treated mice.

This thesis focuses on several objectives to address this hypothesis:

1. Develop a behavioural battery appropriate to evaluate mouse models for a variety of FASD-relevant behavioural and cognitive phenotypes.
2. Evaluate behavioural changes resulting from a binge-like dose of ethanol during synaptogenesis as a model for FASD. Included in this are the effects of timing and the cumulative effect of a multi-day treatment.
3. Establish if a binge-like dose of ethanol during synaptogenesis results in acute changes to the transcriptome of the developing brain.
4. Determine if ethanol treatment during synaptogenesis results in long-term changes to mRNA and miRNA expression in the adult brain.
5. Perform gene set analysis of the genes identified in (3) and (4) to evaluate associations with biological pathways and relevance to brain development and behaviour.

Chapter 2

MATERIALS AND METHODS

2.1 Animal care

All animal protocols were approved by the Animal Use Subcommittee at the University of Western Ontario (London, ON) and complied with the ethical standards established by the Canadian Council on Animal Care. Male and female C57BL/6J (B6) mice were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and subsequently bred and maintained in the Health Sciences Animal Care Facility at the University of Western Ontario. Prior to breeding, mice were housed in same-sex colonies of two to four mice with *ad libitum* access to water and food. Environmental factors (such as cage type and size, colony size, bedding, nestlets, and environmental enrichment) were standardized between cages. Colony rooms were maintained in a controlled environment on a 14/10-hour light/dark cycle (2000 h to 0600 h dark) with 40% to 60% humidity and a temperature range of 21°C to 24°C.

2.2 Breeding and ethanol treatments

Nulliparous female B6 mice of approximately 8 weeks of age were individually housed and mated overnight with 8 to 12-week old B6 males. Males were removed the subsequent morning and, during gestation, females were housed individually in standard caging. Towards the end of gestation (gestational days 17-22) cages were checked each evening at 5pm and each morning at 10am for the presence of pups. Day of birth was noted as postnatal day (P) 0. Litters were culled to contain a maximum of 10 pups and litters containing less than 4 pups were excluded from further study. At P0, pups from each litter were assigned to one of three treatment groups, each designed to mimic a binge-like ethanol exposure within the human third trimester-equivalent (T3) of neurodevelopment: early T3 at P4, mid T3 at P7, or both early and mid T3 (P4 and P7). On treatment days, dams were temporarily removed from the cage and pups were divided into control and ethanol treatment groups as equally as possible by sex and weight to account for litter effects. Treatments consisted of two subcutaneous injections spaced two hours apart (0 h and 2 h) of 2.5 g/kg ethanol in 0.15 M saline. This paradigm results in peak blood alcohol concentrations of over 0.3 g/dl for 4 to 5 hours following the initial injection (IKONOMIDOU et al. 2000, WOZNIAK et al. 2004). Control mice were injected with an equivalent amount of 0.15 M saline only. Following injection, the tail of each pup was marked with non-toxic ink to allow for identification. Once the injections for all pups were completed, the dam was returned to the cage. Pups remained with their biological dams until weaning at P25, when they were re-housed into same-sex in colonies of 2-4 littermate mice.

2.3 Behavioural phenotyping

When developing a behavioral battery for the assessment of FASD-relevant behavioural phenotypes, I considered several criteria:

1. The assay measured phenotypes in mice that had been consistently observed in children, adolescents, or young adults with FASD.

2. The tests were well-established, appropriate to mouse behaviour, and consistently shown to successfully test phenotypic features relevant to FASD.

3. The battery was conducted from least intrusive and/or cognitively demanding to those that tested higher executive functioning, in order to minimize pre-testing confounds.

Given these criteria, I developed a behavioural battery that tested mice at several stages of development, designed to evaluate a range of FASD-related behaviours. The tests included in the behavioural battery are described below.

2.3.1 Early developmental milestone achievement

The appearance of early postnatal coordination, strength, sensory and motor skills were assessed using a behavioural battery adapted from Wu et al. (WU et al. 1997) and Hill et al.(HILL et al. 2007). Testing was performed from P2 to P21 at 1000 hrs. Dams were removed and placed in a separate clean cage for the duration of the testing. Each pup was weighed then assessed for its ability to achieve specific age-appropriate developmental milestones. Testing lasted approximately four minutes per pup. Tests were performed each day until the pup was able to perform the task on two consecutive days. At the completion of testing, the pup was returned to its home cage. Each developmental milestone task is outlined below and illustrated in Figure 2.1.

2.3.1.1 *Postnatal weight*

Pups were weighed daily from P2 until weaning at P25. Following weaning, pups were weighed at P30 and P35, then every seven days until P70.

2.3.1.2 *Surface righting*

The latency of the pup to right itself with all four paws touching the surface of the table when placed on its back on a plastic surface was evaluated. If the mouse did not respond or complete the task within 15 seconds (s), the test was terminated. The test was continued daily until the pup was able to right itself on two consecutive days in less than 2 s.

2.3.1.3 *Negative Geotaxis*

The pup was placed head-down on a mesh screen set at an incline of 45°. The time required for the pup to turn its body 180° to a head-up position was recorded. The task was considered complete when the mouse was successful on two consecutive days within 30 s.

2.3.1.4 *Cliff aversion*

The pup was positioned at the edge of a plastic box, approximately 3.0 cm above the surface of the table, with the digits of its forepaws and nose over the edge. The day on which the mouse was able to rotate away from the edge and place its body fully on the surface of the box (within 30 s) was recorded.

2.3.1.5 *Forelimb grasp*

The pup was placed with its forelimbs resting on a thin metal rod (1.5 mm diameter) suspended 10 cm above a soft surface (a 5cm thickness of fine wood shavings). Task was completed when the pup was able to grasp the rod and remain suspended for more than one second on two consecutive days.

2.3.1.6 *Auditory startle*

The mouse pup was placed on a flat surface and the day it showed an auditory reflex response (an involuntary startle) to a handclap at a distance of 10 cm from its head was recorded.

2.3.1.7 *Ear twitch*

The reflex of the ear to flatten in response to a tactile stimulus was tested using a fine cotton filament brushed gently against the pup's ear three times. The pup's ability to flatten or twitch its ear in response on two consecutive days indicated a successful achievement of the reflex.

2.3.1.8 *Open field traversal*

The pup was placed in the centre of a circle 13 cm in diameter on a smooth plastic surface. The day the pup was able to extinguish pivoting behavior and move outside of the circle with 30 s was recorded. Success on two consecutive days marked the end of testing.

2.3.1.9 *Air righting*

The pup was gently held upside down, approximately 10 cm above a soft surface (5 cm thickness of fine wood shavings), and released. The day the pup was able to right itself and land with all four paws on the wood shavings on two consecutive days was recorded.

2.3.1.10 *Eye opening*

The first day both eyes were open on each pup was recorded.

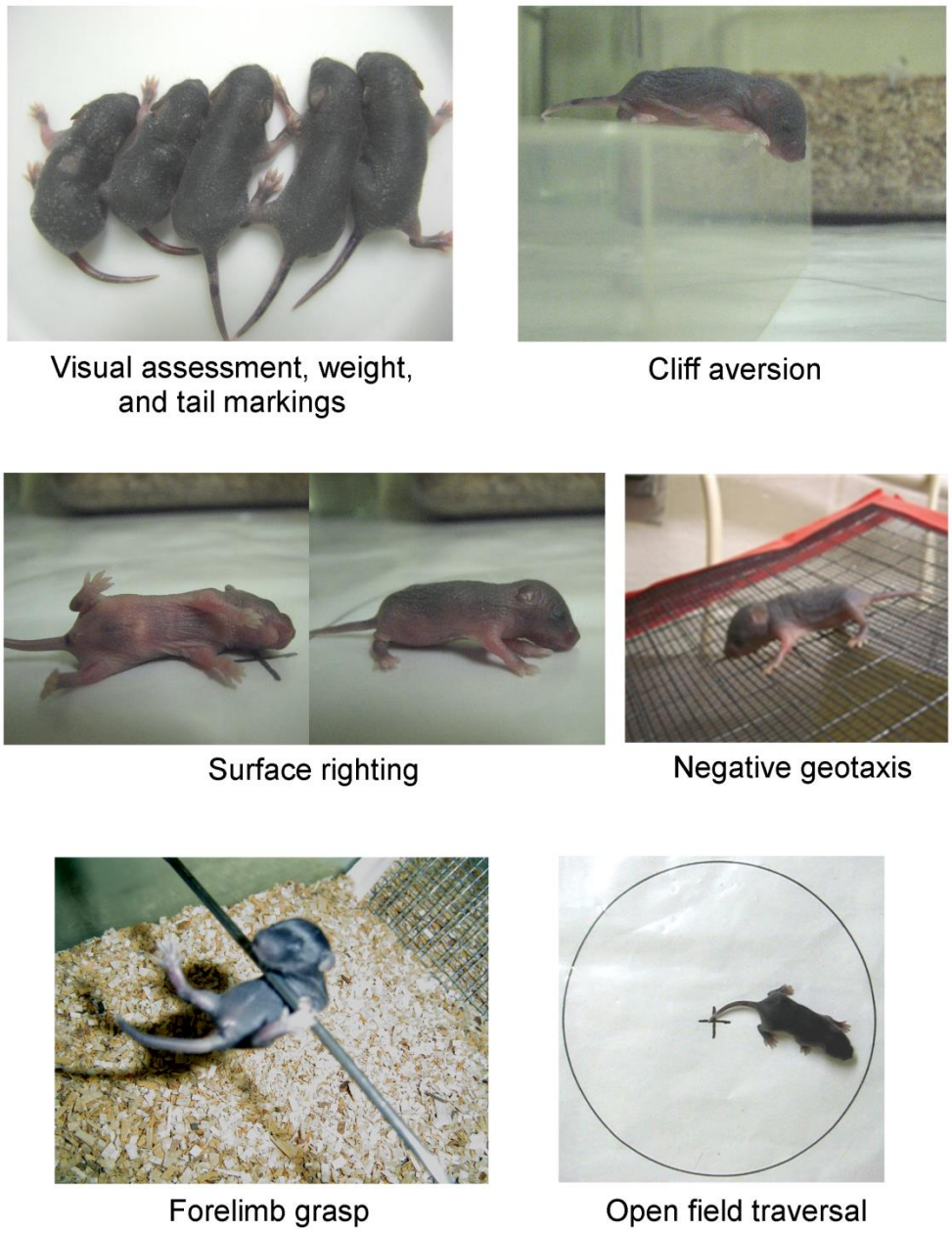


FIGURE 2.1. Images representative of behavioural tasks used to measure the achievement of early postnatal developmental milestones. Pups were tested for their ability to reach age-appropriate tasks assessing neuromuscular coordination and reflexes. Task is indicated below the image.

2.3.2 Juvenile locomotor activity

Locomotor activity in juvenile mice (P26-35) was tested using two methods. The first tested spontaneous daytime locomotor activity in a novel environment, and served as a measure of both activity and anxiety-related traits. The second method tested nocturnal activity in a familiar home cage environment and was a measure of overall activity. All locomotor testing occurred in a room reserved for testing that was separate from the general colony. The experimenter was not present in the room during the testing.

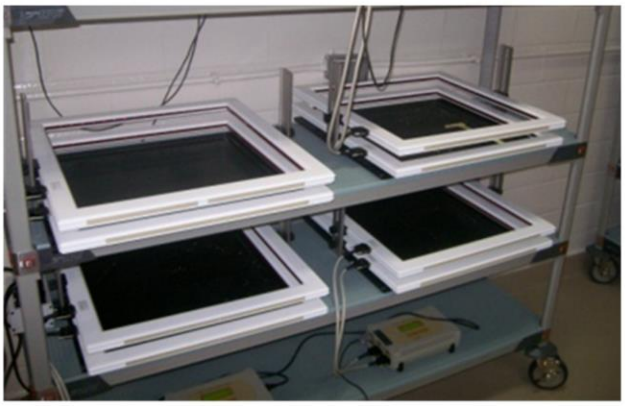
2.3.2.1 *Activity in a novel open-field*

At P26-P28, spontaneous locomotor activity and exploration of a novel environment was assessed using an infrared Actimeter system (Panlab, Barcelona, Spain) (Figure 2.2). The apparatus consisted of a 45 cm (width) x 45 cm (depth) arena of black plexiglass enclosed with four clear acrylic walls (35 cm in height) (Figure 2.2B). A square frame mounted outside of the arena created a 16 x 16 grid of intersecting infrared beams used to track the horizontal movement of each mouse in real-time. A second frame placed above the lower frame was used to track the vertical movement of each mouse, set to detect hind-leg rearing. Locomotor measures were recorded using ActiTrack software (Panlab), which uses infrared beam data to calculate parameters such as overall locomotor activity (total number of beam breaks), distance traveled, and number of rears. Testing was conducted during the light phase between 1030 h and 1230 h for all mice. Mice were brought into the testing room in their home cage a half hour prior to testing. An individual mouse was then placed in the corner of the arena and allowed to freely explore it for 15 min. During testing, the lighting of the arena was 100 lx so as not to inhibit normal exploratory behaviour. At the end of testing, the mouse was removed and returned to its home cage and the surface and walls of the arena were wiped clean with 30% isopropanol.

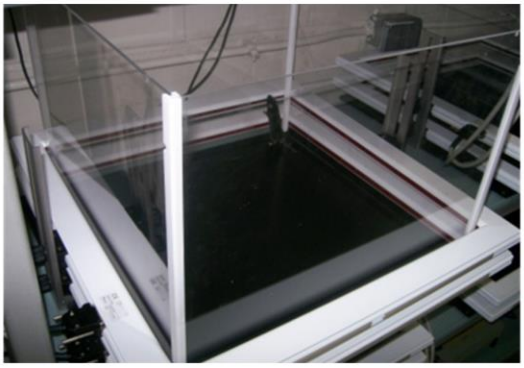
2.3.2.2 *Home cage activity*

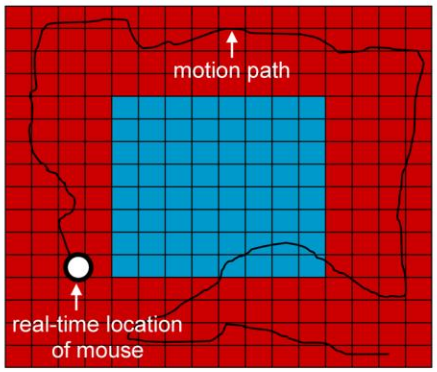
Nocturnal locomotor activity in a familiar, home cage environment was evaluated using the infrared Actimeter system (Panlab, Barcelona, Spain) (see apparatus description above) at age P30-35 (Figure 2.2C). Prior to testing, mice were re-housed individually in 38 cm (length) x 24 cm (width) x 14 cm (height) transparent plastic cages (Innovive, San Diego, CA, USA) with standard woodchip bedding and *ad libitum* access to food and water. After re-housing, mice were moved from the colony room to the testing room. After a 24 h acclimation period, cages were placed within the infrared Actimeter frame at least 3 h prior to the initialization of testing. Testing was conducted over a 12 h-period from 1900 h to 0700h which spanned the dark phase of the light/dark cycle. Data were collected using ActiTrack software (Panlab). Recordings were taken for two consecutive nights and averaged to obtain data for parameters such as locomotor activity per hour, distance traveled, mean speed, and number of rears. Mice were re-housed in their original colony cages at the end of testing.

A. Actimeter locomotor apparatus



B. Novel open field exploration





C. Home cage locomotor activity

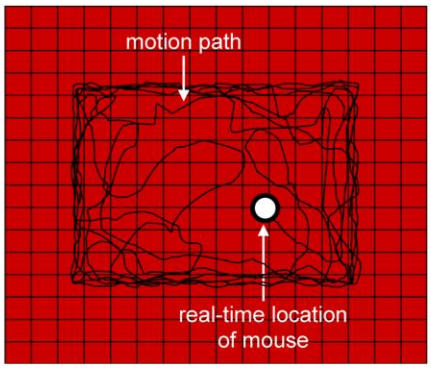


FIGURE 2.2. Infrared Actimeter (Panlab) apparatus for locomotor activity tracking.

(A) Actimeter system four-arena set-up allowing for the concurrent assessment of four animals. Lower frame is used to track horizontal movements, while the upper frame tracks vertical movements (rearing). (B) Novel open field arena apparatus and sample ActiTrack real-time tracking output. (C) Apparatus used for assessment of nocturnal home cage activity, including a sample ActiTrack output.

2.3.3 Anxiety-associated behavioural measures

Anxiety-related traits were assessed using three measures: locomotor activity in a novel open field arena, a light-dark box assay, and an elevated plus maze assay (Figure 2.3). Each test relies on the innate behaviour of mice to avoid open or highly-lit areas as these features are anxiogenic. All treatment groups were analyzed using the open field arena. Only the P4+7 control (saline-treated) and ethanol-treated groups were assessed using the latter two measures due to apparatus availability.

2.3.3.1 *Thigmotaxis in a novel open field*

Tracking of mouse locomotor activity using the Actimeter system (Panlab, Barcelona, Spain) in a novel open-field arena is described above (open-field activity in a novel environment). This open-field data was also analyzed by dividing the arena into a 11.25 cm-wide periphery zone bordering the walls of the arena and a 22.5 cm ×22.5 cm central zone to allow for the evaluation of thigmotaxis, or the tendency of mice to explore mainly along the periphery walls of an enclosed arena rather than to travel directly through the centre (SIMON et al. 1994) (Figure 2.3A). Testing parameters (time of day, presence of experimenter, duration of testing, and ambient lighting of the arena) are described above under section 2.1.2 (Juvenile activity in a novel open-field).

2.3.3.2 *Elevated plus maze*

Mice were evaluated for anxiety-related testing using the elevated plus maze (EPM) at P35-40. Mice were removed from the colony room and brought to the testing room 1 h prior to testing. Mice were placed in the centre of a maze consisting of four adjoining arms that intersected at a 90° angle (Figure 2.3B), constructed from a protocol described in Walf and Frye (2007). The central area was 5 cm (length) x 5 cm (width) and allowed the mouse to move freely between four 30 cm (length) x 5 cm (width) arms that extended from the central region, with two opposite arms enclosed by 16 cm (height) walls along their length. The arms and walls were

constructed of acrylic and plastic materials to facilitate cleaning and the removal of olfactory cues. The maze was elevated atop a wooden support 100 cm above the ground. Maze illumination from above by a bright (220 lx) ceiling-mounted light and a computer-generated 85 dB white noise were used during testing as adversive (anxiogenic) environmental stimuli. Each mouse was allowed to explore the maze freely for one 5 min trial (WALF and FRYE 2007), during which its movements were tracked by a video camera mounted above the centre of the maze. AnyMAZE video tracking software (San Diego Instruments, San Diego, CA, USA) was used to analyze the real-time movements of the mouse including entries into and time spent in the open and closed arms. Following the conclusion of the trial, the mouse was returned to its home cage and the maze was cleaned with 30% isopropanol.

2.3.3.3 *Light/dark box*

Mice were also assessed for anxiety-related traits using the light/dark box test (LDB) at P35-40. The LDB was constructed from acrylic materials according to the protocol outlined from Bourin and Hascoet (BOURIN and HASCOET 2003) (Figure 2.3C). The apparatus consisted of a 45 cm (length) x 26 cm (width) arena enclosed by 30 cm (height) walls. One third of the arena – 18 cm (length) x 26 cm (width) – was enclosed using black acrylic creating the "dark" region. This region was open to a 27 cm (length) x 26 cm (width) uncovered "light" area of the arena via a 7cm (width) x 7 cm (height) opening that provided the mouse free access between each area. At the beginning of each trial, the mouse was transferred from the colony room to the testing room and allowed to acclimatize for 1 h. Following acclimation, the mouse was placed in the light area of the arena facing the opening to the dark area and the mouse was allowed to freely explore the arena for one 5 min trial. Each trial was conducted using a 220 lx ceiling-mounted light and an 85 dB white noise as a mild environmental stressor. The experimenter was not present in the room during testing. Movement was recorded using a ceiling-mounted video camera and analyzed using AnyMAZE video tracking software (San Diego Instruments, San Diego, CA, USA). Measurements recorded included the number of transitions between the light and dark areas, and the time spent in the light versus dark region. Following cessation of the trial, the

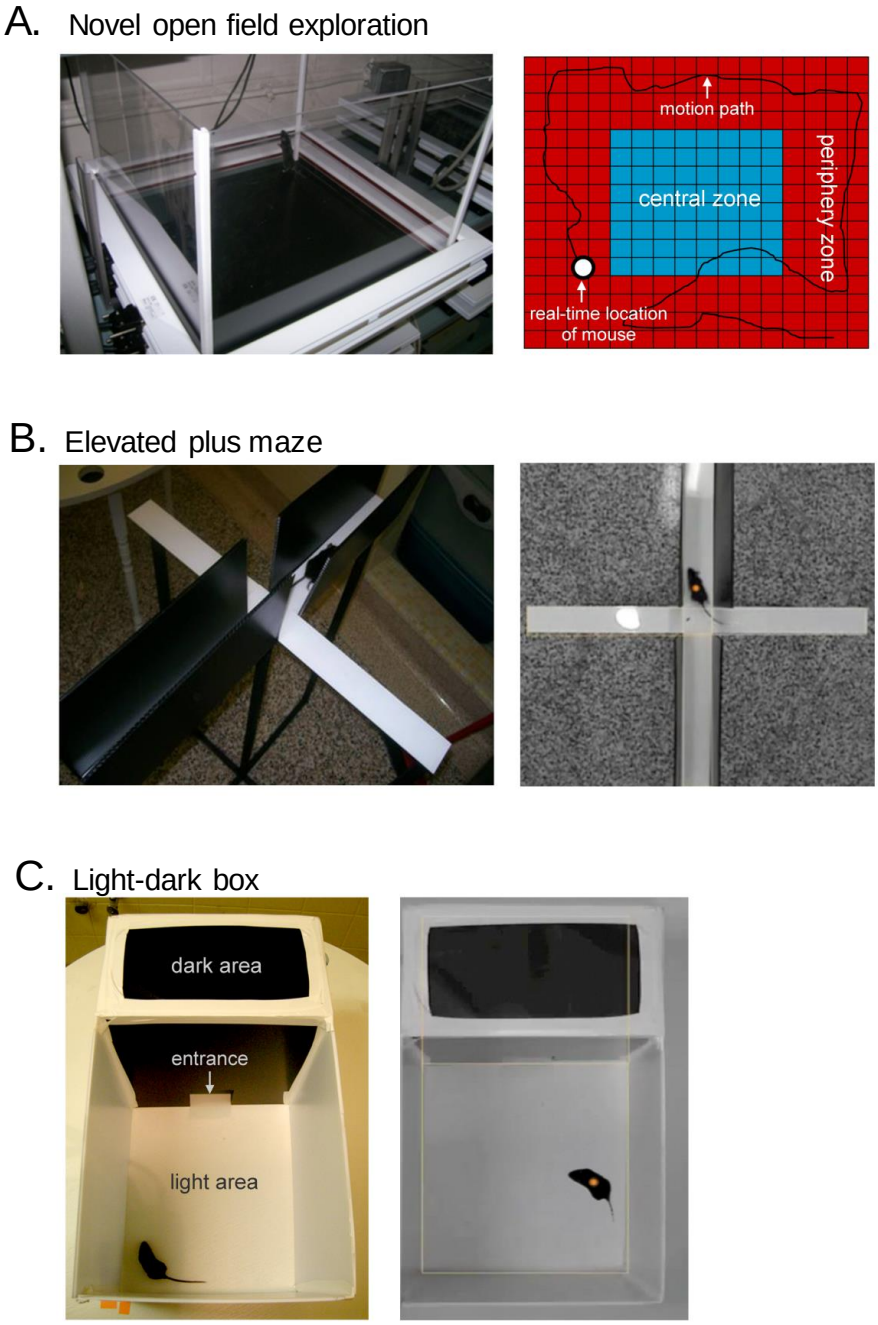


FIGURE 2.3. Apparatus used to assess anxiety-related traits.

(A) Actimeter (Panlab) open field arena (left image) and ActiTrack real-time monitoring of mouse movement (right image), including the separation of the peripheral zone (red) and the central zone (blue) used for analysis of thigmotaxis. (B) Elevated plus maze test consisting of two "open" arms and two enclosed arms (left image). Beside the image of the maze is a screenshot of the AnyMAZE (San Diego Instruments) video tracking output, with the orange dot indicating the location of the mouse. (C) Light-dark box apparatus showing the light (open) area and dark (enclosed) area (left image), as well as AnyMAZE (San Diego Instruments) video tracking screenshot (right image).

mouse was returned to its home cage and returned to the colony room. The arena (light and dark regions) was cleaned with 30% isopropanol between mice.

2.3.4 Assessment of spatial learning and memory

Spatial learning and reference memory were evaluated in mice using a modified Barnes maze constructed according to a protocol described by Sunyer et al. (2007) (Figure 2.4). The apparatus consisted of a white circular platform, 92 cm in diameter, elevated 75 cm from the floor. Along the periphery of the platform were 20 evenly-spaced circular holes of 5 cm in diameter located 2cm from the edge of the table. Each hole was sealed by black card (not visible from the surface of the table) except for one, representing the target hole, under which a black plastic escape box of 13 cm (length) x 13.5 cm (width) x 5 cm (height) was placed. Various large shapes of different colours were placed on the walls of the testing room to serve as extra-maze visual cues. A light ceiling-mounted directly above the centre of the table (220 lx) and an 85 dB computer-generated white noise were used as adversive stimuli to promote escape to the target (SUNYER et al. 2007). Due to the short duration of some trials, the experimenter remained in the room during testing but stood in the same location and was dressed in the same attire for each trial (white laboratory coat). Mouse movements were tracked in real-time using AnyMAZE video tracking software (San Diego Instruments, San Diego, CA, USA) via an overhead-mounted video camera. Testing consisted of two stages: a 4-day acquisition period testing spatial learning and a 2-day probe period testing short and long-term recall memory, described below.

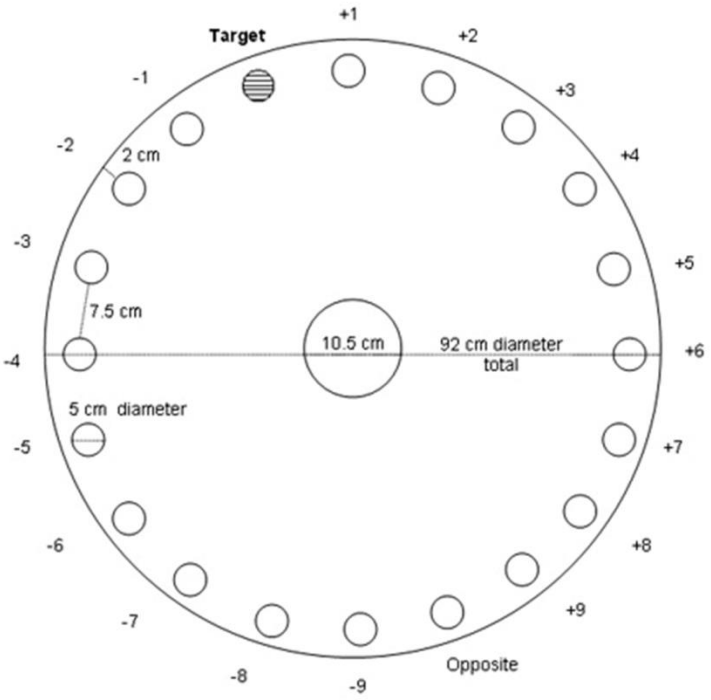
2.3.4.1 *Spatial acquisition learning*

Acquisition training began at P45 (day 1) and consisted of four, 3-min trials spaced 15 min apart per day for four consecutive days. Each testing day, mice to be tested were removed from the colony room and brought to the testing room 1 h prior to the

commencement of testing. Following the 1 h acclimation period, the mouse was placed in a 14 cm (height) x 10 cm (diameter) cylindrical black start chamber in the centre of the platform. The overhead light and the white noise were turned on and, after 10 s, the start chamber was lifted to allow the mouse to freely explore the maze. The trial was concluded once the mouse had entered the escape box, at which the noise and light were turned off and the mouse was left undisturbed in the box for 1 min before it was returned to its home cage. If the mouse had not found or entered the escape after the 3 min trial, the mouse was gently guided to the escape hole by the experimenter using one hand and without lifting the mouse from the table. Again, once the mouse had entered the escape chamber, the overhead light and the white noise were turned off and the mouse was left in the chamber for 1 min before being returned to its home cage. To avoid the influence of olfactory or proximal cues, the table and the escape box were cleaned with 30% isopropanol between mice and the table was rotated around its central axis between each of the four daily trials per mouse. This altered the specific hole that represented the target but not its relationship to the extra-maze visual cues, which remained consistent between trials for each mouse. AnyMAZE video tracking software was used to analyze variables such as latency to reach target and mean speed. Number of errors (visits to non-target holes) and search strategy were analyzed manually using video recordings of each trial by two separate evaluators blind to treatment. Search strategies were defined as the following:

1. Direct: mouse moves directly to the target hole or adjacent hole (+1 or –1 only) before reaching and entering target
2. Serial: the first visit to the target hole is preceded by visiting at least two adjacent holes in a serial manner, in a clockwise or counter-clockwise direction.
3. Mixed: searches to various holes separated by crossing through the centre of the maze or in a general unorganized manner.

A. Schematic of Barnes maze construction specifications



B. Barnes maze apparatus and testing room

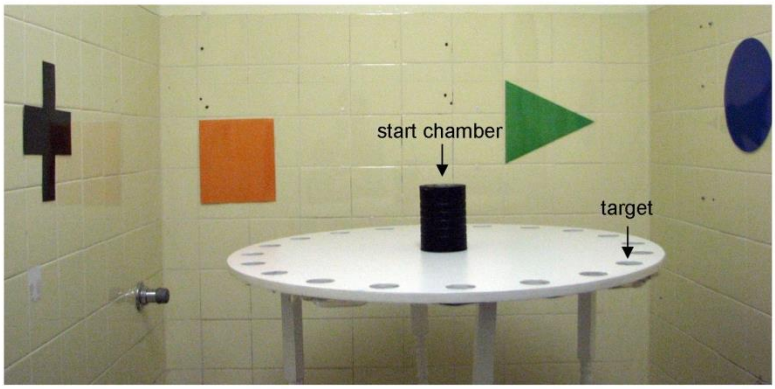


FIGURE 2.4. Barnes maze apparatus for the testing of spatial learning and recall memory.

(A) Specifications used for the construction of the Barnes maze apparatus.

(B) Photograph of the Barnes maze showing the position of the start chamber, the target, and the placement of visual cues used for spatial orientation.

2.3.4.2 *Short and long-term recall memory*

Following the conclusion of the acquisition days (days 1 – 4), the short-term spatial memory of each mouse was tested on the subsequent day (day 5 of testing) and the long-term spatial recall memory was tested 7 days later (day 12 of testing). During these two "probe" trials, the escape box was removed and the target hole was closed with black card. To initiate testing, the mouse was placed in the start chamber and the light and white noise were turned on. After 10 s, the chamber was removed and the mouse was allowed to freely explore the maze for 1 min before being gently removed from the table by the experimenter and returned to its home cage. Behaviours measured (via video recording) included explorations to each hole and were scored manually by two separate evaluators blind to treatment groups.

2.3.5 Statistical analysis of behaviour data

All data were analyzed using SPSS v.16.0 (SPSS Inc., 2007, Chicago, IL, USA). Analysis for developmental milestones were analyzed using Multivariate Analysis of Variance (MANOVA) using sex and treatment as fixed factors and were Bonferroni-corrected for multiple testing. Given the multiple measures tested for during the open-field locomotor assay, these data were subjected to a principal components analysis (PCA). These results revealed two main components, driven by activity (major slope) and rearing (secondary slope). As such, total activity (number of beam breaks) was analyzed as the main measure of activity and evaluated using a MANOVA (open-field activity) or repeated-measure-MANOVA (RM-MANOVA) using sex and treatment as fixed variables and hour as a repeated measure (home cage activity). Anxiety-related data was analyzed using MANOVA (sex and treatment as fixed factors). Barnes maze data were analyzed using a RM-MANOVA (where testing occurred over acquisition days) or MANOVA for sex and treatment as appropriate. Latency measures were cube-root transformed to meet equality of variance between days of testing, then analyzed using RM-MANOVA. Barnes maze error data were analyzed for goodness of

fit to expected explorations per hole using Chi-squared tests. All data are reported as mean ± standard error of the mean (SEM). The null hypothesis was rejected at $p < 0.05$.

2.4 Analysis of ethanol-induced gene expression changes

Brain gene expression was assessed in two cohorts of mice using expression microarray analyses: the first evaluated transcript alterations acutely (4 hours) following ethanol exposure at P7 (short-term effects), and the second assessed mRNA level changes in the adult (P60) brain of mice treated at P4+7 (long-term effects). Only male mice were used for all molecular analyses due to estrous cycle gene expression variation in females (JORGE et al. 2002, KIM et al. 2011). For the short-term cohort, whole brain RNA from 30 male mice were used (18 for array hybridization and 12 for qPCR confirmation). For long-term evaluation of gene expression, 24 mice were used (12 for array hybridization and 12 for qPCR confirmation).

2.4.1 Tissue collection and RNA isolation

Male mice at P7 and P60 were euthanized using CO_2 asphyxiation followed by cervical dislocation. Whole brain tissue was isolated within 2 min of euthanization and snap-frozen in liquid nitrogen and stored at -80° C. Total RNA was isolated using Trizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and cleaned using RNeasy Mini kit (QIAGEN, Valencia, CA, USA). The quality and quantity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA samples used for all analyses had an optical density $OD_{260/280}$ ratios of 2.0-2.1 nm and an RNA integrity number (RIN) of 8.0 – 10.0.

2.4.2 RNA sample preparation for expression array analysis

For the analysis of immediate (4 hours post-treatment) gene expression alterations induced by ethanol treatment, a total of six biological replicates – four control and two ethanol-treated – were used. For sample preparation, whole brain RNA from 18 male mice (12 control and 6 ethanol-treated) was diluted to 200 ng/µl and RNA from three non-littermate P7 males were pooled to create each biological replicate to reduce potential expression differences due to litter effects. For each control (saline-treated) RNA sample within a replicate, a littermate ethanol-treated RNA sample was included in the comparative replicate.

For the analysis of long-term (P60, 53 days post-treatment) changes to gene expression induced by neurodevelopmental ethanol exposure, four biological replicates – two control and two ethanol-treated – were used. Whole brain RNA from 6 saline-treated and 6 ethanol-treated males were diluted to 200 ng/µl and three non-littermate RNA samples was pooled to create each biological replicate. For each control RNA sample included within a replicate, a littermate ethanol-treated male was included in the comparative replicate.

2.4.3 Microarray hybridization

Pooled whole-brain RNA samples were rechecked for concentration (200 ng/µl) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Subsequent sample preparation and hybridization steps were performed at the London Regional Genomics Centre (Robarts Research Institute, London, ON, CA). Briefly, single-stranded complementary DNA (sscDNA) was synthesized using 200 ng of total RNA using the Ambion WT Expression Kit for Affymetrix GeneChip® Whole Transcript WT Expression Arrays (Applied Biosystems, Carlsbad, CA, USA) and the Affymetrix GeneChip® WT Terminal Labeling kit according to the protocol outlined in the hybridization manual (Affymetrix, Santa Clara, CA, USA). First-cycle cDNA was transcribed *in vitro* to cRNA, which was used to

synthesize 5.5 µg of sscDNA that was subsequently biotin-end-labeled and hybridized for 16 h at 45°C to Affymetrix Mouse Gene 1.0 ST expression arrays. The arrays were then stained using streptavidin-phycoethyrin prior to scanning. All liquid-handling steps were performed by a GeneChip® Fluidics Station 450 and arrays were scanned using the GeneChip® Scanner 3000 7G using Command Console v1.1 (Affymetrix, Santa Clara, CA, USA). Command Console v1.1 was used to calculate the intensity value per array cell based on the pixel intensity of each cell from the array scans (DAT) file. These data was converted to cell-based intensity calculations and exported as .CEL files.

2.4.4 Microarray data analysis and gene identification

Probe-level (.CEL) data were imported into Partek Genomics Suite software v.6.6 (Partek Inc., St. Louis, MO, USA) for summarization to gene-level data and quality-control analyses. Data were background-corrected, quantile-normalized, summarized using the GeneChip®-Robust Multiarray Averaging (GC-RMA) algorithm to take into account probe sequence (GC content) (IRIZARRY et al. 2003), and $\log_2$-transformed. The Partek Suite was also used to determine gene-level ANOVA *p*-values and fold changes. Genes meeting the criteria of a 1.2-fold change with a false-discovery rate (FDR)-corrected p value of < 0.05 were considered for further analyses. Unannotated genes and standards used for array normalization were removed from gene lists used for clustering and pathway analyses. Genes meeting the criteria for significance were subjected to hierarchical clustering analysis using Euclidean distance and average linkage to assess the consistency across replicates and to evaluate the general (visual) trends in changes to gene expression across each treatment. All data files from the array experiments have been deposited in the National Center for Biotechnology Information (NCBI) Gene expression Omnibus (GEO) and can be found under the accessions GSE59512 (short-term P7 data) and GSE34549 (long-term P60 gene expression data). Complete gene lists of transcripts meeting the criteria for significance are given in Appendices A and B.

2.4.5 Gene set analysis of differentially expressed transcripts

To examine the biological roles of genes identified as affected by ethanol, the gene lists generated from Partek were analyzed using publically and commercially available bioinformatic tools to assess over-represented Gene Ontology (GO) biological functions, molecular processes, canonical pathway associations, biological pathway associations, and gene interaction networks. Gene ontology biological functions term enrichment was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (DENNIS et al. 2003). DAVID is a freely-available bioinformatic tool which extracts biological themes and clusters genes into functional groups from an input gene list and assigns significance of gene-enrichment in each annotation term via a modified Fisher exact *p*-value. The biological functions associated with differentially expressed genes were also evaluated using Ingenuity® Pathway Analysis, which clusters genes by functional annotation using QIAGEN's Ingenuity Ontology 'Knowledge Base' and assigns significance to over-represented functions using a right-tailed Fisher exact test. The Knowledge Base differs from standard gene ontology analyses in that it incorporates processes to resolve synonyms and homographs in order to maintain term identity and remove duplicate or redundant terms. Ingenuity® software was also used to evaluate the gene lists for significant associations to canonical biological pathways, as well as to perform gene network analysis, which incorporates differentially expressed genes into predicted and networks involving annotated interacting genes and proteins.

2.4.6 Confirmation of mRNA levels by qRT-PCR

Quantitative reverse-transcription PCR (qRT-PCR) was performed to confirm the expression of select genes identified by the short-term and long-term array analyses. Whole brain RNA isolated from six non-littermate male mice not used for array hybridization were used for confirmation. Complimentary DNA (cDNA) was

synthesized from 2 µg of total brain RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. PCR reactions were run using Gene-specific TaqMan® Assay Reagents and TaqMan® Gene Expression Assay products in a StepOne™ Real Time PCR System cycler (Applied Biosystems, Foster City, CA, USA). Gene-specific primers and were obtained from Applied Biosystems Inventoried Assays and used according to the instructions supplied by the manufacturer. Genes were selected for confirmation based on their functional relevance within significantly-identified pathways affected by ethanol treatment, as identified during functional clustering and pathway bioinformatic analyses, and are listed in Table 2.1. All reactions were multiplexed with *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) (long-term genes) or *Actin, beta* (*Actb*) (short-term genes) as internal controls. Target gene-specific probes were labeled with a 5' 6-carboxyfluorescein (FAM) fluorophore and a 3' tetramethylrhodamine (TAMRA) quencher. Control gene probes were labeled with a 5' VIC fluorophore and a 3' TAMRA quencher. Reactions were performed using a standard ramp speed protocol using 10 µl volumes of cDNA. PCR cycling consisted of an initial denaturing at 95°C for 10 min, followed by 40 cycles of a 15 sec 95°C denaturation stage and an anneal and extension at 60°C for 60 seconds. Three biological replicates per treatment group and three technical replicates per sample were used (total N=6). Relative expression was calculated according to the comparative C_T method (SCHMITTGEN and LIVAK 2008) using StepOne™ v.2.0 software (Applied Biosystems). Significant differences were assessed using a two-tailed student's t-test, assessed using SPSS v.16 (SPSS Inc., Chicago, IL, USA).

Table 2.1. Genes selected for mRNA level confirmation by quantitative RT-PCR

Treatment group	Gene symbol	Gene name	Reference gene*
P7 (short term)	*Dusp1*	*dual specificity phophatase 1*	*Actb*
	Jun	*jun proto-oncogene*	*Actb*
	Nr4a1	*nuclear receptor subfamily 4, group A, member 1*	*Actb*
	Pomc	*pro-opiomelanocortin-alpha*	*Actb*
P4+7 (long term)	*Cnr1*	*cannabinoid receptor 1 (brain)*	*Gapdh*
	Grin2b	*glutamate receptor, ionotropic, NMDA2B (epsilon 2)*	*Gapdh*
	Htr5b	*5-hydroxytryptamine (serotonin) receptor 5B*	*Gapdh*
	Pomc	*pro-opiomelanocortin-alpha*	*Gapdh*
	miR-26b	*microRNA 26b*	*Actb*

*Reference genes used for qRT-PCR: *Actb: Actin, beta; Gapdh*: *Glyceraldehyde-3-phosphate dehydrogenase*

2.5 Analysis of microRNA alterations in the adult brain

2.5.1 miRNA array hybridization

To evaluate changes to miRNA levels in the adult brain of mice exposed to ethanol during synaptogenesis, whole brain RNA samples isolated from young adult (P60) mice were evaluated for genome-wide microRNA (miRNA) expression differences using Affymetrix miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Similarly to the mRNA expression arrays, a total of four arrays – two control and two ethanol-treated – were used. Each biological replicate consisted of pooled whole-brain RNA samples from three P60 non-littermate male mice (total N=12 mice). The experimental design was balanced for litter, with an ethanol-treated male matched for weight and litter with each control male. All labeling and hybridization steps were performed at the London Regional Genomics Center (Robarts Research Institute, London, ON, CA). One μg of total brain RNA from each replicate was labeled using the Flash Tag Biotin HSR kit (Genisphere, Hatfield, PA, USA) and hybridized to Affymetrix miRNA 2.0 arrays (Affymetrix, Santa Clara, CA, USA) for 16 hours at 45°C. All liquid-handling steps were performed using a GeneChip® Fluidics Station 450 and arrays were scanned using the GeneChip® Scanner 3000 using Command Console v.1.1 (Affymetrix). The expression data generated from the miRNA arrays were deposited to the NCBI Gene Expression Omnibus database and are accessible under the accession GSE 34413.

2.5.2 miRNA array analysis and mRNA target filtering

Probe-level (.CEL) data were imported into Partek Genomics Suite software v.6.6 (Partek Inc., St. Louis, MO, USA) for summarization to miRNA expression data and quality-control analyses. Differential miRNA expression was evaluated using a one-way ANOVA with treatment as the between-subjects factor. The miRNA genes identified by this analysis were filtered using a 1.2-fold change and a FDR-corrected *p*-value of 0.05. The complete miRNA list is shown in Appendix C. This list was

imported concurrently with the long-term mRNA gene list results into Ingenuity® Pathway Analysis software (Ingenuity Systems, Redwood, CA, USA, www.ingenuity.com) and analyzed using the microRNA Target Filter subprogram to develop a list of potential regulatory interactions between altered mRNA and miRNA transcripts. Results were further filtered based on brain-specific gene expression and an inverse miRNA to target mRNA expression relationship. The subsequent miRNA-mRNA transcript list meeting these criteria was evaluated using Ingenuity® Pathway Analyses for biological function and pathway associations.

2.5.3 miRNA expression confirmation by quantitative RT-PCR

Whole brain RNA samples not used for array hybridization were used to confirm the expression of miRNA *mmu-miR-26b* using quantitative reverse-transcription PCR (qRT-PCR). The altered expression of the putative target of this miRNA, *Cannabinoid receptor 1 (brain)* (*Cnr1*), was also confirmed using qRT-PCR. Two µg of whole brain RNA was used in reverse-transcription reactions using the TaqMan® Small RNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), which includes hairpin stem-loop primers for only one specific non-coding RNA. Small nucleolar RNA 202 (snoRNA 202) was used as an endogenous control (BRATTELID et al. 2011) and it was not identified as differentially expressed between control and ethanol-treated by the miRNA array experiments. Reverse transcription was performed in a StepOne™ Real Time PCR System (Applied Biosystems) according to the protocol outlined by the reverse transcription kit. Non-coding RNA (ncRNA)-specific primers and FAM-labeled TaqMan® probes were selected using the Applied Biosystems search engine to identify gene-specific TaqMan® ncRNA assays. PCR reactions were conducted using TaqMan® Small RNA Assay products on a StepOne™ Real Time PCR System (Applied Biosystems). Reactions for each target ncRNA were run in separate reaction tubes but using the same PCR master mix and assessed within the same PCR plate. Six biological replicates were used for each ncRNA-of-interest, and three technical replicates were included per biological replicate. Reactions were run

following the recommended ramp protocol: an initial 2 min hold at 50°C, a 10 min enzyme activation hold at 95°C, and 40 cycles of a 15 sec denature at 95°C and a 60 sec anneal/extend period at 60°C. Relative expression was determined using the comparative C_T method (SCHMITTGEN and LIVAK 2008) using StepOne™ v.2.0 software (Applied Biosystems) and Excel (Microsoft 2007). All qRT-PCR data are described as mean ± standard error of the mean relative expression values. Significant differences were assessed using a two-tailed student's t-test, assessed using SPSS v.16 (SPSS Inc., Chicago, IL, USA).

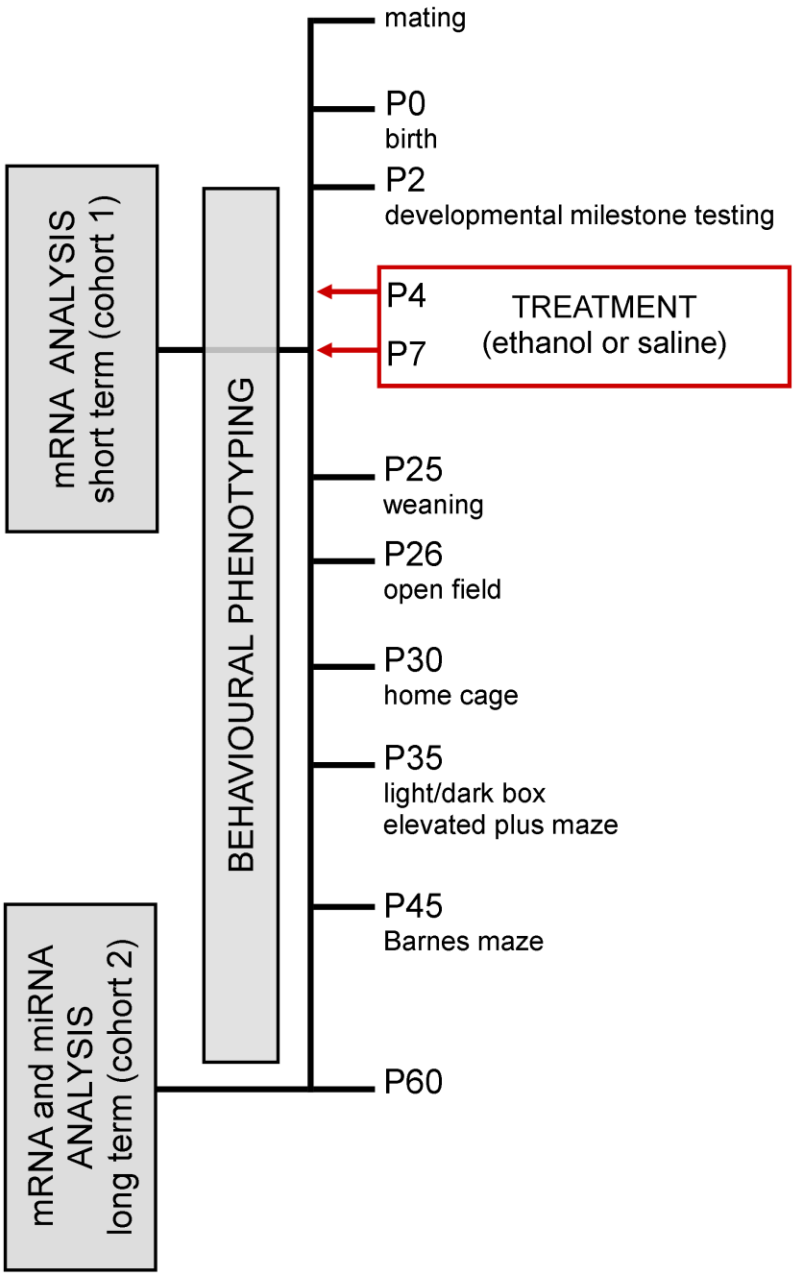


FIGURE 2.5. Experimental design overview.
Important treatment or testing days are outlined and indicated as the postnatal day of age of the pup (P).

Chapter 3

RESULTS

3.1 Effect of ethanol exposure during synaptogenesis on FASD-relevant behaviours

The effect of ethanol exposure during synaptogenesis on behavioural phenotypes were measured from time of treatment extending into early adulthood (P60). Early postnatal characteristics such as weight and the achievement of tasks designed to test the acquisition of neuromuscular coordination were performed given that these features are affected in infants with FASD. As the pups matured, they were then tested for other FASD-relevant behavioural characteristics such as hyperactivity, anxiety-related traits, and learning and memory impairments. The results from these analyses are outlined in the following sections.

3.1.1 Postnatal weight

Mice treated with ethanol at P4, P7, or P4+7 were assessed for differences in weight during postnatal growth and into early adulthood. Weight graphs of each treatment group stratified by sex are shown in Figure 3.1. Analysis using repeated-measures

univariate ANOVA (RM-ANOVA) revealed a significant interaction between postnatal day and sex, as expected, for both the single-treatment groups (P4, $F_{11, 30} = 23.23$, $p < 0.001$; P7, $F_{11, 27} = 20.97$, $p < 0.001$), but no interaction between treatment, sex, or day of testing was detected. A significant interaction between postnatal day and sex ($F_{11, 29} = 35.30$, $p < 0.001$) and between postnatal day and treatment ($F_{11, 29} = 3.27$, $p = 0.005$) was detected for the P4+7 treatment group, with both males and females showing a trend to be underweight as compared to littermate controls of the same sex (Figure 3.1). This difference was most prominent in juveniles and adolescents (P10 to P35) and was not maintained into early adulthood (P56 to P70).

3.1.2 Delayed achievement of early postnatal developmental milestones

Mice treated with ethanol at P4, P7, and P4+7 were assessed during early postnatal development for reflexes, strength, coordination, and the appearance of early neurobehavioural developmental milestones. Multivariate ANOVA (MANOVA) analysis of only the control (saline-treated) mice from all treatment groups showed no overall significant effect of sex ($F_{10, 49} = 0.679$, $p = 0.738$) or day of treatment ($F_{20, 98} = 0.269$, $p = 0.999$), as well as no interaction between the two ($F_{20, 98} = 0.865$, $p = 0.993$) suggesting consistency between the control groups. A number of differences were identified by analysis of developmental milestone achievement between control and ethanol-treated groups (Table 3.1). Generally, ethanol exposure resulted in the delay of achievement of many of the developmental milestones tested. MANOVA analysis using sex and treatment as fixed factors of the P4 treatment group showed an overall significant effect of treatment ($F_{10, 32} = 6.71$, $p < 0.001$). Pups treated with ethanol at P4 showed delayed ability to right themselves when placed on their back (surface righting) ($F_{1, 41} = 6.38$, $p = 0.022$), delayed ability to grasp a metal rod using their forelimbs and support their weight for greater than one second (forelimb grasp) ($F_{1,41} = 7.66$, $p = 0.011$), delayed ear twitch reflex ($F_{1, 41} = 2.77$, $p = 0.015$), delayed extinguishing of pivoting behaviour (open field traversal) in 30 s ($F_{1, 41} = 15.09$, $p = 0.017$) and in 15 s ($F_{1, 41} = 45.45$, $p < 0.001$), delayed eye opening ($F_{1, 41} = 2.77$, $p =$

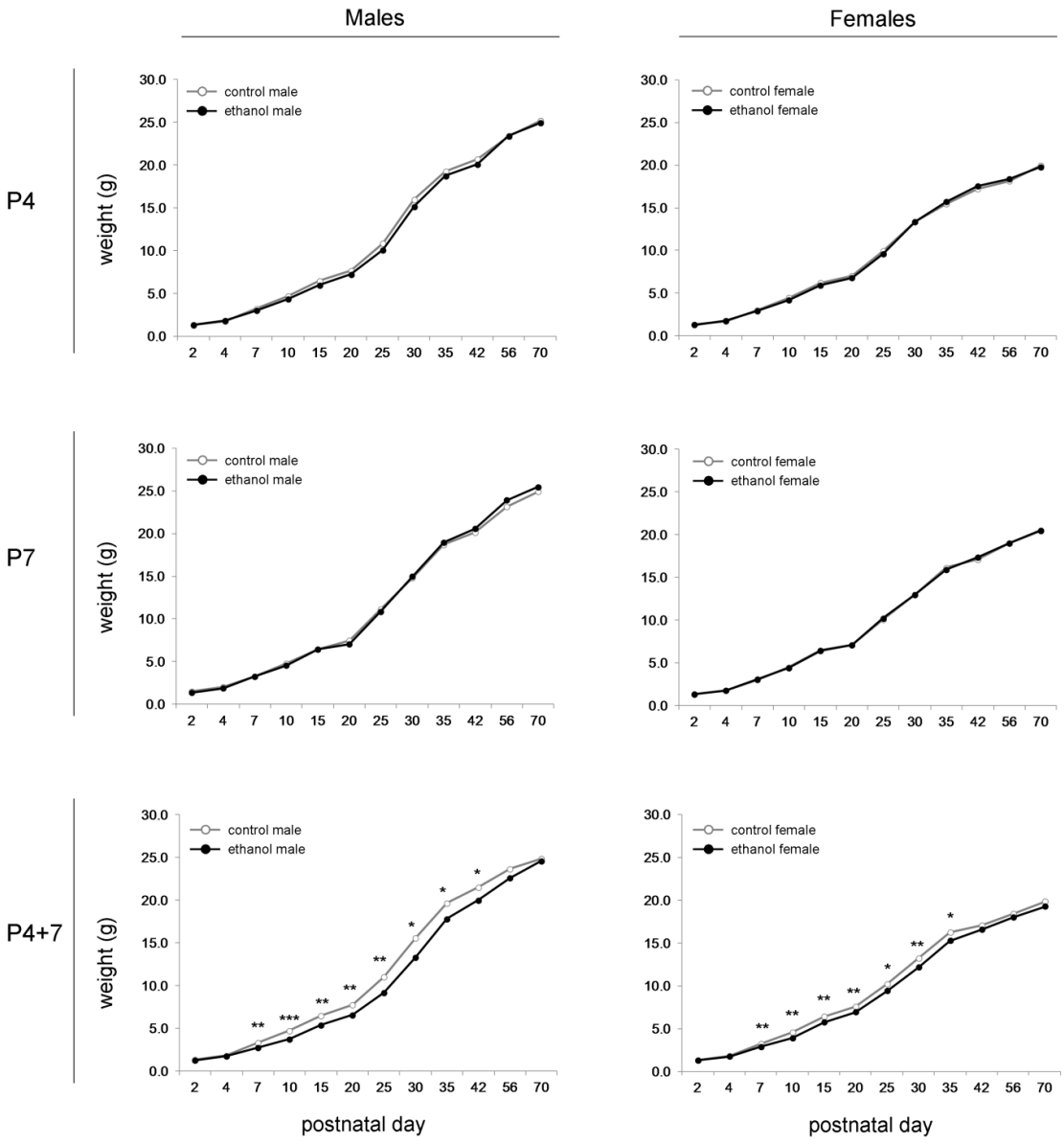


FIGURE 3.1. Weight graphs for mice treated with ethanol during the trimester three-equivalent and matched littermate controls. Mice were treated with ethanol in saline or saline alone (control) at postnatal day 4 (P4), 7 (P7) or 4 and 7 (P4+7). Data is presented as the mean of each group at select postnatal days ((n = 8 – 12 mice per group) and is stratified by sex. Significant effect of treatment: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

0.034), and delayed ability to right themselves in the air (air righting) ($F_{1,41} = 39.98$, $p < 0.001$). Similar analysis of the P7 treatment group as compared to their respective saline-treated controls showed an overall significant effect of treatment ($F_{19,28} = 5.48$, $p < 0.001$), but no significant effect of sex or interaction between sex and treatment. Mice treated with ethanol at P7 showed delays in their ability to extinguish pivoting behaviour and traverse and open field in 30 s ($F_{1,37} = 16.12$, $p = 0.005$) and in 15 s ($F_{1,37} = 51.60$, $p < 0.001$), as well as a delayed ability to right themselves in the air when dropped (air righting) ($F_{1,37} = 68.69$, $p < 0.001$). Finally, a MANOVA revealed a significant overall effect of treatment in mice treated with ethanol on P4+7 ($F_{10,30} = 12.33$, $p < 0.001$), with ethanol-treated mice showing a significant delay in milestone achievement. No significant effect of sex or interaction between sex and treatment were detected. Specifically, a significant effect of treatment was observed for the ability for an ethanol-treated pup to right itself when placed on its back (surface righting) ($F_{1,39} = 10.07$, $p = 0.007$), appearance of an acoustic startle reflex ($F_{1,39} = 7.16$, $p = 0.004$), appearance of an ear twitch reflex ($F_{1,39} = 4.03$, $p = 0.016$), ability to grasp an object and support its own weight for more than 1 s (forelimb grasp) ($F_{1,39} = 9.22$, $p = 0.004$), achieve open field traversal in less than 30 s ($F_{1,39} = 22.01$, $p < 0.001$) or 15 s ($F_{1,39} = 66.47$, $p < 0.001$), achieve eye opening ($F_{1,39} = 5.40$, $p = 0.001$), and achieve the ability to right in the air when dropped from a small distance (air righting) ($F_{1,39} = 109.56$, $p < 0.001$).

In general, pups exposed to alcohol experienced a delay in the ability to achieve developmental milestones. The P4 ethanol-treated group experienced delays in behaviours that perhaps required more motor control, such as surface righting, forelimb grasp, extinguishment of pivoting behaviour (open field traversal), and air righting. The P7-treated group showed some delays, but they were limited to behaviours that required (arguably) substantially more neuromuscular coordination and perhaps reflected delays in later-appearing milestones. Mice treated at P4+7 showed significant delays in the greatest number of tested behaviours (8/10), though the behaviours affected were consistent with the other single-treatment groups.

TABLE 3.1. Postnatal day of achievement of developmental milestone behaviours in control and ethanol-treated mice.

	Control		P4 ethanol		P7 ethanol		P4+7 ethanol	
	Male (n=31)	**Female (n=33)**	**Male (n=12)**	**Female (n=11)**	**Male (n=11)**	**Female (n=10)**	**Male (n=9)**	**Female (n=12)**
Surface righting	5.45 ± 0.13	5.55 ± 0.19	6.42 ± 0.29*	6.36 ± 0.39*	5.50 ± 0.27	5.50 ± 0.22	6.33 ± 0.44*	6.33 ± 0.36*
Negative geotaxis	5.48 ± 0.16	5.64 ± 0.16	5.92 ± 0.23	6.09 ± 0.28	5.50 ± 0.27	5.60 ± 0.34	6.00 ± 0.24	5.92 ± 0.23
Cliff aversion	5.35 ± 0.15	5.39 ± 0.14	5.42 ± 0.15	5.64 ± 0.24	5.64 ± 0.24	5.40 ± 0.40	5.44 ± 0.24	5.75 ± 0.22
Forelimb grasp	10.84 ± 0.15	11.00 ± 0.18	11.83 ± 0.27*	11.73 ± 0.27*	10.82 ± 0.57	10.90 ± 0.43	12.00 ± 0.33*	11.75 ± 0.25*
Ear twitch	9.58 ± 0.12	9.73 ± 0.13	10.08 ± 0.15*	10.18 ± 0.23*	9.91 ± 0.44	10.10 ± 0.23	10.33 ± 0.24*	10.17 ± 0.30*
Auditory startle	10.32 ± 0.16	10.64 ± 0.15	10.50 ± 0.15	10.55 ± 0.25	11.09 ± 0.28	11.30 ± 0.40	11.11 ± 0.31*	11.17 ± 0.21*
Air righting	10.61 ± 0.17	10.64 ± 0.16	12.50 ± 0.45***	12.64 ± 0.47***	13.18 ± 0.48***	13.20 ± 0.57***	14.00 ± 0.55***	13.58 ± 0.48***
Open field traversal (30s)	11.55 ± 0.21	11.58 ± 0.21	12.50 ± 0.51*	12.64 ± 0.49*	12.91 ± 0.34*	12.70 ± 0.42*	13.00 ± 0.29***	13.33 ± 0.36***
Open field traversal (15s)	13.00 ± 0.23	12.97 ± 0.24	14.75 ± 0.52***	15.09 ± 0.28***	15.09 ± 0.51***	14.90 ± 0.57***	15.78 ± 0.46***	15.75 ± 0.33***
Eye opening	14.55 ± 0.09	14.85 ± 0.12	15.08 ± 0.23*	15.27 ± 0.27*	14.64 ± 0.20	14.70 ± 0.21	15.44 ± 0.18*	15.50 ± 0.23*

Significant effect of treatment: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

3.1.3 Ethanol exposure alters juvenile locomotor behaviour

3.1.3.1 *Changes to activity in a novel open-field arena are dependent on treatment day*

The activity of mice treated with ethanol at P4, P7, or P4+7 in an open-field arena was assessed as a measure of novelty-induced exploration (Figure 3.2). ANOVA analysis including only the control groups from all treatment paradigms detected a significant effect of sex, with control males generally exhibiting less infrared beam-breaks than control females across treatment groups ($F_{1, 54}$ = 6.88, p = 0.01). No effect of treatment day ($F_{2, 54}$ = 1.62, p = 0.21) nor an interaction between sex and treatment day ($F_{2, 54}$ = 1.07, p = 0.35) was detected, suggesting consistency between the control groups.

No significant difference in locomotor activity levels (all $p \geq 0.124$) or rearing frequency (all $p \geq 0.128$) was detected between control and ethanol-treated mice within the P4 treatment group. Similarly, within the group treated on P7, no significant effect of ethanol treatment on activity was observed. A significant effect of treatment ($F_{1,37}$ = 14.77, $p < 0.001$) on rearing frequency, however, was detected, with both male and female ethanol-treated mice displaying more vertical rears (total mean = 156.67 ± 7.79) than saline-treated controls (total mean = 117.40 ± 6.56). Within the P4+7 treatment group, an interesting interaction between treatment and sex was observed ($F_{1,38}$ = 5.05, p = 0.031) with female ethanol-treated mice displaying less activity (mean = 2650 ± 106) than control females (mean = 3180 ± 135). A main effect of ethanol treatment on rearing behaviour was also observed ($F_{1,38}$ = 4.13, p = 0.049), driven by the increased rearing activity of ethanol-treated females (mean = 170.46 ± 8.74) as compared to controls (mean = 126.42 ± 10.64).

3.1.3.2 *Ethanol exposure induces nocturnal home cage hyperactivity*

To further evaluate effects of trimester three-equivalent ethanol exposure on locomotor activity levels as well as an indicator of possible alterations to Circadian

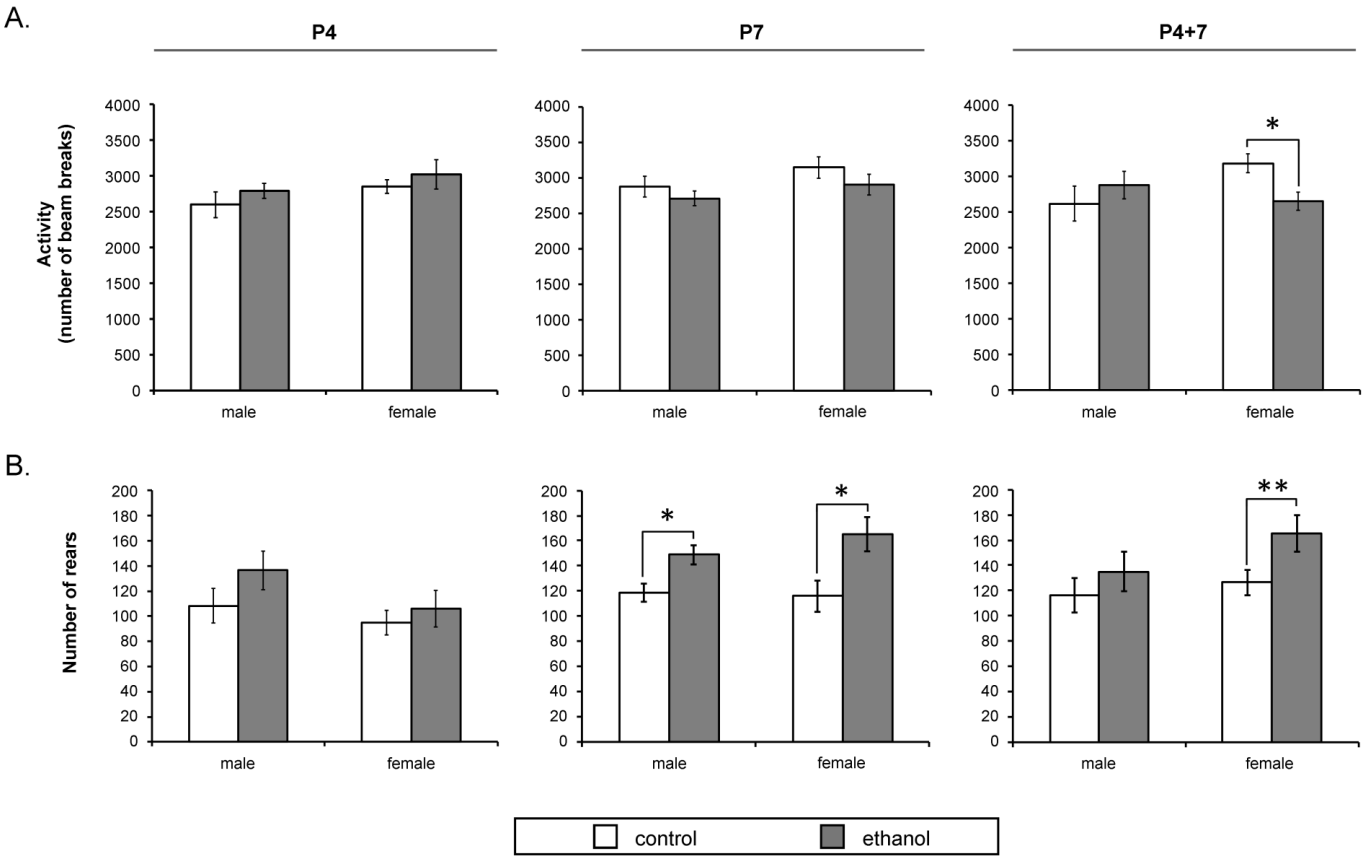


FIGURE 3.2. Open field activity of adolescent mice treated with ethanol during the trimester three-equivalent. Treatment day (P4, P7, or P4+7) is indicated above the appropriate bar graphs showing average infrared beam breaks (A) and rearing frequency (B) of control and ethanol-treated male and female mice within a 15 min period. Data are presented as mean ± SEM (n = 8 – 12 mice per group). Significance is indicated: *, $p < 0.05$; **$p < 0.01$.

rhythm, adolescent mice were assessed for nocturnal activity in a familiar, home cage environment. Due to the number of different measurements recorded by the Actimeter system, a correlation matrix principal components analysis (PCA) was conducted using the control data from each of the three treatment groups (P4, P7, and P4+7) to see if the measurements were correlated with one another and a single measure could accurately reflect the overall activity data trends. Measures included in PCA analysis were overall activity (total number of infrared beam breaks), locomotion (horizontal non-repetitive beam breaks), mean velocity, distance traveled, and vertical rearing. Bartlett's test of sphericity indicated that the dataset was significantly collinear and that factor analysis could be performed ($\chi^2 = 474.22$, df = 10, $p < 0.001$). Also, Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy showed that the degree of common correlation between the variables was strong (KMO = 0.622), indicating that the factors extracted by data reduction would account for a substantial amount of variance in the data. The eigenvalues associated with each extracted component determined that 90.10% of the variance in the data could be explained by two components (Table 3.2, Figure 3.3) and that all measures were positively correlated (Table 3.3). The component matrix indicated that two main extracted components were driven primarily by a significant correlation between total activity, locomotion, mean velocity, and distance travelled (component 1) and number of rears (component 2) (Table 3.3). Given that overall activity (total number of beam breaks) was significantly correlated with each of the other four measures (Table 3.3), hourly activity was used as the measure of home cage activity in all subsequent analyses.

Univariate ANOVA analysis of total nocturnal activity across the three control groups from each treatment paradigm indicated no overall significant effect of sex ($F_{1,50} = 0.184$, $p = 0.670$), day of saline treatment ($F_{2,50} = 1.835$, $p = 0.170$), or an interaction between the two ($F_{2,50} = 0.199$, $p = 0.821$), suggesting statistical consistency across control groups. Given that nocturnal activity of mice varies considerably from hour to hour (TANG et al. 2002), activity was measured in 12 one-hour bins from 19:00h to 07:00h rather than one 12-hour period.

TABLE 3.2. Total variance explained by each extracted correlation matrix PCA component for measures of home cage locomotor activity.

	Initial Eigenvalues		
Component	**Total**	**% of Variance**	**Cumulative %**
1	3.404	68.083	68.083
2	1.101	22.022	90.105
3	0.475	9.505	99.610
4	0.015	0.300	99.910
5	0.004	0.089	100.000

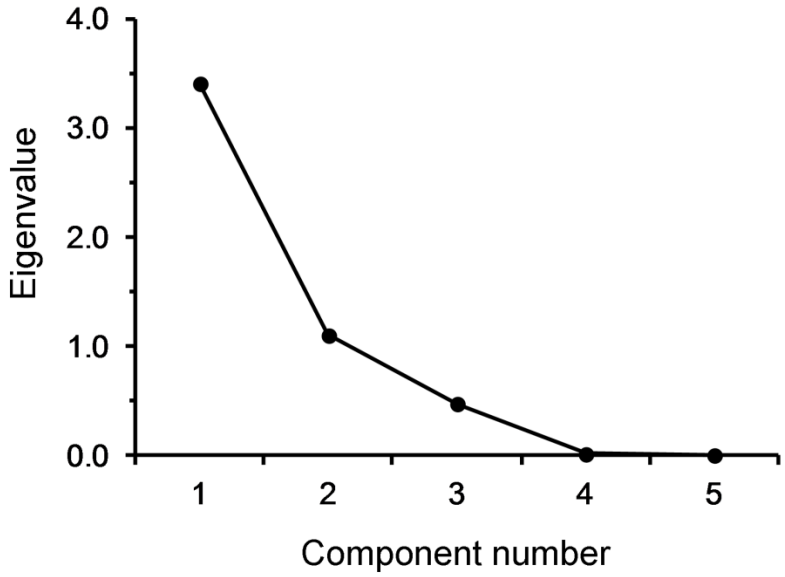


FIGURE 3.3. Scree plot showing the total amount of variance (eigenvalue) in the home cage activity dataset measures that can be accounted for by each component extracted by a correlation matrix PCA. Five indicators of locomotor activity were used in the analysis (N=56).

TABLE 3.3. Correlation matrix showing the relationship between five indicators of home cage locomotor activity (N=56).

		total activity	**locomotor activity**	**mean velocity**	**distance travelled**	**number of rears**
total activity	*Pearson correlation*	1.000	0.990	0.666	0.638	0.491
	Significance (p)	-	< 0.001	< 0.001	< 0.001	< 0.001
locomotor activity	*Pearson correlation*	0.990	1.000	0.588	0.563	0.500
	Significance (p)	< 0.001	-	< 0.001	< 0.001	< 0.001
mean velocity	*Pearson correlation*	0.666	0.588	1.000	0.985	0.166
	Significance (p)	< 0.001	< 0.001	-	< 0.001	0.110
distance travelled	*Pearson correlation*	0.638	0.563	0.985	1.000	0.176
	Significance (p)	< 0.001	< 0.001	< 0.001	-	0.097
number of rears	*Pearson correlation*	0.491	0.500	0.166	0.176	1.000
	Significance (p)	< 0.001	< 0.001	0.110	0.097	-

Repeated-measures ANOVA (RM-ANOVA) of the P4 ethanol treatment group and controls using "hour" as the within-subjects factor and sex and treatment as between-subjects factors indicated a significant effect of "hour" on activity, as expected ($F_{11,24}$ = 33.06, $p < 0.001$) (Figure 3.4). However, ethanol treatment at P4 did not significantly affect activity levels in the home cage envinronment, and no significant interaction between hour and sex, hour and treatment, or hour and sex and treatment was detected.

Analysis of home cage nocturnal activity in male and female mice treated with ethanol at P7 indicated that ethanol treatment at P7 significantly increased nocturnal home cage activity. The data show a trend for female mice show a more pronounced increase in activity, and this difference was evident primarily from 21:00h to 01:00h. There also seemed to be a trend for increased activity towards the end dark phase and beginning of the light phase of the light/dark cycle. Where control animals showed a marked reduction in activity from 06:00h to 07:00h (mean = 1596.7 ± 159.76 beam breaks per hour), the ethanol-treated mice remained significantly more active (mean = 5833.9 ± 608.14 beam breaks per hour). RM-ANOVA indicated a significant effect of hour on activity ($F_{11,21}$ = 44.29, $p < 0.001$) and a significant interaction between hour and treatment ($F_{11, 21}$ = 18.11, $p < 0.001$) (Figure 3.5). No significant interaction between hour and sex ($F_{11,21}$ = 1.81, $p = 0.12$) was observed. Interestingly, however, a significant interaction between hour and sex and treatment ($F_{11,21}$ = 4.10, $p = 0.003$) on activity levels was observed.

Lastly, RM-ANOVA was performed on the activity levels of the group treated at P4+7 (Figure 3.6). Again, a significant effect of hour on activity was observed ($F_{11,29}$ = 63.79, $p < 0.001$), as well as an interaction between hour and treatment ($F_{11,29}$ = 7.38, $p < 0.001$), with ethanol-treated animals showing an increase in activity as compared to controls. No interaction between hour and sex or hour, sex, and treatment was observed. The pattern of increased activity was similar to the P7 treatment paradigm, with much of the increase in activity towards the latter hours of the dark phase,

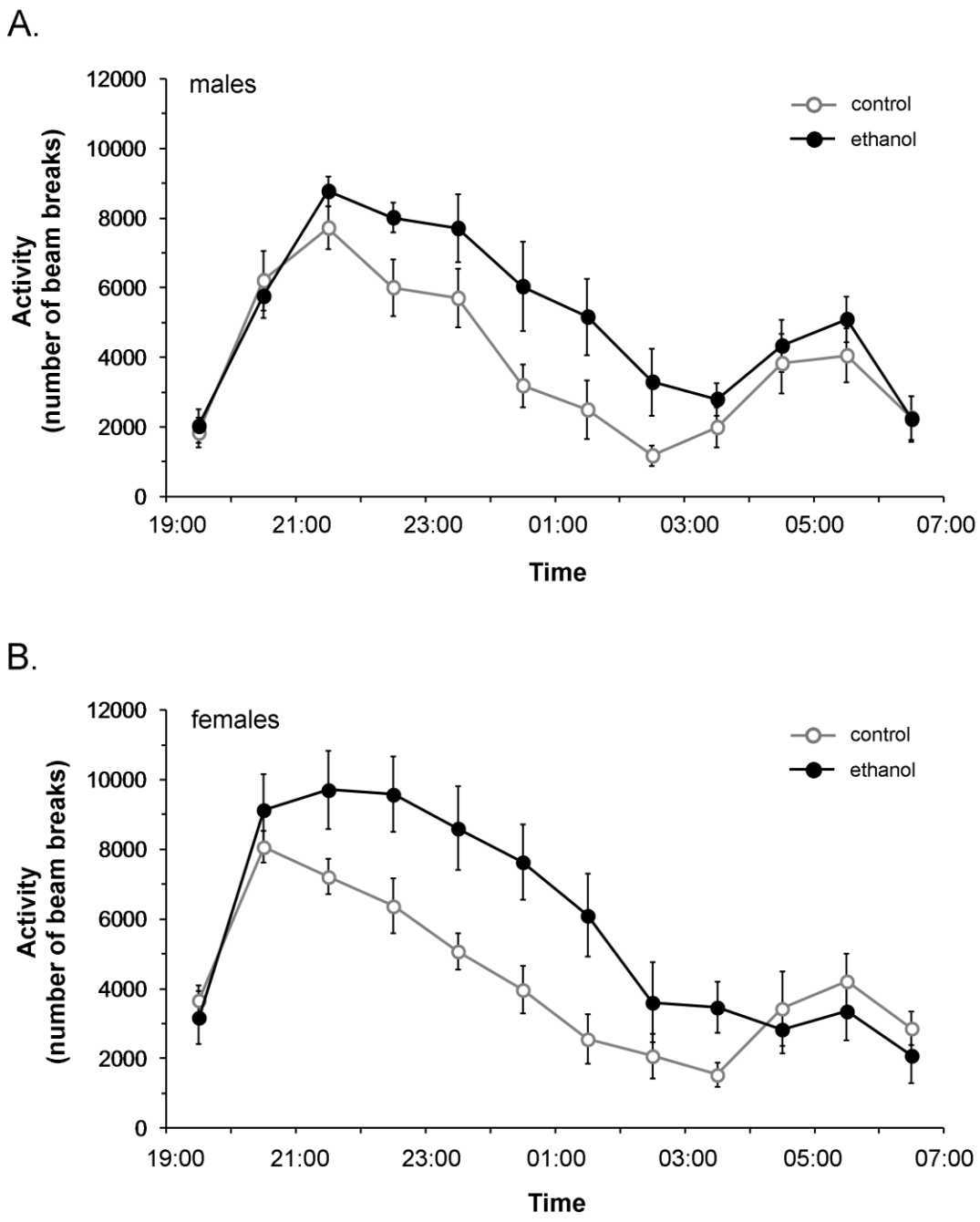


FIGURE 3.4. Locomotor activity of mice treated with ethanol at P4 in a familiar home cage environment. Data shown represent the mean (± SEM) infrared beam breaks by ethanol-exposed and control males (A) and females (B) over a 12-hour period during the dark phase (20:00h to 06:00h) and the last and first hour of the light phase of the light/dark cycle (n= 8-11 mice per group). No significant differences between control or ethanol-treated male or females were detected.

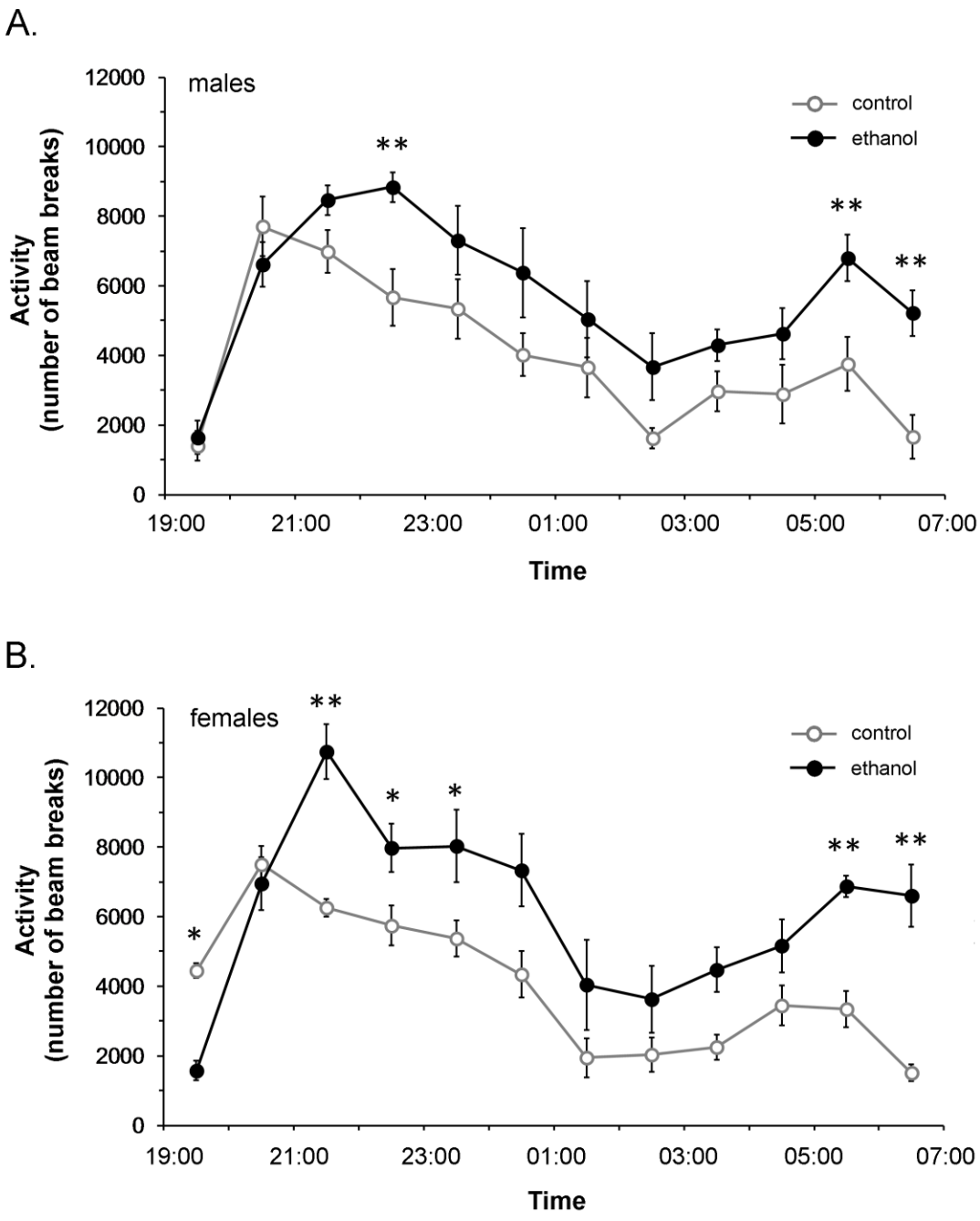


FIGURE 3.5. Locomotor activity of mice treated with ethanol on P7 in a familiar home cage environment. Data shown represent the mean (± SEM) infrared beam breaks by ethanol-exposed and control males (A) and females (B) over a 12-hour period during the dark phase (20:00h to 06:00h) and the last and first hour of the light phase of the light/dark cycle (n= 7-10 mice per group). *, $p < 0.05$; **, $p < 0.01$.

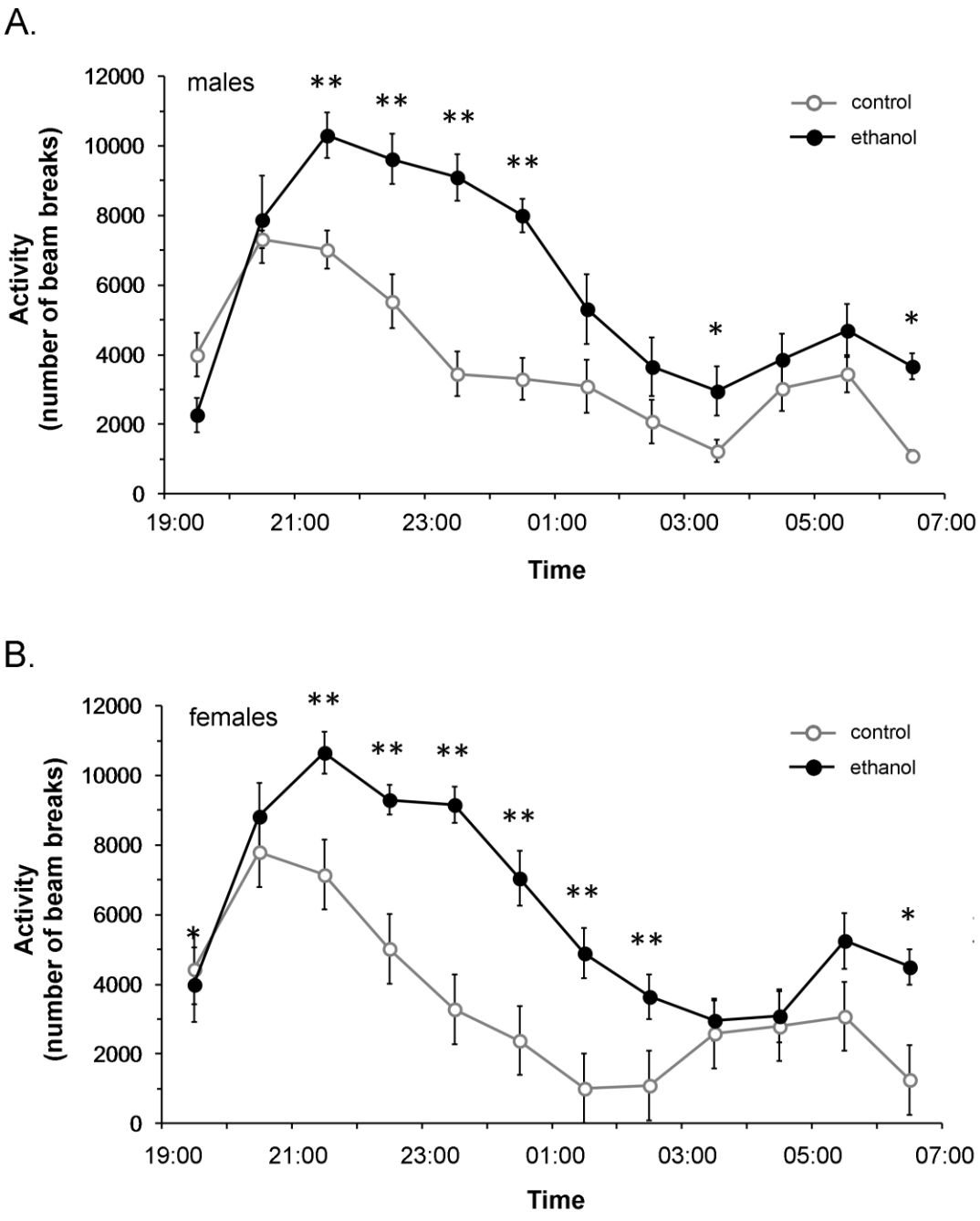


FIGURE 3.6. Locomotor activity of mice treated with ethanol on P4 and P7 in a familiar home cage environment. Data shown represent the mean (± SEM) infrared beam breaks by ethanol-exposed and control males (A) and females (B) over a 12-hour period during the dark phase (20:00h to 06:00h) and the last and first hour of the light phase of the light/dark cycle (n= 9-12 mice per group). *, $p < 0.05$; **, $p < 0.01$.

occurring between 21:00h and 02:00h. Also, similar to the P7 treatment group, there was an increase in activity towards the latter hours of the dark phase and, particularly, during the first hour of the light phase of the light/dark cycle (06:00h to 07:00h control mean = 1183.82 ± 178.23, ethanol mean = 4080.25 ± 340.88 beam breaks).

3.1.4 Ethanol exposure increases anxiety-related traits in adolescent mice

3.1.4.1 *Increased open field thigmotaxis is dependent on treatment day*

Decreased tendency to explore the inner area of a novel open field arena was used as an indicator of increased thigmotaxis as an anxiety-related behaviour. Univariate ANOVA analysis of only the control groups for time spent in the centre zone, latency to enter the center zone, and number of entries into the centre zone using "sex" and "day of treatment" as between-subjects factors indicated no significant effect of treatment day (P4, P7, or P4+7) between control groups. A significant effect of sex was detected for amount of time spent in the centre zone ($F_{1,54} = 4.39$, $p = 0.04$) and latency to enter the centre zone ($F_{1,54} = 5{,}75$, $p = 0.02$), with female mice (mean = 58.20 ± 3.70 seconds) spending less time in the centre than males (mean = 71.39 ± 4.82 seconds) as well as an increased latency to enter the centre zone (female mean = 102.71 ± 10.74 seconds, male mean = 72.79 ± 7.02 seconds). Female mice also showed a fewer number of entries into the centre zone (mean = 31.62 ± 2.44) as compared to male mice (mean = 37.42 ± 2.38) but this trend did not reach significance ($F_{1,54} = 2.64$, $p = 0.11$).

Analysis of thigmotaxis measures in adolescent control and ethanol-exposed mice treated at P4 showed no significant effect of treatment or interaction between sex and treatment were observed for any measures. Mice treated with ethanol at P7 showed a trend for females towards increased thigmotaxic behaviours, but also detected some significant effects of ethanol treatment (Figure 3.8). ANOVA analysis of time spend in the centre showed a significant effect of sex ($F_{1,37} = 7.31$, $p = 0.010$) and a

significant effect of treatment ($F_{1,37}$ = 6..25, p = 0.017), with ethanol-treated males and females showing a decreased amount of time in the centre zone. Effects of treatment on latency to enter the centre zone ($F_{1,28}$ = 5.70, p = 0.022) and number of entries into the centre zone ($F_{1,38}$ = 4.42, p = 0.042) were both detected, with ethanol-treated mice showing an increased latency to enter the centre and a reduction in the number of times each explored the centre zone as compared to their control littermates (Figure 3.8). No significant interaction between sex and treatment for any measures were detected. Analysis of the P4+7 treatment group showed the most pronounced effect of ethanol treatment on anxiety-related measures (Figure 3.9). Ethanol-treated mice spend less time within the centre zone ($F_{1,38}$ = 5.86, p = 0.020), and increased latency to enter the centre zone ($F_{1,38}$ = 5.70, p = 0.022), and a decreased number of entries into the centre ($F_{1,38}$ = 4.42, p = 0.42). No significant interaction between sex and treatment was detected for any measures within the P4+7 treatment group.

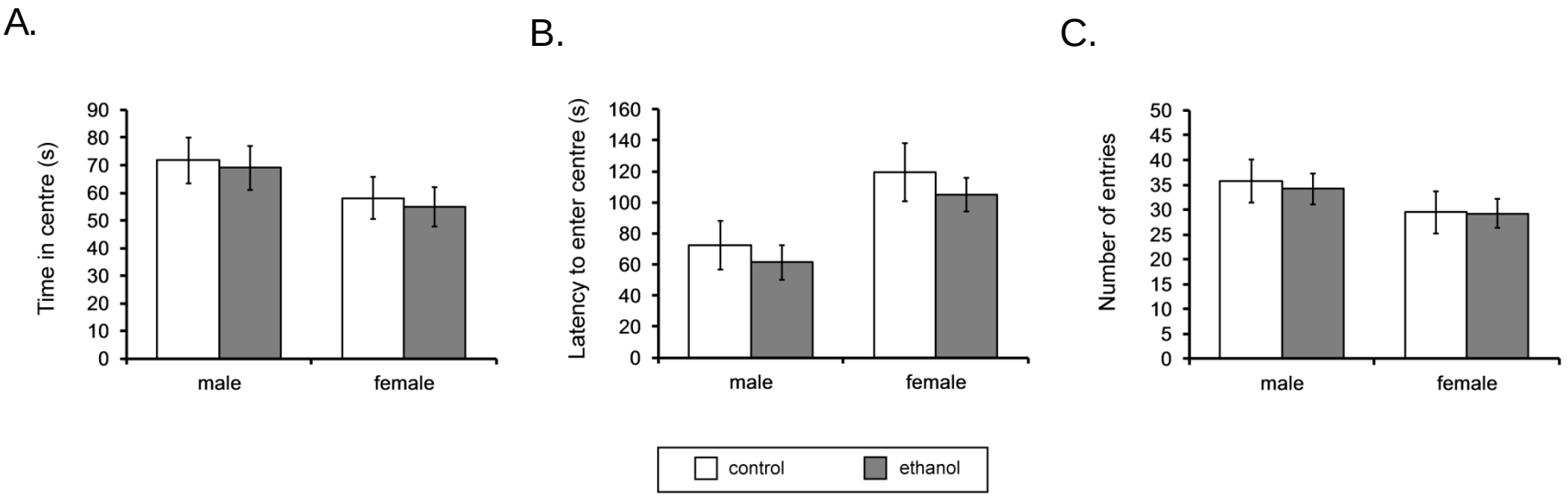


FIGURE 3.7. Anxiety-related behaviours in a novel open field arena in mice treated at P4 with ethanol. Data shown represent the mean (± SEM) (A) time spent in the centre zone of the open field arena, (B) latency to enter the centre, and (C) Number of entries into the centre zone, stratified by sex (n = 8-11 mice per group). No effect of treatment was detected for any measure.

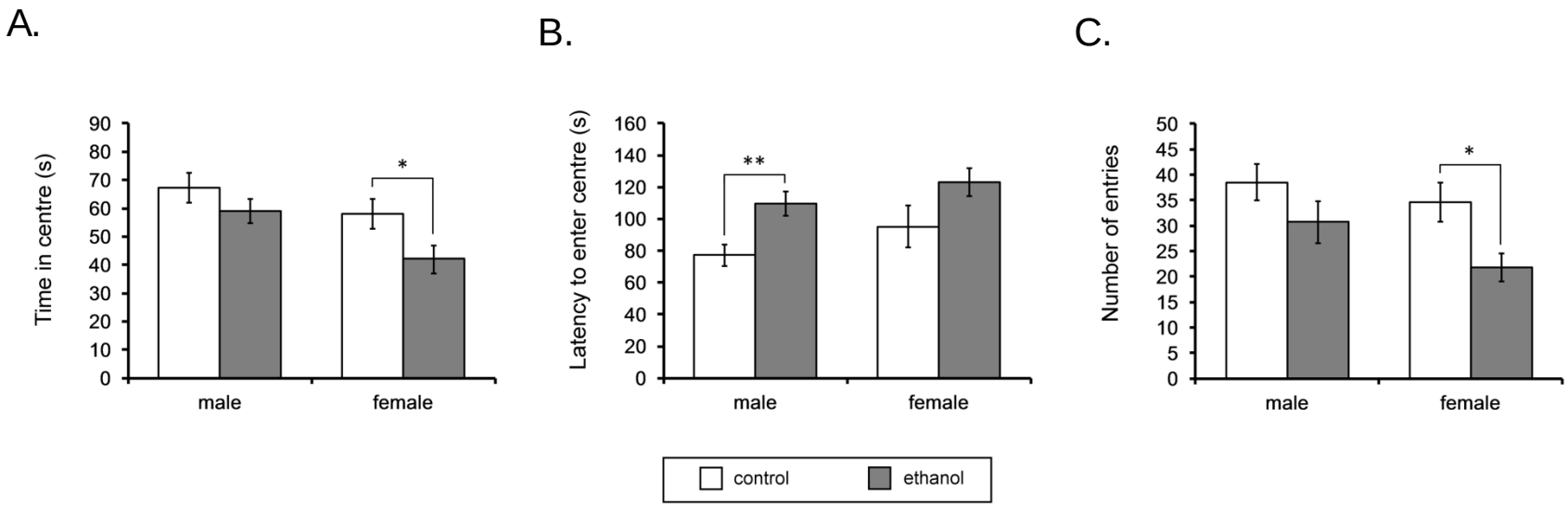


FIGURE 3.8. Anxiety-related behaviours in a novel open field arena in mice treated at P7 with ethanol. Data shown represent the mean (± SEM) (A) time spent in the centre zone of the open field arena, (B) latency to enter the centre, and (C) Number of entries into the centre zone, stratified by sex (n = 7-10 mice per group). *, $p < 0.05$; **, $p < 0.01$.

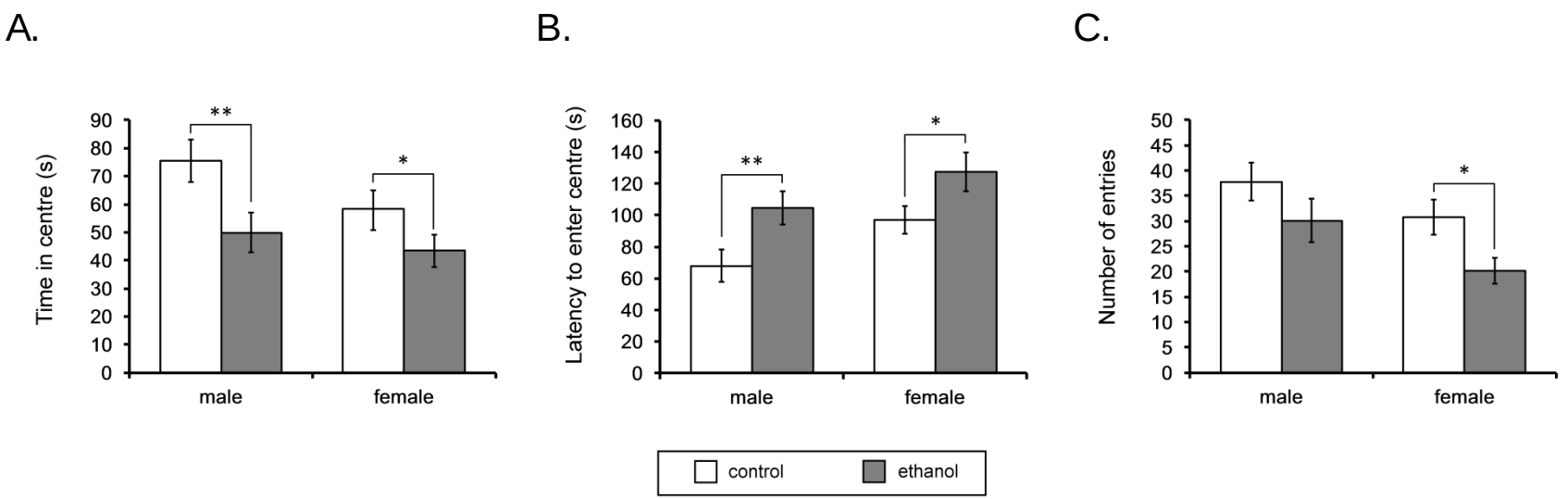


FIGURE 3.9. Anxiety-related behaviours in a novel open field arena in mice treated at P4 and P7 with ethanol. Data shown represent the mean (± SEM) (A) time spent in the centre zone of the open field arena, (B) latency to enter the centre, and (C) Number of entries into the centre zone within a 15 min testing period, stratified by sex (n = 9-12 mice per group). *, $p < 0.05$; **, $p < 0.01$.

3.1.4.2 *Ethanol exposure results in sex-specific anxiety-related behaviour in the light/dark box and elevated plus maze*

Given the subtlety of the increased anxiety-related traits exhibited by ethanol-treated mice in the open field test and the availability of other assays, the P4+7 treatment group was also tested in two other assays designed to assess anxiety-related phenotypes: the light/dark box and the elevated plus maze. For the light/dark box test, univariate ANOVAs of the P4+7 group using "sex" and "treatment" as between-subjects factor were performed on the time spent in the light section of the apparatus and number of entries into the light section as a measure of movement between the light and dark regions. Results indicated a significant interaction between sex and treatment ($F_{1,39}$ = 5.13, p = 0.029), with both ethanol treated males and females showing a reduced amount of time spent in the light as compared to controls (Figure 3.10A). This interaction was driven by the significant decrease observed in female ethanol-treated mice, which, while this also appeared as a trend in ethanol-treated males, did not reach significance. Analysis of the number of entries into the light region indicated significant main effects of both sex ($F_{1,39}$ = 5.50, p = 0.024) and treatment ($F_{1,39}$ = 12.86, p = 0.001). Female mice tended to be less explorative of the light/dark box and move less between light and dark regions than males, and ethanol treatment appeared to reduce movement between the light and dark regions further in both sexes (Figure 3.10B).

The results from the light/dark box were consistent with those from the elevated plus maze, indicating an increase in anxiety-related phenotypes resulting from ethanol exposure that was more pronounced in females. Significant main effects of sex ($F_{1,39}$ = 11.93, p = 0.001) and treatment ($F_{1,39}$ = 22.88, p < 0.001) were detected by ANOVA analysis of time spent in the open arms, with ethanol-treated mice (mean = 50.20 ± 5.43 s) spending less time in the open arms than controls (mean = 78.73 ± 3.49 s). Similar main effects of sex ($F_{1,39}$ = 6.44, p = 0.015) and treatment ($F_{1,39}$ = 13.17, p = 0.001) were found for the number of entries into the open arms, again with ethanol-treated mice showing a reduction in the number of transitions (mean = 5.90 ± 0.61) as compared to controls (mean = 8.73 ± 0.50).

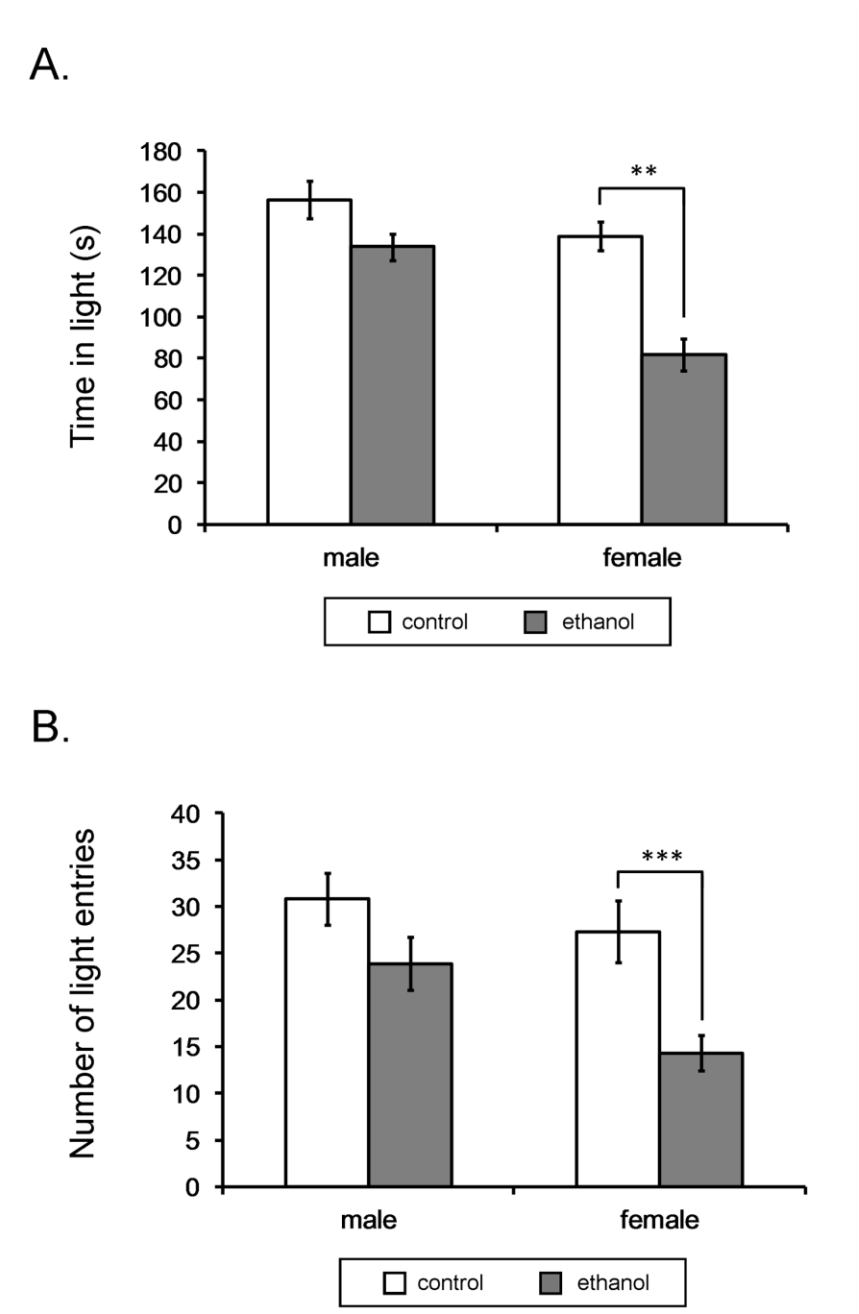


FIGURE 3.10. Light/dark box assay of anxiety-related phenotypes. Mice treated with ethanol or saline (control) at P4+7 were tested over a 5 min period for (A) time spent in the light region and (B) number of entries into the light region. Data presented represent mean ± SEM (n = 9-12 mice per group). **, $p < 0.01$; ***, $p < 0.001$.

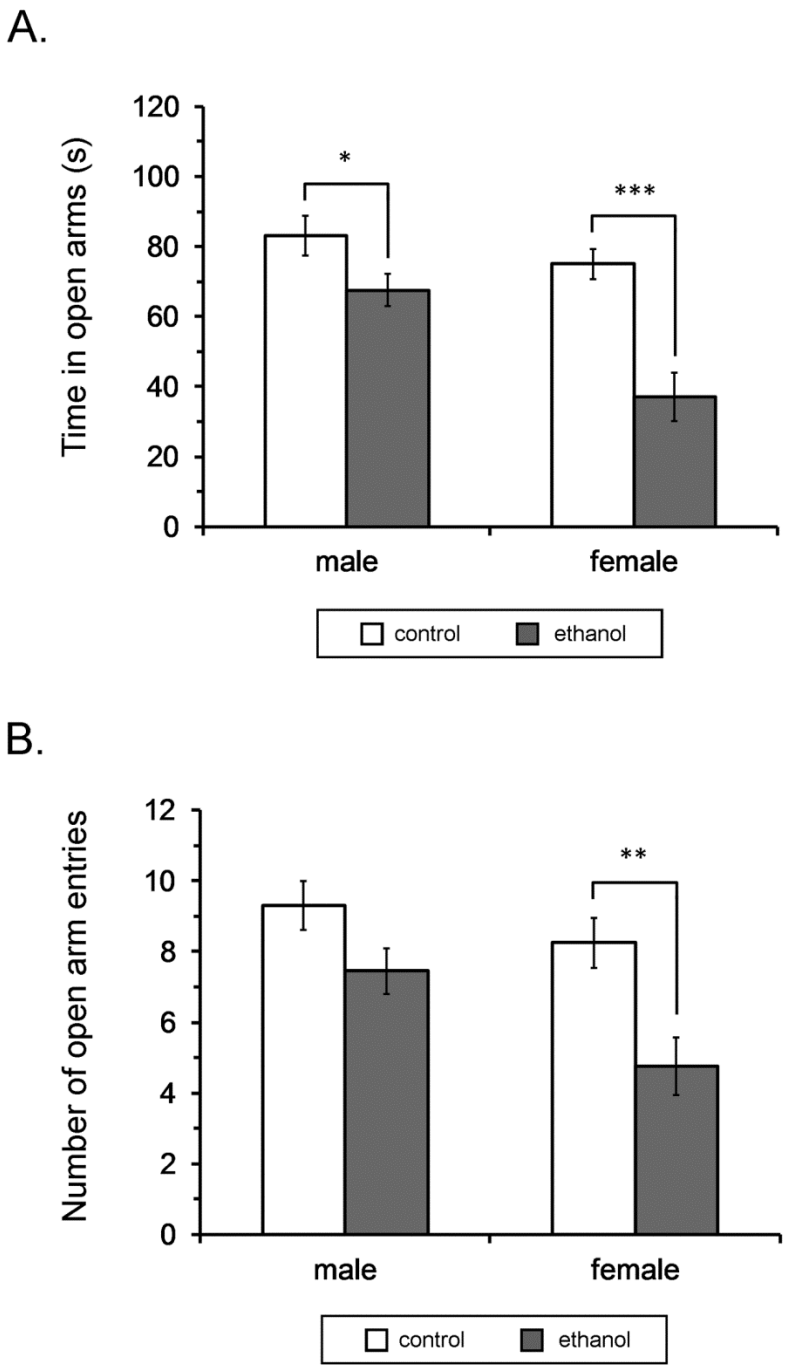


FIGURE 3.11. Elevated plus maze assay of anxiety-related phenotypes. Mice treated with ethanol or saline (control) at P4+7 were tested over a 5 min period for (A) time spent in open arms and (B) number of entries into open arms. Data presented represent mean ± SEM (n = 9-12 mice per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.1.5 Ethanol exposure results in deficits in spatial learning and memory

Mice treated at P4, P7 or P4+7 with ethanol or saline were assessed for learning deficits in a mouse-oriented spatial learning task, the Barnes maze. The primary measure of learning used was latency to reach the target (a measure of escape time), though search strategy used to find the escape target was also analyzed for the four acquisition (learning) days. Short and long-term recall memory were assessed using number of explorations to each hole when the location of the "escape" hole was blocked. It should be noted that due to the variance differences between latency measures depending on day of testing, latency measures were natural-log (ln)-transformed prior to analyses to improve the homoscedasticity of the data to meet the assumptions of general linear models. Following transformation, Box's M test of homogeneity of covariance matrices and Mauchly's sphericity test indicated that the within-group covariance matrices of the latency data was not significantly different (p values > 0.05) and that a RM-ANOVA could be applied appropriately.

3.1.5.1 *Spatial acquisition is impaired by ethanol exposure*

First, the control groups from each treatment paradigm were compared to ensure consistency between latency measures. Escape latencies for the control groups ranged from 77.39 ± 4.86 to 72.15 ± 6.68 seconds on the first learning day to 8.34 ± 0.73 to 7.85 ± 0.62 seconds (mean ± SEM) on the last training day (day 4). Repeated-measures ANOVA of ln-transformed latency to reach the target hole using "sex" and "treatment day" (or "acquisition day") as the between-subjects factors and "day of testing" as the within-subjects factor showed a significant effect of testing day, as expected ($F_{3,171} = 678.97$, $p < 0.001$). No significant interaction between testing day and sex ($F_{3,171} = 0.423$, $p = 0.737$), interaction between testing day and day of treatment ($F_{3,171} = 0.385$, $p = 0.888$), or interaction between testing day, sex, and treatment day ($F_{3,171} = 0.503$, $p = 0.806$) on latency to reach the target were detected for the control groups. This suggested consistency between the control groups of each treatment paradigm.

Latency to reach the escape hole in control and experimental mice treated at P4 were assessed using a repeated-measures ANOVA using "sex" and "treatment" as between-subjects factors and "day of testing" as the within-subjects factor. Mean escape latencies of control and ethanol-treated mice across the four acquisition trial days are shown in Figure 3.12A. Analysis indicated a significant effect of day of testing ($F_{3,117}$ = 389.37, $p < 0.001$), as expected, as well as a significant interaction between day of testing and treatment ($F_{3,117}$ = 6.84, $p < 0.001$). No interaction between sex and day of testing or sex, treatment, and day of testing was detected. Post-hoc univariate ANOVA analyses were conducted with the *a priori* intention of comparing the control and treatment group not stratified by sex (which was not indicated as significant by the RM-ANOVA) at each particular testing day rather than performing a full set of pairwise contrasts. These tests revealed that ethanol exposure at P4 resulted in a significant increase in Barnes maze escape latency in young adult mice on acquisition testing day 3 ($F_{1,41}$ = 15.17, $p < 0.001$) and testing day 4 ($F_{1,41}$ = 24.74, $p < 0.001$).

A similar approach was used to assess the escape latencies of mice treated with ethanol or saline at P7. Mean escape latencies of P7-treated control and ethanol-exposed mice across the four acquisition trial days are shown in Figure 3.12B. A significant effect of testing day was detected ($F_{3,114}$ = 313.24, $p < 0.001$), as well as a significant interaction between treatment and testing day ($F_{3,144}$ = 4.74, $p = 0.004$). No significant interactions between sex and day of testing and sex, treatment, and day of testing were indicated. Post-hoc univariate ANOVA analyses indicated that ethanol treatment on P7 delayed the the latency to reach the target on acquisition day 2 ($F_{1,41}$ = 8.10, $p = 0.007$), day 3 ($F_{1,41}$ = 20.37, $p < 0.001$) and day 4 ($F_{1,41}$ = 55.11, $p < 0.001$).

Analysis of the latency to escape of mice treated with ethanol at both P4+7 indicated a more pronounced delay to reach the target than the ethanol-treated mice of the single-day P4 or P7 treatment groups (Figure 3.12C). RM-ANOVA of the ln-transformed latencies to reach the target indicated a significant effect of acquisition testing day ($F_{3,117}$ = 270.29, $p < 0.001$) and a significant interaction between testing day and treatment ($F_{3,117}$ = 10.12, $p < 0.001$). No other interactions between factors were significant. Post-hoc univariate ANOVAs indicated that ethanol-treated mice

showed delays in reaching the target on acquisition day 2 ($F_{1,41} = 15.44$, $p < 0.001$), day 3 ($F_{1,41} = 47.51$, $p < 0.001$), and day 4 ($F_{1,41} = 41.20$, $p < 0.001$). While control animals tended to reach the target more quickly than ethanol-treated mice on the later trials of acquisition day 1, the average between the two groups across all four trials on the first day of testing did not significantly differ ($F_{1,41} = 3.60$, $p = 0.065$).

3.1.5.2 *Ethanol exposure alters learning strategy*

The effect of trimester three-equivalent ethanol exposure on acquisition learning was also evaluated using the search strategy used to find the target hole during the Barnes maze trials. Each trial (four per acquisition day) was scored for one of three search strategies: direct, serial, or mixed. The total number of trials using each strategy in both control and ethanol-treated mice was evaluated using a chi-squared test to determine if learning strategy was associated with treatment. Percentage of trials using each learning strategy for control and ethanol-treated mice is shown in Figure 3.13. Generally, mice treated with ethanol at P4, P7, or P4+7 showed a reduced tendency to use a direct search strategy as compared to controls. Ethanol treatment resulted in a prolonged use of a mixed strategy through acquisition training and increased use of a serial strategy towards the end of training. Mice treated at P4 showed a significant association between treatment and learning strategy on acquisition testing day 3 ($\chi^2 = 7.35$, df = 5, $p = 0.03$) and day 4 ($\chi^2 = 11.00$, df = 5, $p = 0.004$). This trend held consistent for mice treated at P7, again with a significant association between search strategy used and treatment at testing day 3 ($\chi^2 = 7.38$, df = 5, $p = 0.02$) and day 4 ($\chi^2 = 15.08$, df = 5, $p < 0.001$). Significant associations between treatment and search strategy was detected in the P4 and P7-treated group at testing day 1 ($\chi^2 = 6.20$, df = 5, $p = 0.045$), day 3 ($\chi^2 = 18.86$, df = 5, $p < 0.001$) and day 4 ($\chi^2 = 30.82$, df = 5, $p < 0.001$).

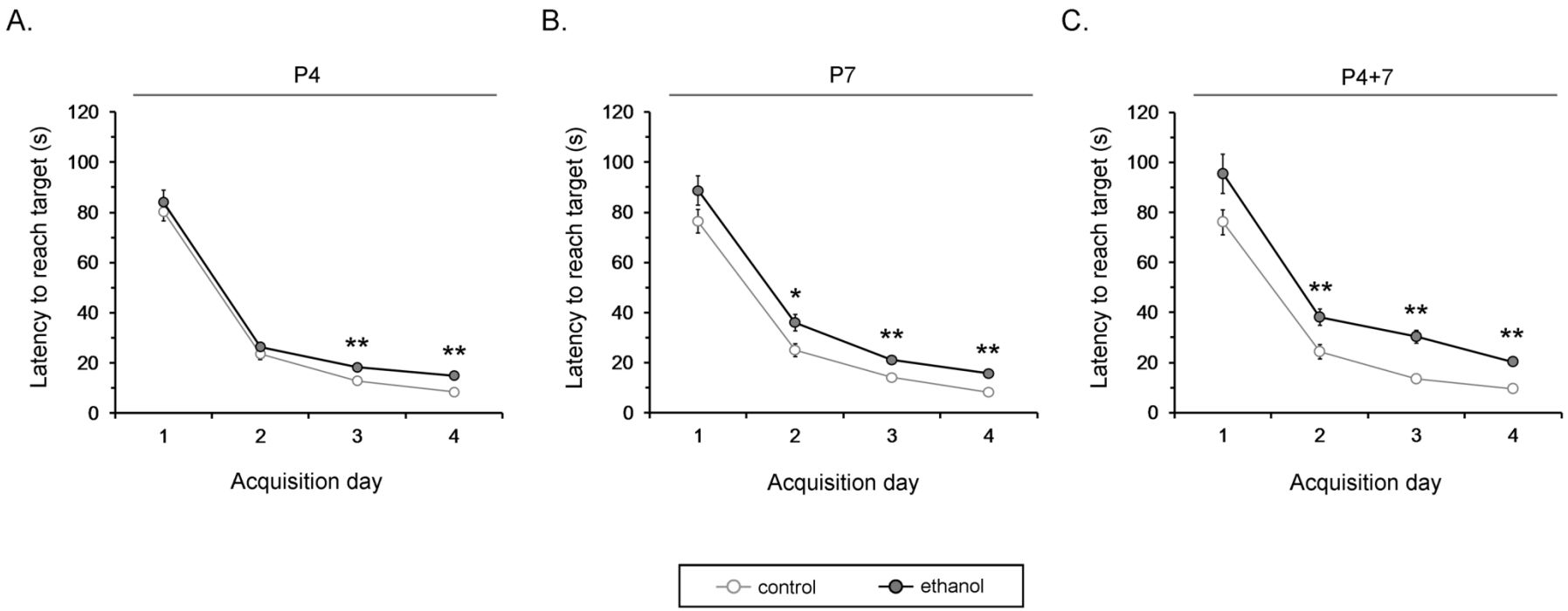


FIGURE 3.12. Latency to escape in the Barnes maze task for spatial learning. The mean (±SEM) time (seconds) to reach the target for mice treated with saline (control) or ethanol at P4 (A), P7 (B), or P4 and P7 (C). Data represent the average of four trials per day of each animal across four consecutive acquisition (learning) days. Data shown are collapsed across sex (n = 20-23 per group). *, $p < 0.01$; **, $p < 0.001$.

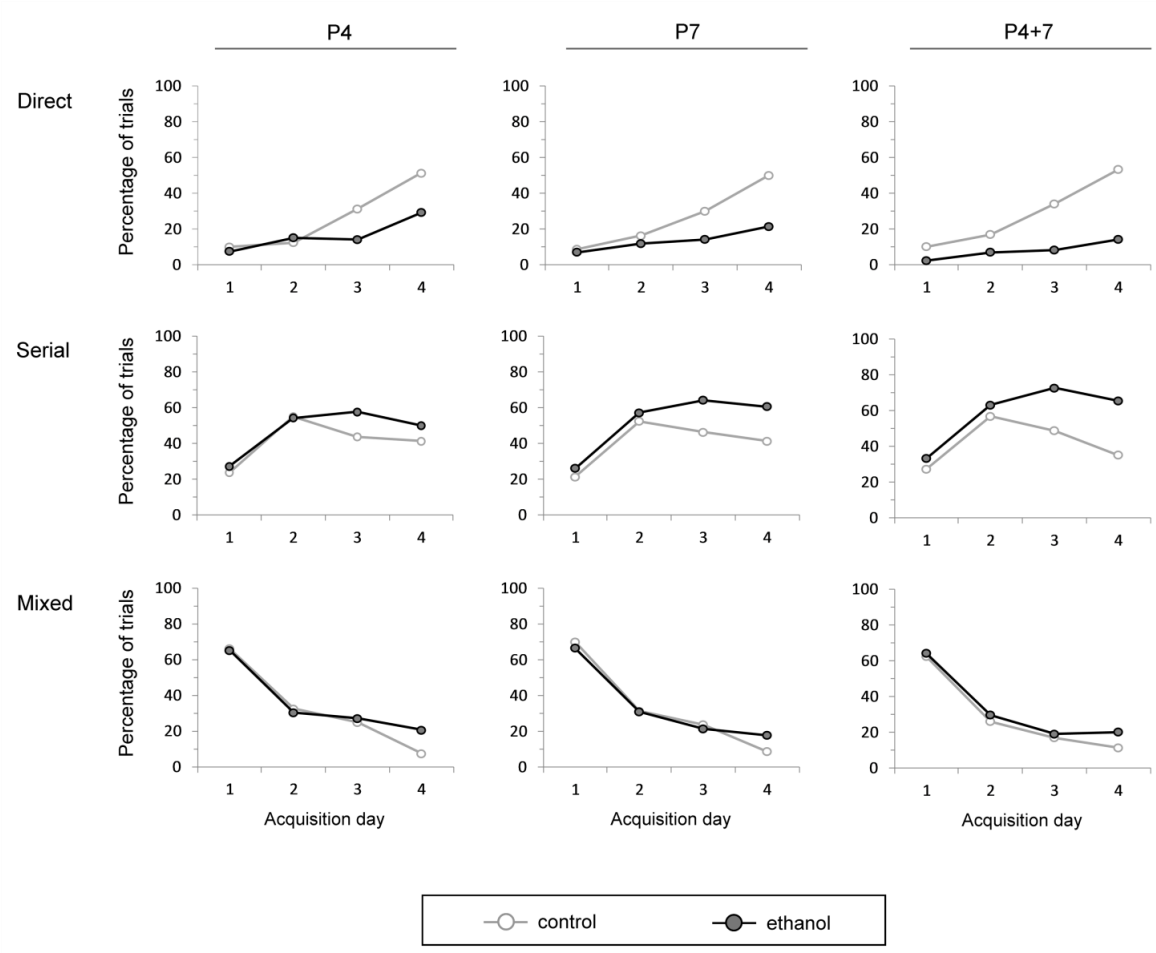


FIGURE 3.13. Type of search strategy used to locate the escape in the Barnes maze task. Data represent the percentage of trials mice treated with saline (control) or ethanol at P4, P7, or P4 and P7 (P4+7) used a direct, serial, or mixed search strategy on each acquisition (testing) day. Data shown are collapsed across sex (n = 20-23 per group).

3.1.5.3 *Short and long-term recall memory are impaired by ethanol exposure*

Following the acquisition learning phase of the Barnes maze testing, each mouse was tested for their ability to recall the location of the escape when all holes were closed. This was accomplished by assessing the number of explorations to each hole during a one minute testing period at testing day 5 (short-term recall memory) and day 12 (long-term recall memory).

Multivariate ANOVA analysis of the number of explorations to each hole using only the control mice from all treatment groups (saline exposure at P4, P7, and P4+7) at day 5 indicated no significant main effect of sex ($F_{20,37}$ = 1.18, p = 0.324), treatment day ($F_{40,76}$ = 1.09, p = 0.367) or interaction between the two ($F_{40,76}$ = 0.77, p = 0.812), indicating consistency between the control groups. Also, no significant difference was detected in the total number of explorations between each group (mean = 37.64 ± 1.76 to 41.55 ± 2.05), including no main effect of sex ($F_{1,56}$ = 1.18, p = 0.282), treatment day ($F_{2,56}$ = 1.23, p = 0.301) or interaction between sex and treatment day ($F_{2,56}$ = 1.17, p = 0.318).

Analysis of mice treated at P4 with ethanol or saline revealed a trend for the ethanol-treated mice to exhibit less explorations to the target and surrounding holes, but this trend did not reach significance (Figure 3.14A). No main effect of sex or interaction between sex and treatment was detected ($p > 0.05$). No significant difference in total number of explorations between control and ethanol-treated animals was detected ($F_{1,39}$ = 0.20, p = 0.654). Conversely, multivariate analysis of mice treated at P7 indicated a significant effect of treatment ($F_{20,18}$ = 2.50, p = 0.028), with ethanol-treated mice showing significantly less explorations to the target (mean = 2.95 ± 0.24 explorations) than controls (mean = 5.12 ± 0.23) and more explorations to holes opposite to the target (Figure 3.14B). No significant effect of sex or interaction between sex and treatment was detected. Further, no significant difference in number of explorations between sexes or between treatment groups was detected ($p > 0.05$). Ethanol treatment at P4+7 resulted in a pronounced difference between control and ethanol-treated groups, with a significant main effect of treatment ($F_{20,20}$

= 3.57, p = 0.003) detected. Control mice showed more explorations to the target (mean = 6.85 ± 0.33 explorations) than ethanol-treated mice (mean = 3.76 ± 0.28) and less explorations to opposite holes (Figure 3.14C). No significant effect of sex or interaction between sex and treatment was detected ($p > 0.05$). Also, no significant differences between the total number of explorations was indicated.

To evaluate if this deficit in recall memory persisted past the first week of testing, mice were re-tested for long-term recall of the location of the escape during the second week (at day 12) without further acquisition training. Multivariate analysis of only the control mice of all treatment paradigms detected no effect of day of testing ($F_{40,76} = 0.76$, $p = 0.828$). Also, no significant effect of sex or interaction between sex and treatment was detected ($p > 0.05$). Analysis of total number of explorations detected no significant effect of treatment day ($F_{2,56} = 1.27$, $p = 0.288$), as well as no significant main effect of sex or interaction between sex and day of treatment ($p > 0.05$).

Analysis of the long-term (day 12) recall memory of mice treated with ethanol or saline at P4 detected no significant effect of sex, treatment, or interaction between the two ($p > 0.05$) across the average number of explorations to each hole (Figure 3.15A). No significant main effects or interactions were detected in the total number of explorations between sexes or treatment groups. Mice treated with ethanol at P7 did show significantly less explorations to the target (mean = 3.52 ± 0.0.22) as compared to controls (mean 5.02 ± 0.28) ($F_{20,18} = 3.96$, $p = 0.002$) during the long-term recall testing (Figure 3.15B). No significant main effect of sex or interaction between sex and treatment on hole exploration was detected, as well as no significant main effects or interactions on the total number of explorations. A significant main effect of treatment on the overall pattern of number of explorations to each hole during the day 12 long-term recall trial was also detected for animals treated at P4+7 ($F_{20,20} = 3.51$, $p = 0.004$). Control animals showed significantly more explorations to the target (mean = 5.10 ± 0.23) than ethanol-treated mice (mean = 3.10 ± 0.27) and fewer explorations at the opposite side of the Barnes maze platform (Figure 3.15C). Again,

no significant effect of sex or interaction between sex and treatment were detected. There were no significant differences in total number of explorations.

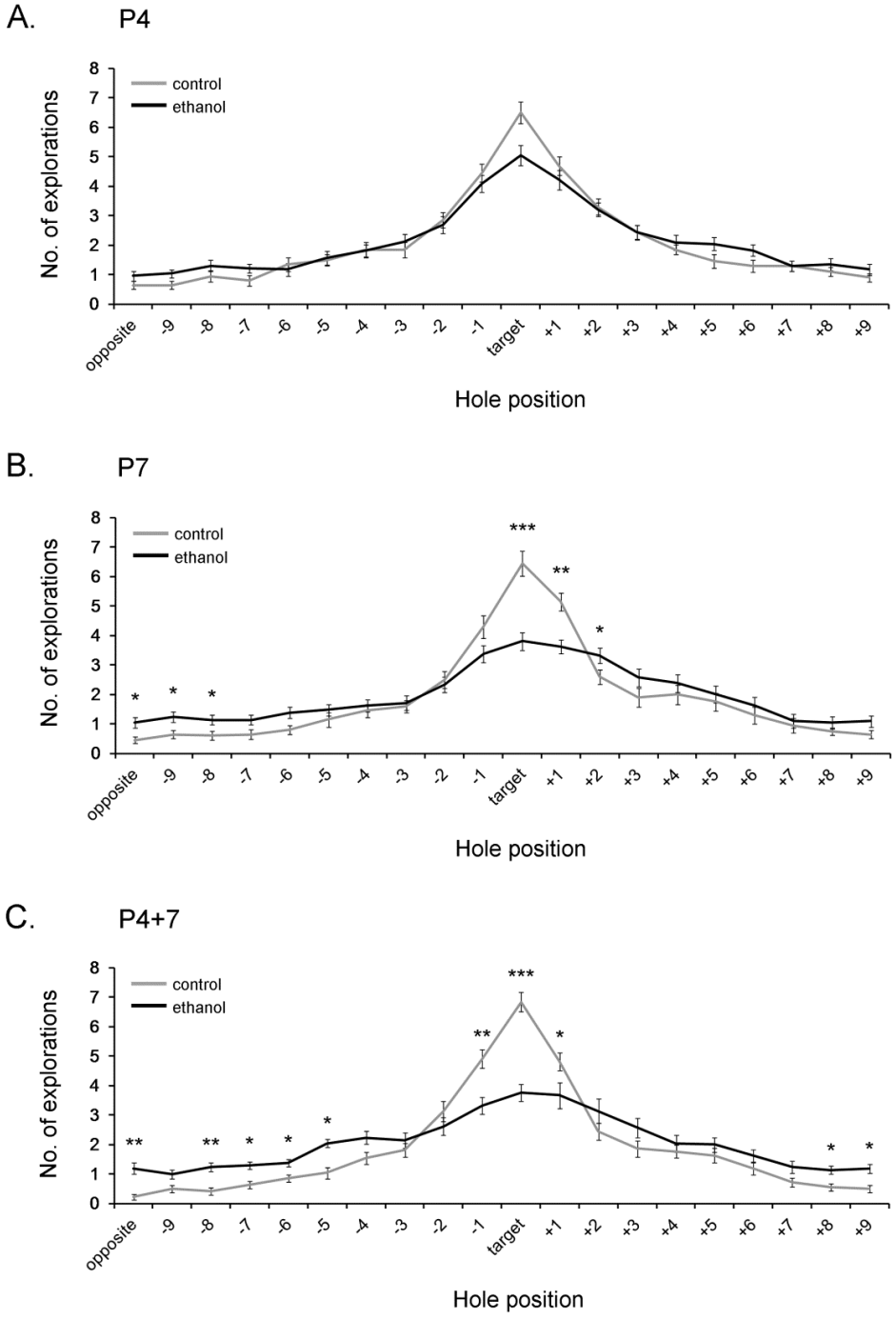


FIGURE 3.14. Number of explorations to each Barnes maze hole during short-term recall memory trials. The mean number of explorations (±SEM) to each hole located around the periphery of the Barnes maze is indicated for mice treated with saline (control) or ethanol at P4 (A), P7 (B), or P4+7 (C). Data represent short-term recall memory, tested on day 5 following four days of learning (acquisition) trials. Data shown are collapsed across sex (n = 20-23 per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

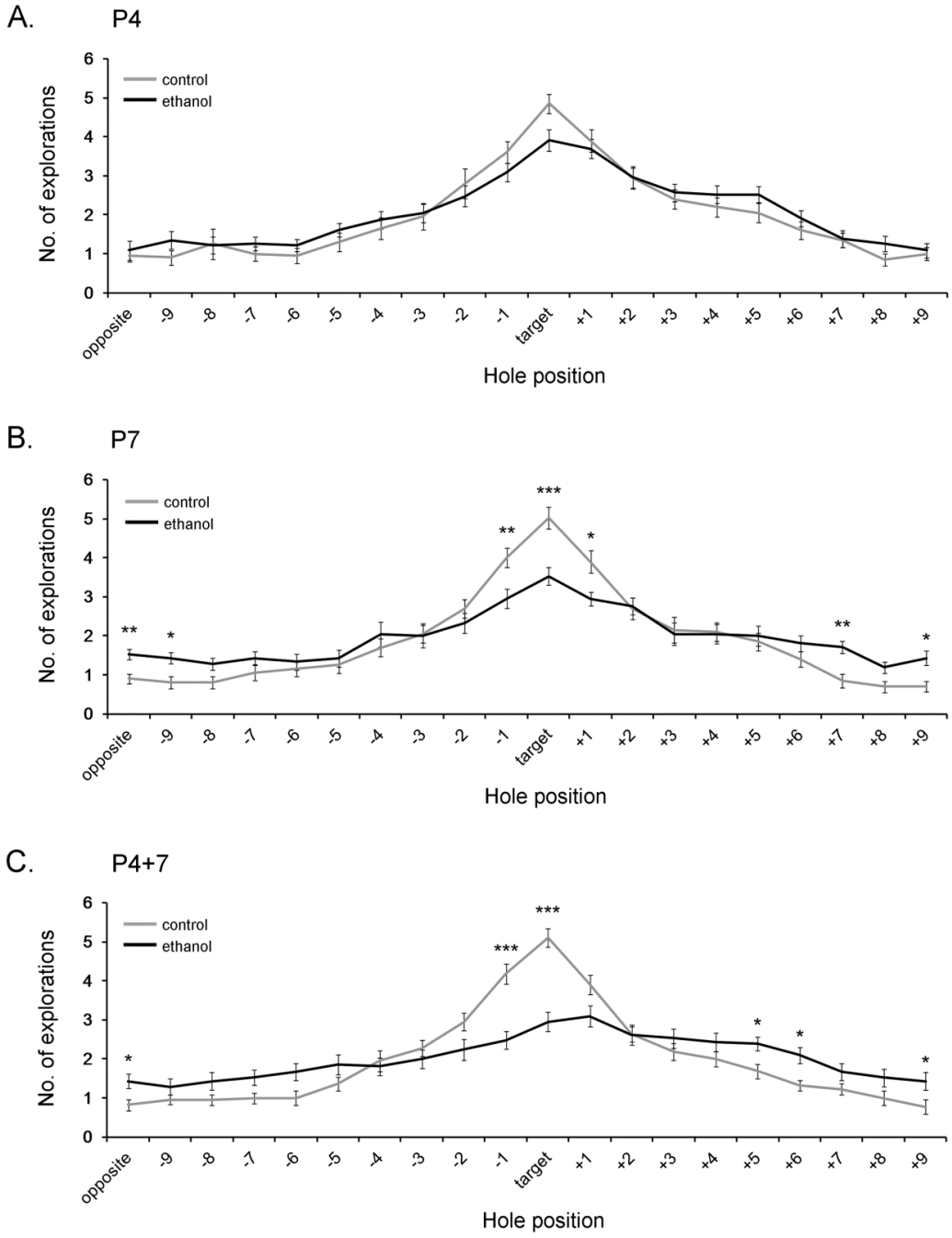


FIGURE 3.15. Number of explorations to each Barnes maze hole during long-term recall memory trials. The mean number of explorations (±SEM) to each hole located around the periphery of the Barnes maze is indicated for mice treated with saline (control) or ethanol at P4 (A), P7 (B), or P4+7 (C). Data represent short-term recall memory, tested on day 12 following four days of learning (acquisition) trials. Data shown are collapsed across sex (n = 20-23 per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.1.6 Summary of behaviour results

Exposure to ethanol during the third trimester-equivalent resulted in pronounced changes in behavioural phenotypes that are apparent during early and late postnatal development, adolescence and early adulthood. Interestingly, the effect of ethanol on these phenotypes appeared to be dependent on the timing of ethanol exposure, and some consistent differences emerged between animals treated at P4 versus P7 (Table 3.4). Treatment at P4 appeared to affect early neuromuscular reflexes and coordination, with ethanol-treated mice showing a delay in their ability to achieve many developmental milestones. Ethanol treatment later during the trimester three-equivalent at P7 resulted in increased home-cage nocturnal activity levels and increased anxiety-related traits in a novel open-field, the light/dark box, and the elevated plus maze (particularly in females). All ethanol treatment paradigms resulted in impaired learning during the Barnes maze acquisition trials; however, the P7 and P4+7 treatment groups showed marked increases in latency to escape the Barnes maze. This trend was consistent when assessing the search strategy used to navigate the Barnes maze: while control mice were quickly able to travel directly towards the escape, ethanol-treated mice showed a preference for a serial strategy in which they would travel to numerous holes in a clockwise or counter-clockwise fashion prior to locating the target. Also, the ethanol-treated mice showed an increased use of a serial strategy and a reduced use of a direct strategy. This was reflected in the short and long-term memory trials, with mice treated with ethanol at P7 or at P4+7 showed a decreased tendency to explore the previous location of the target hole. This difference was apparent at day 5 (short-term) as well as a week later during the long-term recall memory trial (day 12). Taken together, ethanol treatment during the trimester three-equivalent results in behavioural phenotypes consistent with those observed in human children, adolescents, and young adults with FASD.

TABLE 3.4. Summary of behavioural consequences of ethanol exposure during the third trimester-equivalent.

			P4[a]		P7[a]		P4+7[a]	
Behaviour	**Assay[b]**	**Day of testing[c]**	**male**	**female**	**male**	**female**	**male**	**female**
Coordination and reflexes	Developmental milestones	2-21	↓ (7/10)	↓ (7/10)	↓ (3/10)	↓ (3/10)	↓ (8/10)	↓ (8/10)
Activity	Novel open field	26-28	NS	NS	NS	NS	NS	↓
	Home cage	30-35	NS	NS	↑	↑	↑	↑
Anxiety	Novel open field	26-28	NS	NS	↑	↑	↑	↑
	LDB	35-40	-	-	-	-	↑	↑
	EPM	35-40	-	-	-	-	↑	↑
Spatial learning	Barnes maze	45-48	↓	↓	↓	↓	↓	↓
Learning strategy	Barnes maze	42-48	↓ direct ↑ serial	↓ direct ↑ serial	↓ direct ↑ serial	↓ direct ↑serial	↓ direct ↑ serial	↓ direct ↑ serial
Short-term memory	Barnes maze	49	NS	NS	↓	↓	↓	↓
Long-term memory	Barnes maze	56	NS	NS	↓	↓	↓	↓

[a]Changes are relative to control mice: ↑: increased relative to control; ↓: decreased relative to control; NS: no significant difference relative to controls
[b]LDB: light/dark box; EPM: elevated plus maze
[c]Postnatal day testing was conducted

3.2 Gene expression changes following ethanol exposure during synaptogenesis

To evaluate the changes to gene expression induced by ethanol exposure during the trimester three-equivalent and how they may lead to the observed emergent behavioural phenotypes, I examined the alterations to whole-brain gene expression patterns acutely following ethanol exposure at postnatal day 7 (4-hours post-treatment) (short-term) as well as those transcript changes that persisted into adulthood at postnatal day 60 (long-term) using expression microarrays. The results of these analyses comprise the following section of this chapter.

3.2.1 Gene expression changes acutely following ethanol treatment

The changes to whole brain gene expression following ethanol exposure at P7 four hours post-injection were assessed using expression microarray analysis. This time point was chosen due to the multi-phenotype behavioural alterations detected by the behavioural battery after ethanol treatment at P7 as compared to P4. Ethanol exposure at P7 resulted in the acute alteration of 315 transcripts at a fold-change of 1.2 (FDR-corrected $p < 0.05$), with 138 (44%) showing up-regulation four hours after ethanol exposure. A complete gene list using these stringency criteria is shown in Appendix A. Changes ranged from -1.73-fold (*Dual specificity phosphatase 6*) to 2.63-fold (*Thioredoxin interacting protein*), though most gene expression changes were subtle, with 78 per cent (245/315) of gene expression changes showing less than a 1.3-fold difference.

3.2.2 Array hierarchical clustering and functional annotation of genes altered acutely following ethanol exposure

Hierarchical clustering of probe signal intensity values showed good consistency between short-term arrays, with the two ethanol-treatment arrays clustering together distinctly from the four control arrays (Figure 3.16). The clustering also revealed four subsets of genes that responded similarly to ethanol treatment. Some of these groups were not large enough to be subjected to meaningful gene functional clustering analysis, so the groups that showed up-regulation (one and four) or down-regulation (two and three) in response to ethanol treatment were submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (DENNIS et al. 2003) to assess Gene Ontology (GO) biological process terms common between similarly-responsive genes.

Genes showing up-regulation four hours following ethanol exposure at P7 were significantly enriched for GO terms associated with positive regulation of apoptosis, which included genes such as *Cysteine-serine-rich nuclear protein 3* (*Csrnp3*), a transcriptional activator involved in apoptotic genes, *Glutamate (NMDA) receptor subunit epsilon-2* (*Grin2b*), growth factors (*Tgfb3*, *Igfbp3*), and *Bcl2-like 11 (apoptosis facilitator)* (*Bcl2l11*) (Table 3.5). Interestingly, a large cluster of genes (61 transcripts) were associated with cell membrane integrity. Also significant were genes with GO terms associated with post-synaptic density, amino acid dephosphorylation, glycosylation, and regulation of synaptic transmission.

Down-regulated genes showed enrichment for GO terms related to ribosome biogenesis, mitotic cell cycle regulation, MAP kinase phosphatase activity, transcriptional regulation, and hormone activity, including a significant down-regulation in genes associated with steroid hormone receptor activity (Table 3.5). Other significantly reduced transcripts were associated with chromosomal remodeling or a constituent of chromatin or DNA structure, such as Ube2a, required for DNA damage repair, *histone deacetylase 1* (*Hdac1*), and *Nuclear FMRP-interacting protein 1* (*Nufip1*).

To further characterize the biological functions targeted by ethanol identified by the broad GO analyses, the up- and down-regulated transcripts were also evaluated independently for biological function clusters using Ingenuity Pathway Analysis™ (IPA) (Ingenuity Systems, Redwood, CA, www.ingenuity.com). The results confirmed and extended the biological functions identified by DAVID and showed that many genes altered by ethanol exposure influenced cell survival and development, particularly those processes involving neuronal apoptosis and plasticity (Table 3.6). IPA analysis identified abnormal expression of genes associated with dopaminergic neuron development and neuroglia function. Also, this analysis identified alterations to genes associated with endocrine system development and function including the hydroxylation of melatonin, corticosterone secretion, phospholipid metabolism, and calcium mobilization and influx.

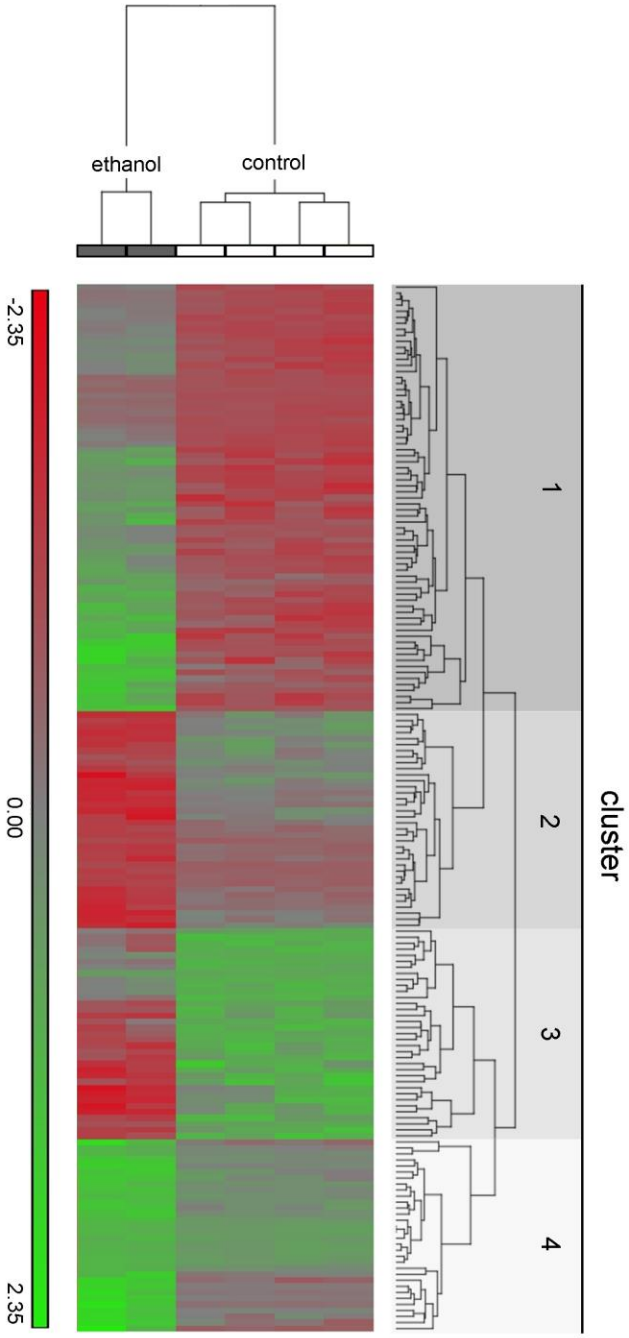


FIGURE 3.16. Heat map hierarchical clustering of arrays representing transcripts altered in the brain acutely following ethanol treatment on P7 based on normalized intensity of the probe sets. Heat map was generated by Partek® Genomics Suite software based on ANOVA-calculated significance levels and fold-changes. Clusters of up-regulated (green) and down-regulated (red) genes are indicated.

TABLE 3.5. Gene Ontology (GO) biological function annotation clustering of transcripts altered in the brain acutely following ethanol exposure at P7.

GO Term	GO ID	No. genes	*p*-value	Up-regulated genes
positive regulation of apoptosis	0043065	10	0.000170	*Csrnp3, Grin2a, Tgfb3, Trp53inp1, Foxo3, Sp110, Perp, Igfbp3, Bcl2l11, Jmy*
integral to membrane	0016021	61	0.00643	*Mchr1, Slc16a14, B3galt5, Osmr, Slc44a5, Ints2, Sdc4, Megf10, Pigm, Clec4e, Slc24a4, Grin2b, Slc25a24, Kcnk6, Slc2a1, Atp8b2, Htr1d, Htr5a, Slc43a2, Gpr139, Vmn2r33, Pigv, Lifr, Grin2a, Vmn2r37, Ntsr1, Pik3ip1, Parp16, Folh1, Cldn1, Mfsd11, Steap2, Vmn2r43, Gcnt2, Tmem194b, Enpp3, Aph1b, St8sia1, Gpr6, Csmd3, Igsf10, Igf1r, Prrg4, B3gnt5, Pcdhb18, Scn9a, Tmc7, Fam26e, Rnf144b, Fktn, St6gal1, Abca8b, Acer2, Bcg1, Cdhb21, Kctd6, Slc7a3, Slc16a9, Itpripl2, Mertk, Perp*
postsynaptic density	0014069	4	0.00761	*Grin2b, Grin2a, Psd3, Cpeb1*
protein amino acid dephosphorylation	0006470	5	0.0139	*Dusp1, Ppmik, Ptpn4, Dusp16, Dusp8*
glycosylation	0070085	4	0.0366	*St6gal1, B3gnt5, B3galt5, St8sia1*
regulation of synaptic transmission	0050804	4	0.0464	*Grin2b, Edn1, Grin2a, Cpeb1*
				Down-regulated genes
ribosome biogenesis	0042254	7	0.0000618	*Utp11l, Tbl3, Gtpbp4, Nop2, Utp18, Mphosph10, Nmd3*
mitotic cell cycle	0000278	8	0.000719	*Ccnd1, E2f4, Incenp, Aurka, Pmf1, Sirt7, Nup43, Rbbp8*
MAP kinase phosphatase activity	0033549	3	0.00280	*Dusp4, Dusp6, Dusp7*
regulation of transcription	0045449	25	0.00293	*E2f4, Nufip1, Zbtb17, Cited4, Med27, Per2, Olig2, Cry1, Etv5, Etv4, Klf6, Egr3, Egr4, Snapc1, Rxrg, Nr4a1, Mcm2, Pmf1, Sirt7, Nrob1, Inhba, Hdac1, Vegfa, Zfp143, Hdac9*
chromosomal part	0044427	8	0.00363	*Ube2a, Hdac1, Incenp, Nufip1, Pmf1, Mcm2, Sirt7, Nup43*
hormone activity	0005179	4	0.0401	*Inhba, Pomc, Apln, Adcyap1*
steroid hormone receptor activity	0003707	3	0.0425	*Rxrg, Nr4a1, Nrob1*

TABLE 3.6. Ingenuity Pathway Analysis™ biological functions associated with ethanol-induced gene expression changes at P7.

Category	Function annotation	*p*-value	No. genes	Up-regulated genes	Down-regulated genes
Cell death and survival	apoptosis of neurons	0.000497	17	*Agt, Bcl2l11, Btg2, Dusp1, Grin2b, Igf1r, Jun, Mt1e, Mt1h, Perp, St8sia1, Tgfb3, Zfp110/Zfp369*	*Adcyap1, Hdac1, Klf6, Vegfa*
Nervous system development and function	proliferation of pituitary cells	0.00220	3	*Tgfb3*	*Inhba, Srsf2*
	proliferation of neuroglia	0.0155	5	*Cpeb1, Foxo3*	*Adcyap1, Ccnd1, Etv5*
	abnormal morphology of astrocytes	0.0252	3	*Mt1e, Mt1h*	*Ldlr*
	quantity of neurons	0.0334	10	*Dusp1, Igf1r*	*Adcyap1, Egr3, Inhba, Lifr, Olig2, Pomc, Trpa1, Vegfa*
	plasticity of neuronal synapse	0.0371	2	*Cpeb1, Grin2b*	
Tissue development	regeneration of nerves	0.000544	3	*Agt, Jun, St8sia1*	-
Cell cycle	mitosis	0.00175	3	*Edn1, Tgfb3*	*Ccnd1*
	mitogenesis of central nervous system cells	0.00216	2	*Edn1, Tgfb3*	-
Lipid metabolism	hydrolysis of phospholipid	0.0181	2	*Agt, Edn1*	-
	turnover of phospholipid	0.00216	2	*Edn1*	*Adcyap1*
	metabolism of membrane lipid derivative	0.0462	2	*St8sia1*	*Pomc*
Neurological disease	neuromuscular disease	0.0116	15	*Agt, Mertk, Mt1e, Mt1h, Sdc4, Slc16a9, Zfp932*	*Egr4, Etv5, Gabra5, Hla-b, Lifr, Nr4a1, Rgs4, Rxrg*
	disorder of basal ganglia	0.0171	14	*Mertk, Mt1e, Mt1h, Sdc4, Slc19a9, Zfp932*	*Egr4, Etv5, Gabra5, Hla-b, Lifr, Nr4a1, Rgs4, Rxrg*
	neuropathy of brain	0.00216	2	*Mt1e, Mt1h*	-
	damage of brain	0.0479	4	*Edn1, Folh1, Grin2a, Grin2b*	-

TABLE 3.6. continued…

Category	Function annotation	*p*-value	No. genes	Up-regulated genes	Down-regulated genes
Cellular growth and proliferation	proliferation of cells	0.0197	13	*Cpeb1, Foxo3, Tgfb3*	*Adcyap1, Ccnd1, Etv5, Gnl3, Gtpbp4, Inhba, Klf6, Pomc, Srsf3, Vegfa*
Vitamin and mineral metabolism	mobilization of Ca2+	0.0122	2	-	*Adcyap1, Trpa1*
	influx of Ca2+	0.0371	2	*Grin2b*	*Trpa1*
Cell-to-cell signaling and interaction	damage of dopaminergic neurons	0.00356	2	*Mt1e, Mt1h*	-
	plasticity of neuronal synapse	0.0371	2	*Cpeb1, Grin2b*	-
Endocrine system development and function	proliferation of pituitary cells	0.00220	3	*Tgfb3*	*Inhba, Srsf2,*
	hydroxylation of melatonin	0.0193	1	*Cyp1b1*	-
	production of L-triiodothyronine	0.0193	1	-	*Dio2*
	secretion of corticosterone	0.0193	1	-	*Pomc*

3.2.3 Canonical signaling pathways affected acutely following ethanol exposure

To assess biological pathways that may be affected immediately following ethanol treatment at P7, the genes identified by the array analysis were subjected to IPA's canonical pathway analysis to identify pathways associated with altered gene expression and the identified biological functions. The canonical pathways identified were also consistent with the gene ontology and biological functions analyses. Canonical pathways that possessed a significant number of altered genes are shown in (Table 3.7). Significantly affected pathways included p53 signaling (p = 0.0002), which included the upregulated transcription factor *jun proto-oncogene* (*Jun*), classically associated with early cellular stress response, and a down-regulation of the histone deacetylase *Hdac1*. Circadian rhythm signaling was also significantly-affected (p = 0.0002) and included altered genes that control the proper timing of cellular and metabolic events such as glutamate neurotransmission (*Grin2a*, *Grin2b*), period genes (*Per1*, *Per2*) and the cryptochrome protein *Cry1*. Other canonical pathways identified as significantly affected acutely following ethanol exposure at P7 were associated with cell cycle: G1/S checkpoint regulation (p = 0.0008), which is consistent with the cell cycle-associate genes identified by the IPA biological functions analysis and included the growth factor *transforming growth factor beta-3* (*Tgfb3*) as well as the histone deacetylases *Hdac1* and *Hdac9*. Other pathways of note included phosphoinositide biosynthesis (p = 0.11), associated with membrane integrity, retinoic acid-associated TR/RXR activation (p = 0.017), and the related canonical PTEN signaling pathway (p = 0.02), associated with regulation of cell growth and mitotic progression.

TABLE 3.7. Ingenuity® canonical pathways associated with transcripts altered by ethanol exposure at P7.

Canonical pathway	*p*-value	No. molecules	Up-regulated genes	Down-regulated genes
p53 signaling	0.00023	8	*Jmy, Jun, Perp, Pik3r1, Tp53inp1*	*Ccnd1, Gnl3, Hdac1*
Circadian rhythm signaling	0.00024	6	*Grin2a, Grin2b, Per1*	*Adcyap1, Cry1, Per2*
Cell cycle: G1/S checkpoint regulation	0.00077	6	*Tgfb3*	*Ccnd1, E2f4, Gnl3, Hdac1, Hdac9*
Chronic myeloid leukemia signaling	0.00698	9	*Pik3r1, Tgfb3*	*Ccdn1, E2f4, Hdac1, Hdac9*
3-phosphoinositide biosynthesis	0.00927	7	*Dusp1, Dusp8, Dusp16, Nudt11, Pik3r1, Ppm1k*	*Pptc7*
D-myoinositol tetraphosphate biosynthesis	0.0110	6	*Dusp1, Dusp8, Dusp16, Nudt11, Ppm1k*	*Pptc7*
Cyclins and cell cycle regulation	0.0132	5	*Tgfb3*	*Ccnd1, E2f4, Gnl3, Hdac1, Hdac9*
ErbB2-ErbB3 signaling	0.0165	4	*Jun, Pik3r1*	*Ccnd1, Etv4*
TR/RXR activation	0.0172	5	*Pik3r1, Slc2a1*	*Dio2, Ldlr, Rxrg*
PTEN signaling	0.0203	5	*Pik3r1, Slc2a1*	*Dio2, Ldlr, Rxrg*

3.2.4 Interacting gene networks involving genes altered by ethanol exposure

To evaluate how the ethanol-responsive genes altered acutely following ethanol treatment at P7 may interact with one another, I utilized the gene network analysis tool within the Ingenuity® software platform to predict interacting molecular networks (algorithmically generated pathways). The molecular networks represented pleiotropic, broadly-acting functions and correlated well with the GO functions and the canonical pathways identified by the previous analyses. Network analysis identified three significant networks, shown in Figures 3.15, 3.16, 3.17. The returned networks were associated with 'cellular development, cellular growth and proliferation, tumor morphology' (CDCG), which contained 17 focus molecules identified by the array (IPA network score = 20) (Figure 3.15), 'tissue morphology, cell-to-cell signaling and interaction, cellular growth and proliferation' (TMCS) (focus molecules = 13; score = 13) (Figure 3.16), and 'behaviour, cell-to-cell signaling and interaction, nervous system development and function' (BCSN) (focus molecules = 11; score = 10) (Figure 3.17). As the network descriptors suggests, these networks were highly interrelated. The CDCG network centralized on three highly connected 'hub' molecules: *jun proto-oncogene* (*Jun*), which was detected as highly up-regulated in the ethanol-treatment arrays as compared to controls, and *cAMP responsive element binding protein 1* (*Creb1*) and *FBJ osteosarcoma oncogene* (*Fos*), which were not detected as differentially expressed but served as connector molecules between multiple up- and down-regulated transcripts (Figure 3.17). The TMCS also included *Jun*, as well as *tumor necrosis factor* (*Tnf*) (not differentially expressed) and *transforming growth factor, beta 1* (*Tgfb1*)(up-regulated) as hub molecules, as well as interleukins 15 (down-regulated) (Figure 3.18). Also relevant to this network was *adenylate cyclase activating polypeptide 1* (*Adcyap1*) (down-regulated) that acted as a molecular hub connecting the down-regulated *pro-opiomelanocortin-alpha* (*Pomc*) to *tumor necrosis factor* (*Tnf*) via expression regulation (E) and co-localization (LO) annotations. Finally, the BCSN network was characterized by the presence of multiple glutamate receptor subunits, including *glutamate receptor, ionotropic, N-methyl*

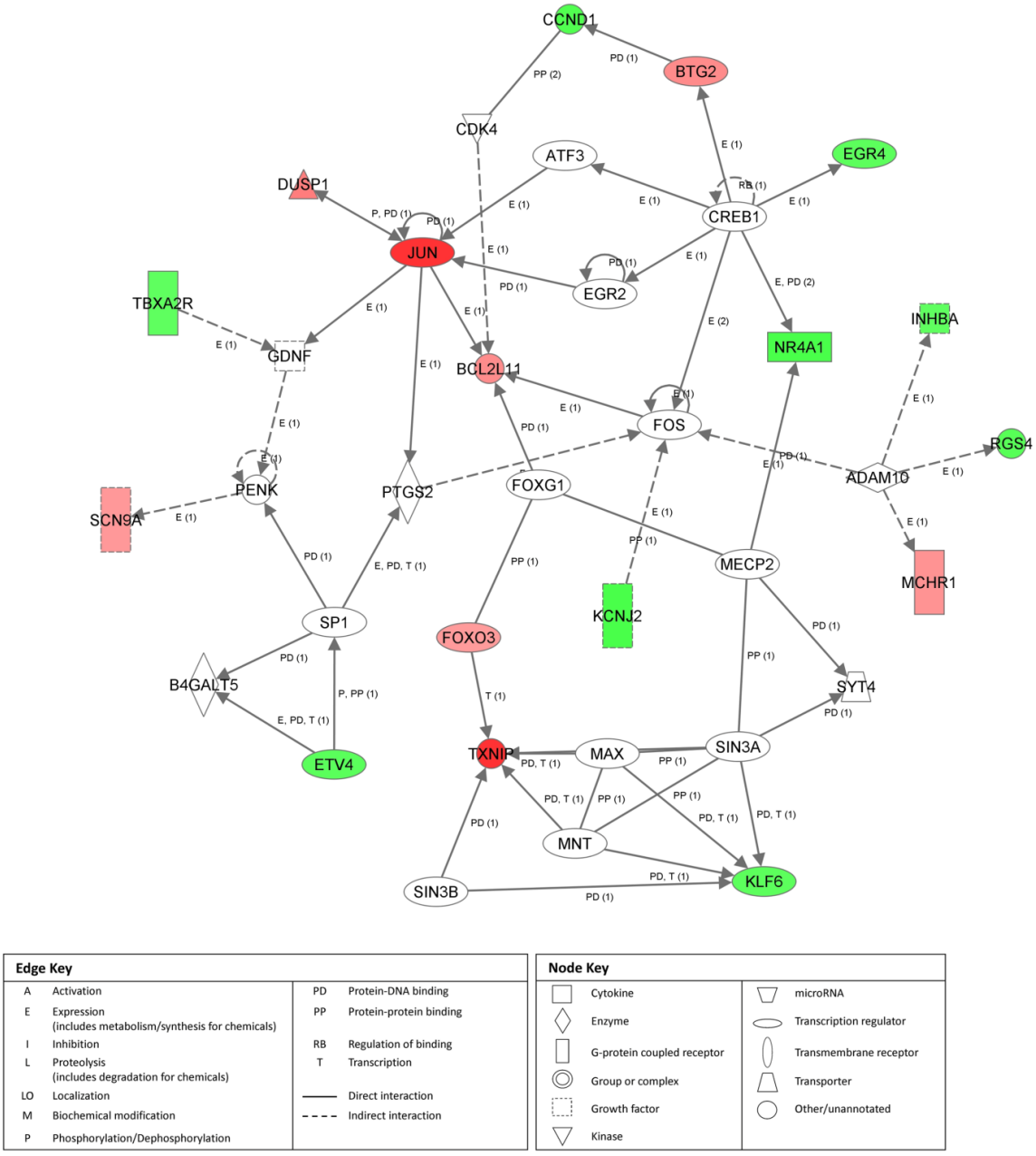


FIGURE 3.17. Ingenuity Pathway Analysis™ network analysis indicating annotated interactions between transcripts altered acutely following ethanol exposure at P7 associated with 'cellular development, cellular growth and proliferation, and tumor morphology' (CDCG). Up (red) and down (green) regulated genes are indicated. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

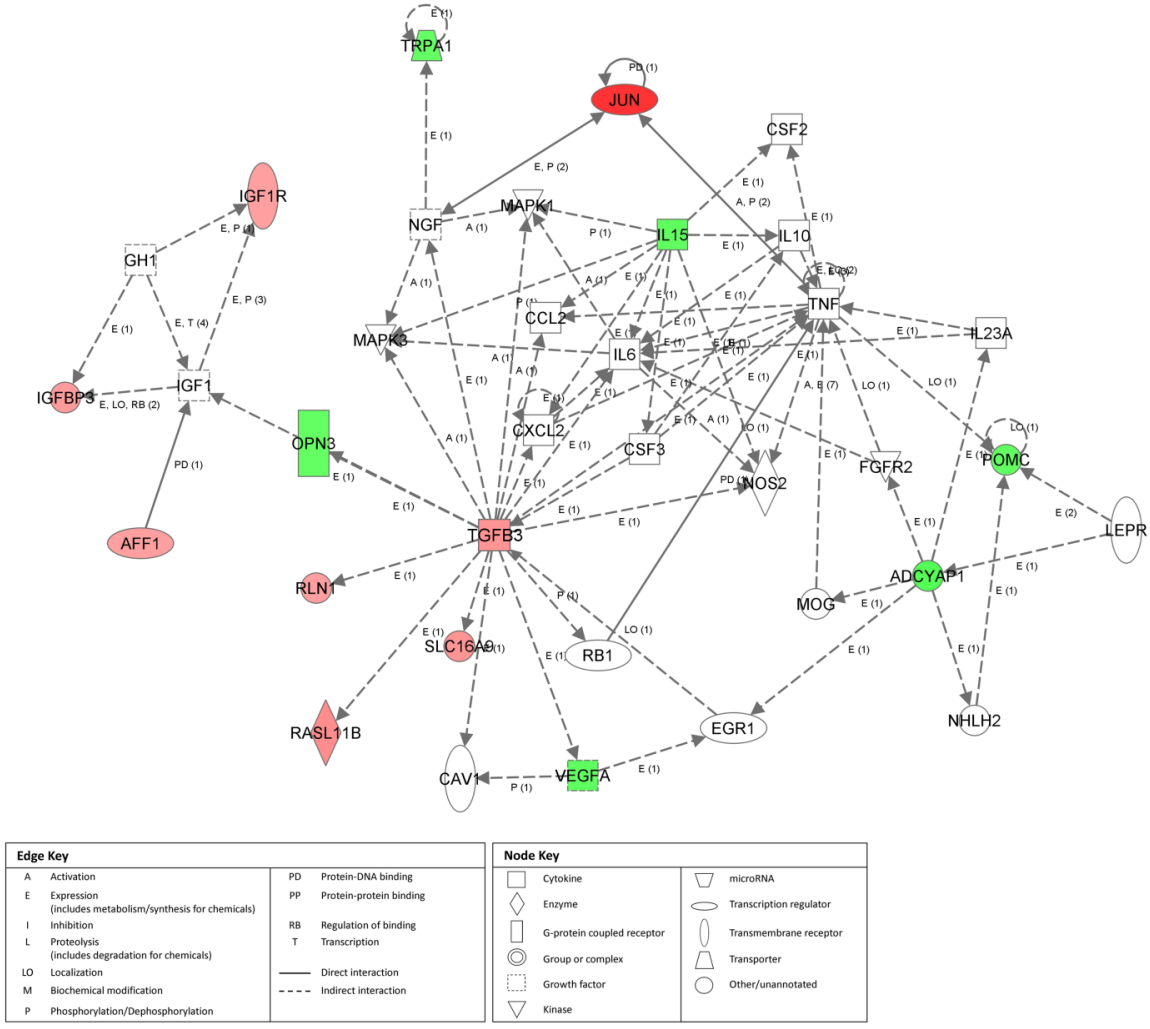


FIGURE 3.18. Ingenuity Pathway Analysis™ network analysis indicating annotated interactions between transcripts altered acutely following ethanol exposure at P7 associated with 'tissue morphology, cell-to-cell signaling and interaction, cellular growth and proliferation' (TMCS). Up (red) and down (green) regulated genes are indicated. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

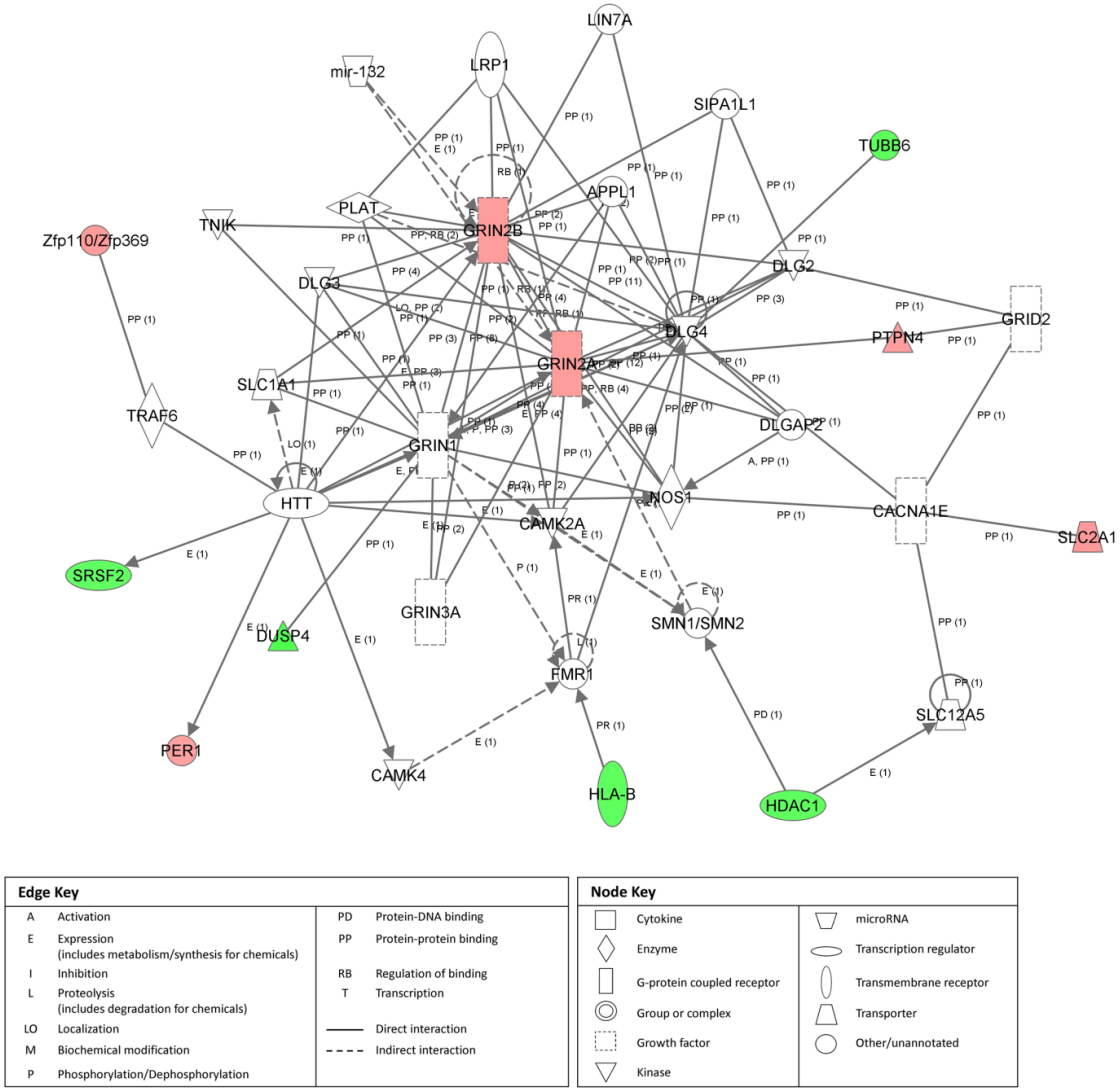


FIGURE 3.19. Ingenuity Pathway Analysis™ network analysis indicating annotated interactions between transcripts altered acutely following ethanol exposure at postnatal day 7 associated with 'behavior, cell-to-cell signaling and interaction, nervous system development and function' (BCSN). Up (red) and down (green) regulated genes are indicated. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

D-aspartate 2A (*Grin2a*) and *Grin2b*, both up-regulated (Figure 3.19). Further, this network included components of circadian rhythm patterning (*Per1*) and histone modification (*Hdac1*). Interestingly, *Huntingtin* (*Htt*) was implicated as a major hub of this network which, though it was not detected as differentially expressed, interacts with differentially expressed genes including *Per1* (up-regulated), glutamate receptor subunits, serotonin transporter genes (*Slc1a1*), and the RNA spliceosome protein *Srsf2* (down-regulated).

To identify molecules that may be key effectors of the ethanol-induced changes in gene expression, the three networks were merged to identify hub molecules that may unify the interactions of all three (Figure 3.20). Early immediate transcriptional regulators controlling cell cycle and proliferation, such as cell-cycle control genes such as *Jun*, *Fos*, *early growth response 1* (*Egr1*) and *Egr2*, and *Creb1*, appeared as central hubs. Also highly-represented are signaling molecules such as cytokines and growth hormones, including *growth hormone 1* (*Gh1*), nerve growth factor (*Ngf*), *Tumor necrosis factor* (*Tnf*), *Transforming growth factor, beta 1* (*Tgfb1*), and *interleukin 6* (*Il6*). The glutamate receptors (*Grin2a*, *Grin2b*, and *Grin1*) prominent in the BCSN network remain as hubs in the merged model of molecular interactions, as does *Htt*. Finally, an interesting interaction emerged between *Adcyap1*, *Pomc*, and *nuclear receptor subfamily 4, group A, member 1* (*Nr4a1*), all of which showed reduced mRNA levels. Taken together, these genes and their interactions may be viewed as the primary response of developing brain cells to ethanol exposure at synaptogenesis and illustrate a balance between the role of cell cycle genes, genes critical to synaptic formation and maintenance, and the expression of early response growth factors.

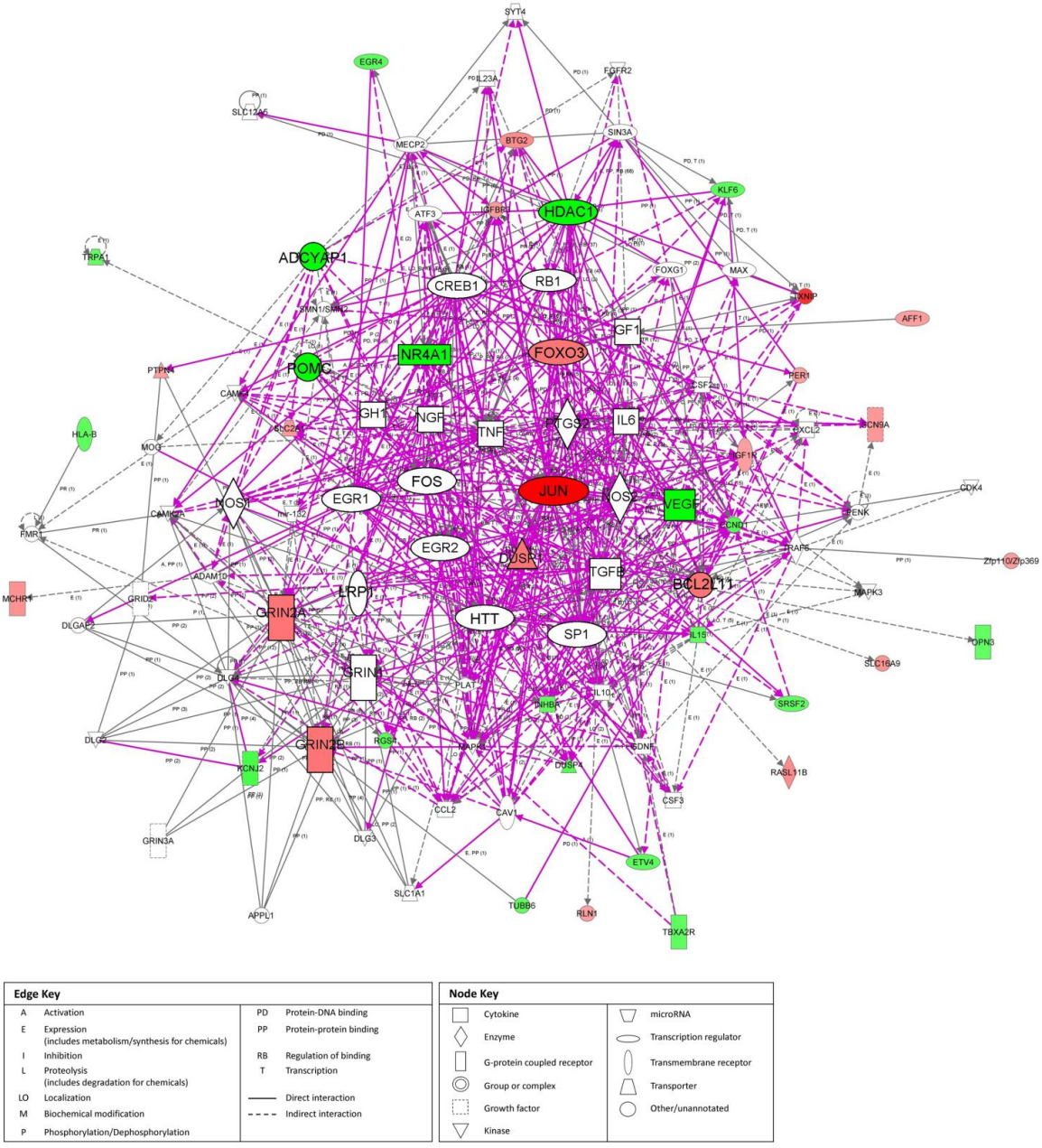


FIGURE 3.20. Merged Ingenuity Pathway Analysis™ networks showing interactions between the most significant gene networks (see Figure 3.15, 3.16, and 3.17) affected acutely following ethanol exposure at postnatal day 7. Up (red) and down (green) regulated genes are indicated. Centralized "hub" molecules linking multiple interacting genes are enlarged. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

3.2.5 Validation of array-identified mRNA levels by quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCT) expression analysis was performed for four genes, selected based on their fold changes and prominence in biological functions and networks identified by bioinformatics analyses. Genes chosen for qRT-PCR analysis were: *Dual-specificity phosphatase 1* (*Dusp1*), *jun proto-oncogene* (*Jun*), *nuclear receptor subfamily 4, group A, member 1* (*Nr4a1*), and *pro-opiomelanocortin-alpha* (*Pomc*). Real-time results as compared to array fold-changes are shown in Figure 3.21. Fold-changes calculated from deltaCt values showed good consistency with the array results. All genes confirmed (student's *t*-test, $p < 0.05$) with the exception of *Pomc*, which approached but did not reach significance ($t = 2.078$, $p = 0.071$).

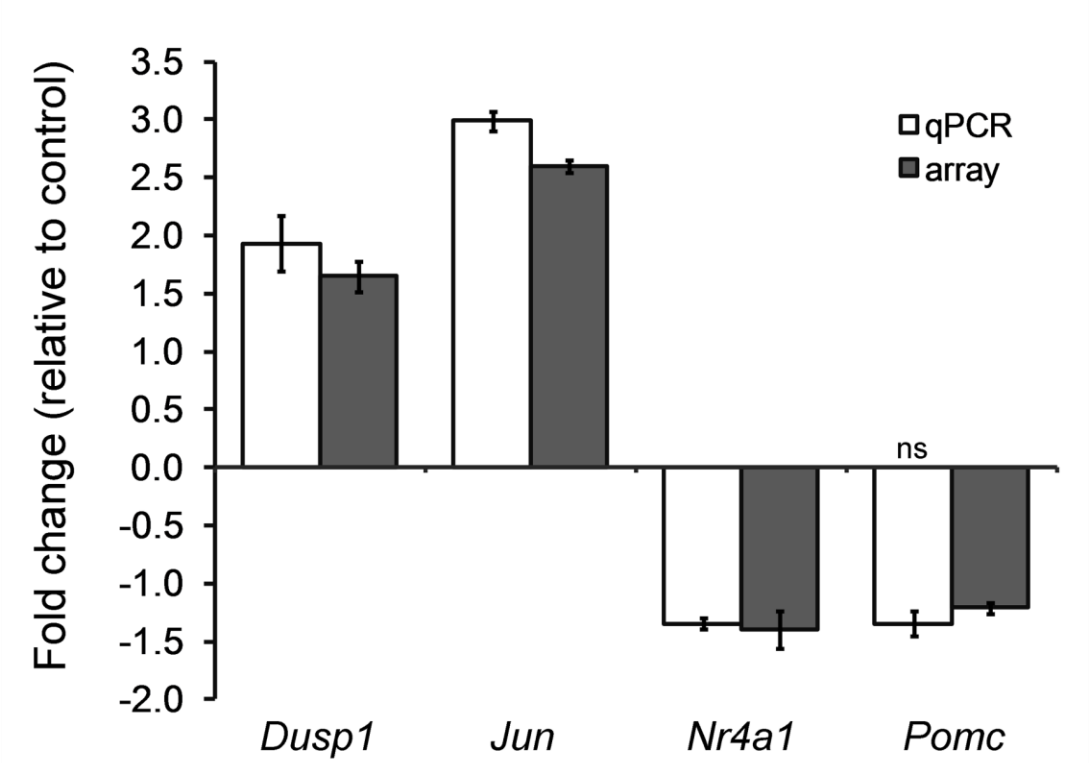


FIGURE 3.21. Quantitative RT-PCR confirmation of array-identified genes altered acutely following ethanol treatment at P7. Data are shown as mean (±SEM). Genes are listed alphabetically: *Dual-specificity phosphatase 1* (*Dusp1*), *jun proto-oncogene* (*Jun*), *nuclear receptor subfamily 4, group A, member 1* (*Nr4a1*), and *pro-opiomelanocortin-alpha* (*Pomc*). All genes were significantly different from controls (students' *t*-test, $p < 0.05$) with the exception of *Pomc*. ns: not significantly different from controls.

3.3 Long-term alterations to brain gene expression following ethanol exposure during synaptogenesis

In addition to identifying genes that were acutely affected by third-trimester ethanol exposure (4 hours post-injection at P7), I examined the brain gene expression changes in the adult brain (P60) resulting from ethanol exposure during synaptogenesis, or the human third trimester-equivalent (at postnatal days 4 and 7). These results represent alterations to the transcriptome that are initiated during neurodevelopment but persist into adulthood in mice displaying behavioural phenotypes relevant to FASD.

3.3.1 Gene expression changes in the adult brain

Expression microarray analysis identified 376 transcripts as differentially expressed between ethanol-treated mice and controls (1.2-fold change, FDR-correct $p < 0.05$). Changes to gene expression were relatively subtle, ranging from -2.21-fold (*Triadin*) to 3.3-fold (*Proline-rich protein 2*). Interestingly, the distribution of up- and down-regulated genes was not equal, with 92% of genes (361/376) identified as down-regulated as compared to controls. Further, the gene expression changes were subtle with 173/376 (47%) of genes showing less than a 1.3-fold change. A full list of genes meeting the stringency parameters is provided in Appendix B. This list was used for subsequent biological functions, pathway, and gene network analyses.

3.3.2 Array hierarchical clustering and functional annotation of genes altered in the adult brain

Hierarchical clustering analyses of each array indicated that the consistency between arrays was good, with the ethanol and control arrays cleanly pairing when grouped by probe intensity (Figure 3.22). When subjected to statistical stringency measures

for fold-change and *p*-value cutoffs, genes detected as up-regulated in the ethanol-treated samples as compared to controls were greatly reduced, indicating that many up-regulated genes showed low fold-changes and/or *p*-value significance. Due to the low number of significant up-regulated transcripts, the total gene list (both up- and down-regulated transcripts) was analyzed using DAVID (DENNIS et al. 2003) for over-represented gene ontology processes. DAVID analysis identified a number of biological processes associated with synaptic function, including glutamate receptor activity, synaptic transmission, and regulation of synaptic plasticity (Table 3.8). These annotations were comprised of a number of altered glutamate receptor subunits, including *Grin2b*, *Grin2c*, *Grin2d*, *Grin3b*, *Grik1*, and *Grik3*. These gene ontology functions shared multiple genes with other significant functions related to behaviour, such as "adult behaviour", "learning", and "locomotory behaviour". Interestingly, a significant number of genes were associated with folic acid metabolism, including *5, 10-methenyltetrahydrofolate synthetase* (*Mthfs*), and *folate hydrolase 1* (*Folh1*), and *5-methyltetrahydrofolate-homocysteine methyltransferase* (*Mtr*), involved in folate usage and one-carbon metabolism. Other gene clusters were also related to epigenetic processes such as chromatin organization and histone modification, indicating that these processes may be disrupted by trimester three-equivalent ethanol exposure. Other significant clusters of genes were associated with cytoskeletal transport and cell adhesion, protein phosphorylation, and mitogen-activated protein (MAP) kinase signaling cascade.

The transcripts identified were independently analyzed for over-representation within biological functions using Ingenuity Pathway Analysis™ (IPA) (Ingenuity Systems, Redwood, CA, www.ingenuity.com) (Table 3.9). The results supported the gene ontology biological functions and identified a significant number of differentially expressed genes associated with neurotransmission and synaptic plasticity, including again the glutamate receptor subunits *Grik1* and *Grin2b*. Also identified were a number of genes associated with retinoic acid signaling (*Fadd, Casp3, Rarb, Tnfsf10, Rxrb, Irf1*), lipid metabolism and accumulation, involving the hormone precursor

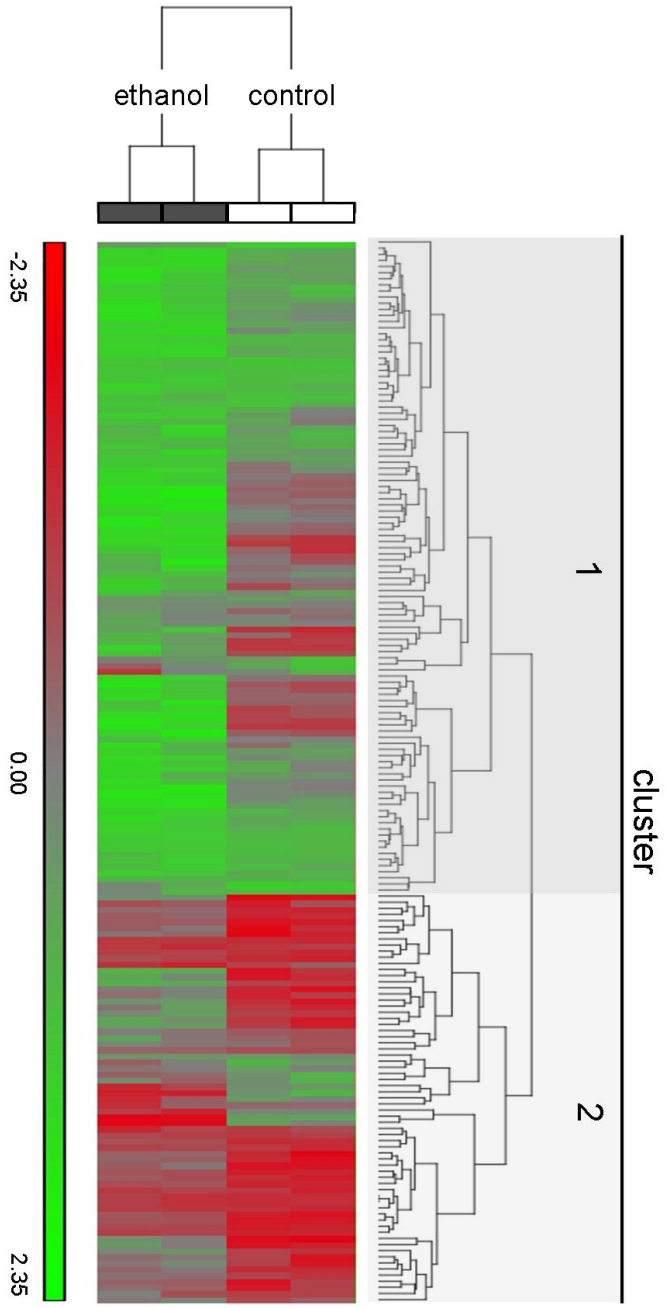


FIGURE 3.22. Heat map hierarchical clustering of arrays representing transcripts altered in the adult (P60) brain of control (n=2) and ethanol-treated (n=2) mice exposed at P4 +7 based on the normalized intensity of the probe sets. Heat map was generated by Partek® Genomics Suite software based on ANOVA-calculated significance levels and fold-changes. Clusters of up-regulated (green) and down-regulated (red) genes are indicated.

TABLE 3.8. Gene Ontology (GO) biological function annotation clustering of transcripts altered in the adult brain of mice treated with ethanol at P4+7.

GO term	GO ID	No. genes	*p*-value	Genes
glutamate receptor activity	0008066	9	5.84E-7	*Grm4, Grin2b, Grik1, Grin2c, Grik3, Grin2d, Ubc, Grin3b, Grind1*
synaptic transmission	0007268	16	0.000012	*Grik1, Ntf3, Nlgn1, Grm4, Grin2b, Grin2c, Grin2d, Chrm1, Cnr1, Pcdhb16, P2rx3, Dmd, Syngap1, Cacna1c, Sv2c, Apba1*
regulation of synaptic plasticity	0048167	8	0.00035	*Apoe, Grin2b, Grik1, Grin2c, Htt, P2rx3, Syngap1, Syngr1*
cellular ion homeostasis	0006873	15	0.00071	*Ftl1, Grik1, Ntf3, Htt, Grik3, Hexa, Pex5l, Vdr, Grin2b, Grin2c, Apoe, Grin2d, Pth, Cacna1c, Cln5*
folic acid and derivative metabolic process	0006760	4	0.00189	*Mthfs, Folh1, Mtr, Aldh1l2*
chromatin organization	0006325	15	0.00408	*Hist1h2bf, Crebbp, Hdac10, Dmap1, Mbd3, Hltf, Nap1l5, Hist2h4, Hist1h2bm, Hist1h3c, Hist1h4d, Kdm5a, Rbm14, Hdac8, Myst1, Kdm6b*
histone modification	0016570	7	0.00982	*Crebbp, Hdac10, Dmap1, Mbd3, Rbm14, Hdac8, Kdm6b*
regulation of protein kinase cascade	0045449	9	0.0120	*Traf3ip1, Spry1, Grin2b, Iltifb, Fgf9, Mdfic, Map3k1, Itsn1, Syngap1*
locomotory behaviour	0007626	11	0.0207	*Cmtm2a, Npas3, Grin2c, Htt, Hexa, Grin2d, Pcdh15, Gpr77, Cacna1c, Apba1, Itgam*
regulation of transcription, DNA-dependent	0006355	41	0.0220	*Nkap, Kchn1, Irx5, Pax3, Dmap1, Vdr, Trp53bp1, Npas3, Hsf1, Tgif2lx1, Elk4, Mdfic, Tead4, Pth, Etv2, Yeats2, Rarb, Nfatc1, Maf, Ntf3, Rxrb, Crebbp, Gsx1, Mbd3, Zfp59, Six4, Hltf, Mycn, Traf3ip1, Myt1l, Mef2d, Zfp9, Cmtm2a, Foxf1a, Irf5, Iltifb, Thrap3, Irf1, Rbm14, Kdm6b, Tbx19*

TABLE 3.8 continued…

GO term	GO ID	No. genes	*p*-value	Genes
learning	0007612	5	0.0262	*Grm4, Grin2b, Htt, Cacna1c, Syngap1*
cytoskeleton-dependent intracellular transport	0030705	4	0.0203	*Htt, Rhot2, Dst, Hap1*
neuron differentiation	0030182	15	0.0278	*Irx5, Sox1, Ntf3, Htt, Wnt3a, Gsx1, Ntng2, Pcdh15, Pax3, Ephb1, Tulp3, Dmd, Efna5, Syngap1, Dst*
regulation of phosphorylation	0042325	12	0.0298	*Egfr, Traf3ip1, Casp3, Spry1, Vav3, Iltifb, Mdfic, Map3k1, Tlr1, Il34, Dnajc3, Kitl*
regulation of MAPKKK cascade	0043408	6	0.0372	*Spry1, Grin2b, Fgf9, Mdfic, Map3k1, Syngap1*
adult behaviour	0030534	6	0.0373	*Grik11, Adh1, Hexa, Grin2d, Pcdh15, Cacna1c*
regulation of cell adhesion	0030155	6	0.0388	*Lama4, Vav3, Prlr, Srcin1, Fbln2, Cml5*

TABLE 3.9. Ingenuity Pathway Analysis™ biological functions associated with gene expression changes in the adult brain of mice treated with ethanol at P4+7.

Category	Function annotation	*p*-value	No. genes	Genes
Cell-to-cell signaling and interaction	neurotransmission	0.0000829	10	*Apoe, Chrm1, Cnr1, Ephb1, Grik1, Grin2b, Htr1a, Htt, Nlgn1, Ntf3*
Cell growth and proliferation	proliferation of cells	0.000378	17	*Alk, Anxa2, Cnr1, Efnb2, Egfr, Htt, Irx5, Lama4, mir-221, Mknk1, Mycn, Nbn, Npas3, Ntf3, Pomc, Spry1, Wnt3a*
Cell death	retinoic acid-mediated apoptosis signaling	0.00055	6	*Fadd, Casp3, Rarb, Tnfsf10, Rxrb, Irf1*
Cell death	cell viability of cerebral cortex cells	0.00141	5	*Alk, Apoe, Htt, Ksr1,Ntf3*
Lipid metabolism	secretion of steroid	0.00181	3	*Apoe, Pomc*
Lipid metabolism	accumulation of lipid	0.00360	3	*Chrm1, Cnr1. Htt*
Neurological disease	movement disorder	0.00534	9	*Anxa2, Atxn3, Capn3, Casp3, Ftl, Htt, Nbn, Ptges3, Ran*
Carbohydrate metabolism	quantity of carbohydrate	0.00553	3	*Htt, Inppl1, Insr*
Cell death	neuronal cell death	0.00707	21	*Alka, Apoe, Casp3, Cnr1, Efnb2, Fadd, Grin2b, Hsf1, Htt, Inppl1, Insr, Itsn1, Kitlg, Ksr1, Map3k1, Mycn, Nbn, Ntf3, Smpd2, Tyk2, Xrcc5*
Cell-to-cell signaling and interaction	plasticity of synapse	0.0144	6	*Apoe, Grik1, Grin2b, Htt, Inppl1, Syngap1*
Nervous system development and function	quantity of microglia	0.0197	2	*Apoe, Htt*

TABLE 3.9 continued…

Category	Function annotation	*p*-value	No. genes	Genes
Cell morphology	formation of cellular protrusions	0.0329	17	*Apoe, Dst, Efna5, Efnb2, Egfr, Ephb1, Grm4, Htt, Kif23, Nbl1, Ntf3, Ntng2, Pcdh15, Rhog, Syne1, Syngap1, Wnt3a*
Cell death	cell death of dopaminergic neurons	0.0344	3	*Casp3, Efnb2, Fadd*
Neurological disease	gliosis of hippocampus	0.0400	2	*Apoe, Htt*
Nucleic acid metabolism	concentration of cyclic AMP	0.0400	2	*Htt, Pth*
Cellular assembly and organization	transport of synaptic vesicles	0.0443	3	*Apba1, Htt, Itsn1*
Cellular assembly and organization	microtubule dynamics	0.0446	18	*Apoe, Dst, Efna5,Efnb2, Egfr, Ephb1, Grm4, Htr1a, Htt, Kif23, Nbl1, Ntf3, Ntng2, Pcdh15, Rhog, Syne1, Syngap1, Wnt3a*

protein *pro-opiomelanocortin-alpha* (*Pomc*), muscarinic cholinergic and cannabinoid receptors *Chrm1* and *Cnr1*, as well as groups of genes associated with cytoskeletal function including genes important for the transport of synaptic vesicles and microtubule dynamics.

3.3.3 Canonical signaling pathways associated with gene expression changes in the adult brain

Genes differentially expressed between control versus ethanol-treated adults were analyzed using the IPA for associations to canonical biological pathways (Table 3.10). Consistent with the biological functions analyses, glutamate receptor signaling and synaptic long-term potentiation were identified, driven by the altered expression of a number of glutamate receptor subunits. Also identified was a significant cluster of genes associated with retinoic acid signaling, ephrin receptor signaling, calcium signaling, and neuronal nitric oxide synthase (nNOS) signaling. Interestingly, Circadian rhythm signaling and serotonin receptor signaling were also identified, indicating that both glutamate and serotonergic-based neurotransmission may be affected by trimester three-equivalent ethanol exposure. Other pathways of interest were folate-mediated one-carbon metabolism, also identified by the biological functions analyses, and mammalian target of rapamycin (mTOR) signaling.

3.3.4 Interacting gene networks involved in gene expression changes in the adult brain

The genes detected as altered in the brain of adult mice exposed to alcohol at P4 and P7 were analyzed using the gene network analysis tool within the Ingenuity® software platform to predict interacting molecular networks. The predicted networks were highly interrelated and focused around various glutamate receptor subunits. While five networks were identified as significant, most of the interactions

between altered transcripts were described by the first two, associated with the functions of "neurological disease, psychological disorders, behaviour" (NPD) (focus molecules = 17) and "cell morphology, cell-to-cell signaling and interaction, nervous system development and function" (CCN) (focus molecules = 14). These networks are shown in Figures 3.23 and 3.24. The NPD network contained *huntingtin* (*Htt*) as a hub molecule, which was not differentially expressed acutely following ethanol exposure but was identified as a major hub of the short-term gene network. Other hub molecules included *glutamate receptor, ionotropic, N-methyl D-aspartate 2B* (*Grin2b*) and the apoptosis enzyme *caspase 3* (*Casp3*), both of which were also down-regulated. The CCN network also contained a number of glutamate receptor subunits and *Grin2b* appeared again as a network hub molecule. Other hub molecules included the synaptic protein *discs, large homolog 4 (Drosophila)* (*Dlg4)* and, interestingly, *retinoic acid receptor, alpha* (*Rara*). Given that the functions associated with these networks were interrelated, the networks were merged to evaluate molecules that may be effectors of the neurological changes associated with third trimester ethanol exposure (Figure 3.25). The merged networks implicated *Htt* as a central hub, as well as the glutamate receptor proteins *Grin2b* and *glutamate receptor, ionotropic, AMPA1 (alpha 1)* (*Gria1*). *Neurotrophin 3* (*Ntf3*), which appeared within many gene clusters identified by the biological functions analyses, also appeared within the merged network as a central hub molecule, as did *apolipoprotein E* (*Apoe*).

TABLE 3.10. Ingenuity® canonical pathways associated with transcripts altered in the adult brain of mice treated with ethanol at P4+7.

Canonical pathway	*p*-value	No. molecules	Genes
Glutamate receptor signaling	0.0000074	9	*Grin2b, Grin3b, Gnb3, Grid1, Grin2c, Grin2d, Grik3, Grm4, Grik1*
Retinoic acid-mediated apoptosis signaling	0.000550	6	*Fadd, Casp3, Rarb, Tnfsr10, Rxrb, Irf1*
Ephrin Receptor Signaling	0.00224	11	*Grin2b, Efnb2, Ephb1, Grin3b, Gnb3, Itsn1, Grin2c, Efna5, Grin2d, Vegfc, Figf*
Calcium Signaling	0.00417	10	*Rcan1, Grin2b, Grin3b, Hdac8, Grin2c, Grin2d, Mef2d, Trdn, Hdac10, Grik1*
nNOS Signaling in Neurons	0.00447	5	*Grin2b, Grin3b, Grin2c, Grin2d, Capn3*
Aminosugars Metabolism	0.00501	6	*Gnpnat1, Gnpda1, Pde12, Mdp1, Hexa, Gfpt2*
Circadian Rhythm Signaling	0.00692	4	*Grin2b, Grin3b, Grin2c, Grin2d*
One Carbon Pool by Folate	0.00794	3	*Tyms, Mthfs, Mtr*
CREB Signaling in Neurons	0.0170	9	*Grin2b, Gnb3, Polr2d, Grid1, Grin2c, Grin2d, Grik3, Grm4, Grik1*
mTOR Signaling	0.0288	14	*Fau, Rhog, Rps10, Rhot2, Rptor, Vegfc, Figf, Insr, Rpsa, Efnb2, Ephb1, Gnb3, Itsn1, Vav3*
Ephrin B Signaling	0.0295	5	*Efnb2, Ephb1, Gnb3, Itsn1, Vav3*
Serotonin Receptor Signaling	0.0347	3	*Htr5b, Htr1a, Htr1d*
Synaptic Long Term Potentiation	0.0347	6	*Grin2b, Grin3b, Grin2c, Grin2d, Cacna1c, Grm4*

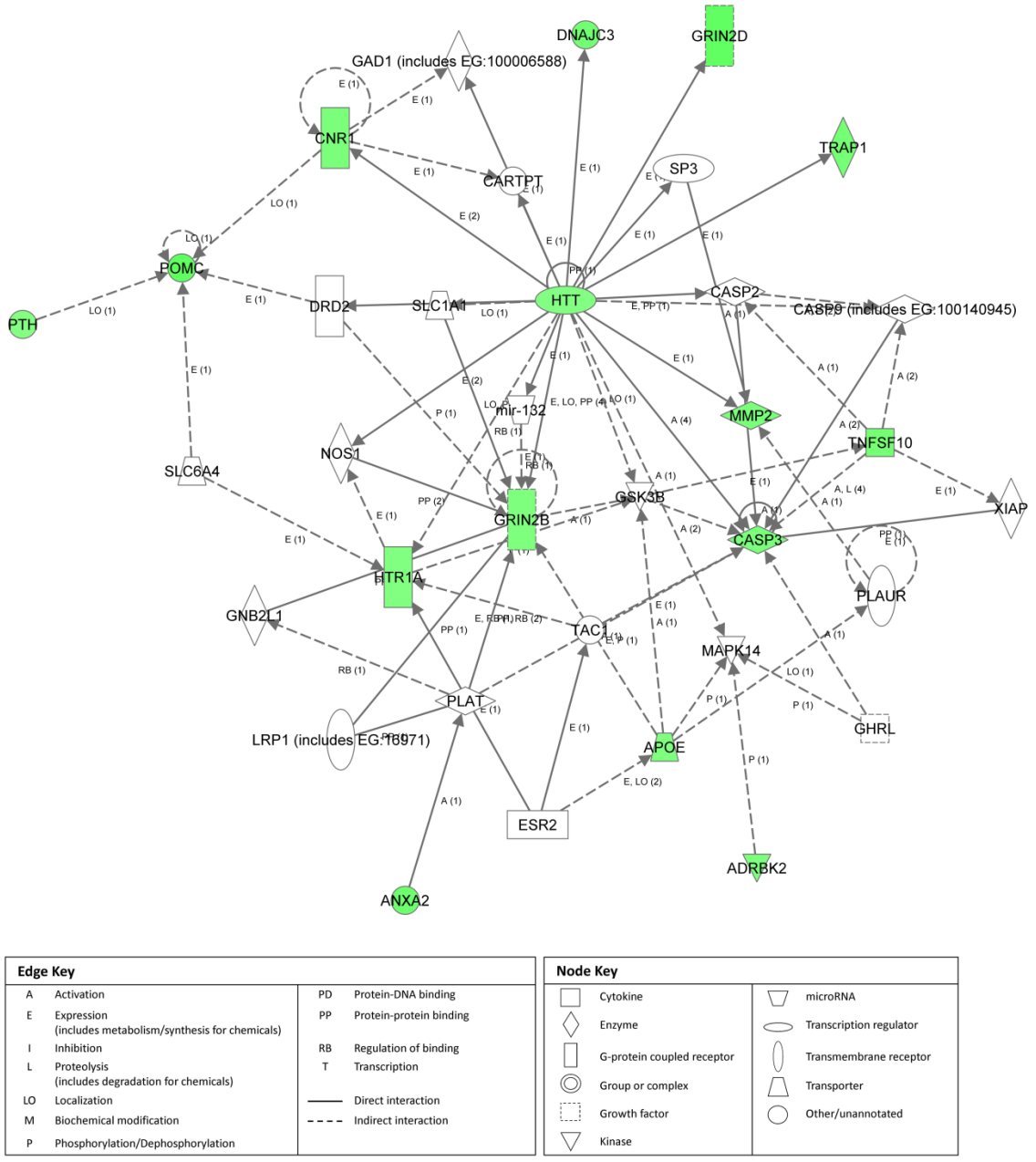


FIGURE 3.23. Ingenuity Pathway Analysis™ network analysis indicating annotated interactions between transcripts altered in the adult brain by ethanol exposure at P4+7 associated with 'neurological disease, psychological disorders, behaviour' (NPD). Down-regulated (green) genes are indicated. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

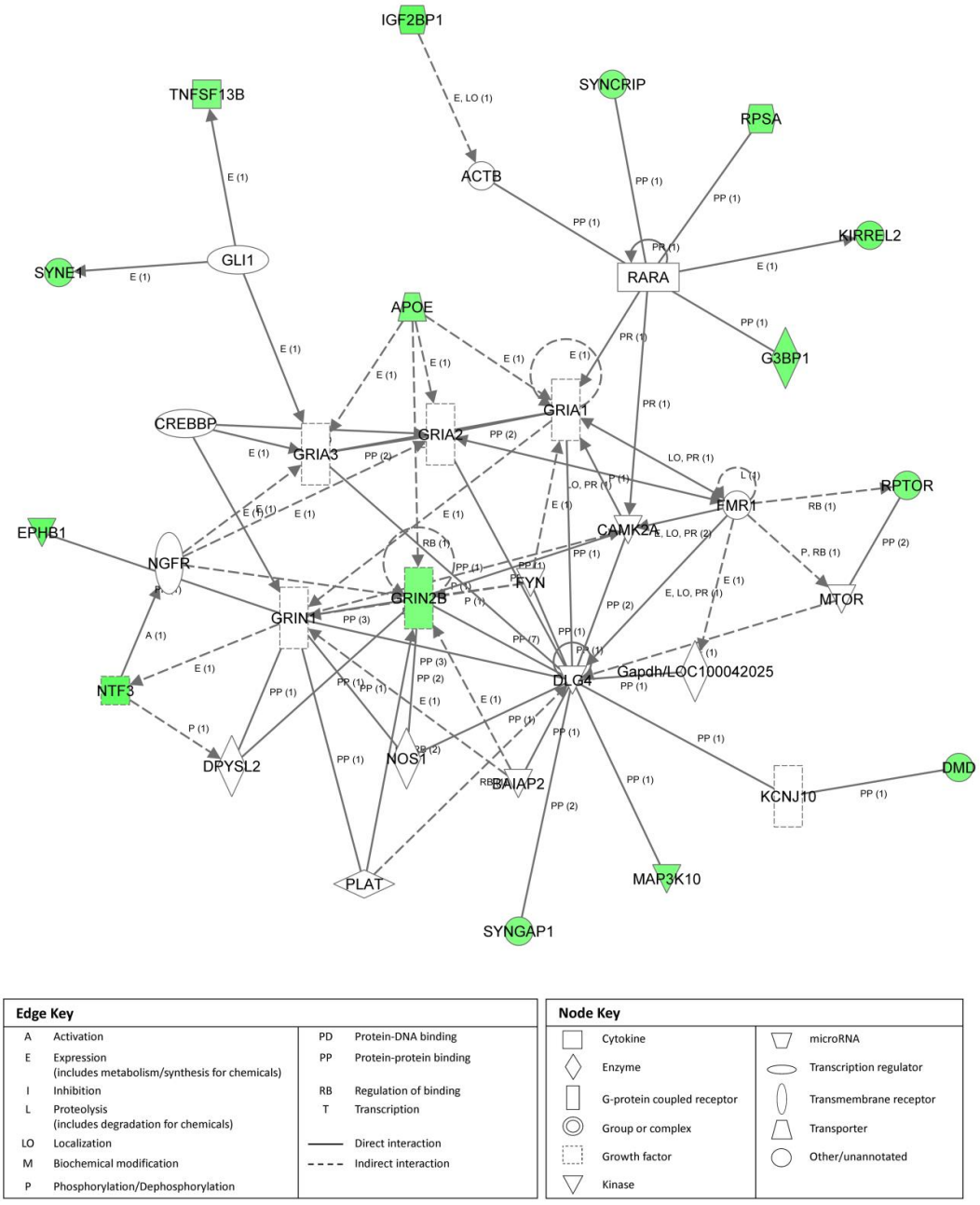
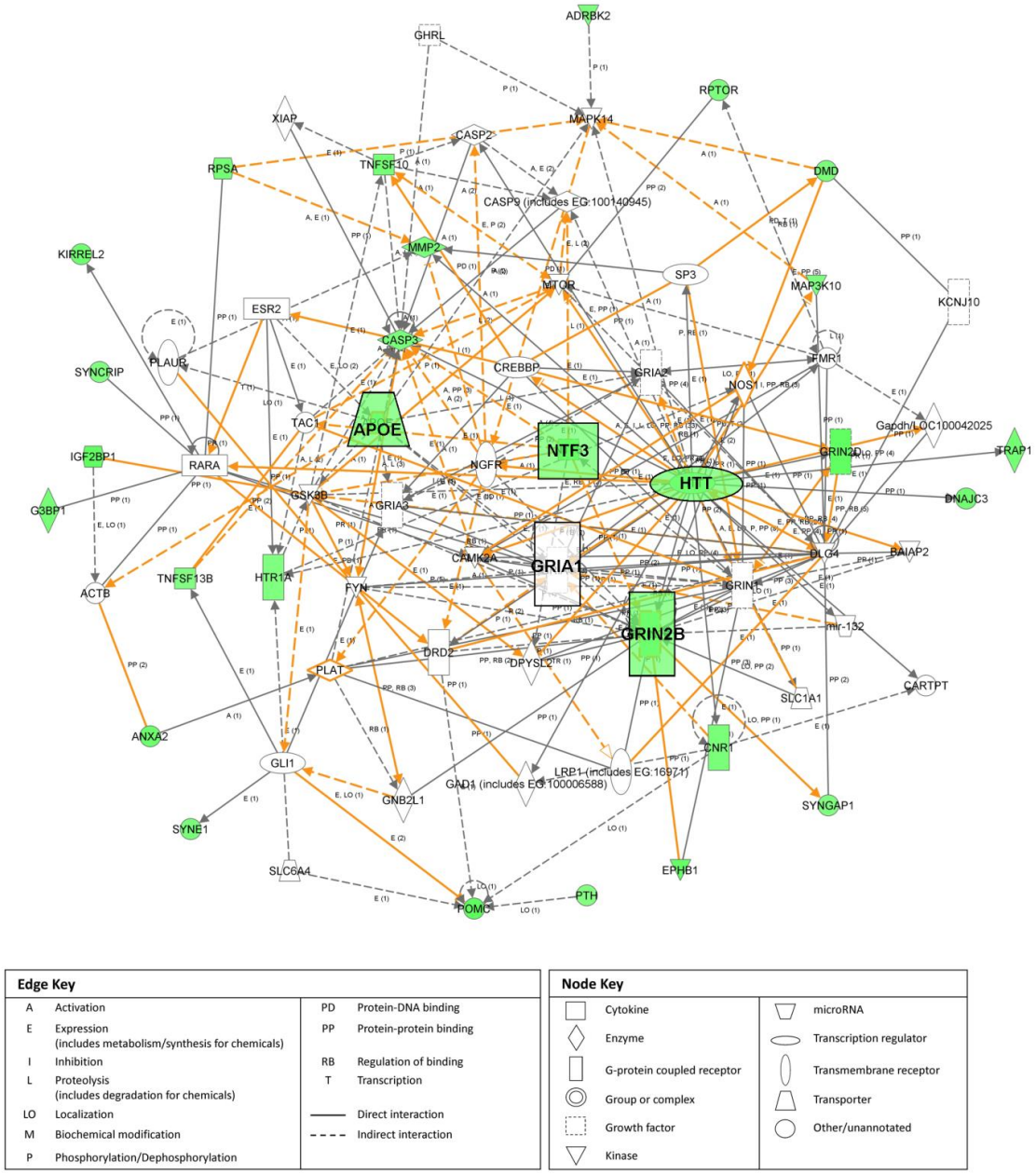


FIGURE 3.24. Ingenuity Pathway Analysis™ network analysis indicating annotated interactions between transcripts altered in the adult brain by ethanol exposure at P4+7 associated with 'cell morphology, cell-to-cell signaling and interaction, nervous system development and function' (CCN). Down-regulated (green) genes are indicated. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.



Edge Key

A	Activation	PD	Protein-DNA binding
E	Expression (includes metabolism/synthesis for chemicals)	PP	Protein-protein binding
I	Inhibition	RB	Regulation of binding
L	Proteolysis (includes degradation for chemicals)	T	Transcription
LO	Localization	———	Direct interaction
M	Biochemical modification	- - -	Indirect interaction
P	Phosphorylation/Dephosphorylation		

Node Key

Cytokine	microRNA
Enzyme	Transcription regulator
G-protein coupled receptor	Transmembrane receptor
Group or complex	Transporter
Growth factor	Other/unannotated
Kinase	

FIGURE 3.25. Merged Ingenuity Pathway Analysis™ networks showing interactions between the most significant gene networks (see Figures 3.23 and 3.24) affected in the adult brain following ethanol exposure at P4+7. Down-regulated (green) genes are indicated. Centralized "hub" molecules linking multiple interacting genes are enlarged. Arrowhead indicates the direction of action. Notations in brackets adjacent to the edge key labels indicate the number of lines of evidence (publically available data sourced by Ingenuity®) supporting the interaction.

3.3.5 Quantitative RT-PCR confirmation of array-identified mRNA levels

Four genes detected by the array analysis were chosen for confirmation by real-time quantitative RT-PCR (qRT-PCT), selected based on their fold changes and prominence in biological functions and networks identified as affected following ethanol treatment. Genes chosen for qRT-PCR analysis were: *cannabinoid receptor 1 (brain)* (*Cnr1*), *glutamate, ionotropic, N-methyl D-aspartate 2B* (*Grin2b*), *5-hydroxytryptamine (serotonin) receptor 5B* (*Htr5b*), and *pro-opiomelanocortin-alpha* (*Pomc*). Real-time results as compared to array fold-changes are shown in Figure 3.26. Fold-changes calculated from deltaCt values showed good consistency with the array results. All genes confirmed as significantly different from controls (student's *t*-test, $p < 0.05$).

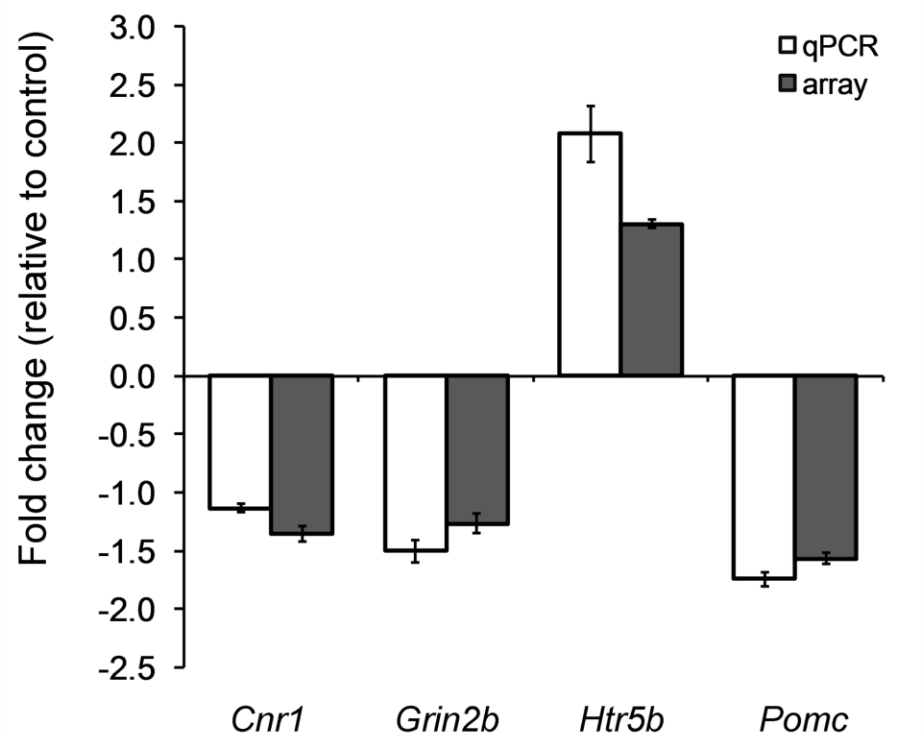


FIGURE 3.26. Quantitative RT-PCR confirmation of array-identified changes in mRNA levels in the adult (P60) brain of mice treated with ethanol at P4+7. Data are shown as mean (±SEM). Genes are listed alphabetically: *cannabinoid receptor 1 (brain)* (*Cnr1*), *glutamate receptor, ionotropic, N-methyl D-aspartate 2B* (*Grin2b*), *5-hydroxytryptamine (serotonin) receptor 5B* (*Htr5b*), and *pro-opiomelanocortin-alpha* (*Pomc*). Expression of all genes was significantly different from controls (students' *t*-test, $p < 0.05$).

3.4 Comparison of acute and long-term gene expression changes resulting from ethanol exposure during synaptogenesis

The gene expression changes that were identified by the short-term microarray analysis (4 hours post-treatment) were compared to the transcript list generated by adult gene expression analysis to evaluate potential common genes and pathways that may represent the molecular adaptation of neural cells to developmental ethanol exposure. Twelve transcripts that showed altered expression acutely following ethanol exposure at P7 were also detected as differentially expressed in the adult brain (Table 3.11). Five of these genes showed down-regulation in both samples, while the remaining seven showed an inverse relationship (upregulated acutely following ethanol exposure but down-regulated in the adult brain samples) between the acute versus long-term gene expression changes. Among these were genes critical to both neurodevelopment and adult brain function, including glutamate signaling (*Folh1* and *Grin2b*), serotonin signaling (*Htr1d*, *Slc16a6*), hypothalamic-pituitary-adrenal stress response (*Pomc*), and cellular organization (*Hmcn1*, *Pcdhb18*). Other genes identified have been previously implicated in neurological phenotypes, such as *seizure threshold 2* (*Szt2*) and *XRCC6 binding protein 1* (*Xrcc6bp1*), or have roles as zinc finger-containing transcription factors (*Zbtb10* and *Zfp9*).

The DAVID gene ontology biological functions, IPA gene ontology and IPA canonical pathways associated with the altered short- and long-term transcripts were compared to identify common potentially-targeted pathways that may represent some continuity between the acute and the persistent effects of ethanol exposure during synaptogenesis. This analysis revealed that, while the number of overlapping transcripts identified by both the short- and long-term gene arrays was limited, there were a number of closely associated biological functions and canonical pathways represented within both gene lists (Table 3.12). Genes associated with synaptic transmission and plasticity were present in both the short- and long-term gene lists, with both showing a number of glutamate receptor subunit transcripts including

TABLE 3.11. Genes altered by alcohol exposure acutely following treatment (at P7) that remain altered during early adulthood (at P60).

Accession No.	Gene Symbol	Gene Name	Chr. Location	Fold Change	
				acute (P7)	long-term (P60)
NM_016770	*Folh1*	*folate hydrolase 1*	7 D1-D2	1.44	-1.28
NM_008171	*Grin2b*	*glutamate receptor, ionotropic, NMDA2B (epsilon 2)*	6 G1	1.25	-1.27
NM_144835	*Heatr1*	*HEAT repeat containing 1*	13 A1	-1.31	-1.33
NM_001285482.1	*Htr1d*	*5-hydroxytryptamine (serotonin) receptor 1D*	4 D3	1.23	-1.27
NM_001024720	*Hmcn1*	*hemicentin 1*	1 G1	-1.20	-1.28
NM_053143	*Pcdhb18*	*protocadherin beta 18*	18 B3	1.21	-1.43
NM_008895	*Pomc*	*pro-opiomelanocortin-alpha*	12 A1	-1.21	-1.57
NM_001029842	*Slc16a6*	*solute carrier family 16 (monocarboxylic acid transporters), member 6*	11 E1	1.35	-1.45
NM_198170	*Szt2*	*seizure threshold 2*	4 D2	-1.22	-1.24
NM_026858	*Xrcc6bp1*	*XRCC6 binding protein 1*	10 D3	-1.20	-1.36
NM_177660	*Zbtb10*	*zinc finger and BTB domain containing 10*	3 A1	1.38	-1.30
NM_011763	*Zfp9*	*zinc finger protein 9*	6 F1	1.25	-1.29

TABLE 3.12. Related gene ontology biological functions and canonical pathways associated with transcripts altered acutely following ethanol treatment at P7 and those altered in the adult (P60) brain of mice treated at P4+7.

Short term response				*Long term response*			
Function	Identified by[a]	*p*-value	Genes	Function	Identified by[a]	*p*-value	Genes
regulation of synaptic transmission	DAVID	0.0464	*Grin2b, Edn1, Grin2a, Cpeb1*	synaptic transmission	IPA GO	0.00014	*Apoe, Chrm1, Cnr1, Ephb2, Grik1, Grin2b, Htr1a, Nrgn1, Ntf3*
plasticity of neuronal synapse	IPA GO	0.0371	*Cpeb1, Grin2b*	plasticity of synapse	IPA GO	0.0144	*Apoe, Grik1, Grin2b, Htt, Inppl1, Syngap1*
				glutamate receptor signaling	IPA Canonical pathway	0.000007	*Grin2b, Grin3b, Gnb3, Grid1, Grin2c, Grin2d, Grik3, Grm4, Grik1*
steroid hormone receptor activity	DAVID	0.0425	*Rxrg, Nr4a1, Nrob1*	secretion of steroid	IPA GO	0.0018	*Apoe, Pomc*
hormone activity	DAVID	0.0401	*Inhba, Pomc, Apln, Adcyap1*				
secretion of corticosterone	IPA GO	0.0193	*Pomc*				
Circadian rhythm signaling	IPA Canonical pathway	0.000244	*Adcyap1, Cry1, Grin2a, Grin2b, Per1, Per2*	Circadian rhythm signaling	IPA Canonical pathway	0.00692	*Grin2b, Grin3b, Grin2c, Grin2d*
TR/RXR activation	IPA Canonical pathway	0.0172	*Dio2, Ldlr, Pik3r1, Rxrg, Slc2a1*	retinoic acid-mediated apoptosis signaling	IPA Canonical pathway	0.00055	*Fadd, Casp3, Rarb, Tnfsf10, Rxrb, Irf1*
PTEN signaling	IPA Canonical pathway	0.0203	*Dio2, Ldlr, Pik3r1, Rxrg, Slc2a1*	mTOR Signaling	IPA Canonical pathway	0.0288	*Fau, Rhog, Rps10, Rptor, Vegfc, Figf, Insr, Rpsa*

[a]Biological functions or canonical pathways identified by DAVID gene ontology functional clustering, IPA gene ontology functional clustering, or IPA canonical pathways analysis.

Grin2a, *Grin2b*, and *Grik1* . Also common between the short- and long-term lists were GO functions related to hormone activity and signaling, specifically steroid and corticosterone secretion and, interestingly, Circadian rhythm signaling. Finally, retinoic acid signaling and the canonical PTEN/ATK/mTOR signaling pathway were identified as associated with transcripts within both the short- and long term gene lists.

3.5 microRNA expression changes in the adult brain following ethanol exposure during synaptogenesis

Given that the long-term changes to mRNA levels may be mediated by epigenetic regulatory mechanisms, I evaluated the changes to microRNA (miRNA) expression in the adult brain (P60) following ethanol exposure at P4 and P7 as one possible regulatory influence for the adult gene expression observations. miRNA expression array analysis identified 33 miRNA transcripts that were differentially expressed between control and ethanol-treated whole brain RNA (fold change > 1.2, FDR-corrected $p = 0.05$) (Appendix C). Fold changes ranged from -4.17 (*mmu-miR-704*) to 2.44 (*mmu-miR-721*). Again, most miRNAs were down-regulated following ethanol exposure, with 73% (24/33) showing a negative fold-change as compared to saline-treated controls. A complete miRNA list showing differential expression between control and ethanol-treated samples is provided in Appendix C.

3.5.1 Relationship between miRNA and predicted target mRNA expression changes in the adult brain

Ingenuity Target Filtering™ analysis was performed using the gene lists from the miRNA array and the long-term (P60) mRNA expression array to assess potential regulatory interactions between altered miRNAs and mRNAs in the adult brain of mice exposed to ethanol at P4+7. High-confidence (experimentally shown or strongly

predicted based on sequence) interactions showing an inverse relationship between altered miRNA and mRNA expression were included. A number of miRNAs were paired with a number of potential mRNA targets (Table 3.13), suggesting that a single miRNA may have multiple targets and exert an effect on multiple biological pathways, shown in Table 3.13. These genes were associated with a number of previously-identified gene ontology functions and canonical pathways including axonal guidance (*Adam9*, *L ingo1*, *Sema6d*, *Shank2*, *Prkcb*, and *Efnb2*), cytoskeletal organization (*Arhgap6*, *Shank2*, *Rhou*), intracellular signaling (*Lingo1*, *Lrrk2*, *Map3k7*, *Mras*, *Prkcb*, *Rhou*, and *Ppm1b*), chromatin organization and structure (*Ino80, Ncor2*), and transcriptional regulation (*Rbm9, Spen, Ncor2, Ncoa3*). Interestingly, hormonal signaling-related transcripts were abundant among the potential mRNA targets showing altered expression in the adult brain, including corticotrophin-releasing hormone signaling, glucocorticoid receptor signaling, estrogen, and androgen receptor signaling (*Adam9*, *Cnr1*, *Mras*, *Ncor2*, *Ncoa3*, *Prkcb*, *Ppargc1a*, *Rbm9*, and *Spen*). Lastly, altered mRNA transcripts identified as potential targets of miR-721 were associated with retinoid acid signaling (*Ncor2*, *Irf1*, *Ncoa3*, *Ppargc1*) and the canonical PI3K/AKT/mTOR signaling pathway (*Tsc1*).

TABLE 3.13. miRNAs and putative mRNA transcript targets showing altered inverse expression in the adult (P60) brain following ethanol exposure at P4+7 and associated biological pathways.

miRNA ID	miRNA fold change	Target gene ID	Target mRNA fold change	Associated pathways
mmu-miR-26b	1.284	*Adam9*	-1.114	Axonal Guidance Signaling
mmu-miR-26b	1.284	*Cnr1*	-1.332	Corticotropin Releasing Hormone Signaling G-Protein Coupled Receptor Signaling Reelin Signaling in Neurons cAMP-mediated signaling
mmu-miR-26b	1.284	*Exoc8*	-1.221	Cdc42 Signaling
mmu-miR-26b	1.284	*Hs6st1*	-1.523	Cysteine Metabolism LPS/IL-1 Mediated Inhibition of RXR Function Xenobiotic Metabolism Signaling
mmu-miR-26b	1.284	*Lingo1*	-1.153	Axonal Guidance Signaling
mmu-miR-26b	1.284	*Map3k7*	-1.193	Acute Phase Response Signaling BMP signaling pathway
mmu-miR-26b	1.284	*Mras*	-1.200	14-3-3-mediated Signaling AMPK Signaling Actin Cytoskeleton Signaling Acute Phase Response Signaling Androgen Signaling
mmu-miR-26b	1.284	*Pfkfb3*	-1.143	AMPK Signaling Fructose and Mannose Metabolism
mmu-miR-26b	1.284	*Ppm1b*	-1.429	AMPK Signaling
mmu-miR-26b	1.284	*Rhou*	-1.250	CXCR4 Signaling Glioblastoma Multiforme Signaling Glioma Invasiveness Signaling HMGB1 Signaling
mmu-miR-26b	1.284	*Sema6d*	-1.181	Axonal Guidance Signaling
mmu-miR-26b	1.284	*Shank2*	-1.291	Axonal Guidance Signaling
mmu-miR-26b	1.284	*Tab3*	-1.253	NF-κB Signaling
mmu-miR-26b	1.284	*Tdrd7*	-1.568	Pluripotency
mmu-miR-26b	1.284	*Ube2j1*	-1.151	Hypoxia Signaling Protein Ubiquitination Pathway

Table 3.13 continued...

miRNA ID	miRNA fold change	Target gene ID	Target mRNA fold change	Associated pathways
mmu-miR-184	1.485	*Ncor2*	-1.273	Aryl Hydrocarbon Receptor Signaling Estrogen Receptor Signaling Glucocorticoid Receptor Signaling Huntington's Disease Signaling LXR/RXR Activation PPAR Signaling PPARα/RXRα Activation RAR Activation
mmu-miR-184	1.485	*Prkcb*	-1.115	14-3-3-mediated Signaling Aldosterone Signaling Androgen Signaling Axonal Guidance Signaling β-adrenergic Signaling Protein Kinase A Signaling cAMP-mediated signaling
mmu-miR-721	2.437	*Akap11*	-1.111	
mmu-miR-721	2.437	*B4galt5*	-1.257	N-Glycan Biosynthesis
mmu-miR-721	2.437	*Cnr1*	-1.332	Corticotropin Releasing Hormone Signaling G-Protein Coupled Receptor Signaling Reelin Signaling in Neurons cAMP-mediated signaling
mmu-miR-721	2.437	*Efnb2*	-1.261	Axonal Guidance Signaling Ephrin Receptor Signaling
mmu-miR-721	2.437	*Fam20b*	-1.271	Inositol Phosphate Metabolism Nicotinate and Nicotinamide Metabolism
mmu-miR-721	2.437	*Ino80*	-1.247	Purine Metabolism
mmu-miR-721	2.437	*Irf1*	-1.323	Production of Nitric Oxide and Reactive Oxygen Species Retinoic acid Mediated Apoptosis Signaling Role of PKR in Interferon Induction
mmu-miR-721	2.437	*Lrrk2*	-1.196	Mitochondrial Dysfunction
mmu-miR-721	2.437	*Ncoa3*	-1.247	Aryl Hydrocarbon Receptor Signaling Estrogen Receptor Signaling Glucocorticoid Receptor Signaling PPARα/RXRα Activation Role of Wnt/GSK-3β Signaling TR/RXR Activation VDR/RXR Activation

Table 3.13 continued…

miRNA ID	miRNA fold change	Target gene ID	Target mRNA fold change	Associated pathways
mmu-miR-721	2.437	*Pfkfb3*	-1.143	AMPK Signaling Fructose and Mannose Metabolism
mmu-miR-721	2.437	*Ppargc1a*	-1.123	Estrogen Receptor Signaling FXR/RXR Activation PPAR Signaling PPARα/RXRα Activation PXR/RXR Activation RAR Activation TR/RXR Activation Xenobiotic Metabolism Signaling
mmu-miR-721	2.437	*Rbm9*	-1.311	Estrogen Receptor Signaling
mmu-miR-721	2.437	*Shank2*	-1.291	Axonal Guidance Signaling
mmu-miR-721	2.437	*Spen*	-1.140	Estrogen Receptor Signaling
mmu-miR-721	2.437	*Sphk2*	-1.304	Ceramide Signaling PDGF Signaling Sphingolipid Metabolism
mmu-miR-721	2.437	*Tsc1*	-1.381	14-3-3-mediated Signaling AMPK Signaling Glioblastoma Multiforme Signaling PI3K/AKT Signaling mTOR Signaling
mmu-miR-721	2.437	*Wdfy3*	-1.327	N-Glycan Biosynthesis
mmu-miR-1970	-1.694	*Arhgap6*	1.188	RhoA Signaling

3.5.2 Confirmation of *miR-26b* and putative mRNA target *Cnr1* inverse expression by quantitative RT-PCR

Given the roles of *cannabinoid receptor 1* (*Cnr1*) in neurological phenotypes including corticotropin releasing hormone signaling, the relationship between *Cnr1* and its putative regulatory miRNA *miR-26b* were confirmed by qRT-PCR. The down-regulation of *Cnr1* was confirmed successfully, showing reduced transcript levels in the brain of adult (P60) mice treated with ethanol at P4 and P7 as compared to saline-treated controls (fold-change = -1.13 ± 0.04, p = 0.0042) (Figure 3.26). The up-regulation of *mmu-miR-26b* also confirmed successfully, showing increased transcript levels (fold-change = +3.70 ± 0.20) in the adult brain of mice treated with ethanol during the trimester three-equivalent ($p < 0.001$).

Chapter 4
DISCUSSION

This project was initiated to evaluate if human FASD could be effectively modeled using mice and to utilize that model to investigate the molecular underpinnings of the range of neural and phenotypic abnormalities associated with these spectrum disorders. The work included in this thesis sought to assess if a binge-like dose of ethanol during third trimester-equivalent could produce, in mice, behavioural and cognitive phenotypes consistent with human FASD, and if that model could then be used to elucidate molecular alterations in the brain that may initiate and maintain these phenotypes. The results presented indicate that a trimester three-equivalent binge exposure to alcohol is sufficient to produce multiple behavioural phenotypes that reflect those commonly observed in human children, adolescents, and adults prenatally exposed to alcohol. Further, there is substantial specificity and consistency with regard to the genes, biological pathways, and processes that are associated with this exposure paradigm both acutely following ethanol exposure and those that persist into adulthood. Finally, utilization of the process outlined in this thesis is discussed in terms of its efficacy to model human FASD, its contributions furthering our understanding of the

disorder, and to evaluate the results within the context of potential diagnostic or therapeutic strategies.

4.1 Effects of ethanol exposure during synaptogenesis on behaviour

The results presented indicate that a binge-like dose of ethanol within a narrow neurodevelopmental period during the trimester three-equivalent in mice is sufficient to produce a number of behavioural and cognitive phenotypes relevant to FASD in humans.

This finding is interesting given that a large proportion of prenatally-exposed infants are likely exposed via low to moderate consumption (1-2 drinks) at multiple points during gestation (KESMODEL and OLSEN 2001, ZELNER and KOREN 2013). Binge drinking is even more rare, and only a small percentage of women (approximately 16%) of binge-drinkers self-report on binge-like alcohol consumption after the first trimester (GLADSTONE et al. 1997). As such, most FASD-related research has focused on physiological or cognitive consequences of early-gestation exposure. High doses of alcohol consumed during the first trimester of pregnancy are associated with distinctive cranio-facial abnormalities, decreases in brain volume, and detectable changes to neuronal tract connectivity in numerous brain regions (ARCHIBALD et al. 2001, LEBEL et al. 2008, NORMAN et al. 2009, LIPINSKI et al. 2012). These features may assist in the identification of alcohol-exposed children; however, the majority of prenatally- exposed offspring show changes in cognitive function and behaviour in the absence of any physiological abnormalities (MATTSON et al. 1998, GREEN et al. 2009). It is therefore important to understand the effects of alcohol on the developing brain at all developmental stages to understand how timing contributes to specific phenotypic changes, even in the absence of physiological markers. Further, it is prudent to understand the biological mechanisms that underlie certain windows

of ethanol vulnerability such as synaptogenesis, which is a critical period for establishing the basis of neuronal communication that supports adult brain function.

4.1.1 Effects of ethanol exposure on neonatal development

Early neonatal indications of prenatal alcohol exposure are useful both diagnostically and prognostically, as they can facilitate FASD identification and intervention to perhaps improve the later-life secondary behavioural and cognitive outcomes associated with these disorders (STREISSGUTH et al. 2004). The weight of pups treated with ethanol at P4 or P7 was not affected by ethanol treatment, while exposure at both postnatal days (P4 and P7) did significantly decrease the weight of neonates compared to their saline-treated controls. This is likely attributable to reduced feeding ability and nutritional intake rather than a direct effect of alcohol (BARRON et al. 1991), however it is interesting that this difference persisted well into adolescence. Low birth weight has epidemiologically associated with moderate to high alcohol consumption, including late-gestational binges (WHITEHEAD and LIPSCOMB 2003), though, studies including self-reports of binge-like alcohol consumption during the third trimester in humans are rare. Animal studies corroborate these findings, indicating that high, punctuated ethanol exposures producing low neonatal weight (ABEL 1996, WOZNIAK et al. 2004); although, the pattern of ethanol administration between studies varies considerably making comparisons difficult. Reduced birth and neonatal weight generally appears to be dose-dependent with no clear threshold, but is consistently associated with prenatal alcohol exposure.

Prenatal alcohol exposure has been linked to infant delays in muscle tone, motor coordination, reflexes and balance (STAISEY and FRIED 1983, COLES et al. 1985, JACOBSON et al. 1998, KALBERG et al. 2006), but literature on the effects of alcohol exposure on early-life coordination and neuromuscular reflexes is somewhat

lacking in animal studies, particularly when compared to quantity of research examining other behaviours such as learning. It is reasonable to believe that the abnormalities consistently observed in higher cognitive functions would be reflected in the disruption of basic neurodevelopmental achievement. Therefore, a more detailed evaluation of the early postnatal development of ethanol-exposed neonates was a goal of this investigation. Ethanol exposure did not have a strong effect on reflexive behaviours that appear early in postnatal development such as negative geotaxis, cliff aversion, and auditory startle (Table 3.1). However, milestones that rely on the concurrent development of strength and coordination were affected in mice treated at P4 or P4+7, such as surface righting and forelimb grasp. Also affected in these two groups were the later development of an ear-twitch reflex and delayed eye opening, suggesting that ethanol was able to differentially target multiple developmental systems.

These milestone delays were not observed in P7-treated mice, suggesting that treatment earlier during the trimester three-equivalent synaptogenesis period affected the development of these traits but that the neuromuscular coordination, acoustic startle reflex, and ocular maturation processes responsible for the achievement of these milestones were sufficiently established by P7. Alcohol exposure at P7 resulted in the delay of more complex coordination-dependent milestones such as air righting and open field traversal. During the early period of neonatal development in mice (as well as in humans), forelimb coordination and strength develops prior to hindlimb and interlimb coordination (ALTMAN and SUDARSHAN 1975, THELEN 1985). Given that these milestones are dependent on this rostal-caudal gradient of limb maturation, the results suggests that ethanol exposure may significantly delay this ability to coordinate reflexes with full limb control. As motor development and motor learning are largely determined by the cerebellum (ITO 2000), these delays in developmental milestone achievement may be due to ethanol's teratogenic effect on this brain region. Indeed, the cerebellum matures along a protracted timeline, including a rapid period of growth during synaptogenesis (VOLPE 2009), and has been shown to be

exquisitely sensitive to ethanol-induced apoptosis during this period (IKONOMIDOU et al. 2000, OLNEY et al. 2002a). This is consistent with reports of reduced cerebellar volume in children with FASD (RILEY et al. 2004, NORMAN et al. 2009, MEINTJES et al. 2014). Additionally, cerebellar Purkinje cells that survive third trimester-equivalent ethanol exposure show altered GABA subunit expression, affecting GABAergic tone of remaining neurons (DIAZ et al. 2014b). Interestingly, delayed motor development may be indicative of cognitive performance given that the cerebellum is also involved in higher-level cognitive processes (DIAMOND 2000, BEN-YEHUDAH and FIEZ 2008, BELLEBAUM and DAUM 2011). At the least, neonatal developmental assessment was able to reveal consistent delays in early postpartum milestone achievement in ethanol-treated pups, a hallmark of which was delayed limb coordination that may be an indicator of third trimester-equivalent exposure and also, potentially, of performance at older ages on more complex behavioural tasks.

4.1.2 Ethanol exposure during synaptogenesis results in environment-dependent hyperactivity

Hyperactivity, impulsivity, and inattention has been consistently associated with children prenatally exposed to alcohol, with significant differences from non-exposed children detectable at very young ages (BHATARA et al. 2006, NASH et al. 2008, ALVIK et al. 2013). There is further evidence that these traits show some gender specificity, with males more likely to be diagnosed with attention deficit/hyperactivity disorder (ADHD), a common psychiatric co-morbidity to FASD (HERMAN et al. 2008). Literature is conflicted, however, on the effect of prenatal ethanol exposure on locomotor activity in animal models. Depending on animal model, ethanol dosage, and timing of administration, studies have found hyperactivity, hypoactivity, or a lack of effect of ethanol (TRAN et al. 2000, ALLAN et al. 2003, DOWNING et al. 2009, MARCHE et al. 2010, MANTHA et al. 2013). We have published data supporting that the timing and method of ethanol exposure is

critical to this phenotype, suggesting there are windows of vulnerability (KLEIBER et al. 2011, MANTHA et al. 2013). The results generated from the novel open field exploration assay were subtle, and showed that individual ethanol treatments on P4 or P7 alone did not significantly alter locomotor activity in a novel open field arena, and that treatment on both days (P4 and P7) resulted in hypoactivation in females only (Figure 3.2). This was contrasted by an increased number of rears in both the P7 group and the P4+7 females. Given the increased rearing activity and the sex-specificity of the hypoactivity in P4+7 females, it is possible that the lack of significant effect may have been confounded by neophobia caused by exposure to a novel environment. This was supported by the trend towards decreased time spent in the centre zone by ethanol-treated animals (Figure 3.6), an indicator of anxiety-associated behaviour. Consequently, I examined the nocturnal locomotor activity of ethanol-exposed and control mice in a familiar, home cage environment to evaluate activity in the absence of novelty-induced stress.

The infrared Actritrack system is able to record a variety of measures related to locomotor activity, including total number of infrared beam breaks, which includes locomotor activity and stereotypic movement, velocity of motion, distance travelled, and rearing behaviour. In young children and adolescents with FASD, hyperactivity is among the most consistently-associated phenotype, but a recent meta-analysis has also suggested impairments in balance and coordination (LUCAS et al. 2014), which is certainly consistent with the ability of ethanol-treated mice to achieve early developmental milestones (Table 3.1). As such, I sought to assess the locomotor behaviour of control and ethanol-treated mice, including changes in other activity and motor-related features captured by the Actimeter system, over the course of the nocturnal dark cycle. Following PCA analysis, the Actimeter measures were found to be all significantly positively correlated with total activity (Table 3.3), suggesting that this measure could give an adequate indication of the overnight behaviour of control and ethanol-treated mice. Increases in activity were detected during the early part of the dark cycle in

ethanol-treated mice, significant in mice treated at P7 and P4 and P7, suggesting that treatment at P7 was critical to this behaviour. There is also evidence that hyperactivity is associated with altered glutamate and GABA signalling in the neural pathways linking the hippocampus, accumbens, and subpallidal region (MOGENSON and NIELSEN 1984). The hippocampus and areas of the mesolimbic pathway are particularly susceptible to ethanol exposure during the brain growth spurt. In particular, binge-like ethanol exposure during the third trimester equivalent results in drastic reductions of the CA1 region of the hippocampus (OLNEY et al. 2002a) and reduced expression of numerous glutamate receptor subunits in the adult brain (KLEIBER et al. 2013), presumably altering the efficiency of glutamatergic synaptic transmission involving this region – a feature that is also a hallmark of animal models of hyperactivity (JENSEN et al. 2009).

Interestingly, mice treated at P7 and P4+7 also show increased activity at the end of the dark cycle and beginning of the light cycle (Figures 3.5, 3.6). Whereas control mice typically show extremely low levels of activity during this period, ethanol-exposed mice continue to remain active. Altered activity patterns during light/dark cycling may be an indication of impairments in suprachiasmatic nuclei control of circadian clock function (HOUBEN et al. 2014). Prenatal alcohol exposure is a strong predictor of sleep disturbance in children that are characterized by frequent sleep disruption, shortened sleep duration, and increased sleep anxiety as compared to non-ethanol exposed children (STRATTON et al. 1996, WENGEL et al. 2011, CHEN et al. 2012). While the association between altered activity levels during the early light cycle and circadian rhythm disruption in this study may be speculative, it is intriguing given the strong evidence from animal models that ethanol exposure during the third trimester-equivalent leads to shortened sleep-wake cycles, delayed sleep onset, and delayed sleep cycle transitions (EARNEST et al. 1997, SAKATA-HAGA et al. 2006, VOLGIN and KUBIN 2012). These phenotypes may be attributed to a number of biological mechanisms, including vulnerability of the suprachiasmatic nuclei to ethanol teratogenesis leading to the dampening of BDNF rhythmicity (EARNEST et al. 1997, EARNEST et al.

2001), reduction in serotonergic tone and neuronal density (TAJUDDIN and DRUSE 1999, SARI and ZHOU 2004), and, as reported by this study, altered mRNA levels of serotonin receptor subunit genes, *Period* genes, and *Cryptochome* genes that regulate Circadian function (CHEN et al. 2006a, KLEIBER et al. 2013, KLEIBER et al. 2014b). Given this biological and epidemiological data, it is evident that the third trimester equivalent may be a window of vulnerability for phenotypes associated with alterations in activity levels and circadian function resulting from ethanol's ability to target those systems governing these behaviours. While evaluating sleep disturbance and circadian cycling was not directly assessed by this study, evidence suggests that altered circadian function may play a role to, in the least, exacerbate risk for other phenotypes such as hyperactivity, anxiety, and cognitive impairments that are hallmarks of fetal alcohol exposure, and that later gestational exposures appear to be a stronger window of vulnerability as compared to early exposures (TRAN et al. 2000, O'LEARY-MOORE et al. 2006).

4.1.3 Ethanol exposure results in anxiety-related phenotypes

In the assessment of anxiety-related phenotypes, this study utilized three well-established assays that are indicative of behavioural responses to perceived environmental stress: thigmotaxis in a novel open field, exploratory behaviour in the light region of a light/dark box (LDB) assay, and willingness to explore a narrow, elevated open arm of the elevated plus maze (EPM). Unfortunately, the latter two assays became available late during the course of this study and only the double-treatment (P4 + 7) group was able to be tested using all three assays, but the results revealed some interesting features regarding anxiety-related phenotypes and third trimester-equivalent ethanol exposure.

Thigmotaxic behaviour was observed in mice treated with ethanol at P7 (Figure 3.8) and at P4+7 (Figure 3.9). These data indicated that ethanol exposure during this neurodevelopmental period may contribute to anxiety-related behaviours, and that the severity of these behaviours may be dose-dependent. Also, they

suggested a possible sex difference in vulnerability to anxiety-related traits. As such, mice treated at P4+7 were also assessed in the LDB and EPM, both of which are more anxiogenic tasks than open field exploration, to perhaps more sensitively assess anxiety-related phenotypes related to third trimester-equivalent exposure. Interestingly, results from the LDB assay showed that while both males and females showed a decrease in the amount of time spent in the light region and a reduction in the number of entries into the light zone, a clear distinction between males and females emerged with females showing significantly less light zone exploration than their control counterparts (Figure 3.10). This trend was echoed in the EPM assay, with both males and females showing increases in anxiety-related open arm exploration, but with females showing a marked decrease in the time spent in the open arms and number of open arm entries (Figure 3.11).

These findings are interesting given that maternal binge drinking during pregnancy confers risk for adult anxiety and depressive disorders in ethanol-exposed individuals (FAMY et al. 1998, BARR et al. 2006). These disorders are frequently linked to altered hypothalamic-pituitary-adrenal (HPA) axis function, either via altered cortisol or adrenocorticotropin (ACTH) levels, or altered expression of HPA axis regulator genes at basal or under stressful conditions (DE KLOET et al. 2005). Prenatal alcohol exposure is linked to hypothalamic-pituitary-adrenal (HPA) dysregulation, with heavy drinking during pregnancy associated with higher basal cortisol levels and elevated post-stress cortisol (JACOBSON et al. 1999). Further, the appearance of anxiety-related behaviours seems to be dependent on timing of exposure as mice exposed to alcohol during the first trimester-equivalent show no differences from control mice in the LDB or EPM assays, while exposure during the second trimester equivalent leads to a decrease in anxiety-related phenotypes (MANTHA et al. 2013). This is in contrast to the findings of this study, which suggest that late-gestation ethanol exposure may result in an increase in some anxiety-related phenotypes in mice.

HPA axis hyper-responsiveness has been identified in both male and female animal models of prenatal alcohol exposure; however, sexually dimorphic phenotypes have also been consistently reported. The findings within this study corroborate other reports of higher instances of anxiety-related behaviour in the EPM in females (OSBORN et al. 1998), potentially due to higher stress hormone reactivity in female rodents (CHOI et al. 2008). Further, Brocardo et al. (2012) showed that prenatal alcohol exposure resulted in anxiety and depressive-like behaviours in both male and female mice; however, only males showed an improvement in these phenotypes following voluntary exercise. In humans, a strong effect of sex has been reported among a sample of adults with fetal alcohol exposure; a study by Famy *et al.* (1998) (FAMY et al. 1998) reported 50% of women diagnosed with anxiety disorder co-morbidity as compared to no instances in men. These results may be attributed to the interaction between alcohol and the development of the HPA axis and activational effects related to gonadal hormonal differences (VIAU and MEANEY 1991, KOFMAN 2002, KUDWA et al. 2014).

4.1.4 Ethanol exposure affects spatial learning and learning strategy

Learning deficits are a hallmark of FASD and are well-documented in human and animal studies of prenatal alcohol exposure (COLES et al. 1991, CLARREN et al. 1992, KODITUWAKKU 2009, MATTSON et al. 2010, BRADY et al. 2011). Human studies have emphasized the role of impairments in "executive control" in individuals with FASD (COLES et al. 1997, GREEN et al. 2009, MATTSON et al. 2010), which depends on inhibition of task-inappropriate responses and a working memory of the goal. In particular, prenatal alcohol exposure appears to affect executive function, spatial processing, attention, and memory, with binge-like exposures more detrimental to these processes than mild-to-moderate exposures (MATTSON et al. 2010, FLAK et al. 2014). Given that cognitive behaviours such as learning and attention are such a widely-reported consequence of prenatal alcohol exposure, it is important that any animal model of the disorder replicates these phenotypes. The findings of this

study corroborate this human literature and indicate that spatial learning is impaired, in a timing and dose-dependent manner, in mice exposed to alcohol during the third trimester equivalent (Figure 3.12). Ethanol treatment at P4 resulted in minor delays in reaching the target on acquisition days three and four, whereas exposure at day 7 resulted in more severe deficits beginning at day 1 and significantly lasting throughout days 2 to 4. This may reflect the sensitivity of hippocampal-dependent learning circuitry to ethanol at later stages of neurodevelopment, particularly at the peak of the brain growth spurt that occurs postnatally in mice but during the third trimester in humans (LIVY et al. 2003, WOZNIAK et al. 2004). This finding is consistent with other studies that suggest that ethanol treatment at a specific neurodevelopmental window (P7) can produce significant and enduring impairments in spatial learning (SULIK 2005, WAGNER et al. 2014). Learning ability appeared to be susceptible to dose effects as multiple ethanol exposures at P4+7 appeared to increase the severity of spatial learning deficits during adulthood (Figure 3.12). These deficits may be attributed to the effect of ethanol on learning-associated brain structures, such as apoptosis to diencephalic structures that form the hippocampal circuit (OLNEY et al. 2002a, WOZNIAK et al. 2004) as well as decreases in cell volume in the within CA1 and CA3 pyramidal cells and dentate gyrus neurons (LIVY et al. 2003, TRAN and KELLY 2003).

It is important to note that all mice – ethanol and saline treated alike – showed evidence of learning in the Barnes maze task. The delayed latency to reach the target during the later acquisition days in ethanol-treated mice may be attributed to differences in learning strategy. This is perhaps most evident following analysis of the type of search strategy used by ethanol and control mice during the acquisition learning trials (Figure 3.13). Interestingly, control mice were rapidly able to utilize extra-maze cues to learn the precise location of the target, and the number of trials during which they utilized a "direct" search strategy, that is, travelled directly to the target hole following release from the central starting zone, dramatically increased during acquisition days 2 to 4. Conversely, ethanol-treated mice did not exhibit the same increase in "direct" search strategies;

observationally, they tended to travel toward the quadrant of the maze in which the target was placed, but often the initial hole visited was two or more holes away from the target and they progressively searched, hole by hole, in a clockwise or counterclockwise manner until they had located the target. This finding is illustrated by the divergence between control and ethanol-treated animals in the increase in "direct" search strategy in controls and the increase in "serial" search strategy in ethanol-treated mice in later acquisition days (Figure 3.13). It is important to note that both groups showed clear reductions in their use of a "mixed" strategy, suggesting that, in both groups, the search for the target was not random. However, the differences in search strategy used may indicate that prenatal ethanol exposure may alter spatial learning acquisition styles.

The spatial learning deficits apparent during the acquisition trails were reflected in both the short- and long-term memory recall trials on day 5 and day 12 of Barnes maze testing (Figure 3.14, Figure 3.15). Mice treated with ethanol at P4 showed a small and non-significant reduction in explorations to the target hole location at day 5, but treatment at P7 or at P4+7 resulted in a significant reduction of explorations at or around the target, which was not driven by a decrease in the total number of explorations. These trends persisted during the long-term recall task at testing day 12. These results suggest that ethanol exposure may not only impair learning during task acquisition, but also the ability of the mouse to recall the relationship between the maze layout, the extra-maze cues, and the target during spatial memory trials. Further, the increase in explorations to non-target holes opposite the target in ethanol-treated mice may reflect the learning strategy acquired during the acquisition phase of testing: while control mice tended to travel back and forth remaining around the previous location of the target, the ethanol-treated mice persisted in searching each hole on the Barnes maze platform in a clock-wise or counter-clockwise direction, as they had during acquisition.

There is evidence that certain types of learning occur similarly in ethanol-exposed and non-exposed children but learning in FASD individuals is benefitted by

increased repetition and more frequent positive reinforcement (ROEBUCK-SPENCER and MATTSON 2004, ENGLE and KERNS 2011). Other studies have suggested that learning and memory strategies do differ between exposed and non-exposed children, including variations in visual versus verbal learning styles, deficits in attention, problems with planning and spatial working memory, and deficits in cognitive flexibility, planning, and strategy use (RASMUSSEN 2005, GREEN et al. 2009, RASMUSSEN et al. 2009). Results from this study suggest that ethanol treatment during the third trimester-equivalent results in alterations to both learning, memory, and overall strategy in a spatial learning task, consistent with findings from the human studies. Also, while these findings show that prenatal alcohol exposure does not prevent learning altogether, they do indicate that ethanol induces changes to attention and learning style that are detectable in early adulthood. In humans with FASD, this may establish a pattern of educational disadvantage that, when matched with poor early environmental enrichment or educational support that recognizes these changes to learning styles, may ultimately lead to poor long-term educational and socio-economic outcomes (STREISSGUTH et al. 1994, FAMY et al. 1998, JIRIKOWIC et al. 2008b).

4.1.5 Ethanol exposure during synaptogenesis in mice as a model for human FASD: a summary

Modeling complex human disorders in animals requires careful attention to behavioural endophenotypes commonly observed in humans with the disorder and an appropriate choice of animal and behavioural battery that can most accurately represent these phenotypes. Most neurobehavioural or psychiatric disorders are extremely complex via their mode of causation, their effect on multiple neurological pathways, and their interaction with environmental factors. Usually, modeling them in in their entirety in animals is not possible either conceptually or practically (e.g., (O'NEIL and MOORE 2003, O'TUATHAIGH and WADDINGTON 2015)); however, it is possible to model smaller aspects of a larger

phenotype, with the results to be used with caution when extending behaviours observed in an animal model to any human disorder, particularly one that involves changes to behavioural and cognitive phenotypes (NESTLER and HYMAN 2010b, KOOB 2012).

As compared to other neurobehavioural disorders, the causation of FASD is clear. As such, any model involving ethanol exposure during neurodevelopment has a certain etiological validity. In humans, however, the timing and dose of ethanol exposure is extremely variable from case-to-case, and not likely to be accurately reported (HANNIGAN et al. 2010, LANGE et al. 2014). It is difficult to ascertain, therefore, the relationship between ethanol exposure and the numerous behavioural phenotypes that may result. I thought it important that a behavioural battery attempting to evaluate a mouse model of FASD should test a number of relevant phenotypes reliably and with regard to the innate behaviour of mice.

The behavioural battery used in this study was designed to evaluate behaviours that are common in humans with FASD as they appear across infanthood, childhood, adolescence, and early adulthood within the confines of a mouse model system. This study was the first to use a thorough evaluation of early postnatal coordination and reflexive behaviours, given that infants with FASD often show delays in the appearance of these behaviours. Also, this is the first behavioural paradigm to take into account the emergence of various behavioural changes across development and maturation. The results indicate that the analysis of early behaviours are indeed informative, and may be predictive of later-life neuromotor and cognitive impairments. Further, single binge-like exposures during the third trimester-equivalent were able to reliably produce changes to activity, anxiety-related traits, and learning ability, which often were made more severe by a subsequent second ethanol treatment. The behavioural battery is certainly not exhaustive, and there are a number of behavioural phenotypes exhibited by humans with FASD that cannot accurately be modeled in mice, but the results suggest that at least some of the behaviours exhibited in humans can be replicated by this paradigm. Also, this particular type of treatment paradigm itself may have

limited face validity in that it is likely an uncommon practice for women to consume a substantial quantity of alcohol only during late gestation; however, it serves as a mechanism to evaluate the specific changes that may occur resulting from third trimester ethanol exposure in the absence of confounding previous exposures. Also, it serves to show that there may be temporal windows of neurodevelopmental vulnerability of brain regions responsible for specific later-life emergent phenotypes. Finally, while this thesis is focused on a third-trimester paradigm, we have previously shown that the model developed here may be applied to other exposure paradigms to evaluate and compare the effects of dose and timing of exposure (KLEIBER et al. 2011, MANTHA et al. 2013). These are etiologically valid models that then can be used within a larger experimental and theoretical framework to evaluate underlying biological mechanisms including developmental trajectories of genetic and epigenetic changes that result from neurodevelopmental alcohol exposure (KLEIBER et al. 2012, KLEIBER et al. 2013, LAUFER et al. 2013, KLEIBER et al. 2014b, MANTHA et al. 2014b).

As a whole, the results presented suggest that this trimester three-equivalent exposure paradigm can provide an effective model for the evaluation of behavioural and cognitive phenotypes associated with FASD that may be particularly vulnerable to late-gestation alcohol exposures in humans. Also, the data suggests that this multi-phenotype behavioural battery is a useful strategy to evaluate phenotypes that reflect a more holistic neurocognitive profile common to human individuals prenatally exposed to ethanol. As with any model, its value lies not only in its ability to recapitulate behaviours observed in humans, but also if it can be used to examine the underlying biological causes of those behaviours. This second goal forms the focus of the following sections of discussion.

4.2 Disruption of gene expression during neurodevelopment by ethanol and its role in FASD

This study sought to evaluate the changes in gene expression that occur in the developing brain acutely following a binge-like dose of ethanol during synaptogenesis in the B6 mouse strain. These changes represent the *in vivo* response within a previously naïve biological system that is highly dynamic and acutely sensitive to the effects of ethanol. The response also represents a discontinuity in normal developmental processes that may result in apoptosis (IKONOMIDOU et al. 2000, OLNEY et al. 2002a, DIKRANIAN et al. 2005) or, in surviving cells, a 'molecular footprint' of neurodevelopmental disruption that may persist long past the initial ethanol insult (SANDERSON et al. 2009, PUGLIA and VALENZUELA 2010, KLEIBER et al. 2013). The discussion below evaluates how the mRNAs detected by array analysis of short and long-term gene expression changes resulting from ethanol exposure may affect neurodevelopment, adult brain function, and how they may be reflected in the cognitive and behavioural phenotypes associated with FASD.

4.2.1. Effects of ethanol exposure on gene expression in the postnatal brain

Ethanol is known to affect the expression of a large subset of genes in the brain acutely following exposure (TREADWELL and SINGH 2004), including in the developing brain (HARD et al. 2005, GREEN et al. 2007). I analyzed the effects of ethanol exposure at P7 in mice, a time neurodevelopmentally equivalent to the human third trimester, and known to be particularly sensitive to ethanol (OLNEY et al. 2002a). The 315 transcripts affected acutely (4h) following ethanol exposure (Appendix A) were categorized into broader biological functions and gene networks, identifying over-represented affected molecular processes. The genes differentially expressed between control and ethanol-treated mice and the

associated biological processes were perhaps unsurprising given that the ethanol insult occurred at such a vulnerable developmental time for synapse formation and neuronal pruning (DOBBING 1974). The ability of ethanol to affect the expression of ionotropic glutamate receptor subunits likely has some impact on excitotoxic cell death triggered by abnormal glutamate signaling. Not all cells, however, succumb to cellular death; many of the identified changes to the transcriptome were certainly categorized by an acute stress response, but there appeared to be a precarious balance between cell death and survival cues, including alterations to genes associated with cell cycle signaling, cell proliferation and maturation, neurotransmitter signaling, cell membrane integrity, and protein modification.

4.2.1.1 *Acute stress response, apoptosis and cell survival*

Up- and down-regulated gene lists were separated and subsequently submitted as two gene groups to DAVID for over-represented biological functions, which allowed for the evaluation of processes that may be activated or inhibited by ethanol exposure. Up-regulated transcripts were strongly associated with 'positive regulation of apoptosis' and 'cell death and survival' (Table 3.5, 3.6). These results corroborate physiological evidence that this binge-like ethanol treatment at synaptogenesis results in wide-spread cell death across the cortex, hippocampus, cerebellum, and other brain regions (OLNEY et al. 2002a, WOZNIAK et al. 2004, DIKRANIAN et al. 2005). A number of GO annotations and genes present within these annotations suggest a series of events by which ethanol may trigger this apoptotic response, including a number of stress-related mechanisms. Several studies have shown that ethanol exposure during rodent neurodevelopment, particularly during the early postnatal period, can interfere with a number of neurotrophic factors (HEATON et al. 2000b, CLIMENT et al. 2002, MOORE et al. 2004) that play key roles in neuronal survival and maturation including normal apoptosis of cells during brain maturation (KIRSTEIN and FARINAS

2002, LYKISSAS et al. 2007). The results from this study corroborate these findings, with a number of pro-survival and pro-maturation factors growth and transcription factors showing down-regulation, including *Etv4*, *E2f4*, *Egr3*, *Egr4*, and *Tgfb3*, *Vegfa* (Table 3.5, 3.6). Other studies using early postnatal ethanol exposure paradigms have also implicated the nerve growth factor receptor *tropomyosin receptor kinase A* (*TrkA*), *brain-derived neurotrophic factor* (*Bdnf*) and its receptor *TrkB*, *nerve growth factor* (*Ngf*), and the neurotrophin p75 receptor (HEATON et al. 2000b, HEATON et al. 2003, TOESCA et al. 2003). These molecules have well-established roles in multiple developmental processes and their reduced expression may alter the maturation of neurons, extent of dendritic branching, synapse formation, and neuronal plasticity (RUZHYNSKY et al. 2007, ROSENSTEIN et al. 2010, PEREZ-CADAHIA et al. 2011). Alterations in levels of survival factors can alter the signaling function of cell survival pathways, including mitogen-activated protein kinase (MAPK/ERK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways, which is associated with increased levels of apoptosis in the developing cortex (CLIMENT et al. 2002).

It is interesting that a number of up-regulated transcripts associated with 'protein amino acid dephosphorylation' and down-regulated transcripts under the annotation 'MAP kinase phosphatase activity' (Table 3.5) are dual-specificity phosphatase (*Dusp*) genes. A number of these genes are present in the genome, each with different target specificity, cellular, and tissue localization, and are rapidly activated in response to oxidative stress or growth factor signaling (induced as early response genes). They function to inactivate various MAPK isoforms, negatively regulating cellular proliferation (reviewed in (LANG et al. 2006)). The regulation of *Dusp* genes is somewhat gene-dependent, with *Dusp* genes acting through the inhibition of the MAPK/ERK pathway, the mammalian target of rapamycin (mTOR) pathway or both (BERMUDEZ et al. 2008, LAWAN et al. 2011, RASTOGI et al. 2013). An increase in transcription in some *Dusp* genes and a down-regulation of others suggest a fast-acting induced response to cellular stress that may indicate a balance between pro- and anti-apoptotic pathways to

decide cell fate. Further, the abnormal increased or decreased presence of these genes may also affect the regulation of other genes via epigenetic mechanisms, as they have also been shown to have the ability to alter histone phosphorylation states (LAWAN et al. 2011). The simultaneous up- and down-regulation of members of this gene family may mediate a balance between pro-survival and pro-apoptotic signaling through the activation and deactivation of specific MAPK signaling pathways.

Cellular stress response leading to cell cycle arrest and potential cell death via MAPK/ERK and PI3K/AKT/mTOR pathways is also indicated by the up-regulation of the p53 pathway-associated proteins *Jun proto-oncogene* (*Jun*), *PERP, TP53 apoptosis effector* (*Perp*), and *Junction-mediating and regulatory protein* (*Jmy*). The p53 pathway may be initiated by both oxidative and osmotic stress, both of which are induced in the developing brain by high doses of ethanol (GOLDSTEIN 1986, BROCARDO et al. 2011). *Jun* is an immediate early gene (IEG) canonically associated with the p53 pathway, but may act as a pro-apoptotic or pro-survival cue, depending on other signals present in the cell (HUGHES et al. 1999, SCHREIBER et al. 1999). However, *Perp* is expressed in a p53-dependent manner at high levels in apoptotic cells (ATTARDI et al. 2000), while *Jmy*, a p53 cofactor, has been shown to be a negative regulator of neuritogenesis (FIRAT-KARALAR et al. 2011) (Table 3.7) and has also been identified by array analysis of gene expression changes associated with trimester two-equivalent ethanol exposure (MANTHA et al. 2014a).

Also indicative of changes in MAPK and PI3K/AKT signaling is the up-regulation of Forkhead transcription factor *Foxo3*, which has been shown to be involved oxidative stress-induced apoptosis in mouse neurons (Table 3.5, 3.6). In the neonatal mouse brain, the expression and apoptotic function of *Foxo3* is associated with Jun N-terminal kinase 3 activity (PIRIANOV et al. 2007), phosphorylation and activation of Jun and, interestingly, has recently shown to be exerted via its association with histone deacetylase 2 (*Hdac2*) (PENG et al. 2015). While *Hdac2* was not identified in this study, the expression of *Hdac1* and *Hdac9* were found to be down-regulated. Given the sequence similarity of *Hdac* family

genes, it is likely that Forkhead transcription factors may also be regulated by other *Hdac* molecules. In support of this, *Hdac1* was found to be sufficient to activate Forkhead family transcription factors and trigger apoptosis associated with muscle fiber atrophy (BEHARRY et al. 2014).

While the genes identified in this study represent a snap-shot of gene response four hours following a binge-like dose of ethanol across the multitude of cell types found in whole brain tissue, it is clear that ethanol triggers a strong cellular stress response in the postnatal brain that likely involves a number of pathways. Further, there appears to be changes to molecules associated with both pro-survival and pro-apoptotic cues. Overall, these results support other studies reporting that ethanol exposure during this early neonatal period instigates an acute stress response that leads to apoptotic neurodegeneration in a large number of brain regions (IKONOMIDOU et al. 2000, OLNEY et al. 2002a). Also, these results suggest that a number of cells that undergo this stress response and experience an interruption to normal developmental processes, but do not succumb to apoptosis. It is these cells that form the foundation for further neurodevelopment.

4.2.1.2 *Maintenance of cell viability*

A number of altered transcripts may be functionally grouped as having an association with the maintenance of cell integrity, including an up-regulation of 'integral to membrane'-associated transcripts and the down-regulation of notoriously energetically costly processes, such as 'ribosome biogenesis', 'mitotic cell cycle' progression, 'cellular growth and proliferation', and 'regulation of transcription' (Table 3.5, 3.6). While 'integral to membrane' is a fairly broad Gene Ontology classification and the transcripts identified within this group play numerous roles with regard to their association to membrane dynamics, cell membrane composition can strongly affect the function of intrinsic membrane proteins. High alcohol concentrations have been shown to force the

transmembrane proteins into unfavorable conformations affecting the chemical and physical functioning of the cell, including at synaptic plasma membranes (AVDULOV et al. 1994). In support of this, a number of up-regulated genes are involved with the maintenance of cell membrane-associated molecules such as oligosaccharides and cell-surface carbohydrates (*Bgalt3*, *St6gal1*), phosphatidylinositol (*Pigm*, *Pigv*, *Pik3ip1*, *Itprip2*), sphingosine (*Acer2*), and aminophospholipids (*Atp8b2*). Coinciding with this, the up-regulation of genes associated with glycosylation (Table 3.5) may be related to reinstating membrane integrity as glycoproteins are developmentally integral to cell membrane stability, cell-cell recognition and interaction (KLEENE and SCHACHNER 2004), as well as regulating excitatory synapse development and modulation of synaptic efficacy (DE WIT et al. 2013). These findings agree with previous reports that ethanol targets glycosylation machinery (TOMAS et al. 2002).

Conversely, the down-regulation of genes associated with ribosome biogenesis may also be a response to re-direct cellular energy to stress response and cell survival mechanisms. The synthesis of ribosomal protein mRNAs requires a large proportion of the cell's energy, and is balanced by the growth requirements of the cell, and is regulated by nutrient availability, cell cycle, and cell stress (THOMAS 2000, MOIR and WILLIS 2013). Similarly, down-regulation of cell cycle progression, proliferation, and differentiation-associated transcription factors, growth factors, and cyclins may provide a comparable function (Table 3.5, 3.6, 3.7) and act to halt developmental processes to deal with the acute cellular insult.

4.2.1.3 *Neurotransmitter signaling systems*

In addition to the prominent effect of ethanol on cell survival and stress response mechanisms, a number of transcripts were identified that, while they may have roles in cellular stress, also have demonstrated roles in neurodevelopment and have been implicated in long-term risk for neurological disorders. Particularly notable is alteration of genes associated with major excitatory and inhibitory

neurotransmitter systems, including glutamate and γ-aminobutyric acid signaling (Table 3.5, 3.6; Figure 3.17). Among the genes affected by ethanol treatment were *Cpeb1*, *Gabra5*, *Grin2a*, and *Grin2b* all of which are canonically critical to synaptic formation and maintenance (Table 3.6). *Cpeb1* regulates translation at synapses and reduced or elimination of expression during development results in poor synaptic efficacy leading to reduced long-term potentiation (ALARCON et al. 2004) and, in adulthood, rapid extinction of hippocampal-dependent spatial learning memories (BERGER-SWEENEY et al. 2006). *Cpeb1* protein is activated in response to N-methyl-D-aspartate ionotropic (NMDA) glutamate receptor stimulation to facilitate activity-dependent gene transcription (WELLS et al. 2000). Further, during early postnatal development, the formation of functional circuit formation is dependent on the synchronous activity of depolarization of $GABA_A$ receptors and activation of NMDA receptors (WANG and KRIEGSTEIN 2008, CSEREP et al. 2012).

In this study, *γ-aminobutyric acid (GABA) A receptor, subunit alpha 5* (*Gabra5*), *glutamate receptor, ionotropic, NMDA2A (epsilon 2)* (*Grin2a*), and *Grin2b* transcript levels were identified as altered following ethanol exposure, with *Gabra5* down-regulated and the glutamate subunits up-regulated (Table 3.5, 3.6)). While these genes are associated with the major inhibitory (GABA) and excitatory (glutamate) neurotransmitter systems in the adult brain, it has also been established that they play key roles in the neurodevelopmental patterning of synaptic networks (LUJAN et al. 2005). Both NR2A, encoded by *Grin2a*, and NR2B, encoded by *Grin2b* are modulatory NMDA receptor subunits, with *Grin2a* widely expressed in the postnatal brain and *Grin2b* expressed in multiple regions embryonically, but restricted to the forebrain during postnatal stages (LUJAN et al. 2005), the transition of which occurs during synaptogenesis (YASHIRO and PHILPOT 2008). NR2B-containing receptors are important for circuit formation and establishment, and the developmental switch to NR2A-containining receptors results in excitatory postsynaptic currents with a more rapid decay (VAN ZUNDERT et al. 2004). It is possible that the up-regulation of both *Grin2a* and *Grin2b* modulatory subunits may alter the sensitivity of developing synapses,

contributing to abnormal synapse formation and arborization, as well as (in some cells) excitotoxic cell death. Other studies have shown that the subunit composition (NR2A versus NR2B) of NMDA receptors can control neurodevelopmentally-critical processes such as dendritic spine motility, synapse formation and stabilization (GAMBRILL and BARRIA 2011). The *Gabra5* subunit is expressed in the brain throughout pre-and postnatal development (KILLISCH et al. 1991). The coincident down-regulation of inhibitory neurotransmitter receptor subunit *Gabra5* and the up-regulation of excitatory neurotransmitter receptor subunits *Grin2a* and *Grin2b* suggest that ethanol exposure during the third trimester-equivalent may lead to general over-excitatory signaling within the developing brain. This would have broad effects on multiple brain regions, given that role of NMDA and GABA receptors in the formation, maturation, and pruning of synapses has been well-established (BOLTON et al. 2000, COHEN-CORY 2002, LUJAN et al. 2005, CSEREP et al. 2012). Disruption of regulatory control of subunit expression by ethanol at this developmental stage has potent consequences for neural network connectivity, consequently affecting brain function not only acutely but establish an abnormal foundation for adult cellular communication and plasticity (MALENKA and BEAR 2004), a crucial impairment associated with prenatal alcohol exposure.

4.2.1.4 *Hormone signaling and endocrine system development*

The DAVID analyses identified hormone activity and hormone receptor activity as biological functions with a significantly over-represented proportion of altered transcripts (Table 3.5), most of which were significantly down-regulated following ethanol exposure. These genes included *Adcyap1* and *Pomc*, as well as *Nuclear receptor subfamily 4, group A, member 1* (*Nr4a1*), *Nuclear receptor subfamily 0, group B, member 1* (*Nrob1*) and *Retinoid X receptor gamma* (*Rxrg*). These genes play varying roles in the development of the brain, most notably in the proper function of the HPA axis (VAZQUEZ 1998) including altered stress

reactivity, a consistent endophenotype associated with fetal alcohol spectrum disorders (HELLEMANS et al. 2008, WEINBERG et al. 2008, HELLEMANS et al. 2010). It is possible that this period of neural development is critically susceptible to molecular changes that affect HPA connectivity and maturation, leading to stress vulnerability in prenatally-exposed adolescents and adults (WEINSTOCK 2010). The down-regulation of *Nr4a1* is interesting given that it is able to mediate both pro- and anti-apoptotic effects (LI et al. 2006), as well as mediating thyrotropin-releasing hormone stimulation within the pituitary (NAKAJIMA et al. 2012). Also relevant to HPA axis function is the reduced expression of *Rxrg*. This gene is part of the family of retinoic acid receptors that mediate the anti-proliferative effects of retinoic acid and act as ligand-dependent transcriptional regulators (DAWSON and XIA 2012). These results follow other studies that have implicated retinoic acid signaling in the effects of prenatal alcohol exposure (GRUMMER and ZACHMAN 1995, KANE et al. 2010, KUMAR et al. 2010), the reduced expression of which can delay cellular differentiation and myelination (HUANG et al. 2011), most notably in the HPA axis structures but also in the cerebellum and hippocampus, affecting developmental and behavioural phenotypes (LU et al. 2001).

4.2.1.5 *Gene network interactions highlight the impact of ethanol on stress response and neurotransmission*

In addition to functional classification and grouping of the transcripts identified as altered following ethanol exposure at P7, the genes were also assessed for known interactions to provide further information for how these genes may interact to regulate each other, and via which other molecules and pathways. Figures 3.17, 3.18 and 3.19 represent significant networks identified by Ingenuity Pathway Analysis™ and the findings from these analyses reinforced the involvement of the cellular processes identified by the functional annotation clustering results in the acute transcriptional response to ethanol. It is evident that ethanol exposure results in a cellular stress response through the up-

regulation of key effector molecules such as *Jun* and its interactions with *Dusp*-family genes, and the mitochondria-associated apoptosis regulator *Bcl2l11* (Figure 3.17). These genes are central modulators of oxidative stress-related neuronal apoptosis, a process which includes the Wnt and Akt pathways that act on downstream effectors such as Forkhead family transcription factors and Bcl-family genes (CHONG et al. 2005) (Figure 3.17). This analysis also indicated the complex balance of factors that act to direct or halt cell growth and proliferation, such as the down-regulation of the tumor suppressor transcription factor *Klf6* and coincident down-regulation the *Etv4*, a transcriptional activator that acts to drive cell proliferation. Similarly, cell cycle arrest is indicated by the up-regulation of *Btg2*, an antiproliferation protein that interacts with NGF to regulate G1/S cell cycle transition via the down-regulation of cyclin *Ccnd1* (MONTAGNOLI et al. 1996, GUARDAVACCARO et al. 2000); however, the steroid-thyroid hormone and retinoid receptor *Nr4a1* can act as a powerful instigator of programmed cell death (MOLL et al. 2006), but was found to be down-regulated.

Network analysis also highlighted the role of factors affecting cellular growth and tissue morphology in ethanol response (Figure 3.18), including the up-regulation of growth factor *Tgfb3*, which appears to act as a hub molecule linking other altered growth factors such as *Vegfa*, insulin-like growth factor (*Igf*) family genes, and implicating interleukin and MAP kinase molecules in *Jun*-associated cellular stress response. We have previously reported that trimester two-equivalent ethanol exposure results in an inflammatory response, including an alteration to the expression of interleukin genes (MANTHA et al. 2014b). The expression of interleukin 15 has also been implicated as having a role in neural stem cells and differentiating neurons, and that down-regulation may increase *microtubule-associated protein 2* (*Map2*) protein levels to support neurite outgrowth (HUANG et al. 2009).

Also included in this network are genes associated with HPA axis development and function, including a down-regulation of *Pomc* and *Adcyap1* (Figure 3.18). *Pomc* is a polypeptide hormonal precursor that yields multiple biologically active

peptides such as corticotrophin, α-melanotropin, and β-endorphin, with diverse functions. Early postnatal reductions of *Pomc*, including those associated with ethanol exposure, is associated with apoptosis, altered Circadian regulation, and altered stress reactivity in later-life (CHEN et al. 2006a, GREENMAN et al. 2013, AGAPITO et al. 2014, LIU et al. 2014). *Adcyap1* is also a secreted peptide that is further processed into a number of neurologically active molecules. Evidence from *Adcyap1*-null mice suggest that reductions in *Adcyap1* signaling plays an important role in neurodevelopmental-associated psychiatric behaviours such as altered psychomotor behaviour, stress response, memory, ethanol sensitivity, and circadian rhythm (HASHIMOTO et al. 2006, YAMADA et al. 2010). The alteration of these HPA axis-associated genes by ethanol may be directly related to the altered expression of growth factors, cytokines, and interleukins, given that these molecules can act to affect HPA axis gene expression and function (DUNN 2000). Though the precise impact on long-term phenotype is speculative, it is evident that the cellular stress response triggered by a binge-like dose of ethanol has consequences for the development of brain regions that are sensitive to cellular stress-associated molecules, leading to long-term changes to the architecture of brain circuits and setting up the physiological basis for long-term changes to behavioural phenotypes.

Network analysis also highlighted the prominent role of glutamate signaling and NMDA receptor expression in the acute response to ethanol at P7 (Figure 3.19). Up-regulation of *Grin2a* and *Grin2b* may be a response to ethanol's ability to act as a NMDA receptor antagonist, and an attempt to curb the excitotoxic response in developing cells in a number of regions within the postnatal brain (IKONOMIDOU et al. 1999). However, this analysis also indicated the interaction between glutamate receptor subunit genes and other biological processes. Of note are the interaction of *Grin* genes with *Tubb6*, a tubulin isoform, through *Dlg4*. Increases in *Tubb6* expression can profoundly destabilize microtubules and block cell proliferation (BHATTACHARYA and CABRAL 2004), suggesting that the down-regulation may help stabilize microtubule formation that support axonal

branching and synapse formation occurring during this developmental stage. This is supported by observations that prenatal alcohol exposure increases neurite branching in the cerebellar macroneurons (ZOU et al. 1993) and disorganized dentritic morphology in the medial prefrontal cortex (LAWRENCE et al. 2012). This network also outlines the potential interaction between glutamate receptor up-regulation and the up-regulation of the Circadian rhythm pacemaker gene *Per1* through *huntingtin* (*Htt*) as well as down-regulation of *histone deacetylase 1* (*Hdac1*) via *survival of motor neuron* genes *Smn1*/*Smn2* (Figure 3.19). Given that regions of the hypothalamus are extremely vulnerable to apoptosis caused by ethanol's ability to block NMDA receptors during the postnatal period (IKONOMIDOU et al. 2000), it is logical ethanol exposure at this time may also affect genes expressed within the suprachiasmatic nucleus such as Period genes. However, it is likely that the up-regulation of *Per1* is associated with its roles in cellular stress and rather than its canonical circadian clock function: *Per1* mRNA is a target for the unfolded protein stress response, which can be induced by ethanol-associated oxidative stress, and increasing its expression is associated with increased cell survival and reduced tumorigenesis in certain carcinomas (PLUQUET et al. 2013).

The gene network analysis of genes acutely altered in the brain of mice treated with ethanol at P7 further reinforced the findings of the functional annotation analysis, and a summary of genes and processes implicated in this ethanol response are further highlighted following a merging of these individual gene networks (Figure 3.20). The major gene hubs may be categorized into two main functions: stress response and apoptosis, and cellular signaling and interaction. These functions are certainly not disparate, as evidenced by the substantial interaction between all molecules, and a number of these genes play critical roles during synaptogenesis. The most prominent hub gene appears to be *Jun*, which appears to serve as a mediator between numerous growth factors (such as *Gh1*, *Ngf*, *Tnf*, *Tgfb*, and *Vegf*), early growth response (EGR) genes, apoptosis-associated molecules (such as *Bcl2l11*), and a variety of developmentally-important

transcription factors (such as *Sp1* and *Foxo3*). Related both to ethanol excitotoxicity and synaptic transmission are the marked up-regulation of glutamate receptor subunit genes (*Grin2a*, *Grin2b*), suggesting that ethanol exposure at P7 may alter the balance of excitatory neurotransmission that is extremely critical for synaptic stabilization and pruning at this neurodevelopmental stage. Finally, an interesting relationship emerged between HPA axis-associated genes *Pomc*, *Adcyap1*, and *Nr4a1*, all of which were down-regulated. Given their modulatory roles in HPA axis development and function, their responsiveness suggests that the developing HPA axis is particularly vulnerable to trimester three-equivalent ethanol exposure.

4.2.2. Effects of ethanol exposure on gene expression in the adult brain

In addition to examining the gene expression profile immediately following ethanol exposure during the trimester three-equivalent, an investigation into the effects of neurodevelopmental ethanol exposure on adult gene expression became intriguing given the observed long-term behavioural effects exhibited by adolescent and adult mice. It is clear that some developing cells succumb to ethanol-induced apoptosis (OLNEY et al. 2000); however, some cells encounter teratogenic adversity and undergo molecular adaptation, and form the foundation for further brain development. These surviving cells then undergo further mitosis, differentiation, migration, and establish synaptic connectivity to form the final cellular network of the mature brain. These cells that survive represent a population that must reinitiate neurodevelopment and adjust their developmental trajectories to recoup, at least somewhat, the functionality of those cells that are lost. These cells must do so within a relatively limited amount of time, via alterations to gene expression patterns. These expression changes may represent an adaptive response, involving the reorganization of gene expression in surviving cells undergoing development. These changes set up a pattern of

long-lasting genomic dysregulation that can be detected in the adult brain, long after the cessation of alcohol exposure. This analysis was performed to examine how this interruption to developmental cues during the third trimester-equivalent may lead to an altered pattern of gene expression and genomic regulation that may be persistent throughout the lifetime of the individual (Appendix B). Also, it facilitates the evaluation of those biological processes that remain affected in the adult brain of mice exposed to ethanol during neurodevelopment that may contribute to the persistence of altered brain function, resulting in long-term cognitive and behavioural phenotypes that are observed in individuals with FASD.

4.2.2.1 *Glutamate-associated synaptic neurotransmission*

The expression differences identified in the adult brain were much more subtle than the acute ethanol response and biased heavily towards a significant down-regulation of transcripts (Appendix B). Gene ontology functional annotation of these transcripts showed some similarities to the short-term analyses, particularly with respect to the number of transcripts associated with glutamate-associated neurotransmission and synaptic function (Table 3.8). A large number of glutamate receptor subunits showed altered expression, including both metabotropic and ionotropic type members of the glutamate receptor family. It is interesting that exposure to ethanol during synaptogenesis has such a long-lasting effect on glutamate receptor expression, given that regulation of subunit expression can greatly impact synaptic function, plasticity, and maintenance (AAMODT and CONSTANTINE-PATON 1999, CLINE and HAAS 2008, YASHIRO and PHILPOT 2008). This analysis indicates that not only can ethanol alter glutamate-based regulation of developing neurons, including during initial synapse establishment, but that alterations to this process have long-term repercussions on adult glutamate receptor subunit expression and, likely, glutamate neurotransmission. Gene ontology, Ingenuity® biological annotation clustering, and Ingenuity®

canonical pathway analyses all identified glutamate receptor activity, neurotransmission, and signaling as the most significant biological process over-represented among altered transcripts (Table 3.8, 3.9, 3.10), suggesting that altered glutamate signaling may be among the most pervasive long-term consequences of ethanol exposure during synaptogenesis. The implications of this with regard to behavioural phenotypes is likely quite broad given that glutamate is the predominant excitatory neurotransmitter in the mammalian brain (HAYASHI 1954), and altered glutamate signaling has been implicated in a number of cognitive and psychiatric phenotypes. Reduced brain volume and connectivity in cortical and limbic regions, and consequently reduced glutamatergic signaling, underlie the "neuroplasticity hypothesis" of anxiety and mood disorders (PRICE and DREVETS 2010, DUMAN and AGHAJANIAN 2012). Given that regions such as the prefrontal cortex and the limbic system are vulnerable to ethanol-induced apoptosis, particularly during the third trimester-equivalent (IKONOMIDOU et al. 2000), the reduction in brain volume and dendritic reorganization observed in individuals with FASD could contribute to reduced glutamate signaling in the adult brain. Also, interference with the developmental and functional properties of synapse formation is likely to lead to the formation of abnormal brain circuits and contribute to complex behavioural phenotypes, as has been observed in schizophrenia and autism spectrum disorders (SIGURDSSON et al. 2010, CANITANO 2014).

In addition to reduced brain volume as a potential cause for reduced glutamate receptor expression, other molecules that impact the stability of synapse formation, maintenance of synaptic strength, and synaptic plasticity were also found to be affected in the adult brain of ethanol-exposed mice. These include membrane receptors such as ephrins *Ephb1, Efnb2*, and *Efna5* (Table 3.10), which are critical to axon guidance and target recognition during the formation of dendritic spines (FLANAGAN and VANDERHAEGHEN 1998), as well as drive NMDA-type glutamate receptor clustering and enhance NMDA receptor calcium influx (DALVA et al. 2000, TAKASU et al. 2002). Ephrin receptors are therefore important

for NMDA-dependent synaptic plasticity and long-term potentiation, and a reduction in the expression of these genes may imply reduced dentritic spine density and reduced glutamate receptor clustering on remaining synapses. Studies have indicated that mice lacking *Ephb1* expression also show fewer hippocampal neural progenitor cells and abnormal positioning of CA3 hippocampal pyramidal neurons (CHUMLEY et al. 2007), which has implications for performance on learning and memory tasks – a phenotype frequently associated with prenatal alcohol exposure and an impairment observed in adult mice treated with ethanol within this study.

Other genes of note associated synaptic function are *Cacna1c* and the postsynaptic density proteins *Syngap1* and *Syngr1*, all of which were also found to be down-regulated (Table 3.8). *Cacna1c* encodes a subunit of a voltage-dependent calcium channel that has been identified by unbiased genome-wide association studies as a risk gene in multiple psychiatric disorders (BHAT et al. 2012, YOSHIMIZU et al. 2015). Animal studies have shown that hippocampal and forebrain-specific knock-out of *Cacna1c* in mice results in a severe impairment of NMDA-receptor-independent spatial memory and long-term potentiation, leading to a decrease in MAP/ERK signaling and a sustained reduction in CREB-dependent transcription (MOOSMANG et al. 2005). CREB signaling has a well-documented role in neuronal plasticity and long-term memory formation (SILVA et al. 1998, WON and SILVA 2008), and was identified by this study as an over-represented canonical pathway associated with transcripts altered in the adult brain (Table 3.10). Consistent with this, *Cacna1c* knockout animals exhibit deficits in hippocampus-dependent spatial learning and memory in maze testing paradigms (WHITE et al. 2008).

Syngap1 is a neuron-specific Ras-GTP activating protein that is expressed in glutamatergic neurons and is highly enriched at excitatory synapses where it co-localizes with postsynaptic density proteins and NMDA receptors to regulate excitatory synaptic function and dendritic spine size (KIM et al. 1998, CLEMENT et al. 2012). Altered expression or function of *Syngap1* has been identified in numerous whole-genome studies evaluating the contribution of DNA variants to

neurodevelopmental psychiatric disorders including as autism, schizophrenia, and intellectual disability (KREPISCHI et al. 2010, HAMDAN et al. 2011a, HAMDAN et al. 2011b, RAUCH et al. 2012, STEFANSSON et al. 2014). Further, reductions in S*yngap1* expression has a multitude of pathological consequences on the dynamics and structure of postsynaptic structures that guide not only the initial development of cortical circuitry, but that also provide the foundation for synaptic plasticity associated with learning (ACETI et al. 2015). *Syngap1* was not identified as acutely altered following ethanol exposure, raising the question of whether changes in its expression in a mature brain can have a significant impact on synaptic function, or if the effects of abnormal *Syngap1* expression are restricted to neurodevelopment. Interestingly, reductions in *Syngap1* expression initiated in adulthood using a Cre/loxP knockout resulted in disorganized glutamatergic receptor localization and a decline in spontaneous excitatory currents. Phenotypically, this resulted in impairments in spatial recognition memory and elevated spontaneous locomotor activity in an open field assay (MUHIA et al. 2012), suggesting that reduced *Syngap1* expression in the adult brain of mice exposed to ethanol during the third trimester-equivalent may contribute to the observed deficits in spatial learning and memory tasks (Figure 3.12, 3.14).

Similarly, *Syngr1* encodes a membrane protein that is abundant in presynaptic vesicles (WIEDENMANN and FRANKE 1985) and is a candidate susceptibility gene for psychiatric disorders such as schizophrenia and bipolar disorder (VERMA et al. 2005, IATROPOULOS et al. 2009). While the specific function of *Syngr1* is not completely elucidated and it may have a redundant function with other synaptogyrin and synaptophysin proteins (MCMAHON et al. 1996), it has been linked to impairments in long term potentiation and synaptic plasticity without being directly related to the probability of presynaptic neuro transmitter release (JANZ et al. 1999).

It is clear that ethanol exposure during the third trimester-equivalent in mice leads to long-term alterations to glutamate neurotransmission systems. Alterations to glutamate signaling has profound implications to brain structure

and function, particularly within cortical and hippocampal structures, and could certainly explain a number of phenotypic observations such as impaired learning and memory and changes to locomotor behaviour. Interestingly, these extensive changes to glutamate receptor subunit and associated gene expression seems to be specific to third trimester-equivalent exposure as these genes were not identified as altered in the adult brain of mice treated during the first or second trimester equivalent (KLEIBER et al. 2013). This suggests that altered glutamate neurotransmission and synaptic function is an important consequence of exposure at this developmental stage, with long-term and broad consequences to neurological function.

4.2.2.2 *Retinoic acid signaling*

A number of gene clustering and functional annotation analyses identified an over-representation of transcripts with roles in both retinoic acid and folate-associated processes. The importance of vitamin A and its derivative retinoic acid to the developing brain is well-established (ROSS et al. 2000), and numerous studies have implicated disruptions to retinoic acid signaling in fetal alcohol spectrum-associated cognitive and physiological abnormalities (GRUMMER and ZACHMAN 1995, KANE et al. 2010, KUMAR et al. 2010). Retinoid signaling is also important for aspects of adult brain function, however, and its disruption may impact the regulation of a number of neuronal genes. Genes known to be responsive to retinoic acid include transporters, metabolic enzymes, receptors, and signaling molecules associated with acetylcholine, dopamine, glutamate, and GABA, as well as oxytocin, nerve growth factor, neural cell adhesion molecule, and apolipoprotein E (reviewed in (LANE and BAILEY 2005).

The genes identified as down-regulated in the adult brain were specifically associated with retinoic acid-mediated apoptosis pathways (Table 3.9, 3.10). The pleiotropic actions of retinoic acid are mediated through retinoic acid receptors or retinoid X receptors, and both *retinoic acid receptor, beta* (*Rarb*) and *retinoid X*

receptor, beta (*Rxrb*) were found to be down-regulated in the adult brain of mice treated with ethanol during synaptogenesis. These genes are members of the steroid/thyroid hormone superfamily of transcriptional activators and act as ligand-dependent transcription factors. Upon binding to retinoic acid, they are able to alter the expression of a large number of genes including those associated with cell cycle arrest and apoptosis (BASTIEN and ROCHETTE-EGLY 2004). Retinoic acid can trigger apoptosis through the increased expression of interferons and TNF-related apoptosis inducing ligands (TRAILs), which bind to their associated death receptors leading to the recruitment of *Fas (Tnfrsf6)-associated death domain* (*Fadd*) that serves as a molecular bridge to Caspase 8, initiating caspase-mediated mitochondrial apoptotic processes (NOY 2010). Consistent with evidence that ethanol exposure disrupts retinoic acid signaling is the reduced expression of a number of genes involved in this pathway in addition to the retinoic acid receptors, including *interferon regulatory factor 1* (*Irf1*), *Fadd*, *Tumor necrosis factor (ligand) superfamily, member 10* (*Tnfsf10*) (which, when bound to TRAIL protein receptors, can recruit *Fadd*), and *caspase 3* (*Casp3*). It is interesting to hypothesize how ethanol exposure during the third trimester-equivalent can lead to such an extensive footprint on adult retinoic acid signaling pathways, but it may be a result of ethanol's ability to alter neurodevelopmental retinoic acid signaling through a variety of mechanisms, including mobilizing hepatic retinol stores, altering enzyme activity including its ability to compete as a substrate for alcohol dehydrogenase and acetalydehyde dehydrogenase, changing retinoid-binding protein expression, or disrupting retinoic acid receptors (GRUMMER and ZACHMAN 1995, DUESTER 1996, LIU et al. 2002). Regardless, adult deficiencies in retinoic acid signaling and, specifically, reduced retinoic acid receptor expression, is associated with synaptic efficacy including impaired hippocampal long-term potentiation and long-term depression of synaptic function (CHIANG et al. 1998). Hippocampal long-term potentiation was found to be absent in mice lacking *Rarb*, suggesting a specific role of *Rarb*-containing receptors in learning and memory formation. Reduced expression of this retinoic acid receptor isoform may have a

role in the learning deficits observed in adult mice exposed to ethanol during synaptogenesis.

4.2.2.3 *Folate metabolism*

A number of transcripts altered in the adult brain were associated with folate metabolism and the 'one carbon pool by folate' canonical pathway (Table 3.8, 3.10). Folate is a B-vitamin that acts as an enzyme cofactor that chemically activate 1-carbons, which are required for the synthesis of purine and thymidine nucleotides, and for the remethylation of homocysteine to methionine. Methionine can be used for protein synthesis or also adenosylated to S-adenosylmethionine (SAM) that acts as the major methyl donor for numerous processes including the methylation of proteins such as histones and cytosine bases within DNA (SELHUB 2002). The genes identified as altered by neurodevelopmental ethanol exposure play a role in the metabolism of folate into molecules that are used in both DNA repair and DNA methylation processes. The enzyme *thymidylate synthetase* (*Tyms*) catalyzes the creation of deoxythymidylate using 5,10-methylenyltetrahydrofolate as a cofactor, which maintains the dTMP (thymidine-5-prime-monophosphate) pool essential for DNA replication and repair (NAZKI et al. 2014). Also, the enzyme *5,10-methenyltetrahydrofolate synthetase* (*Mthfs*) catalyzes tetrahydrofolate to 5,10-methenyltetrahyrofolate, a precursor to methionine produced by *5-methyltetrahydrofolate-homocysteine methyltransferase* (*Mtr*). This methionine is then converted to SAM to methylate DNA at CpG sites (NAZKI et al. 2014). Down-regulation of *Mthfs* nd *Mts* likely has an impact on methionine availability for this process. Further, as a consequence of methylation, SAM is converted into homocysteine, a potent neurotoxin that must be excreted or drawn back into the methionine cycle by *Mts* and additional folate (NAZKI et al. 2014). Reduced expression of *Mts* may result in increased concentrations of homocysteine.

Folate availability is critical during neurodevelopment for DNA replication, repair, and appropriate epigenetic patterning. It has been shown that alcohol

consumption during pregnancy can affect the transfer of dietary folate to the developing fetus (HUTSON et al. 2012), leading to an array of developmental abnormalities (BEAUDIN and STOVER 2009); however, these results suggest that neurodevelopmental interruptions to folate metabolism can lead to a long-term effect on the expression of a number of genes involved in this process. It is interesting that our lab and others have observed global hypermethylation across the genome of mice exposed to ethanol during neurodevelopment (LAUFER et al. 2013, KHALID et al. 2014), a phenotype that is consistent with a long-term lack of dietary methyl donors (POGRIBNY et al. 2008). This hypermethylation is also consistent with the bias towards down-regulation observed in the expression of genes in the adult brain observed in this study.

Phenotypically, there is evidence that impairments in folate metabolism can lead to neurological abnormalities including adult cognitive function (SHEA and ROGERS 2014). In the adult brain, SAM-dependent histone methylation impacts learning and memory (JAROME and LUBIN 2013). Alterations to histone methylation/demethylation cycles that control memory cycles are associated with a spectrum of neurological disorders, such as intellectual disability, autism, depression, fear and anxiety, and schizophrenia (SHULHA et al. 2012, JAROME and LUBIN 2013, ISHII et al. 2014, MORISHITA et al. 2015), suggesting that factors that affect methylation-associated processes during neurodevelopment, including prenatal alcohol exposure, can set up altered gene expression patterning that persists into adulthood and may alter cognition and behaviour.

4.2.2.4 *Chromatin organization and histone modification*

In keeping with the hypothesis that alterations to epigenetic regulation of genes in the adult brain drive the persistent changes to gene expression, a number of genes identified as altered were involved in histone modification and chromatin remodeling (Table 3.8). These included genes found within the large histone gene cluster 1 located on chromosome 13 in mice such as *Hist1h2bf*, *Hist1h2bm*,

Hist1h3c, and *Hist1h4d*, which are replication-dependent histones that are predominantly expressed during S phase (MARZLUFF et al. 2002). Also affected were histone deacetylase genes *Hdac8* and *Hdac10* and the histone acetyl transferase gene *CREB binding protein* (*Crebbp*). These results support previous findings that perinatal alcohol exposure decreases the expression of *Crebbp* in the cerebellum (GUO et al. 2011), which was associated with decreased lysine acetylation on histones H3 and H4 with motor activity changes observed in individuals with FASD. Other down-regulated genes critical to epigenetic control of gene regulation included *DNA methyltransferase 1 associated protein 1* (*Dmap1*), a co-repressor that supports the maintenance as well as the de novo methylation by *DNA methyltransferase 1* (LEE et al.). The down-regulation of this gene is interesting due to the general hypermethylation observed in the genome of ethanol-exposed individuals; however, knock-down of *Dmap1* has been shown to inhibit cellular proliferation via activation (demethylation) of the *p16* tumor suppressor gene pathway, as well as lead to hypomethylation of regions controlling DNA repair products (LEE et al. 2010). By contrast, *methyl-CpG binding domain protein 3* (*Mbd3*) is a strong inducer of demethylation (BROWN et al. 2008) and was also identified as down-regulated in the brain of adult ethanol-exposed mice. Down-regulation of *Mbd3* was found to be associated with global hypermethylation and increased methylation in CpG islands associated with the control of cell cycle genes (CUI and IRUDAYARAJ 2015). Other studies have examined the effects of neurodevelopmental alcohol exposure on epigenetic patterning in the brain and suggest that ethanol impairs spatiotemporal establishment of appropriate epigenetic marks that guide differentiation and cellular maturation, and that these changes are correlated with neurobehavioural phenotypes associated with FASD such as stress vulnerability, social interaction, and learning and memory deficits (CHEN et al. 2013, VEAZEY et al. 2013, BEKDASH et al. 2014, SUBBANNA et al. 2015). Results from our lab also suggest that these epigenetic changes are genomically wide-spread and may provide a mechanism for long-term gene expression dysregulation (LAUFER et al. 2013). The identification of a number of genes associated with chromatin structure by this

study suggests that ethanol may affect the subtle interplay between epigenetic control mechanisms, including DNA methylation and histone modifications, exerting a global change in regulation of gene transcription.

4.2.2.5 *Gene network interactions highlight alterations to neurotransmission*

A gene network analysis was performed on genes identified as altered in the adult brain of mice treated with ethanol on P4 and P7 to predict interacting molecules and evaluate and predominant biological pathways that may integrate the observations gathered by the GO and IPA gene enrichment analyses. All the genes included in these networks were down-regulated, which reflects the bias observed from the array results (Appendix B). Also, the significant networks were less intricate than those generated from the short-term analyses and showed a number of common molecules (Figures 3.23, 3.24). Glutamate receptor subunit *Grin2b* appeared as a major hub in all networks, consistent with previous analyses suggesting that glutamate neurotransmission may be among the most significant processes affected by ethanol exposure during the third trimester-equivalent. Interestingly, *huntingtin* (*Htt*) appears as a central hub both in the 'neurological disease, psychological disorders, behaviour' (NPD) network (Figure 3.23) and in the merged network (Figure 3.25). We have observed that *Htt* is consistently identified as a major hub in gene network analyses of multiple ethanol treatment paradigms (KLEIBER et al. 2013). While the involvement of *Htt* with regard to prenatal ethanol exposure is, as far as we are aware, unreported in prenatal alcohol exposure literature, it is known that *Htt* is widely expressed in the developing brain and has roles in cell survival, proliferation, and migration, and it is essential for the formation of normal excitatory synapses (TONG et al. 2011, MCKINSTRY et al. 2014). In the adult brain, mouse knock-down models have shown that reduced huntingtin levels were associated with neuronal loss and degeneration in the subthalamic nucleus and global pallidus, resulting in increased motor activity and cognitive deficits (NASIR et al. 1995, O'KUSKY et al. 1999). Despite the well-established role of *Htt* in neuropathology, its relationship

with neurodevelopmental alcohol exposure remains an unexplored avenue, though its down-regulation and consistent appearance as a 'hub' gene in multiple treatment paradigms is intriguing.

Also prominent in the NPD network (Figure 3.23) and in the merged network (Figure 3.25) is *5-hydroxytryptamine (serotonin) receptor 1A* (*Htr1a*). Serotonin receptors *Htr1a*, *Htr5b*, and *Htr1d* were all identified as down-regulated in the adult brain (Table 3.10). This is consistent with studies reporting impaired serotonin neuron innervation in the forebrain, hypothalamus, frontal and parietal cortices, and hippocampus in mice prenatally exposed to alcohol (ZHOU et al. 2001, ZHOU et al. 2005). Reduced expression of human *HTR1A* is associated with a number of psychopathological states such as increased anxiety, stress reactivity, and depression (ALBERT 2012). In mice, *Htr1a* knock-out results in heightened stress reactivity and anxiety-like phenotypes (HEISLER et al. 1998, PARKS et al. 1998), which can be ameliorated by postnatal handling (ZANETTINI et al. 2010). This is consistent with findings that *Htra1* and other serotonin receptors may be sensitive to gene-environment interactions that result in altered epigenetic marks that impact transcriptional control of these genes (ALBERT and FIORI 2014, HOLLOWAY and GONZALEZ-MAESO 2015). Also associated with altered anxiety-related phenotypes is *apolipoprotein E* (*Apoe*), which was identified as down-regulated in the adult brain of mice exposed to ethanol at P4 and P7, and appears within all three gene networks (Figures 3.23, 3.24, 3.25). *Apoe* plays an important role in the metabolism and redistribution of lipoproteins and cholesterol (HUANG and MAHLEY 2014). Further, *Apoe*-deficient mice show age-dependent structural and functional changes to the cortex and hippocampus (MASLIAH et al. 1995, BUTTINI et al. 1999), as well as age-dependent dysregulation of the HPA axis via increased levels adrenal corticosterone both at the basal level and following restraint stress (RABER et al. 2000). This was associated with increased anxiety-related behaviours in the elevated plus maze, reduced activity in a novel open field arena, and learning deficits (RABER et al. 2000, GROOTENDORST et al. 2002). Persistently reduced expression of *Apoe* in the adult brain certainly may

contribute to the maintenance of anxiety and learning-related phenotypes observed in mice exposed to ethanol during the third trimester-equivalent. Also, it is interesting that *Apoe* and *Pomc*, which appears in the NPD gene network as well as the merged network (Figures 3.23, 3.25), were identified as altered following third trimester-equivalent ethanol exposure but not following other treatment paradigms (KLEIBER et al. 2013), suggesting that late gestation may be a critical window of vulnerability for HPA axis-associated processes. Interestingly, there is evidence that ethanol-induced gene expression changes associated with HPA axis dysfunction are the result of altered epigenetic mechanisms that are malleable and can be corrected (BEKDASH et al. 2013, GANGISETTY et al. 2014).

The genes identified as altered in the adult brain of mice exposed to ethanol during neurodevelopment at P4 and P7 suggest changes to critical neural processes that are important for behaviours observed in these animals, such as altered glutamate neurotransmission, changes to serotonin signaling, and HPA axis dysfunction. These processes are certainly interrelated, and also associated with the altered expression of genes related to apoptosis (*Casp3*), neurotrophic signaling (*Ntf3*), extracellular matrix integrity and signal transduction (*Mmp2*, *Map3k10*) (Figure 3.25). While these processes are consistent with the behavioural and physiological phenotypes observed in individuals with FASD, it remains to be evaluated how the altered expression of these genes may impact cellular function and contribute to these phenotypes.

4.2.3 Molecular pathways acutely affected by ethanol exposure during synaptogenesis that remain modified into adulthood

Given the similarities between biological process affected both acutely following ethanol exposure during synaptogenesis and those altered in the adult brain, it is intriguing to make comparisons between the two gene sets. Such a comparison is

valuable in the understanding of the events that establish long-term neural dysfunction resulting from neurodevelopmental ethanol exposure, which ultimately manifests as long-lasting cognitive and neurobehavioural phenotypes. The results included indicate that binge-like ethanol exposure alters two sets of genes: the first represents a rapid cellular stress response that leads to the apoptosis of vulnerable cells. Ultimately, this modified expression profile impacts the further developmental trajectory of surviving cells, resulting in a 'molecular footprint' of third trimester-equivalent ethanol exposure observed in adult mice that may, in part, account for the behavioural deficits presented by these animals (KLEIBER et al. 2013, MANTHA et al. 2013).

While few transcripts were identified as altered in both analyses (Table 3.11), there is a great deal of overlap in the biological processes associated with genes altered at P7 and in adulthood at P60 (Table 3.12). Genes identified as altered both acutely following ethanol exposure and within the adult brain suggest that third trimester-equivalent ethanol exposure has a significant impact on the regulation of synaptic transmission and plasticity, involving the altered expression of a number of glutamate receptor subunit genes as well as genes involved in synapse formation, organization, and maintenance. Glutamate neurotransmission is essential for the formation of functional neural circuits (AAMODT and CONSTANTINE-PATON 1999, BOLTON et al. 2000), and is a major target of ethanol, which is able to act as a strong NMDA receptor antagonist (IKONOMIDOU et al. 2000). Altered glutamate neurotransmission also plays a role in an array of cognitive and behavioural phenotypes, including those associated with FASD such as motor coordination delays, hyperactivity, increased anxiety, and impaired learning (KIYAMA et al. 1998, MOHN et al. 1999, JENSEN et al. 2009, BARKUS et al. 2010, ARAKAWA et al. 2014). Important to this study, synaptogenesis seems to be a critical period of vulnerability to ethanol-induced changes to glutamate neurotransmission (OLNEY et al. 2002a, PUGLIA and VALENZUELA 2010, KLEIBER et al. 2013), which has led us to the hypothesis that neurodevelopmental processes that are active at the time of exposure are most responsive to environmental

interference, leading to long-term alterations in sets of genes that are involved in these processes (or the 'molecular footprint' of timing of exposure) (KLEIBER et al. 2014a).

Also implicated as a consequence of third trimester-equivalent ethanol exposure by both short- and long-term gene sets were alterations in steroid hormone signaling and potentially abnormal HPA axis development and function in the adult brain. Genes involved have well-established roles in stress response and glucocorticoid regulation, including *pro-opiomelanocortin* (*Pomc*), apolipoprotein E (*Apoe*), and the steroid-thyroid hormone-retinoid receptor *nuclear receptor subfamily 4, group A, member 1* (*Nr4a1*). Interestingly, this is associated with altered Circadian rhythm signaling as identified in both the short-term and long-term canonical pathways. We observed altered expression of Period genes *Per1* and *Per2*, as well as long-term altered expression of *pro-opiomelanocortin* (*Pomc*) (CHEN et al. 2006a). Altered sleep-wake cycles have previously been observed in human infants and adolescents exposed to alcohol during neurodevelopment (CHEN et al. 2006a). Consistent with this study and others, these effects are most pronounced in animal models when exposure occurs during the brain-growth-spurt period (EARNEST et al. 2001, SAKATA-HAGA et al. 2006). Altered Circadian rhythm, and corresponding altered HPA axis corticosterone signaling, are further associated in FASD individuals or models with increased vulnerability to stress, depression, hyperactivity, and diminished cognitive function (EARNEST et al. 2001, SAKATA-HAGA et al. 2006, GIROTTI et al. 2007, WEINSTOCK 2010).

A comparison between the biological processes associated with both the short-term and long-term gene sets also identified thyroid hormone/retinoid X receptor signaling (TR/RXR) pathway as affected by trimester three-equivalent ethanol exposure (Table 3.12). Some genes implicated were also associated with altered hormonal signaling given that RXRs are nuclear receptors that mediate retinoic acid-induced gene expression through the formation of homo or heterodimers with other receptors including thyroid hormone receptor (TR), among many others (CHAMBON 1996, BASTIEN and ROCHETTE-EGLY 2004). Again, these processes

seem to be affected primarily by ethanol exposure during this developmental period as they were not identified by analyses of first or second trimester-equivalent exposure paradigms (KLEIBER et al. 2013, MANTHA et al. 2014b), suggesting that late-gestation may be a critical period for the appropriate development and function of systems reliant on retinoic acid receptor signaling.

Lastly, the PTEN canonical pathway was identified as significantly affected in the short-term and long-term effects of ethanol (Table 3.12). Given the broad intracellular signaling functions of the PTEN and associated PI3K/AKT/mTOR pathway and its ability to respond to nutrient availability, stress, hormones, and growth factors to modulate protein synthesis, a comprehensive discussion of this pathway with relevance to prenatal alcohol exposure would be assumptive. The pathway is worth attention, however, in light of recent findings implicating that this pathway may be a consistent target of neurodevelopmental alcohol exposure given its consistent identification in the analysis of brain expression profiles and methylation alterations resulting from multiple treatment paradigms (KLEIBER et al. 2013, LAUFER et al. 2013).

4.2.4 Maintenance of long-term gene expression changes, altered miRNA expression, and epigenetic patterning

The results included in this study offer an evaluation of potential epigenetic mechanisms that may help establish a long-term footprint of altered gene expression following ethanol exposure during synaptogenesis. There has been increasing evidence that epigenetic processes established during neurodevelopment, such as DNA methylation, chromatin structure, and miRNA expression are altered by prenatal ethanol exposure (MIRANDA 2011, BEKDASH et al. 2013, VEAZEY et al. 2013). Histone deacetylases (*Hdac1*, *Hdac9*) appear as target molecules within multiple canonical pathways identified as altered acutely (at P7) following ethanol exposure (Table 3.5). These genes play an important

role in controlling gene expression and cell signaling events via histone modifications and chromatin remodeling, and play an established role in a number of neurodegenerative disorders including fetal alcohol spectrum disorders (BUTLER and BATES 2006, LAUFER et al. 2013, VOGEL-CIERNIA and WOOD 2013). These genes act by not only altering chromatin structure but also in interacting with tissue-specific transcription factors to control apoptosis and differentiation (PARRA and VERDIN 2010, CONWAY et al. 2012).

Evaluation of miRNA changes in the adult brain of mice exposed to alcohol at P4 and P7, within the brain "growth spurt" period, identified a number of miRNAs as altered at P60 following ethanol exposure at synaptogenesis (Appendix C). The genes identified as potential targets of some of these miRNAs play important roles in the function of mature neuronal communication and signaling (Table 3.13). Interestingly, many of the biological pathways associated with these miRNAs and their targets showed similarities with the pathways affiliated with the modified short-term (P7) transcripts and relevance to synaptogenesis. Further, these pathways showed higher relevance to synaptic function and neural function rather than cell survival processes, as may be expected given that these results highlight the residual effects of exposure at synaptogenesis rather than immediate stress response. Of note are the miRNAs posed to regulate genes associated with HPA-associated corticotrophin and retinoic acid signaling such as *miR-26b* and its putative cannabinoid receptor target (*Cnr1*), *miR-184* and its putative target *Ncor2*, *miR-721* and its targets *Ppargc1a* and *Ncoa3* (Table 3.13). The effect of ethanol exposure on *Cnr1* expression in the developing brain has previously been reported. Subbanna *et al*. (2013) reported that ethanol exposure at P7 resulted in increased *Cnr1* expression in the hippocampus and cortex that was associated with Erk1/2 phosphorylation and neurodegeneration. Further, *Cnr1* knock-out eliminated ethanol-induced neurodegeneration, inhibited Erk1/2 phosphorylation, and rescued normal adult synaptic plasticity and novel object recognition (SUBBANNA et al. 2013). *Nuclear receptor coactivator 3* (*Ncoa3*) and *nuclear receptor corepressor 2* (*Ncor2*) both are thyroid hormone and retinoic acid

receptor-associated proteins that act to activate or repress, respectively, transcription of certain gene sets. A recent study suggested that *Ncoa3* is itself an important regulator of miRNA action by promoting the expression of Argonaute proteins, core components of miRNA-induced silencing complex (miRISC), and regulates dendritic complexity and spine maturation in the hippocampus (STORCHEL et al. 2015). NCOR proteins have well-established roles in neural function and memory, with disruption of NCOR-regulated gene expression associated with cognitive and memory impairments in both humans and rodent models (XING et al. 2006, HAWK et al. 2012, GILBERT and LASLEY 2013, LYST et al. 2013). Ncor2 has been experimentally shown to be regulated by miR-184 (WU et al. 2011) and itself regulates gene expression via its interactions with histone deacetylases (SUN et al. 2013).

Correspondingly, the Target Filter™ results suggested that PI3K/AKT/mTOR signaling may be altered via aberrant miRNA regulation of the tumor suppressor protein *Tubular sclerosis 1* (*Tsc1*) (Table 3.13). The up-regulation of *miR-721* and subsequent down-regulation of *Tsc1* is significant with relevance to FASD-related abnormalities given current evidence that knock-down of *Tsc1* or its functional partner, *huntingtin-associated protein 1* (*Hap1*), profoundly impairs the positioning of pyramidal neurons in the hippocampus (MEJIA et al. 2013), leading to long-term neurological impairment including cognitive deficits and learning impairments (ORLOVA and CRINO 2010).

Taken together, these results argue that altered epigenetic mechanisms, including histone modifications and miRNA regulatory control, may reflect consequences of neurodevelopmental ethanol exposure that do not have a specific relationship to cellular stress response but nonetheless exert long-term functional changes within the adult brain, and have roles associated with cognitive and behavioural phenotypes observed in individuals with FASD. While these results are preliminary in nature and require further functional examination to confirm miRNA/mRNA interactions in given cell types and specific consequences on cellular function, the findings are interesting and certainly indicate that long-term

miRNA regulation is altered as a consequence of neurodevelopmental alcohol exposure. These findings are also consistent with recent literature suggesting that miRNAs play multiple roles in both the acute and long-term effects of alcohol in the developing and adult brain (SATHYAN et al. 2007, WANG et al. 2009, BALARAMAN et al. 2013, IGNACIO et al. 2014, CHEN et al. 2015).

4.3 Caveats to assessing FASD in animal models

4.3.1 Limitations to the use of mice in modeling FASD

There are some limitations to the current study which should be noted. A broad observation relevant to many studies that utilize any animal model system to assess neurobehavioural or cognitive disorders is that it cannot be ignored that one is trying to recapitulate a complex human phenotype in an animal model that, by its nature, will not be able to fully model all aspects of that disorder as it presents in human patients. In this sense, the validity of the model is reliant on its ability to effectively map the etiology, behaviour, and biological mechanisms of human psychopathology. FASD is different from many neurological disorders in that it has a clear etiology: exposure to alcohol during neurodevelopment. This known causation provides legitimacy to animal models of FASD in that it is relatively simple to treat pregnant dams with ethanol, either via voluntary ingestion, gavage, or injection of known ethanol doses, allowing clear relevance of the model to human FASD. Animal models must also focus on the presentation of neurobehavioural phenotypes that are relevant to the disorder they are seeking to emulate, either cognitive, which in animal models must be inferred, or behavioural, which is an observable response to a given assay. In this study, I believed it was important that mice exposed to ethanol during neurodevelopment displayed a cluster of endophenotypes that were commonly associated with human FASD, namely developmental delay, activity alterations, anxiety-related phenotypes, and learning deficits. A combined behavioural and molecular

approach may help define genetic contributions to endophenotypes in mental disorders (GOTTESMAN and GOULD 2003). The presented model successfully displayed many endophenotypes that can be compared to human FASD behavioural phenotypes, and there was a great deal of consistency with the genes and pathways that were implicated as involved in both neonate and adult mice. These included pathways that have previously been identified as involved in alcohol response and fetal alcohol spectrum effects including glutamate neurotransmission and genes associated with synaptic plasticity, cell cycle control and stress response, neurotrophic molecules, and genes associated with HPA axis function.

Humans and mice – while genetically and physiologically similar – are certainly not identical, and they have different evolutionary histories that cannot be discounted. The neurodevelopmental timeline of mice and humans have been well-characterized and well-correlated; however, based on the human neurodevelopmental timeline, the developmental processes that occur during the third trimester also occur in the mouse from roughly postnatal days 2 to 14 (DOBBING 1974, DOBBING and SANDS 1979, CLANCY et al. 2001). Thus, to mimic an ethanol exposure during this neurodevelopmental time, ethanol must be administered to a neonate pup rather than to an *in utero* fetus, eliminating interactions with maternal genotype and maternal ethanol metabolism. This could be viewed as a potential advantage, given that these maternal confounding factors can be avoided and the blood alcohol concentration of the animal can be more easily controlled. In this study, the timing of ethanol exposure also allowed us to balance the treatment groups such that we were able to pair each ethanol-treated pup with a weight and sex-matched littermate control, eliminating the potential confounds associated with litter effects. There are certainly other animal models such as non-human primates, other rodent species, and non-mammalian species (PATTEN et al. 2014), many of which are more precocial at birth, that have been successfully used to evaluate behavioural, physiological, or molecular phenotypes associated with FASD. In this study, mice were chosen due

to their amenability to ethanol effects, range of well-validated behavioural assays, and broad range of available genetic information and tools. In mice, these resources have been shown to be useful when evaluating how molecular processes contribute to changes in brain function in neurological disorders, which is not possible to assess in humans (CRYAN and HOLMES 2005, NESTLER and HYMAN 2010a).

With regards to the model presented here that sought to interrogate the specific effects of late-gestational ethanol exposure, it should also be noted that, epidemiologically, it is certainly uncommon that a pregnant female would ingest a high dose of alcohol during the third trimester only (GLADSTONE et al. 1997, ETHEN et al. 2009). Neurodevelopment is a lengthy and complex process, and without controlled models of exposures of specific times, it is difficult to differentiate the resulting molecular and, subsequently, the phenotypic effects that result from exposures at each stage of development. The purpose of this third trimester-equivalent exposure paradigm was to evaluate how exposure during the 'brain growth spurt' – a period of rapid neural development and synapse formation – disrupts certain genes or pathways known to be involved in these processes. Also, certain regions of the brain that have relevance to FASD-related phenotypes, such as learning, stress reactivity, hyperactivity and attention, and motor coordination, show a strong vulnerability to ethanol-induced apoptosis or damage during this period of neurodevelopment (IKONOMIDOU et al. 2000, OLNEY 2004, DIKRANIAN et al. 2005). Evaluations of third trimester-equivalent models of FASD are becoming increasingly prevalent in the literature due to the brain's vulnerability at this stage. Studies have established that ethanol exposure at P7 results in robust phenotypic consequences, such as profound learning deficits, altered stress-reactivity and altered Circadian rhythm dysfunction (EARNEST et al. 2001, WOZNIAK et al. 2004, BROCARDO et al. 2012). This is also why we have employed various treatment paradigms in our research, including 'binge'-like exposures at distinct neurodevelopmental times and a moderate chronic exposure paradigm that exposes the developing pup to ethanol throughout gestation via voluntary

maternal consumption of a 10% ethanol solution (KLEIBER et al. 2011, MANTHA et al. 2013). While it is clear that there is no one exposure time that is independently responsible for the range of behaviours associated with FASD, it is important to discern the mechanisms that may underlie the subtle differences in behavioural and molecular phenotypes generated from each treatment paradigm.

4.3.2 Technical considerations

Aside from considerations regarding the model itself, there are other caveats to this research that should be noted that were associated with technical aspects of the methodology. First, the gene expression changes identified in this study were evaluated using whole-brain homogenates. It is recognized that the developing and adult brain are complex tissues with a number of regions that undergo neurodevelopmental processes at a varying timeframes. While synapse formation may the predominant process occurring during the first two weeks of postnatal mouse development (corresponding to the processes occurring during the third trimester equivalent), there are also a number of other critical processes occurring at this time depending on region, including neurogenesis, glial development, apoptotic pruning, and myelination (DOBBING and SANDS 1973). The results we have generated have relevance to synapse-related processes, particularly in regions such as the corpus callosum, the limbic system, the HPA axis, the cerebellum, and the cortices. Coincidentally, these are the regions that are particularly vulnerable to ethanol-induced neurodegeneration and show altered developmental trajectories following trimester three-equivalent ethanol exposure (BONTHIUS and WEST 1990, IKONOMIDOU et al. 2000, OLNEY 2004, DIAZ et al. 2014a). We hypothesize that the brain regions most vulnerable to ethanol will be those cells that are actively undergoing developmental processes and are particularly sensitive to intracellular and extracellular cues, such as ethanol. As such, the results generated in this study can only be interpreted on a broad scale, perhaps suggesting that common alterations to neurodevelopmental trajectories

may happen in some varying brain regions that have a similar developmental timeline. Regardless, future studies examining region-specific changes in gene expression, protein expression, and physiological structure would be extremely useful and will provide a more detailed perspective of those genes and pathways that may be affected by ethanol within those regions.

Secondly, experimenters using microarrays as investigative tools have attempted to achieve a standardization of data quality with the suggestion of requirements, known as the 'minimum information about a microarray experiment' (MIAME) that may be needed to interpret the results of the experiment and potentially reproduce its findings (BRAZMA et al. 2001). Currently, many journals and funding agencies require that microarray experiments adhere to MIAME guidelines, though there has been criticism (GALBRAITH 2006, SHIELDS 2006). While the array experiments included in this study have attempted to meet MIAME standards and the information regarding all experiments have been curated to NCBI's Gene Expression Omnibus (GEO) database , I acknowledge that the sample sizes used by the microarray studies were low, resulting in less than ideal statistical power to identify low-fold gene expression changes. The use of an FDR-corrected *p*-value in the identification of differentially expressed genes certainly assisted in reducing array noise, though it cannot be discounted that a proportion of the genes identified were false positives, and equally importantly, a number of genes affected were likely not identified by this study (false negatives). It has been reported that the use of inbred animals, as in this study, reduces the need for a greater number of array replicates (WEI et al. 2004). Also, I had success in confirming three of four genes identified by the short-term array, and four of four genes identified by the long-term analysis, which may speak to the reliability of the array results using qPCR, though the confirmation of more genes would certainly add to confidence in the array results. Further, the cost of arrays since 2001 has substantially decreased and continues to decline, allowing current studies to employ more arrays or even make use of next-generation technology, such as RNA-seq (MARIONI et al. 2008, MORTAZAVI et al. 2008).

Lastly, while behavioural analyses used both male and female cohorts, all samples used for RNA and miRNA expression analysis were derived from male mice. Sex-specific differences in certain behaviours, such as activity in a novel open field arena (Figure 3.2) and anxiety-related behaviours (Figures 3.8, 3.9, 3.10, 3.11) were identified by this study. As mentioned, these findings are consistent with other studies suggesting that prenatal alcohol exposure may affect individuals in a sexually-dimorphic manner, particularly with regard to stress vulnerability, HPA axis function, serotonin response, and hippocampal function (GABRIEL et al. 2006, HOFMANN et al. 2007, MCMURRAY et al. 2008, HELLEMANS et al. 2010, PRZYBYCIEN-SZYMANSKA et al. 2010, ZAJAC et al. 2010). It would be prudent to evaluate sex-specific differences in gene expression, particularly with regard to genes that were identified and are critical to HPA axis development and function such as *pro-opiomelanocortin* (*Pomc*) and *apolipoprotein E* (*Apoe*), and to further assess how ethanol affects neurodevelopment in conjunction with sex-specific hormonal signaling (HELLEMANS et al. 2008, WEINBERG et al. 2008, KUDWA et al. 2014).

4.4 Evaluation of behavioural and gene expression changes across treatment paradigms

Upon the initiation of this study, there were few models of FASD that were being utilized to evaluate the molecular effects of prenatal alcohol exposure in the brain. These models varied in treatment dose, timing, treatment method, and model system, making comparisons between them difficult. A major goal of this research was to develop a comprehensive animal model that evaluated the behavioural and genetic effects of ethanol exposure during neurodevelopment using controlled dosage paradigms across multiple trimester equivalents. These studies have provided a mechanism to compare the behaviours and genes that may be affected

by alcohol consumption at specific stages of human neurodevelopment (KLEIBER et al. 2013, MANTHA et al. 2013, KLEIBER et al. 2014b, MANTHA et al. 2014a).

4.4.1 Behavioural changes and timing of exposure

The results included in this thesis focus on findings resulting from a heavy binge-like exposure during early postnatal period, disrupting neurodevelopmental processes that are roughly equivalent to those occurring in the human brain during the third trimester. The first goal of this research was to establish a battery of behavioural assays that was relevant to major behavioural and cognitive phenotypes observed in human FASD and that could be used to test the development of these features as they emerged throughout the course of the maturation from neonate to adult. The behavioural battery, while certainly not an exhaustive evaluation of all FASD-relevant behaviours, was able to successfully identify changes to early neuromuscular coordination, activity levels, anxiety-related traits, and learning ability, strategy, and spatial memory retention resulting from early postnatal ethanol exposure.

This battery was also able to identify behavioural differences between treatment paradigms that used an equivalent ethanol dose administered at the trimester one, trimester two, or trimester three-equivalent (MANTHA et al. 2013). Findings across treatment paradigms were intriguing, and while similar behaviours were observed within all models, there were distinctive differences in the subset or severity of the phenotypes observed. For example, while differences in nocturnal overnight activity were observed in all paradigms, the pattern varied considerably with very little difference observed following trimester one-equivalent exposure, an intermediate phenotype observed following trimester two-equivalent exposure, and a pronounced difference – specifically during the early period of the light/dark cycle - following third trimester-equivalent ethanol exposure. Further, the emergence of anxiety-related traits seemed to be specific to late-gestational (trimester two and three-equivalents) ethanol exposure. Lastly, the pattern of

spatial learning was interestingly variable between treatment times, with trimester one-equivalent-treated mice showing a pronounced difference in latency to locate the target of the Barnes maze between control and ethanol-treated mice only during the latter testing days. Conversely, trimester two-equivalent-treated mice showed differences in latency during the first day of training, but appeared to 'catch up' to their control counterparts – eliminating differences in latency to locate the target - by testing days two to four. As presented within this thesis, ethanol treatment during the third trimester-equivalent resulted in the most severe spatial learning deficits as tested by the Barnes maze, which were more pronounced when postnatal day 7 was included as a treatment day. These findings suggest that there may be windows of vulnerability for the later emergence of certain phenotypes that may be dependent on the sensitivity of certain neural regions that govern these behaviours. This would also imply that certain behaviours are likely to appear co-morbidly given that brain regions interact to influence multiple cognitive behaviours, such as the hippocampus and its role in learning and memory and the influence of HPA axis-associated stress reactivity on learning behaviour (McEwen 2003, Wingenfeld and Wolf 2014). The sensitivity of particular brain regions to ethanol at varying neurodevelopmental times also may speak to the phenotypic heterogeneity of FASD. Its classification as a "spectrum" disorder certainly reflects the broad range and severity of phenotypes that are associated with prenatal alcohol exposure, and factors including (though not limited to) timing and dose of ethanol may contribute to this variability. Finally, as early gestational exposure has been biologically associated with distinctive craniofacial and other physiological abnormalities that categorize FAS (Sulik 2005), the appearance of a subset of abnormal behaviours in an infant, child, or adolescent with prenatal alcohol exposure may offer a modest estimate of the timing and/or dosage of ethanol exposure, though this hypothesis certainly would require a much more thorough evaluation at both the animal model and clinical level given the complex socio-economic factors that influence outcome in humans.

4.4.2 Genome-wide gene expression changes as an indicator of altered neurodevelopmental and adult neural processes

Many studies have examined the ability of ethanol to elicit excitotoxic cell death in immature brain regions depending on timing and dosage (IKONOMIDOU et al. 2000, GOODLETT and HORN 2001, DIKRANIAN et al. 2005, NOWOSLAWSKI et al. 2005). Fewer studies, however, have examined the long-term molecular effects of ethanol exposure – those effects that do not lead to apoptosis but to an interruption of normal neurodevelopmental processes that causes the cell to respond, adapt, and continue on a developmental trajectory towards maturation. Analysis of gene expression changes acutely (4 hours) following ethanol exposure at P7 suggested that a number of cellular stress-associated mechanisms underlie the response of developing neural cells to a binge-like ethanol insult. While a number of genes associated with apoptotic cell death were upregulated, a number of biological processes were identified that were consistent with optimizing cell survival, such as the down-regulation of molecules involved in energetically costly processes such as ribosome synthesis, cell cycle transition, and a number of transcription factors involved in developmental processes, and the upregulation of molecules integral to cell membrane integrity (Table 3.5). These findings are consistent with results from ethanol treatments administered during the first and second trimester equivalent, which identified the down-regulation of genes associated with ribosomal biosynthesis, cell cycle progression, cellular migration, and neuronal maturation, and the upregulation of genes involved in cellular integrity and adhesion (GREEN et al. 2007, MANTHA et al. 2014a). What seems to be integral to ethanol exposure during the third trimester-equivalent is the changes in expression of a number of genes associated with neurotransmitter systems such as glutamate, GABA, dopamine and serotonin, as well as steroid hormone signaling including retinoic acid and metabolic derivatives of *pro-opiomelanocortin* (*Pomc*) (Tables 3.5, 3.6, 3.8. 3.9. 3.10). This may be related to the timing of exposure and the sensitivity of predominant biological processes that are occurring during this 'brain growth spurt' to external molecular cues,

including the maturation of neural circuitry, maintenance of synaptic connections, and apoptotic synaptic pruning (COHEN-CORY 2002, LUJAN et al. 2005, AKERMAN and CLINE 2007). Interestingly, we have found that ethanol exposure during the first trimester equivalent alters the expression of a number of genes associated with cellular survival, neurulation, and extracellular matrix remodeling, while exposure during the second trimester-equivalent disrupts processes associated with cellular migration and differentiation (KLEIBER et al. 2013, KLEIBER et al. 2014a). This has reinforced our hypothesis that ethanol may target cells that are particularly sensitive to intra- and extracellular cues, as is the case with cells undergoing neurodevelopmental processes that rely heavily on cellular communication, signaling, and appropriate and timely gene expression.

A novel goal of this study was to not only assess the changes to the brain transcriptome acutely following ethanol exposure, but to also evaluate if neurodevelopmental ethanol exposure resulted in long-term changes to brain gene expression that are present in adulthood, potentially driving or at least contributing to the behavioural phenotypes that emerge and persevere in individuals with FASD. Given that long-term gene expression changes were identified regardless of treatment paradigm (KLEIBER et al. 2012, KLEIBER et al. 2013), the question of what may initiate and maintain these gene expression changes over time naturally arises. A clear answer to these questions is not straight-forward and may involve some combination of the primary (acute) and secondary effects of ethanol exposure.

Factors that instigate these changes in gene expression may include ethanol-induced apoptosis of susceptible cell types, leading to an overall change in the cellular composition of the brain and, subsequently, the overall pattern of brain gene expression (IKONOMIDOU et al. 2000, OLNEY et al. 2002a). Given that this study utilized whole brain tissue as a source of RNA, this may certainly contribute to some of the gene expression changes identified. Another contributing mechanism may be the ability of ethanol to disrupt developmental processes that are highly reliant on external cues such as cell proliferation, migration, differentiation, and

communication (GUERRI 1998, RICE and BARONE 2000, CAMARILLO and MIRANDA 2008). The disruption of these processes would represent residual effects of the ability of alcohol to alter cellular identity, structure, or connectivity in a way that alters the physiology of the brain such that the appropriate balance of adult gene expression is not maintained. This hypothesis has been suggested for a number of spectrum disorders, and has been referred to as a neurodevelopmental "footprint" of teratogen exposure (DEMJAHA et al. 2011, DUFOUR-RAINFRAY et al. 2011, KLEIBER et al. 2012). We have observed distinct (though related) patterns of altered gene expression depending on timing and dose of ethanol exposure, supporting that neurodevelopmental ethanol exposure may leave a 'molecular footprint' that is somewhat specific to the timing of exposure and involves altered gene expression and epigenetic patterning (KLEIBER et al. 2012, KLEIBER et al. 2013, LAUFER et al. 2013).

There is increasing evidence that developmental ethanol exposure can affect epigenetic patterning including DNA methylation, histone modifications, and microRNA expression, which can produce long-term and relatively stable changes to the expression of a number of genes (SATHYAN et al. 2007, WANG et al. 2008, LIU et al. 2009, KAMINEN-AHOLA et al. 2010, MIRANDA 2011). Results presented within this thesis support research reporting ethanol's ability to alter the expression of proteins involved in histone modifications (ie, HDACs), proteins involved in one-carbon metabolism and DNA methylation, and a number of microRNAs, suggesting that ethanol exposure during the third trimester equivalent is able to interfere with processes that establish appropriate neural epigenetic programming. This is consistent with results from other treatment paradigms used within our lab; indeed, all treatment methods (binge or voluntary maternal consumption) that we have utilized have been associated with changes to epigenetic programming (LAUFER et al. 2013, KLEIBER et al. 2014b, MANTHA et al. 2014b). This avenue of fetal alcohol research is relatively new but gaining in attention given that it has implications towards the persistence of FASD phenotypes throughout the lifetime of an individual. Developmentally-

established epigenetic programming can often be rendered extremely stable (REIK et al. 2001, HAYCOCK 2009, O'SULLIVAN et al. 2012); however, reprogramming can take place at several points throughout the lifespan in response to changes in environmental conditions, suggesting that if epigenetic factors strongly underlie the behavioural and cognitive consequences of fetal alcohol exposure, they may be amenable to treatment by environmental interventions.

4.4.3 Future directions: understanding FASD etiology and potential for developing diagnostic and treatment strategies

The ultimate goal of any animal model of a human genetic disorder is the utilization of that model to understand the pathogenesis of the disease and assess potential treatment strategies. The mouse model developed in this study, along with other models developed by our lab, interrogate the effects of ethanol dosage and timing on behaviour, brain gene expression, and epigenetic patterning (KLEIBER et al. 2012, KLEIBER et al. 2013, LAUFER et al. 2013, MANTHA et al. 2013, KLEIBER et al. 2014b). Given that the ultimate purpose of these models is to examine the biological underpinnings of human FASD, future studies examining how the findings from this study may translate diagnostically or prognostically to humans would be useful and, importantly, evaluate if similar mechanisms, either genetic or epigenetic, contribute to the development of this disorder in humans. The results submitted as part of this thesis are hopefully an initial step to facilitate these studies and there are some findings included that may direct future research with these goals in mind. As with other array studies involving complex traits and disorders, a large number of gene transcripts – both mRNA and miRNA – were identified including those affected acutely following ethanol exposure (short-term effects) and those that remain altered in the adult brain (long-term effects). This indicates that multiple mechanisms and pathways are involved, and certainly, many have been implicated in FASD not only by this study but also within the expanding FASD-associated literature. This raises the question of how to reduce

this biological complexity and how to most effectively identify candidate target molecules or processes that may aid in diagnosis or treatment.

To explore the molecular relationships between the genes identified within this study and to identify core molecules that could unify the somewhat distinct biological processes associated with them, the transcripts were organized into interconnected gene networks. This approach is a key feature of 'network medicine' that has been applied to the analysis of other complex diseases and is a promising approach to identify potential diagnostic and prognostic biomarkers or pharmaceutical targets from large array-based studies (BENSON and BREITLING 2006, BARABASI et al. 2011). Indeed, the results from this study and other research investigating the molecular consequences of neurodevelopmental alcohol exposure demonstrate that while various genes or gene sets may be identified depending on factors such as timing of exposure or ethanol dosage administered, there is some consistency with regard to the biological pathways and 'hub' molecules that appear following network analyses associated with those gene sets (KLEIBER et al. 2013). Evaluating the role of these specific genes in the developing and adult brain with regard to the influence of alcohol on their expression or function may be a promising initial step to unravelling the complexity of FASD-associated neural dysfunction.

In addition to identifying core molecules that may be key biological effectors of FASD, it is also important to consider that FASDs do not occur in isolation of social, economic, and other environmental factors. FASD prevalence is often considerably higher in populations with low socioeconomic and educational status, increasing the likelihood of factors that exacerbate the severity of FASD phenotypes both prenatally and postnatally, including nutritional deficiencies, comorbid exposure to other teratogenic substances, and a suboptimal early-life environment (LIEBER 2003, SECKL and MEANEY 2004, STREISSGUTH et al. 2004, DWYER et al. 2009, ESPER and FURTADO 2014). While these factors contribute to how FASD phenotypes may present, they are also potential targets to mitigate the effects of prenatal alcohol exposure. Improving the nature of early postnatal care

has been shown to improve a number of phenotypic impairments associated with prenatal alcohol exposure (CHIODO et al. 2009, ORNOY and ERGAZ 2010). Children that have a strong, interdisciplinary support system including parents, teachers, and health care professionals show improved social and learning skills (KALBERG and BUCKLEY 2007). Additionally, these improvements may have a molecular basis including epigenetic reprogramming of genes associated with cognition and stress reactivity (WEAVER et al. 2004). Given that epigenetic alterations appear to be a prominent consequence of prenatal alcohol exposure, the malleability of these features by postnatal environment suggests that positive and enriched early life experiences may ameliorate some prenatal alcohol effects in a stable and long-term manner. There are also pharmaceutical epigenetic treatment opportunities that have proven successful for enhancing learning and memory following traumatic brain injury and within some models of neurodegenerative conditions (HOCKLY et al. 2003, CHEN et al. 2006b, DASH et al. 2009), though the transient effects and non-specific alterations to the epigenome limit this avenue. Technologies such as CRISPR-Cas9-mediated alteration of epigenetic marks are promising in that they may be able to specifically target and alter acetylation or methylation at specific locations throughout the genome (DOUDNA and CHARPENTIER 2014, HILTON et al. 2015). This technology is still being developed, but it has proven effective and may, in the future, facilitate new treatments to improve some phenotypes associated with neurodevelopmental disorders such as FASD.

Finally, while mouse models can be extremely useful in identifying molecular pathways that underlie neurodevelopmental disorders, it must be confirmed that those models reliably and consistently reflect the processes that occur during human neurodevelopment and contribute to the manifestation of any neurological disorder. As an initial step towards translating this research to human FASD, our lab has performed some investigative studies examining if the altered epigenetic patterning that we observed in mice following prenatal alcohol exposure may be present in peripheral human tissues and may be used as a

diagnostic measure in children. Indeed, similar to results obtained from mouse brain tissue, we have observed a great deal of consistency between the methylation patterns of buccal DNA obtained from children with FASD that were distinct from non-exposed children, suggesting that analysis of DNA methylation or other epigenetic marks may provide a novel diagnostic strategy (LAUFER et al. 2015).

4.5 Conclusions

4.5.1 Contributions to FASD research

Neurodevelopmental ethanol exposure results in a complex array of behavioural, genetic and epigenetic changes in the brain. It is remarkable that so little attention was given to such a detrimental, prevalent, and entirely preventable set of disorders by the scientific and medical community until the early 1970s (LEMOINE et al. 1968, JONES and SMITH 1973). Since its recognition, however, literature examining the behavioural, cognitive, physiological, cellular, and genetic effects on the developing brain has steadily grown. The results from these studies are varied as are the strategies used by researchers, but consistent themes are emerging. The results included in this thesis present several contributions to extant FASD literature that, along with other research I have previously published, have hopefully progressed our understanding of the biological mechanisms that contribute to these disorders.

First, a goal of this research was to generate a mouse model that recapitulated a number of phenotypes consistent with FASD that could be assessed at throughout the maturation of the mouse from neonate to adult. To assess this, I developed a behavioural battery designed to follow the maturation of the mouse from neonate to adult to somewhat parallel the emergence of behaviours in children and young adults affected by prenatal alcohol exposure. Further, the battery could be used on cohorts of mice exposed to alcohol using varying treatment paradigms. While

this thesis focuses on a third trimester-equivalent 'binge'-like exposure, we have also used the same battery on mice treated with binge doses at other trimester equivalents as well as by voluntary maternal consumption throughout gestation. This has allowed for the validation of these models in that they do show FASD-relevant behavioural and cognitive phenotypes but also allowed for the comparison of the effects of ethanol exposure at different developmental times and doses. The results from these studies have suggested that there are temporal windows during neurodevelopment that affect the presence or severity of certain behaviours as they appear later in life. The results also suggest that the third trimester-equivalent is an extremely sensitive period for the disruption of brain regions or pathways associated with spatial learning and stress reactivity. These data are consistent with FASD as a 'spectrum' disorder and support that alcohol timing and dose are key factors that contribute to the range of neurobehavioural phenotypes associated with prenatal alcohol exposure.

Second, this study is the first to examine genome-wide changes in gene expression resulting from ethanol exposure during synaptogenesis. Gene expression was examined at two time points: acutely (4 hours) following ethanol exposure to gain insight into how ethanol may alter neurodevelopmental processes, and during adulthood (postnatal day 60) to evaluate how neurodevelopmental alcohol exposure may cause long-lasting changes within the adult brain and how these changes relate to the persistent FASD-associated phenotypes. The acute response was characterized by cellular stress response and the down-regulation of energetically costly cellular processes, consistent with the current literature; however, there were a subset of genes identified associated with glutamate neurotransmission and synaptic formation that were not over-represented in other treatment paradigms. This ability to compare altered gene sets across different treatments has been informative, and has led to our hypothesis that ethanol may most effectively disrupt neurodevelopmental processes that are active at the time of exposure, perhaps due to their sensitivity to environmental cues. Also, while few transcripts were identified as altered in both the short-term

and long-term analyses, there was considerable consistency between the biological processes and pathway associated with them including synaptic plasticity, neurotransmission, and HPA axis function. These processes were consistent with the learning and anxiety-related phenotypes I observed in these animals, particularly with regard to their severity compared to animals treated using other paradigms.

Finally, given that epigenetic mechanisms have been implicated in maintaining the long-term effects of prenatal alcohol exposure, miRNA expression in the adult brain was analyzed to evaluate any correlations between altered miRNA levels and the expression of their putative mRNA targets. A number of miRNAs were identified and showed an inverse direction of expression change as compared to their mRNA target(s). While this analysis requires further confirmation, the processes associated with the mRNA targets again showed an over-representation of a number of processes identified by both mRNA array experiments.

It is important to note that all of these analyses are exploratory and designed toward establishing and testing the utility of a novel animal model strategy in assessing the molecular changes in the brain associated with FASD. The model has proven effective in identifying molecular features of ethanol exposure at different developmental times, and comparing those changes with differences in observed behaviours. Also, there is consistency and a logical agreement between the results from multiple lines of analyses including behaviour, biological functions associated with the short and long-term transcript changes, and miRNA target processes that may point to lucrative avenues for future investigation. This process, while essential and valuable, is an initial step towards understanding the complexity associated with how ethanol can affect the developing brain and lead to a lifetime of behavioural and cognitive changes. These studies leave many more questions regarding the role of these genes in altering the function of cells, brain regions, and the neural interactions that ultimately dictate behaviour, as well as which mechanisms may be effective diagnostic or therapeutic targets. There

likely will be no 'smoking gun' mechanism or course of obvious treatment; however, this research has facilitated ongoing studies that use the models and findings established within this thesis to more deeply explore biological mechanisms that contribute to FASD including epigenetic alterations such as the involvement of DNA methylation and histone modification, how alcohol exposure may interact with postnatal environment to affect behaviour, and translational work involving potential diagnostic strategies in humans.

4.5.2 A working model of FASD

This study was initiated to provide a holistic investigation into the effects of ethanol exposure during neurodevelopment on behaviour, gene expression, and epigenetic patterning. The model allows for the proposal of FASD as a continuum of molecular events that ultimately manifests as long-term cognitive and behavioural phenotypes (Figure 4.1). Ethanol exposure represents an interruption in normal neurodevelopmental processes. Depending on the timing of exposure, certain populations of cells will succumb to cellular stress response and undergo apoptosis, while surviving cells must suspend their normal developmental trajectories and utilize molecular adaptations that include changes in gene expression and epigenetic patterning. These epigenetic alterations likely involve the interaction of DNA methylation, histone modification, and ncRNA regulation. Many of these epigenetic changes are expected to be stably inherited following subsequent neurogenesis, differentiation, and maturation, resulting in not only altered brain structure and neuronal connectivity but also a distinctive pattern of altered gene expression in the mature brain. These transcriptomic and epigenetic changes represent an enduring molecular "footprint" that reflects the timing, dose, and pattern of alcohol exposure as well as their interactions with maternal and fetal genotype and the prenatal environment. This reprogramming of neurogenomic patterning may be further compounded by subsequent ontogenetic factors such as postnatal

environment, which may further exacerbate or ameliorate epigenetic signatures and subsequent gene expression. Postnatal environment is therefore a potential avenue for early-life mitigation of FASD phenotypes. Also, these epigenetic signatures may—if shared by peripheral tissue sources—offer a source of early diagnosis of prenatal alcohol exposure. Given the unlikelihood of total abstinence from alcohol consumption during pregnancy, particularly within Western cultures, evidence that the effects of prenatal alcohol exposure may be amended or reversed at the molecular and, subsequently, at the phenotypic level would represent a significant step towards improving the prognosis of individuals with FASD.

Not even half a century after the recognition that alcohol consumption during pregnancy leads to a set of characteristic physiological and cognitive phenotypes that led Jones and Smith to coin the term 'fetal alcohol syndrome' (JONES et al. 1973), we are making leaps in our understanding of the myriad of effects of alcohol on neurodevelopment, including its eventual manifestation as increased risk for behavioural and cognitive phenotypes that ultimately impair the ability of an ethanol-exposed child to thrive. In addition to contributing to our understanding of the biological etiology of FASD, it is hoped that this work facilitates the development of novel therapeutic and treatment approaches to improve the outcome of individuals with this pervasive and costly disorder. Given the increase in prevalence of disorders of neurodevelopmental origin, this work also provides a model to assist in our understanding of how adverse prenatal environments, including exposure to teratogens such as alcohol, can lead to adverse effects on brain development that contributes to the emergence of later-life psychopathologies.

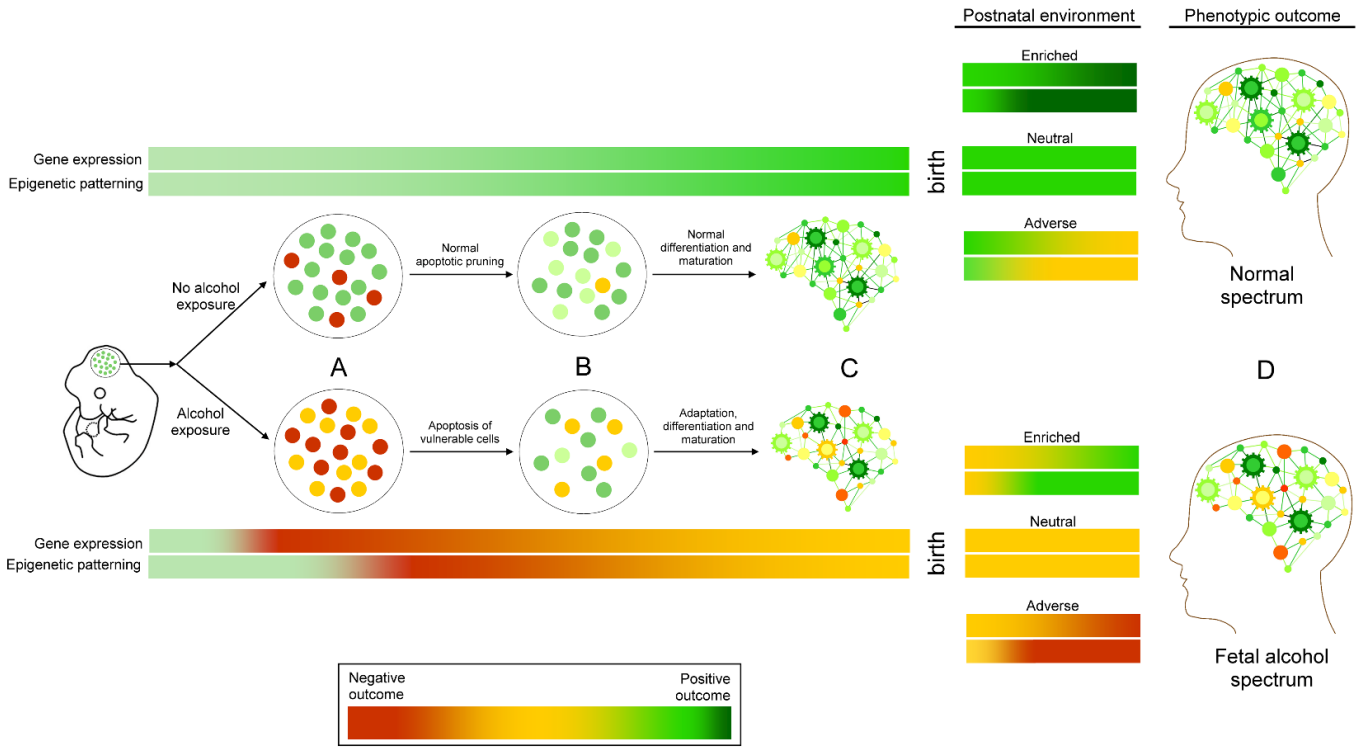


FIGURE 4.1. A working model of FASD as a continuum of genetic and epigenetic events. (A) Ethanol exposure results in cellular stress response leading to the apoptosis of vulnerable cell types. (B) These changes are followed by molecular adaptation in surviving cells that include changes to gene expression, driven in part by changes to epigenetic programming including altered DNA methylation, histone modifications, and ncRNA regulation. (C) These changes may be inherited and maintained throughout further developmental processes such as cellular differentiation and maturation. Additionally, following parturition, epigenetic control of gene expression is vulnerable to exacerbation or amelioration by postnatal environmental experiences. (D) Adverse (red) or positive (green) outcomes likely depend on the interaction of these factors and contribute to the ontogeny of FASD phenotypes.

Chapter 5

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Appendix A. mRNA transcripts identified as altered in the brain of neonate mice (postnatal day 7) four hours following ethanol treatment (fold cutoff ≥ ±1.2, FDR-corrected *p*-value < 0.05). Genes are listed alphabetically by gene symbol.

Transcript ID	Gene symbol	Gene name	RefSeq ID	Chr[a]	Fold change	*p*-value[b]
10539766	Aak1	AP2 associated kinase 1	NM_001040106	6	1.22	0.012
10392484	Abca8b	ATP-binding cassette, sub-family A (ABC1), member 8b	NM_013851	11	1.36	0.009
10443730	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	NM_009593	17	1.21	0.024
10485241	Accs	1-aminocyclopropane-1-carboxylate synthase (non-functional)	NM_183220	2	1.36	0.002
10505779	Acer2	alkaline ceramidase 2	NM_139306	4	1.31	0.003
10397172	Acot6	acyl-CoA thioesterase 6	NM_172580	12	1.25	0.005
10554074	Adamts17	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 17	NM_001033877	7	1.21	0.007
10531195	Adamts3	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 3	NM_177872	5	1.20	0.038
10351551	Adamts4	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4	NM_172845	1	1.21	0.002
10447502	Adcyap1	adenylate cyclase activating polypeptide 1	NM_009625	17	-1.31	0.014
10479351	Adrm1	adhesion regulating molecule 1	NM_019822	2	-1.21	0.012
10523647	Aff1	AF4/FMR2 family, member 1	NM_001080798	5	1.22	0.005
10534889	Agfg2	ArfGAP with FG repeats 2	NM_178162	5	-1.21	0.009
10582658	Agt	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	NM_007428	8	1.23	0.038
10472923	Ak4	adenylate kinase 4	NM_001177602	4	-1.22	0.024
10446214	Alkbh7	alkB, alkylation repair homolog 7 (E. coli)	NM_025538	17	-1.25	0.049
10459389	Amd1	S-adenosylmethionine decarboxylase 1	NM_009665	10	-1.21	0.014
10595668	Ankrd34c	ankyrin repeat domain 34C	NM_207260	9	-1.40	0.022
10583834	Anln	anillin, actin binding protein	NM_028390	9	1.23	0.010
10594631	Aph1b	anterior pharynx defective 1b homolog (C. elegans)	NM_177583	9	1.22	0.003
10604375	Apln	apelin	NM_013912	X	-1.29	0.042
10450796	Armcx5	armadillo repeat containing, X-linked 5	NM_001009575	X	1.40	0.037

10406407	Arrdc3	arrestin domain containing 3	NM_001042591	13	2.22	0.000
10564507	Arrdc4	arrestin domain containing 4	NM_001042592	7	1.65	0.001
10499666	Atp8b2	ATPase, class I, type 8B, member 2	NM_001081182	3	1.29	0.029
10490104	Aurka	aurora kinase A	NM_011497	2	-1.24	0.007
10406456	AY512938	cDNA sequence AY512938	AY512938	UN	1.25	0.003
10437191	B3galt5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	NM_033149	16	1.31	0.008
10434291	B3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	NM_001159407	16	1.23	0.040
10439854	Bbx	bobby sox homolog (Drosophila)	NM_027444	16	1.23	0.001
10587085	BC031353	family with sequence similarity 214, member A	NM_001113283	9	1.21	0.019
10439409	BC031361	cDNA sequence BC031361	NR_033221	16	1.20	0.042
10475866	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	NM_207680	2	1.46	0.002
10357875	Btg2	B cell translocation gene 2, anti-proliferative	NM_007570	1	1.48	0.001
10440419	Btg3	B cell translocation gene 3	NM_009770	16	1.25	0.004
10515026	Cc2d1b	coiled-coil and C2 domain containing 1B	NM_177045	4	1.23	0.011
10578557	Ccdc111	primase and polymerase (DNA-directed)	NM_001001184	8	1.29	0.025
10368083	Ccdc28a	coiled-coil domain containing 28A	NM_144820	10	1.30	0.021
10569646	Ccnd1	cyclin D1	NM_007631	7	-1.34	0.003
10349442	Ccnt2	cyclin T2	NM_028399	1	-1.20	0.024
10474229	Cd59a	CD59a antigen	NM_001111060	2	1.24	0.037
10564573	Chd2	chromodomain helicase DNA binding protein 2	NM_001081345	7	1.34	0.006
10583347	Chordc1	cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein 1	NM_025844	9	-1.28	0.008
10507726	Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	NM_019563	4	-1.26	0.013
10438769	Cldn1	claudin 1	NM_016674	16	1.29	0.018
10547674	Clec4e	C-type lectin domain family 4, member e	AB024717	6	1.20	0.007
10597564	Cmc1	COX assembly mitochondrial protein 1	NM_026442	9	-1.20	0.028
10364593	Cnn2	calponin 2	NM_007725	10	-1.21	0.010

10346298	Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	NM_001039710	1	-1.26	0.014
10565089	Cpeb1	cytoplasmic polyadenylation element binding protein 1	NM_007755	7	1.31	0.011
10388996	Crlf3	cytokine receptor-like factor 3	NM_018776	11	1.21	0.024
10371400	Cry1	cryptochrome 1 (photolyase-like)	NM_007771	10	-1.24	0.001
10428453	Csmd3	CUB and Sushi multiple domains 3	NM_001081391	15	1.21	0.007
10472408	Csrnp3	cysteine-serine-rich nuclear protein 3	NM_153409	2	1.25	0.012
10485580	Cstf3	cleavage stimulation factor, 3' pre-RNA, subunit 3	NM_145529	2	1.31	0.011
10515943	Ctps	cytidine 5'-triphosphate synthase	NM_016748	4	-1.22	0.003
10453057	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	NM_009994	17	1.25	0.011
10514520	Cyp2j9	cytochrome P450, family 2, subfamily j, polypeptide 9	NM_028979	4	1.29	0.001
10489498	Dbndd2	dysbindin (dystrobrevin binding protein 1) domain containing 2	NM_001048227	2	-1.22	0.041
10369290	Ddit4	DNA-damage-inducible transcript 4	NM_029083	10	1.24	0.023
10369630	Ddx21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	NM_019553	10	-1.27	0.001
10470628	Ddx31	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 31	NM_001033294	2	-1.20	0.004
10525751	Ddx55	DEAD (Asp-Glu-Ala-Asp) box polypeptide 55	NM_026409	5	-1.21	0.036
10437664	Dexi	dexamethasone-induced transcript	NM_021428	16	1.21	0.011
10401841	Dio2	deiodinase, iodothyronine, type II	NM_010050	12	-1.21	0.023
10449284	Dusp1	dual specificity phosphatase 1	NM_013642	17	1.65	0.001
10548735	Dusp16	dual specificity phosphatase 16	NM_130447	6	1.27	0.004
10571312	Dusp4	dual specificity phosphatase 4	NM_176933	8	-1.35	0.035
10366043	Dusp6	dual specificity phosphatase 6	NM_026268	10	-1.73	0.000
10588495	Dusp7	dual specificity phosphatase 7	NM_153459	9	-1.21	0.002
10569280	Dusp8	dual specificity phosphatase 8	NM_008748	7	1.21	0.011
10574682	E2f4	E2F transcription factor 4	NM_148952	8	-1.29	0.046
10404783	Edn1	endothelin 1	NM_010104	13	1.29	0.019
10603768	Efhc2	EF-hand domain (C-terminal) containing 2	NM_028916	X	1.23	0.007
10518069	Efhd2	EF hand domain containing 2	NM_025994	4	-1.28	0.007

10416251	Egr3	early growth response 3	NM_018781	14	-1.26	0.003
10545859	Egr4	early growth response 4	NM_020596	6	-1.25	0.040
10368317	Enpp3	ectonucleotide pyrophosphatase/phosphodiesterase 3	NM_134005	10	1.22	0.047
10357164	Epb41l5	erythrocyte membrane protein band 4.1 like 5	NM_145506	1	-1.20	0.027
10391490	Etv4	ets variant 4	NM_008815	11	-1.27	0.016
10438626	Etv5	ets variant 5	NM_023794	16	-1.73	0.000
10545045	Fam13a	family with sequence similarity 13, member A	NM_153574	6	1.30	0.004
10368638	Fam26e	family with sequence similarity 26, member E	NM_178908	10	1.31	0.018
10597088	Fbxw26	F-box and WD-40 domain protein 26	NM_198674	9	1.22	0.049
10524052	Fgfrl1	fibroblast growth factor receptor-like 1	NM_054071	5	-1.26	0.013
10505044	Fktn	fukutin	NM_139309	4	1.25	0.005
10565401	Folh1	folate hydrolase 1	NM_016770	7	1.44	0.003
10368886	Foxo3	forkhead box O3	ENSMUST00000105502	10	1.27	0.000
10553769	Gabra5	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5	NM_176942	7	-1.23	0.024
10404702	Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	NM_023887	13	1.22	0.014
10418480	Gnl3	guanine nucleotide binding protein-like 3 (nucleolar)	NM_153547	14	-1.21	0.002
10493120	Gpatch4	G patch domain containing 4	NM_025663	3	-1.23	0.014
10414278	Gpr137c	G protein-coupled receptor 137C	NM_027518	14	1.32	0.000
10567361	Gpr139	G protein-coupled receptor 139	NM_001024138	7	1.30	0.002
10368780	Gpr6	G protein-coupled receptor 6	NM_199058	10	1.40	0.007
10433101	Gpr84	G protein-coupled receptor 84	NM_030720	15	-1.22	0.029
10437629	Grin2a	glutamate receptor, ionotropic, NMDA2A (epsilon 1)	NM_008170	16	1.25	0.049
10548791	Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)	NM_008171	6	1.25	0.018
10407543	Gtpbp4	GTP binding protein 4	NM_027000	13	-1.27	0.002
10444802	H2-Q2	histocompatibility 2, Q region locus 2	NM_010392	17	-1.23	0.037
10447004	Hdac1	histone deacetylase 1	NM_008228	4	-1.25	0.000
10399973	Hdac9	histone deacetylase 9	NM_024124	12	-1.22	0.008

10403511	Heatr1	HEAT repeat containing 1	NM_144835	13	-1.31	0.001
10560329	Hif3a	hypoxia inducible factor 3, alpha subunit	NM_001162950	7	1.23	0.003
10358609	Hmcn1	hemicentin 1	NM_001024720	1	-1.20	0.039
10435443	Hspbap1	Hspb associated protein 1	NM_175111	16	-1.23	0.004
10509238	Htr1d	5-hydroxytryptamine (serotonin) receptor 1D	NM_008309	4	1.23	0.038
10520355	Htr5a	5-hydroxytryptamine (serotonin) receptor 5A	NM_008314	5	1.22	0.009
10400095	Ifrd1	interferon-related developmental regulator 1	NM_013562	12	-1.29	0.035
10554094	Igf1r	insulin-like growth factor I receptor	NM_010513	7	1.21	0.043
10384223	Igfbp3	insulin-like growth factor binding protein 3	NM_008343	11	1.26	0.013
10498386	Igsf10	immunoglobulin superfamily, member 10	NM_001162884	3	1.34	0.002
10579958	Il15	interleukin 15	NM_008357	8	-1.23	0.005
10413398	Il17rd	interleukin 17 receptor D	NM_134437	14	-1.21	0.043
10465861	Incenp	inner centromere protein	NM_016692	19	-1.21	0.040
10403743	Inhba	inhibin beta-A	NM_008380	13	-1.23	0.006
10389421	Ints2	integrator complexsubunit 2	NM_027421	11	1.26	0.013
10557925	Itgad	integrin, alpha D	ENSMUST00000106237	7	-1.25	0.039
10567297	Itpripl2	inositol 1,4,5-triphosphate receptor interacting protein-like 2	NM_001033380	7	1.24	0.025
10544148	Jhdm1d	lysine (K)-specific demethylase 7A	NM_001033430	6	1.24	0.043
10411126	Jmy	junction-mediating and regulatory protein	NM_021310	13	1.25	0.006
10514466	Jun	jun proto-oncogene	NM_010591	4	2.60	0.000
10382321	Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	NM_008425	11	-1.40	0.008
10561702	Kcnk6	potassium inwardly-rectifying channel, subfamily K, member 6	NM_001033525	7	1.36	0.043
10412650	Kctd6	potassium channel tetramerisation domain containing 6	NM_027782	14	1.27	0.035
10403352	Klf6	Kruppel-like factor 6	NM_011803	13	-1.26	0.011
10396079	Klhdc1	kelch domain containing 1	NM_178253	12	1.25	0.012
10434302	Klhl24	kelch-like 24	NM_029436	16	1.28	0.024
10400572	Klhl28	kelch-like 28	NM_025707	12	1.21	0.025

10519392	Krit1	KRIT1, ankyrin repeat containing	NR_033173	5	-1.21	0.036
10603254	Larp4	La ribonucleoprotein domain family, member 4	NM_001024526	15	-1.31	0.015
10583732	Ldlr	low density lipoprotein receptor	NM_010700	9	-1.26	0.020
10422822	Lifr	leukemia inhibitory factor receptor	NM_013584	15	1.23	0.025
10467139	Lipa	lysosomal acid lipase A	NM_021460	19	1.25	0.024
10578207	Lonrf1	LON peptidase N-terminal domain and ring finger 1	NM_001081150	8	-1.22	0.035
10367931	Ltv1	LTV1 homolog (S. cerevisiae)	NM_181470	10	-1.48	0.005
10521555	Lyar	Ly1 antibody reactive clone	NM_025281	5	-1.28	0.023
10510464	Lzic	leucine zipper and CTNNBIP1 domain containing	NM_026963	4	-1.28	0.032
10465244	Malat1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	NR_002847	19	1.53	0.004
10568780	Mapk1ip1	mitogen-activated protein kinase 1 interacting protein 1	NM_001045483	7	1.20	0.001
10425507	Mchr1	melanin-concentrating hormone receptor 1	NM_145132	15	1.37	0.006
10546163	Mcm2	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)	NM_008564	6	-1.22	0.011
10470696	Med27	mediator complex subunit 27	NM_026896	2	-1.26	0.027
10455826	Megf10	multiple EGF-like-domains 10	NM_001001979	18	1.22	0.020
10475890	Mertk	c-mer proto-oncogene tyrosine kinase	NM_008587	2	1.56	0.001
10382852	Mfsd11	major facilitator superfamily domain containing 11	NM_178620	11	1.23	0.044
10378568	Mir22	microRNA 22	NR_029739	11	-1.36	0.006
10398442	Mir410	microRNA 410	NR_029914	12	-1.47	0.003
10398394	Mir494	microRNA 494	NR_030269	12	-1.22	0.041
10469239	Mir669c	microRNA 669c	NR_030473	2	-1.33	0.029
10433656	Mkl2	MKL/myocardin-like 2	NM_153588	16	1.28	0.029
10401616	Mlh3	mutL homolog 3 (E coli)	NM_175337	12	1.24	0.034
10564313	Mphosph10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	NM_026483	7	-1.34	0.030
10566514	Mrps36	mitochondrial ribosomal protein S36	NM_025369	13	-1.22	0.043
10574027	Mt1	metallothionein 1	NM_013602	8	1.25	0.004
10574023	Mt2	metallothionein 2	NM_008630	8	1.37	0.015

10605421	Mtcp1	mature T cell proliferation 1	NM_001039373	X	1.27	0.000
10545672	Mthfd2	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	NM_008638	6	-1.27	0.012
10361366	Mtrf1l	mitochondrial translational release factor 1-like	NM_175374	10	-1.20	0.022
10601980	Mum1l1	melanoma associated antigen (mutated) 1-like 1	NM_001164630	X	1.25	0.011
10503643	Ndufaf4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 4	NM_026742	4	-1.29	0.005
10488771	Necab3	N-terminal EF-hand calcium binding protein 3	NM_021546	2	-1.20	0.015
10408850	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9	NM_001111324	13	1.20	0.018
10577492	Nek3	NIMA (never in mitosis gene a)-related expressed kinase 3	NM_001162947	8	1.21	0.046
10493114	Nes	nestin	NM_016701	3	-1.22	0.032
10492598	Nmd3	NMD3 homolog (S. cerevisiae)	NM_133787	3	-1.26	0.003
10404827	Nol7	nucleolar protein 7	NM_023554	13	-1.20	0.035
10463535	Nolc1	nucleolar and coiled-body phosphoprotein 1	NM_053086	19	-1.26	0.018
10541845	Nop2	NOP2 nucleolar protein	NM_138747	6	-1.21	0.002
10600707	Nr0b1	nuclear receptor subfamily 0, group B, member 1	NM_007430	X	-1.30	0.025
10427035	Nr4a1	nuclear receptor subfamily 4, group A, member 1	NM_010444	15	-1.40	0.015
10522192	Nsun7	NOL1/NOP2/Sun domain family, member 7	NM_027602	5	1.20	0.032
10479397	Ntsr1	neurotensin receptor 1	NM_018766	2	1.21	0.012
10598236	Nudt11	nudix (nucleoside diphosphate linked moiety X)-type motif 11	NM_021431	X	1.33	0.004
10416510	Nufip1	nuclear fragile X mental retardation protein interacting protein 1	NM_013745	14	-1.22	0.009
10361651	Nup43	nucleoporin 43	NM_145706	10	-1.21	0.014
10466268	Olfr1418	olfactory receptor 1418	NM_001011524	19	-1.24	0.017
10436823	Olig2	oligodendrocyte transcription factor 2	NM_016967	16	-1.30	0.009
10360454	Opn3	opsin 3	NM_010098	1	-1.21	0.035
10346260	Osgepl1	O-sialoglycoprotein endopeptidase-like 1	NM_028091	1	1.29	0.008
10427471	Osmr	oncostatin M receptor	NM_011019	15	1.23	0.033
10498367	P2ry13	purinergic receptor P2Y, G-protein coupled 13	NM_028808	3	-1.20	0.012

10363350	P4ha1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	NM_011030	10	-1.26	0.017
10586347	Parp16	poly (ADP-ribose) polymerase family, member 16	NM_177460	9	1.21	0.035
10455118	Pcdhb18	protocadherin beta 18	NM_053143	18	1.21	0.018
10455135	Pcdhb21	protocadherin beta 21	NM_053146	18	1.22	0.015
10479726	Pcmtd2	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	NM_153594	2	1.30	0.005
10414417	Peli2	pellino 2	NM_033602	14	1.21	0.023
10377439	Per1	period circadian clock 1	NM_001159367	11	1.20	0.000
10356601	Per2	period circadian clock 2	NM_011066	1	-1.26	0.037
10361887	Perp	PERP, TP53 apoptosis effector	NM_022032	10	1.25	0.030
10470959	Phyhd1	phytanoyl-CoA dioxygenase domain containing 1	NM_172267	2	1.22	0.033
10351788	Pigm	phosphatidylinositol glycan anchor biosynthesis, class M	NM_026234	1	1.25	0.040
10441055	Pigp	phosphatidylinositol glycan anchor biosynthesis, class P	NM_001159617	16	-1.21	0.041
10517083	Pigv	phosphatidylinositol glycan anchor biosynthesis, class V	NM_178698	4	1.21	0.008
10373740	Pik3ip1	phosphoinositide-3-kinase interacting protein 1	NM_178149	11	1.51	0.002
10411782	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	NM_001077495	13	1.21	0.005
10515399	Plk3	polo-like kinase 3	NM_013807	4	-1.23	0.006
10499366	Pmf1	polyamine-modulated factor 1	NM_025928	3	-1.21	0.033
10384474	Pno1	partner of NOB1 homolog (S. cerevisiae)	NM_025443	11	-1.28	0.037
10421351	Polr3d	polymerase (RNA) III (DNA directed) polypeptide D	NM_025945	14	-1.27	0.018
10394240	Pomc	pro-opiomelanocortin-alpha	NM_008895	12	-1.21	0.013
10543471	Pot1a	protection of telomeres 1A	NM_133931	6	1.24	0.000
10545001	Ppm1k	protein phosphatase 1K (PP2C domain containing)	NM_175523	6	1.30	0.020
10525374	Pptc7	PTC7 protein phosphatase homolog (S. cerevisiae)	NM_177242	5	-1.20	0.013
10485624	Prrg4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	NM_178695	2	1.27	0.006
10587616	Prss35	protease, serine 35	NM_178738	9	1.41	0.010
10578986	Psd3	pleckstrin and Sec7 domain containing 3	NM_030263	8	1.28	0.021

10535637	Ptcd1	pentatricopeptide repeat domain 1	NM_133735	5	1.20	0.026
10357191	Ptpn4	protein tyrosine phosphatase, non-receptor type 4	NM_019933	1	1.23	0.010
10376163	Rapgef6	Rap guanine nucleotide exchange factor (GEF) 6	NM_175258	11	1.66	0.000
10522467	Rasl11b	RAS-like, family 11, member B	NM_026878	5	1.45	0.003
10558840	Rassf7	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	NM_025886	7	-1.21	0.025
10453867	Rbbp8	retinoblastoma binding protein 8	NM_001081223	18	-1.32	0.001
10410709	Rfesd	Rieske (Fe-S) domain containing	NM_178916	13	1.23	0.003
10359908	Rgs4	regulator of G-protein signaling 4	NM_009062	1	-1.30	0.000
10466935	Rln1	relaxin 1	NM_011272	19	1.22	0.027
10350742	Rnasel	ribonuclease L (2', 5'-oligoisoadenylate synthetase-dependent)	NM_011882	1	1.28	0.003
10404965	Rnf144b	ring finger protein 144B	NM_146042	13	1.30	0.009
10565852	Rnf169	ring finger protein 169	NM_175388	7	1.20	0.020
10381395	Rundc1	RUN domain containing 1	NM_172566	11	-1.22	0.043
10351430	Rxrg	retinoid X receptor gamma	NM_009107	1	-1.24	0.022
10402512	Scarna13	small Cajal body-specific RNA1	NR_028576	12	1.27	0.012
10514219	Scarna8	small Cajal body-specific RNA8	NR_028545	4	1.21	0.015
10483326	Scn9a	sodium channel, voltage-gated, type IX, alpha	NM_018852	2	1.29	0.012
10489484	Sdc4	syndecan 4	NM_011521	2	1.31	0.004
10357072	Serpinb3a	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 3A	NM_009126	1	1.20	0.041
10362811	Sesn1	sestrin 1	NM_001162908	10	1.29	0.001
10469167	Sfmbt2	Scm-like with four mbt domains 2	NM_177386	2	1.29	0.033
10457959	Sft2d3	SFT2 domain containing 3	NM_026006	18	-1.23	0.009
10471505	Sh2d3c	SH2 domain containing 3C	NM_013781	2	1.39	0.000
10393866	Sirt7	sirtuin 7	NM_153056	11	-1.33	0.021
10356240	Slc16a14	solute carrier family 16 (monocarboxylic acid transporters), member 14	NM_027921	1	1.33	0.003
10392440	Slc16a6	solute carrier family 16 (monocarboxylic acid transporters), member 6	NM_001029842	11	1.35	0.006
10363860	Slc16a9	solute carrier family 16 (monocarboxylic acid transporters), member 9	NM_025807	10	1.36	0.010

10397835	Slc24a4	solute carrier family 24 (sodium/potassium/calcium exchanger), member 4	NM_172152	12	1.34	0.000
10495405	Slc25a24	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24	NM_172685	3	1.28	0.015
10507594	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	NM_011400	4	1.30	0.003
10378649	Slc43a2	solute carrier family 43, member 2	NM_173388	11	1.28	0.001
10496975	Slc44a5	solute carrier family 44, member 5	NM_001081263	3	1.41	0.002
10386683	Slc47a1	solute carrier family 47, member 1	NM_026183	11	-1.23	0.020
10605986	Slc7a3	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	NM_007515	X	1.21	0.049
10396936	Smoc1	SPARC related modular calcium binding 1	NM_001146217	12	1.21	0.010
10383731	Smtn	smoothelin	NM_001159284	11	-1.22	0.030
10396442	Snapc1	small nuclear RNA activating complex, polypeptide 1	NM_178392	12	-1.22	0.047
10576216	Snord68	small nucleolar RNA, C/D box 68	NR_028128	8	-1.21	0.010
10582874	Sp110	Sp110 nuclear body protein	NM_175397	1	1.23	0.018
10347933	Sp140	Sp140 nuclear body protein	NM_001013817	1	-1.21	0.046
10387936	Spag7	sperm associated antigen 7	NM_172561	11	-1.22	0.025
10561673	Spred3	sprouty-related, EVH1 domain containing 3	NM_182927	7	-1.35	0.008
10458555	Spry4	sprouty homolog 4 (Drosophila)	NM_011898	18	-1.69	0.001
10393395	Srsf2	serine/arginine-rich splicing factor 2	NM_011358	11	-1.21	0.000
10453102	Srsf7	serine/arginine-rich splicing factor 7	NR_036615	17	-1.20	0.013
10434758	St6gal1	beta galactoside alpha 2,6 sialyltransferase 1	NM_145933	16	1.21	0.020
10549162	St8sia1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	NM_011374	6	1.22	0.013
10519484	Steap2	six transmembrane epithelial antigen of prostate 2	AK141701	5	1.56	0.004
10554863	Sytl2	synaptotagmin-like 2	NM_001040085	7	1.25	0.040
10515706	Szt2	seizure threshold 2	NM_198170	4	-1.22	0.047
10448707	Tbl3	transducin (beta)-like 3	NM_145396	17	-1.21	0.025
10365098	Tbxa2r	thromboxane A2 receptor	NM_009325	10	-1.23	0.022
10365344	Tcp11l2	t-complex11 (mouse) like 2	NM_146008	10	1.22	0.047
10369688	Tet1	tet methylcytosine dioxygenase 1	NM_027384	10	1.22	0.041

10401673	Tgfb3	transforming growth factor, beta 3	NM_009368	12	1.32	0.000
10567316	Tmc7	transmembrane channel-like gene family 7	NM_172476	7	1.25	0.000
10572318	Tmem161a	transmembrane protein 161A	NM_145597	8	-1.24	0.045
10458314	Tmem173	transmembrane protein 173	NM_028261	18	-1.23	0.039
10346224	Tmem194b	transmembrane protein 194B	NM_001142647	1	1.23	0.026
10461214	Tmem223	transmembrane protein 223	NM_025791	19	-1.20	0.040
10572747	Tpm4	tropomyosin 4	NM_001001491	8	-1.20	0.004
10438621	Tra2b	transformer 2 beta homolog (Drosophila)	NM_009186	16	-1.30	0.020
10503259	Trp53inp1	transformation related protein 53 inducible nuclear protein 1	NM_021897	4	1.32	0.001
10353221	Trpa1	transient receptor potential cation channel, subfamily A, member 1	NM_177781	1	-1.20	0.017
10428534	Trps1	trichorhinophalangeal syndrome I (human)	NM_032000	15	1.31	0.011
10483819	Ttc30b	tetratricopeptide repeat domain 30B	NM_028235	2	1.27	0.000
10456400	Tubb6	tubulin, beta 6 class V	NM_026473	18	-1.26	0.009
10494428	Txnip	thioredoxin interacting protein	NM_001009935	3	2.63	0.000
10599222	Ube2a	ubiquitin-conjugating enzyme E2A	NM_019668	X	-1.27	0.046
10416793	Uchl3	ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	NM_016723	14	-1.22	0.039
10469083	Upf2	UPF2 regulator of nonsense transcripts homolog (yeast)	NM_001081132	2	1.32	0.004
10440800	Urb1	URB1 ribosome biogenesis 1 homolog (S. cerevisiae)	NM_029497	16	-1.26	0.011
10516229	Utp11l	UTP11-like, U3 small nucleolar ribonucleoprotein, (yeast)	NM_026031	4	-1.27	0.004
10389842	Utp18	UTP18, small subunit (SSU) processome component, homolog (yeast)	NM_001013375	11	-1.27	0.010
10451198	Vegfa	vascular endothelial growth factor A	NM_001025250	17	-1.24	0.000
10547682	Vmn2r22	vomeronasal 2, receptor 22	NM_001104637	6	1.20	0.007
10559880	Vmn2r33	vomeronasal 2, receptor 33	NM_001105065	7	1.29	0.045
10549904	Vmn2r37	vomeronasal 2, receptor 37	NM_009489	7	1.24	0.034
10559890	Vmn2r43	vomeronasal 2, receptor 43	NM_198961	7	1.28	0.011
10405879	Vmn2r85	vomeronasal 2, receptor 85	NM_001102602	10	1.24	0.026
10552425	Vsig10l	V-set and immunoglobulin domain containing 10 like	BC112410	7	-1.21	0.045

10444098	Wdr46	WD repeat domain 46	NM_020603	17	-1.21	0.005
10346074	Wdr75	WD repeat domain 75	NM_028599	1	-1.21	0.004
10373000	Xrcc6bp1	XRCC6 binding protein 1	NM_026858	10	-1.20	0.020
10433904	Yars2	tyrosyl-tRNA synthetase 2 (mitochondrial)	NM_198246	16	-1.20	0.048
10473349	Ypel4	yippee-like 4 (Drosophila)	NM_001005342	2	1.49	0.018
10446756	Ypel5	yippee-like 5 (Drosophila)	NM_027166	17	1.23	0.006
10406733	Zbed3	zinc finger, BED type containing 3	NM_028106	13	1.30	0.003
10490826	Zbtb10	zinc finger and BTB domain containing 10	NM_177660	3	1.38	0.002
10509998	Zbtb17	zinc finger and BTB domain containing 17	NM_009541	4	-1.25	0.010
10480027	Zbtb2	zinc finger and BTB domain containing 2	NM_001033466	10	-1.28	0.043
10359377	Zbtb37	zinc finger and BTB domain containing 37	NM_173424	1	1.22	0.001
10475946	Zc3h6	zinc finger CCCH type containing 6	NM_178404	2	1.24	0.016
10550029	Zfp110	zinc finger protein 110	NM_022981	7	1.24	0.004
10556246	Zfp143	zinc finger protein 143	NM_009281	7	-1.23	0.015
10591161	Zfp558	zinc finger protein 558	NM_028935	9	1.47	0.006
10547282	Zfp9	zinc finger protein 9	NM_011763	6	1.25	0.008

[a] Chromosomal location of transcript-encoding gene
[b] *p*-values < 0.001 are shown as 0.000

APPENDIX B. mRNA transcripts identified as altered in the adult brain (P60) of mice exposed to ethanol during synaptogenesis (P4+7) (fold cutoff ≥ ±1.2, FDR-corrected *p*-value < 0.05). Genes are listed alphabetically by gene symbol.

Transcript ID	Gene symbol	Gene name	RefSeq	Chr[a]	Fold-change	*p*-value[b]
10389269	Aatf	apoptosis antagonizing transcription factor	NM_019816	11	-1.32	0.001
10546432	Adamts9	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9	NM_175314	6	-1.23	0.000
10496438	Adh1	alcohol dehydrogenase 1 (class I)	NM_007409	3	-1.35	0.000
10532630	Adrbk2	adrenergic receptor kinase, beta 2	NM_177078	5	-1.28	0.000
10554233	Aen	apoptosis enhancing nuclease	NM_026531	7	-1.35	0.000
10371332	Aldh1l2	aldehyde dehydrogenase 1 family, member L2	NM_153543	10	-1.32	0.000
10452734	Alk	anaplastic lymphoma kinase	NM_007439	17	-1.37	0.000
10586744	Anxa2	annexin A2	NM_007585	9	-1.42	0.000
10419977	Ap1g2	adaptor protein complex AP-1, gamma 2 subunit	NM_007455	14	-1.36	0.000
10462113	Apba1	amyloid beta (A4) precursor protein binding, family A, member 1	NM_177034	19	-1.25	0.000
10560624	Apoe	apolipoprotein E	NM_009696	7	-1.26	0.000
10430190	Apol10b	apolipoprotein L 10B	NM_177820	15	-1.27	0.000
10490221	Atp5e	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	NM_025983	2	-1.35	0.000
10587892	Atr	ataxia telangiectasia and Rad3 related	NM_019864	9	-1.37	0.000
10402249	Atxn3	ataxin 3	NM_029705	12	-1.34	0.001
10485700	Bbox1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1 (gamma-butyrobetaine hydroxylase)	NM_130452	2	-1.42	0.000
10400030	Bzw2	basic leucine zipper and W2 domains 2	NM_025840	12	-1.24	0.000
10547322	Cacna1c	calcium channel, voltage-dependent, L type, alpha 1C subunit	NM_001159533	6	-1.24	0.000
10579691	Calr3	calreticulin 3	NM_028500	8	-1.27	0.000
10475144	Capn3	calpain 3	NM_007601	2	-1.25	0.000
10571696	Casp3	caspase 3	NM_009810	8	-1.31	0.001
10457106	Cbln2	cerebellin 2 precursor protein	NM_172633	18	-1.27	0.001
10348999	Cdh20	cadherin 20	NM_011800	1	-1.31	0.000

10513824	Cdk5rap2	CDK5 regulatory subunit associated protein 2	NM_145990	4	-1.26	0.000
10468974	Cdnf	cerebral dopamine neurotrophic factor	NM_177647	2	-1.55	0.000
10466423	Cep78	centrosomal protein 78	NM_198019	19	-1.37	0.000
10470775	Cercam	cerebral endothelial cell adhesion molecule	NM_207298	2	-1.41	0.001
10560063	Chmp2a	charged multivesicular body protein 2A	NM_026885	7	-1.27	0.000
10461143	Chrm1	cholinergic receptor, muscarinic 1, CNS	NM_001112697	19	-1.24	0.000
10542129	Clec2g	C-type lectin domain family 2, member g	NM_027562	6	-1.24	0.000
10402473	Clmn	calmin	NM_053155	12	-1.49	0.000
10416843	Cln5	ceroid-lipofuscinosis, neuronal 5	NM_001033242	14	-1.36	0.001
10484457	Clp1	CLP1, cleavage and polyadenylation factor I subunit	NM_133840	2	-1.20	0.000
10545874	Cml5	camello-like 5	NM_023493	6	-1.47	0.001
10581049	Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A	NM_027022	8	-1.36	0.000
10503902	Cnr1	cannabinoid receptor 1 (brain)	NM_007726	4	-1.30	0.000
10467216	Cpeb3	cytoplasmic polyadenylation element binding protein 3	NM_198300	19	-1.37	0.000
10437330	Crebbp	CREB binding protein	ENSMUST00000100216	16	-1.25	0.000
10526931	Cyp2w1	cytochrome P450, family 2, subfamily w, polypeptide 1	NM_001160265	5	-1.27	0.000
10399732	Dcdc2c	doublecortin domain containing 2C	NM_001177964	12	-1.38	0.000
10560983	Dedd2	death effector domain-containing DNA binding protein 2	NM_207677	7	-1.39	0.000
10577441	Defb9	defensin beta 9	NM_139219	8	-1.41	0.000
10438245	Dgcr14	DiGeorge syndrome critical region gene 14	NM_022408	16	-1.24	0.000
10515481	Dmap1	DNA methyltransferase 1-associated protein 1	NM_023178	4	-1.38	0.001
10600604	Dmd	dystrophin, muscular dystrophy	NM_007868	X	-1.30	0.000
10596231	Dnajc13	DnaJ (Hsp40) homolog, subfamily C, member 13	NM_001163026	9	-1.21	0.001
10417034	Dnajc3	DnaJ (Hsp40) homolog, subfamily C, member 3	NM_008929	14	-1.38	0.000
10345241	Dst	dystonin	NM_134448	1	-1.24	0.000
10506170	Efcab7	EF-hand calcium binding domain 7	NM_145549	4	-1.43	0.000
10452419	Efna5	ephrin A5	NM_207654	17	-1.33	0.000
10576911	Efnb2	ephrin B2	NM_010111	8	-1.30	0.000

10374366	Egfr	epidermal growth factor receptor	NM_207655	11	-1.27	0.000
10400304	Egln3	egl-9 family hypoxia-inducible factor 3	NM_028133	12	1.41	0.001
10349752	Elk4	ELK4, member of ETS oncogene family	NM_007923	1	-1.31	0.000
10596115	Ephb1	Eph receptor B1	AK082061	9	-1.49	0.000
10389990	Epn3	epsin 3	NM_027984	11	-1.41	0.000
10562064	Etv2	ets variant 2	NM_007959	7	-1.23	0.000
10569611	Fadd	Fas (TNFRSF6)-associated via death domain	NM_010175	7	-1.24	0.001
10470298	Fam69b	family with sequence similarity 69, member B	NM_019833	2	-1.28	0.000
10460726	Fau	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)	NM_007990	19	-1.24	0.001
10540085	Fbln2	fibulin 2	NM_007992	6	-1.33	0.000
10557742	Fbxl19	F-box and leucine-rich repeat protein 19	NM_172748	7	-1.38	0.000
10492755	Fga	fibrinogen alpha chain	NM_001111048	3	-1.23	0.000
10415651	Fgf9	fibroblast growth factor 9	NM_013518	14	-1.30	0.000
10481566	Fibcd1	fibrinogen C domain containing 1	NM_178887	2	-1.28	0.000
10603099	Figf	c-fos induced growth factor	NM_010216	X	-1.23	0.001
10565401	Folh1	folate hydrolase 1	NM_016770	7	-1.28	0.000
10576046	Foxf1a	forkhead box F1	NM_010426	8	-1.36	0.000
10504534	Frmpd1	FERM and PDZ domain containing 1	NM_001081172	4	-1.65	0.000
10495596	Frrs1	ferric-chelate reductase 1	NM_001113478	3	-1.24	0.000
10445710	Frs3	fibroblast growth factor receptor substrate 3	NM_144939	17	-1.41	0.000
10530563	Fryl	furry homolog-like (Drosophila)	NM_028194	5	-1.28	0.000
10552964	Ftl1	ferritin light chain 1	NM_010240	7	-1.51	0.000
10581702	Fuk	fucokinase	NM_172283	8	-1.49	0.001
10571233	Fut10	fucosyltransferase 10	NM_134161	8	-1.58	0.000
10376230	G3bp1	GTPase activating protein (SH3 domain) binding protein 1	NM_013716	11	-1.33	0.001
10575616	Gabarapl2	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 2	ENSMUST00000034428	8	-1.51	0.043
10436416	Gabrr3	gamma-aminobutyric acid (GABA) receptor, rho 3	NM_001081190	16	-1.21	0.012
10487190	Galk2	galactokinase 2	NM_175154	2	-1.32	0.000

10578786	Galntl6	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 6	NM_175032	8	1.29	0.001
10526055	Gbas	glioblastoma amplified sequence	NM_008095	5	-1.32	0.001
10406777	Gcnt4	glucosaminyl (N-acetyl) transferase 4, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	NM_001166065	13	-1.44	0.001
10375614	Gfpt2	glutamine fructose-6-phosphate transaminase 2	NM_013529	11	-1.26	0.000
10544588	Gimap3	GTPase, IMAP family member 3	NM_031247	6	-1.23	0.001
10372410	Glipr1	GLI pathogenesis-related 1 (glioma)	NM_028608	10	-1.26	0.000
10558454	Glrx3	glutaredoxin 3	NM_023140	7	-1.87	0.000
10560202	Gltscr1	glioma tumor suppressor candidate region gene 1	NM_001081418	7	-1.30	0.000
10560181	Gltscr2	glioma tumor suppressor candidate region gene 2	NM_133831	7	-1.30	0.001
10490653	Gmeb2	glucocorticoid modulatory element binding protein 2	NM_198169	2	-1.31	0.000
10547858	Gnb3	guanine nucleotide binding protein (G protein), beta 3	NM_013530	6	-1.41	0.000
10458547	Gnpda1	glucosamine-6-phosphate deaminase 1	NM_011937	18	-1.48	0.000
10419216	Gnpnat1	glucosamine-phosphate N-acetyltransferase 1	NM_019425	14	-1.25	0.000
10526838	Got2	glutamatic-oxaloacetic transaminase 2, mitochondrial	NM_010325	8	-1.34	0.000
10598381	Gpkow	G patch domain and KOW motifs	NM_173747	X	1.35	0.000
10560237	Gpr77	G protein-coupled receptor 77	NM_176912	7	-1.28	0.001
10414137	Grid1	glutamate receptor, ionotropic, delta 1	NM_008166	14	-1.31	0.001
10508052	Grik3	glutamate receptor, ionotropic, kainate 3	NM_001081097	4	-1.29	0.002
10548791	Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)	NM_008171	6	-1.27	0.000
10392863	Grin2c	glutamate receptor, ionotropic, NMDA2C (epsilon 3)	NM_010350	11	-1.26	0.002
10563421	Grin2d	glutamate receptor, ionotropic, NMDA2D (epsilon 4)	NM_008172	7	-1.54	0.000
10364583	Grin3b	glutamate receptor, ionotropic, NMDA3B	NM_130455	10	-1.31	0.001
10449343	Grm4	glutamate receptor, metabotropic 4	NM_001013385	17	-1.38	0.004
10521461	Grpel1	GrpE-like 1, mitochondrial	NM_024478	5	-1.38	0.000
10527562	Gsx1	GS homeobox 1	NM_008178	5	-1.37	0.000
10420268	Gzme	granzyme E	NM_010373	14	-1.21	0.027
10391084	Hap1	huntingtin-associated protein 1	NM_010404	11	1.50	0.000
10514201	Haus6	HAUS augmin-like complex, subunit 6	NM_173400	4	-1.42	0.000

10585851	Hcn4	hyperpolarization-activated, cyclic nucleotide-gated K+ 4	NM_001081192	9	-1.53	0.000
10431371	Hdac10	histone deacetylase 10	NM_199198	15	-1.25	0.001
10606088	Hdac8	histone deacetylase 8	NM_027382	X	-1.38	0.000
10403511	Heatr1	HEAT repeat containing 1	NM_144835	13	-1.33	0.001
10507394	Hectd3	HECT domain containing 3	NM_175244	4	-1.32	0.000
10435266	Heg1	HEG homolog 1 (zebrafish)	NM_175256	16	-1.55	0.000
10382152	Helz	helicase with zinc finger domain	NM_198298	11	-1.26	0.000
10542917	Hepacam2	HEPACAM family member 2	NM_178899	6	-1.32	0.000
10585874	Hexa	hexosaminidase A	NM_010421	9	-1.39	0.000
10408210	Hist1h2bf	histone cluster 1, H2bf	NM_178195	13	-1.33	0.000
10403943	Hist1h2bm	histone cluster 1, H2bm	NM_178200	13	-1.53	0.001
10408239	Hist1h3c	histone cluster 1, H3c	NM_175653	13	-1.30	0.001
10404051	Hist1h4d	histone cluster 1, H4d	NM_175654	13	-1.26	0.000
10500333	Hist2h4	histone cluster 2, H4	NM_033596	3	-1.27	0.000
10491014	Hltf	helicase-like transcription factor	NM_009210	3	-1.33	0.000
10358557	Hmcn1	hemicentin 1	NM_001024720	1	-1.28	0.001
10424909	Hsf1	heat shock factor 1	NM_008296	15	-1.22	0.013
10407034	Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	NM_008308	13	-1.25	0.001
10509238	Htr1d	5-hydroxytryptamine (serotonin) receptor 1D	NM_008309	4	-1.28	0.009
10357288	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	NM_010483	1	1.30	0.012
10521261	Htt	huntingtin	NM_010414	5	-1.23	0.001
10569014	Ifitm2	interferon induced transmembrane protein 2	NM_030694	7	-1.43	0.000
10588707	Ifrd2	interferon-related developmental regulator 2	NM_025903	9	-1.21	0.000
10390209	Igf2bp1	insulin-like growth factor 2 mRNA binding protein 1	NM_009951	11	-1.56	0.000
10540679	Il17re	interleukin 17 receptor E	NM_145826	6	-1.28	0.000
10581664	Il34	interleukin 34	NM_029646	8	-1.44	0.000
10372730	Iltifb	interleukin 10-related T cell-derived inducible factor beta	NM_054079	10	-2.20	0.000
10555574	Inppl1	inositol polyphosphate phosphatase-like 1	NM_010567	7	-1.27	0.000

10576696	Insr	insulin receptor	NM_010568	8	-1.30	0.000
10535247	Iqce	IQ motif containing E	NM_028833	5	-1.34	0.000
10385870	Irf1	interferon regulatory factor 1	NM_008390	11	-1.25	0.001
10536898	Irf5	interferon regulatory factor 5	NM_012057	6	-1.25	0.000
10573910	Irx5	Iroquois related homeobox5 (Drosophila)	NM_018826	8	-1.54	0.000
10554240	Isg20	interferon-stimulated protein	NM_020583	7	-1.51	0.001
10557862	Itgam	integrin alpha M	NM_001082960	7	-1.29	0.001
10435305	Itgb5	integrin beta 5	NM_001145884	16	1.37	0.001
10485340	Itpa	inosine triphosphatase (nucleoside triphosphate pyrophosphatase)	NM_025922	2	-1.25	0.001
10436892	Itsn1	intersectin 1 (SH3 domain protein 1A)	NM_010587	16	-1.23	0.000
10352798	Kcnh1	potassium voltage-gated channel, subfamily H (eag-related), member 1	NM_010600	1	-1.27	0.000
10401987	Kcnk10	potassium channel, subfamily K, member 10	NM_029911	12	-1.32	0.000
10541216	Kdm5a	lysine (K)-specific demethylase 5A	NM_145997	6	-1.34	0.000
10377547	Kdm6b	KDM1 lysine (K)-specific demethylase 6B	NM_001017426	11	-1.35	0.000
10594251	Kif23	kinesin family member 23	NM_024245	9	-1.41	0.000
10398814	Kif26a	kinesin family member 26A	NM_001097621	12	-1.22	0.000
10561942	Kirrel2	kin of IRRE like 2 (Drosophila)	NM_172898	7	-1.41	0.000
10366052	Kitl	kit ligand	NM_013598	10	1.33	0.000
10514319	Klhl9	kelch-like 9	NM_172871	4	-1.31	0.000
10552594	Klk1b22	kallikrein 1-related peptidase b22	NM_010114	7	-1.26	0.000
10548450	Klra5	killer cell lectin-like receptor, subfamily A, member 5	NM_008463	6	-1.28	0.001
10379260	Ksr1	kinase suppressor of ras 1	NM_013571	11	-1.26	0.000
10565479	l7Rn6	lethal, Chr 7, Rinchik 6	NM_026304	7	1.32	0.000
10362538	Lama4	laminin, alpha 4	NM_010681	10	-1.25	0.000
10571005	Letm2	leucine zipper-EF-hand containing transmembrane protein 2	NM_173012	8	-1.28	0.000
10502774	Lphn2	latrophilin 2	NM_001081298	3	-1.32	0.000
10445816	Lrfn2	leucine rich repeat and fibronectin type III domain containing 2	NM_027452	17	-1.23	0.001
10537123	Lrguk	leucine-rich repeats and guanylate kinase domain containing	NM_028886	6	-1.32	0.000

10396314	Lrrc9	leucine rich repeat containing 9	NM_001142728	12	-1.41	0.000
10463457	Lzts2	leucine zipper, putative tumor suppressor 2	NM_145503	19	-1.44	0.000
10581992	Maf	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	NM_001025577	8	-1.30	0.000
10412100	Map3k1	mitogen-activated protein kinase kinase kinase 1	NM_011945	13	-1.50	0.000
10561323	Map3k10	mitogen-activated protein kinase kinase kinase 10	NM_001081292	7	-1.25	0.001
10551666	Map4k1	mitogen-activated protein kinase kinase kinase kinase 1	NM_008279	7	-1.21	0.000
10346328	Mars2	methionine-tRNA synthetase 2 (mitochondrial)	BC132343	1	-1.62	0.000
10480432	Mastl	microtubule associated serine/threonine kinase-like	NM_025979	2	-1.43	0.000
10370824	Mbd3	methyl-CpG binding domain protein 3	NM_013595	10	-1.41	0.000
10536472	Mdfic	MyoD family inhibitor domain containing	NM_175088	6	-1.48	0.000
10503723	Mdn1	midasin homolog (yeast)	NM_001081392	4	-1.38	0.000
10420080	Mdp1	magnesium-dependent phosphatase 1	NM_023397	14	-1.37	0.000
10554819	Me3	malic enzyme 3, NADP(+)-dependent, mitochondrial	NM_181407	7	-1.33	0.000
10508686	Mecr	mitochondrial trans-2-enoyl-CoA reductase	NM_025297	4	-1.39	0.000
10493189	Mef2d	myocyte enhancer factor 2D	ENSMUST00000001455	3	-1.26	0.000
10398326	Meg3	maternally expressed 3	NR_003633	12	-1.22	0.001
10378482	Mett10d	methyltransferase like 16	NM_026197	11	-1.34	0.000
10584589	Mir100	microRNA100	NR_029790	9	-1.37	0.000
10472982	Mir10b	microRNA10b	NR_029566	2	-1.42	0.000
10470296	Mir126	microRNA126a	NR_029541	2	-1.24	0.000
10543680	Mir182	microRNA182	NR_029569	6	-1.26	0.000
10352499	Mir194-1	microRNA194-1	NR_029580	1	1.50	0.000
10398812	Mir203	microRNA203	NR_029590	12	-1.32	0.000
10345028	Mir206	microRNA206	NR_029593	1	-1.41	0.001
10375214	Mir218-2	microRNA218-2	NR_029799	11	-1.22	0.000
10603807	Mir222	microRNA222	NR_029807	X	-1.35	0.000
10398382	Mir379	microRNA379	NR_029880	12	-1.58	0.000
10379028	Mir451	microRNA451a	NR_029971	11	-1.33	0.001

10604585	Mir717	microRNA717	NR_030497	X	-1.26	0.000
10507218	Mknk1	MAP kinase-interacting serine/threonine kinase 1	NM_021461	4	-1.21	0.001
10492469	Mlf1	myeloid leukemia factor 1	NM_001039543	3	-1.54	0.000
10515363	Mmachc	methylmalonic aciduria cblC type, with homocystinuria	NM_025962	4	-1.22	0.000
10482486	Mmadhc	methylmalonic aciduria (cobalamin deficiency) cblD type, with homocystinuria	NM_133839	2	-1.27	0.000
10590781	Mmp1b	matrix metallopeptidase 1b (interstitial collagenase)	NM_032007	9	-1.24	0.001
10573924	Mmp2	matrix metallopeptidase 2	NM_008610	8	-1.30	0.001
10553330	Mrgprb13	MAS-related GPR, member B13	XM_003086592	7	-1.62	0.000
10563760	Mrgprb2	MAS-related GPR, member B2	NM_175531	7	-1.43	0.000
10523161	Mthfd2l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	NM_026788	5	-1.47	0.001
10587695	Mthfs	5, 10-methenyltetrahydrofolate synthetase	NM_026829	9	-1.37	0.000
10407709	Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase	NM_001081128	13	-1.29	0.001
10399421	Mycn	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	NM_008709	12	-1.35	0.000
10557831	Myst1	MYST histone acetyltransferase 1	NM_026370	7	-1.36	0.001
10395074	Myt1l	myelin transcription factor 1-like	NM_001093775	12	-1.27	0.000
10480901	Nacc2	nucleus accumbens associated 2, BEN and BTB (POZ) domain containing	NM_001037098	2	-1.24	0.000
10411595	Naip2	NLR family, apoptosis inhibitory protein 2	NM_010872	13	-1.45	0.000
10545041	Nap1l5	nucleosome assembly protein 1-like 5	NM_021432	6	-1.21	0.000
10517677	Nbl1	neuroblastoma, suppression of tumorigenicity 1	NM_008675	4	-1.47	0.000
10503431	Nbn	nibrin	NM_013752	4	-1.33	0.000
10480652	Ndor1	NADPH dependent diflavin oxidoreductase 1	NM_001082476	2	-1.44	0.000
10456254	Nedd4l	neural precursor cell expressed, developmentally down-regulated gene 4-like	NM_001114386	18	-1.20	0.000
10407420	Net1	neuroepithelial cell transforming gene 1	NM_019671	13	-1.52	0.000
10459944	Nfatc1	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1	NM_001164109	18	-1.26	0.000
10553635	Nipa1	non imprinted in Prader-Willi/Angelman syndrome 1 homolog (human)	NM_153578	7	-1.22	0.000
10599232	Nkap	NFKB activating protein	NM_025937	X	-1.21	0.001

10408037	Nkapl	NFKB activating protein-like	NM_025719	13	-1.43	0.001
10491066	Nlgn1	neuroligin 1	NM_138666	3	-1.26	0.000
10494595	Notch2	notch 2	NM_010928	3	-1.24	0.000
10395719	Npas3	neuronal PAS domain protein 3	NM_013780	12	-1.32	0.000
10401519	Npc2	Niemann-Pick type C2	NM_023409	12	-1.27	0.000
10363492	Npffr1	neuropeptide FF receptor 1	NM_001177511	10	-1.30	0.000
10548038	Ntf3	neurotrophin 3	NM_001164035	6	-1.58	0.000
10481349	Ntng2	netrin G2	NM_133501	2	-1.25	0.000
10520111	Nupl2	nucleoporin like 2	NM_153092	5	-1.36	0.000
10351491	Olfml2b	olfactomedin-like 2B	NM_177068	1	-1.26	0.000
10592391	Olfr937	olfactory receptor 937	NM_146439	9	-1.34	0.000
10484514	Olfr993	olfactory receptor 993	NM_146435	2	-1.41	0.000
10479833	Optn	optineurin	NM_181848	2	-1.46	0.000
10484486	P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3	NM_145526.2	2	-1.28	0.000
10378319	P2rx5	purinergic receptor P2X, ligand-gated ion channel, 5	NM_033321	11	-1.29	0.000
10492330	P2ry1	purinergic receptor P2Y, G-protein coupled 1	NM_008772	3	-1.37	0.000
10590983	Panx1	pannexin 1	NM_019482	9	-1.39	0.000
10592355	Panx3	pannexin 3	NM_172454	9	-1.24	0.000
10587871	Paqr9	progestin and adipoQ receptor family member IX	NM_198414	9	-1.40	0.001
10355916	Pax3	paired box 3	NM_008781	1	-1.24	0.000
10363921	Pcdh15	protocadherin 15	NM_023115	10	-1.32	0.000
10455088	Pcdhb11	protocadherin beta 11	NM_053136	18	-1.34	0.000
10455108	Pcdhb16	protocadherin beta 16	NM_053141	18	-1.30	0.000
10455118	Pcdhb18	protocadherin beta 18	NM_053143	18	-1.43	0.000
10455071	Pcdhb7	protocadherin beta 7	NM_053132	18	-1.47	0.000
10418247	Pde12	phosphodiesterase 12	NM_178668	14	1.25	0.000
10497713	Pex5l	peroxisomal biogenesis factor 5-like	NM_021483	3	-1.30	0.000
10445607	Pex6	peroxisomal biogenesis factor 6	NM_145488	17	-1.49	0.000

10550509	Pglyrp1	peptidoglycan recognition protein 1	NM_009402	7	-1.31	0.000
10439710	Phldb2	pleckstrin homology-like domain, family B, member 2	NM_153412	16	-1.26	0.001
10452030	Plin3	perilipin 3	NM_025836	17	-1.23	0.000
10497253	Pmp2	peripheral myelin protein 2	NM_001030305	3	-1.30	0.000
10454478	Polr2d	polymerase (RNA) II (DNA directed) polypeptide D	NM_027002	18	-1.57	0.000
10394240	Pomc	pro-opiomelanocortin-alpha	NM_008895	12	-1.57	0.000
10542983	Pon1	paraoxonase 1	NM_011134	6	-1.25	0.000
10357948	Ppp1r12b	protein phosphatase 1, regulatory (inhibitor) subunit 12B	NM_001081307	1	-1.29	0.001
10607475	Prdx4	peroxiredoxin 4	NM_016764	X	-1.39	0.000
10408412	Prl7b1	prolactin family 7, subfamily b, member 1	NM_029355	13	-1.28	0.000
10408436	Prl7d1	prolactin family 7, subfamily d, member 1	NM_011120	13	-1.22	0.000
10423049	Prlr	prolactin receptor	NM_011169	15	-1.57	0.000
10542239	Prp2	proline rich protein 2	NM_031499	6	3.30	0.000
10407390	Ptbp1	polypyrimidine tract binding protein 1	NM_001077363	10	1.61	0.000
10471411	Ptges2	prostaglandin E synthase 2	NM_133783	2	-1.28	0.001
10367100	Ptges3	prostaglandin E synthase 3 (cytosolic)	NM_019766	10	1.68	0.000
10567034	Pth	parathyroid hormone	NM_020623	7	-1.20	0.000
10513154	Ptpn3	protein tyrosine phosphatase, non-receptor type 3	NM_011207	4	-1.25	0.000
10409414	Rab24	RAB24, member RAS oncogene family	NM_009000	13	-1.26	0.000
10423068	Rad1	RAD1 checkpoint DNA exonuclease	NM_011232	15	-1.55	0.000
10535312	Radil	Ras association and DIL domains	NM_178702	5	-1.35	0.000
10525983	Ran	RAN, member RAS oncogene family	NM_009391	5	-1.26	0.000
10417713	Rarb	retinoic acid receptor, beta	NM_011243	14	-1.44	0.000
10544573	Rarres2	retinoic acid receptor responder (tazarotene induced) 2	NM_027852	6	-1.54	0.000
10526459	Rasa4	RAS p21 protein activator 4	NM_133914	5	-1.24	0.000
10359235	Rasal2	RAS protein activator like 2	NM_177644	1	-1.25	0.000
10464819	Rbm14	RNA binding motif protein 14	NM_019869	19	-1.41	0.000
10440993	Rcan1	regulator of calcineurin 1	NM_001081549	16	-1.27	0.001

10373588	Rdh5	retinol dehydrogenase 5	NM_134006	10	-1.32	0.001
10490736	Rgs19	regulator of G-protein signaling 19	NM_026446	2	-1.37	0.000
10428124	Rgs22	regulator of G-protein signalling 22	NM_001195748	15	-1.47	0.000
10566132	Rhog	ras homolog gene family, member G	NM_019566	7	-1.48	0.001
10449071	Rhot2	ras homolog gene family, member T2	NM_145999	17	-1.23	0.000
10423855	Rims2	regulating synaptic membrane exocytosis 2	NM_053271	15	-1.22	0.001
10507731	Rims3	regulating synaptic membrane exocytosis 3	NM_182929	4	-1.23	0.000
10383200	Rnf213	ring finger protein 213	ENSMUST00000131035	11	-1.37	0.000
10445061	Rnf39	ring finger protein 39	NM_001099632	17	-1.30	0.000
10533106	Rnft2	ring finger protein, transmembrane 2	NM_172998	5	-1.34	0.001
10378768	Rnmtl1	RNA methyltransferase like 1	NM_183263	11	-1.38	0.000
10438564	Rps10	ribosomal protein S10	NM_025963	17	-1.29	0.000
10385034	Rpsa	ribosomal protein SA	NM_011029	9	-1.36	0.000
10383245	Rptor	regulatory associated protein of MTOR, complex1	NM_028898	11	-1.27	0.001
10444137	Rxrb	retinoid X receptor beta	NM_011306	17	-1.41	0.000
10402428	Serpina12	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	NM_026535	12	-1.26	0.000
10574404	Setd6	SET domain containing 6	NM_001035123	8	-1.51	0.000
10344750	Sgk3	serum/glucocorticoid regulated kinase 3	NM_133220	1	-1.26	0.000
10502224	Sgms2	sphingomyelin synthase 2	NM_028943	3	-1.40	0.000
10400971	Six4	sine oculis-related homeobox4	NM_011382	12	-1.26	0.000
10360173	Slamf7	SLAM family member 7	NM_144539	1	-1.25	0.000
10531887	Slc10a6	solute carrier family10 (sodium/bile acid cotransporter family), member 6	NM_029415	5	-1.21	0.000
10474545	Slc12a6	solute carrier family12, member 6	NM_133649	2	-1.28	0.000
10377790	Slc16a11	solute carrier family16 (monocarboxylic acid transporters), member 11	NM_153081	11	-1.50	0.000
10392440	Slc16a6	solute carrier family16 (monocarboxylic acid transporters), member 6	NM_001029842	11	-1.45	0.000
10481634	Slc25a25	solute carrier family25 (mitochondrial carrier, phosphate carrier), member 25	NM_146118	2	-1.35	0.001

10421172	Slc25a37	solute carrier family 25, member 37	NM_026331	14	-1.29	0.001
10451123	Slc29a1	solute carrier family 29 (nucleoside transporters), member 1	NM_022880	17	-1.28	0.000
10510516	Slc2a5	solute carrier family 2 (facilitated glucose transporter), member 5	NM_019741	4	-1.34	0.001
10352548	Slc30a10	solute carrier family 30, member 10	NM_001033286	1	-1.35	0.000
10496324	Slc39a8	solute carrier family 39 (metal ion transporter), member 8	NM_001135149	3	-1.24	0.000
10435993	Slc9a10	solute carrier family 9, subfamily C (Na+-transporting carboxylic acid decarboxylase), member 1	NM_198106	16	-1.31	0.000
10532275	Smndc1	survival motor neuron domain containing 1	NM_172429	19	-1.22	0.000
10368806	Smpd2	sphingomyelin phosphodiesterase 2, neutral	NM_009213	10	-1.35	0.001
10508723	Snora61	small nucleolar RNA, H/ACA box 61	NR_034046	4	-1.34	0.000
10564017	Snord115	small nucleolar RNA, C/D Box 115 cluster	AF357427	7	1.71	0.000
10375499	Snord96a	small nucleolar RNA, C/D box 96A	NR_028563	11	-1.26	0.001
10529515	Sorcs2	sortilin-related VPS10 domain containing receptor 2	NM_030889	5	-1.27	0.000
10570189	Sox1	SRY (sex determining region Y)-box 1	NM_009233	8	-1.57	0.001
10491721	Spry1	sprouty homolog 1 (Drosophila)	NM_011896	3	-1.50	0.000
10541721	Spsb2	splA/ryanodine receptor domain and SOCS box containing 2	NM_013539	6	-1.37	0.000
10390430	Srcin1	SRC kinase signaling inhibitor 1	NM_018873	11	-1.29	0.000
10411274	Sv2c	synaptic vesicle glycoprotein 2c	NM_029210	13	-1.34	0.000
10595604	Syncrip	synaptotagmin binding, cytoplasmic RNA interacting protein	NM_019796	9	-1.24	0.000
10361381	Syne1	spectrin repeat containing, nuclear envelope 1	NM_001079686	10	-1.32	0.001
10443080	Syngap1	synaptic Ras GTPase activating protein 1 homolog (rat)	XM_985548	17	-1.25	0.000
10425335	Syngr1	synaptogyrin 1	NM_207708	15	-1.23	0.001
10515708	Szt2	seizure threshold 2	NM_198170	4	-1.24	0.000
10472240	Tanc1	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	NM_198294	2	-1.39	0.000
10548677	Tas2r140	taste receptor, type 2, member 140	NM_021562	6	-1.39	0.001
10359701	Tbx19	T-box 19	NM_032005	1	-1.27	0.000
10380887	Tcap	titin-cap	NM_011540	11	-1.22	0.000
10548146	Tead4	TEA domain family member 4	NM_011567	6	-1.34	0.000
10557960	Tgfb1i1	transforming growth factor beta 1 induced transcript 1	NM_009365	7	-1.30	0.000

10606548	Tgif2lx1	TGFB-induced factor homeobox2-like, X-linked 1	NM_153109	X	-1.39	0.000
10385518	Tgtp1	T cell specific GTPase 1	NM_011579	11	-1.25	0.000
10516296	Thrap3	thyroid hormone receptor associated protein 3	NM_146153	4	-1.60	0.000
10565081	Timm17a	translocase of inner mitochondrial membrane 17a	NM_011590	1	-1.35	0.000
10512514	Tln1	talin 1	NM_011602	4	-1.25	0.000
10530145	Tlr1	toll-like receptor 1	NM_030682	5	-1.47	0.000
10518714	Tmem201	transmembrane protein 201	NM_001025106	4	-1.38	0.000
10581917	Tmem231	transmembrane protein 231	NM_001033321	8	-1.37	0.000
10491091	Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	NM_009425	3	-1.37	0.001
10576951	Tnfsf13b	tumor necrosis factor (ligand) superfamily, member 13b	NM_033622	8	-1.23	0.001
10580522	Tox3	TOX high mobility group box family member 3	NM_172913	8	-1.22	0.000
10348600	Traf3ip1	TRAF3 interacting protein 1	NM_028718	1	-1.42	0.000
10437311	Trap1	TNF receptor-associated protein 1	NM_026508	16	-1.35	0.000
10362442	Trdn	triadin	NM_029726	10	-2.21	0.000
10356194	Trip12	thyroid hormone receptor interactor 12	NM_133975	1	-1.22	0.001
10534718	Trip6	thyroid hormone receptor interactor 6	NM_011639	5	-1.52	0.000
10424331	Trmt12	tRNA methyltranferase 12	NM_026642	15	-1.28	0.000
10486722	Trp53bp1	transformation related protein 53 binding protein 1	NM_013735	2	-1.25	0.000
10503520	Ttpa	tocopherol (alpha) transfer protein	NM_015767	4	-1.27	0.000
10548163	Tulp3	tubby-like protein 3	NM_011657	6	-1.66	0.000
10591446	Tyk2	tyrosine kinase 2	NM_018793	9	-1.28	0.000
10528915	Tyms	thymidylate synthase	NM_021288	5	-1.57	0.000
10525885	Ubc	ubiquitin C	BC025894	5	-1.34	0.000
10463185	Ubtd1	ubiquitin domain containing 1	NM_145500	19	-1.30	0.000
10436561	Usp25	ubiquitin specific peptidase 25	NM_013918	16	-1.25	0.000
10367843	Utrn	utrophin	NM_011682	10	-1.26	0.000
10495416	Vav3	vav 3 oncogene	NM_020505	3	-1.63	0.000
10432032	Vdr	vitamin D receptor	NM_009504	15	-1.22	0.000

10571788	Vegfc	vascular endothelial growth factor C	NM_009506	8	-1.54	0.000
10559970	Vmn1r69	vomeronasal 1 receptor 69	NM_145842	7	-1.33	0.000
10532285	Vmn2r15	vomeronasal 2, receptor 15	NM_001104626	5	-1.44	0.000
10565373	Vmn2r76	vomeronasal 2, receptor 76	NM_001102580	7	-1.34	0.000
10345546	Vwa3b	von Willebrand factor A domain containing 3B	XM_003086154	1	-1.34	0.000
10526193	Wbscr16	Williams-Beuren syndrome chromosome region 16 homolog (human)	NM_033572	5	-1.36	0.000
10419296	Wdhd1	WD repeat and HMG-box DNA binding protein 1	NM_172598	14	-1.37	0.000
10386376	Wnt3a	wingless-type MMTV integration site family, member 3A	NM_009522	11	-1.25	0.000
10562323	Wtip	WT1-interacting protein	NM_207212	7	1.26	0.000
10468489	Xpnpep1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	NM_133216	19	-1.26	0.000
10347232	Xrcc5	X-ray repair complementing defective repair in Chinese hamster cells 5	NM_009533	1	-1.28	0.000
10373000	Xrcc6bp1	XRCC6 binding protein 1	NM_026858	10	-1.36	0.000
10434313	Yeats2	YEATS domain containing 2	NM_001145930	16	-1.25	0.001
10458755	Ythdc2	YTH domain containing 2	NM_001163013	18	-1.23	0.001
10490826	Zbtb10	zinc finger and BTB domain containing 10	NM_177660	3	-1.30	0.000
10350377	Zbtb41	zinc finger and BTB domain containing 41 homolog	NM_172643	1	-1.32	0.001
10551426	Zfp59	zinc finger protein 59	NM_011762	7	-1.20	0.000
10547282	Zfp9	zinc finger protein 9	NM_011763	6	-1.29	0.003

[a] Chromosomal location of transcript-encoding gene
[b] *p*-values < 0.001 are shown as 0.000

APPENDIX C. microRNAs identified as differentially expressed in the adult (P60) brain of mice treated with ethanol during synaptogenesis (fold cutoff ≥ ±1.2, FDR-corrected $p<0.05$). miRNAs are listed in order of fold change relative to control.

Mature miRNA	Gene ID	Chr[a]	Fold Change	*p*-value
mmu-miR-704	Mir704	6	-4.169	0.003
mmu-miR-1903	Mir1903	8	-2.701	0.035
mmu-miR-10b	Mir10b	2	-2.276	0.036
mmu-miR-1196	Mir1196	14	-2.046	0.019
mmu-miR-335-3p	Mir335	6	-1.930	0.003
mmu-let-7g	Mirlet7g	9	-1.914	0.016
mmu-let-7b	Mirlet7b	15	-1.892	0.022
mmu-miR-297a		ml	-1.880	0.002
mmu-miR-1947	Mir1947	16	-1.755	0.008
mmu-miR-376b	Mir376b	12	-1.702	0.043
mmu-miR-1970	Mir1970	X	-1.694	0.034
mmu-let-7i	Mirlet7i	10	-1.589	0.040
mmu-miR-590-5p	Mir590	unk	-1.562	0.003
mmu-miR-223	Mir223	X	-1.491	0.001
mmu-miR-503*	Mir503	X	-1.479	0.029
mmu-miR-1953	Mir1953	2	-1.401	0.031
mmu-miR-343	Mir343	7	-1.317	0.037
mmu-miR-467a-1	Mir467a-1	2	-1.313	0.037
mmu-miR-34b-5p	Mir34b	9	-1.292	0.006
mmu-miR-1927	Mir1927	1	-1.287	0.029
mmu-miR-544	Mir544	12	-1.256	0.022
mmu-miR-2182	Mir2182	unk	-1.256	0.048
mmu-miR-466i	Mir466i	13	-1.252	0.017
mmu-miR-696	Mir696	unk	-1.231	0.008
mmu-miR-15b	Mir15b	3	-1.214	0.022
mmu-miR-665	Mir665	12	-1.207	0.032
mmu-miR-715	Mir715	unk	1.238	0.042
mmu-miR-878-3p	Mir878	X	1.242	0.013
mmu-miR-467b	Mir467b	2	1.246	0.038
mmu-miR-1195	Mir1195	17	1.272	0.033
mmu-miR-26b	Mir26b	1	1.284	0.036
mmu-miR-93	Mir93	5	1.311	0.006
mmu-miR-184	Mir184	9	1.485	0.035
mmu-miR-1941-5p	Mir1941	15	1.688	0.037
mmu-miR-669a		ml	1.876	0.025
mmu-miR-721	Mir721	5	2.437	0.004

[a] Chromosomal location of miRNA gene; ml: multiple loci; unk: unknown

APPENDIX D. Animal use protocols approval obtained from the Animal Use Subcommittee at the University of Western Ontario in compliance with the ethical standards established by the Canadian Council on Animal Care.

November 1, 2009
*This is the 2nd Renewal of this protocol
*A Full Protocol submission will be required in 2011

Western

Dear Dr. **Singh:**

Your Animal Use Protocol form entitled:

Genetic Regulatory Mechanisms

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **November 1, 2009 to October 31, 2010**

The protocol number for this project remains as **2007-059**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. 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.

c.c. Approved Protocol - S. Singh, W. Lagerwerf, S. Waring
Approval Letter - S. Singh, W. Lagerwerf, S. Waring

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 • FL 519-661-2028 • www.uwo.ca / animal

Western

Nov.1, 2010
*This is the 3rd Renewal of this protocol
*A Full Protocol submission will be required in 2011

Dear Dr. **Singh**

Your Animal Use Protocol form entitled:

Genetic Regulatory Mechanisms: Genes Determining Ethanol Preference in Mice

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **Nov.1, 2010 to Oct.31, 2011**

The protocol number for this project remains as **2007-059**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. 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.

c.c. M. Kleiber, W. Lagerwerf

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Western

2007-059-10::5:

AUP Number: 2007-059-10
AUP Title: Genetic Regulatory Mechanisms: Genes Determining Ethanol Preference in Mice

Approval Date: 10/27/2011

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-059-10 has been approved.

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

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
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APPENDIX E. Copyright permission to include data previously published in the journals *Developmental Neuroscience* and the *Journal of Behavioral and Brain Sciences*. Data previously published within the *Journal of Neurodevelopmental Disorders* included in this thesis did not require permission as the journal allows authors to retain ownership of data.

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Third trimester-equivalent ethanol exposure is characterized by an acute cellular stress response and an ontogenetic disruption of genes critical for synaptic establishment and function in mice.
Dev Neurosci. 2014;36(6):499-519 (DOI: 10.1159/000365549)
Epub 2014 Sep 30. PubMed PMID: 25278313.

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Hopefully, I have been of assistance to you with the above.

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Scientific Research

To whom it may concern:

Regarding the following paper published in the Journal of Behavioral and Brain Sciences:

Neurodevelopmental Timing of Ethanol Exposure May Contribute to Observed Heterogeneity of Behavioral Deficits in a Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

Katarzyna Mantha, Morgan Kleiber, Shiva Singh

PP. 85-999, Pub. Date: February 27, 2013.

DOI: 100.4236/jbbs..2013.31009

The coauthor Morgan Kleiber from the University of Western Ontario is thereby allowed to Include the above paper into her doctoral thesis. All the contents cited from the mentioned paper should be included as references. Use of this paper for other purposes should be submitted to the JBBS editorial board office for permission.

Best Wishes

Karen Zhang

Editorial Assistant of JBBS

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MORGAN L. KLEIBER

CURRICULUM VITAE

EDUCATION

2007-2015	Ph.D. Candidate, Molecular Genetics Department of Biology, University of Western Ontario *Supervisor: Shiva M. Singh*
2005-2007	M.Sc. Candidate, Molecular Genetics Department of Biology, University of Western Ontario *Supervisor: Shiva M. Singh*
2000-2004	B.Sc. (Honors), Genetics Specialization University of Western Ontario

RESEARCH EXPERIENCE

2003-2004	Undergraduate Honors Thesis Student Department of Biology, University of Western Ontario
2003	NSERC Undergraduate Research Student Department of Biology, University of Western Ontario
2001-2002	Summer Research Student The Prevention and Early Intervention Program for Psychosis London Health Sciences Centre, London ON

ACADEMIC HONOURS AND AWARDS

2015-2018	Canadian Institutes of Health Research Postdoctoral Fellowship ($150,000)
2014	Faculty of Science Graduate Teaching Award ($100)
2011-2012	Queen Elizabeth II Scholarship in Science and Technology ($10,000)
2011	Dr. Irene Uchida Fellowship in Life Sciences ($3000)
2010-2011	Queen Elizabeth II Scholarship in Science and Technology ($10,000)
2007-2010	NSERC Postgraduate Scholarship - Doctoral ($63,000)
2010	Genetics Society of Canada Best Oral Presentation, Cell and Molecular ($100)
2010	Society of Graduate Students, Graduate Teaching Award ($500)
2010	Department of Biology Graduate Teaching Award ($100)
2009	Robert and Ruth Lumsden Fellowship in Science ($1000)
2009	Distinction in Comprehensive Examination
2006-2007	NSERC Postgraduate Scholarship – Masters ($17,500)
2004	NSERC Undergraduate Student Research Award ($4,500)
2003-2004	Faculty of Science Scholarship ($700)
2000-2001	Western Scholarship of Excellence ($2000)
2000-2001	Reach for the Top Entrance Scholarship ($1000)

RESEARCH AND TRAVEL GRANTS AWARDED

2014	Western Library Open Access Fund ($1370)
2011	International Society of Psychiatric Genetics Alcohol Travel Award ($2000)
2011	Joint Fund for Graduate Research Award ($700)
2011	University of Western Ontario Biology Travel Award ($400)

2011 Behavioral Genetics Association Student Travel Award ($200)

PUBLISHED MANUSCRIPTS

1. **Kleiber ML,** Laufer BI, Stringer RL, Singh SM. (2014) Third trimester-equivalent ethanol exposure is characterized by an acute cellular stress response and an ontogenetic disruption of genes critical for synaptic establishment and function in mice. *Dev Neurosci* 36(6): 499-519. PMID: 25278313.

2. **Kleiber ML**, Diehl, EJ, Laufer BI, Mantha K, Chokroborty-Hoque A, Alberry B, Singh SM. (2014) Long-term genomic and epigenomic dysregulation as a consequence of prenatal alcohol exposure: a model for fetal alcohol spectrum disorders (Review). *Front Genet* 5:161. PMID: 24917881

3. Stringer RL, Laufer BI, **Kleiber ML**, Singh SM. (2013) Reduced expression of brain cannabinoid receptor 1 (Cnr1) is coupled with an increased complementary micro-RNA (miR-26b) in a mouse model of fetal alcohol spectrum disorders. *Clin Epigenetics* 5:14. PMID: 23915435

4. Laufer BI, Mantha K, **Kleiber ML**, Diehl EJ, Addison SM, and Singh SM. (2013) Long-lasting alterations to DNA methylation and ncRNAs could underlie the effects of fetal alcohol exposure in mice. *Dis Model Mech* 6:977-992. PMID: 23580197

5. **Kleiber ML**, Mantha K, Stringer RL, Singh SM. (2013) Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. *J Neurodev Disord* 5:6. PMID: 23497526

6. Mantha K., **Kleiber M**, and Singh S. (2013) Neurodevelopmental timing of ethanol exposure may contribute to the observed heterogeneity of behavioural deficits in a mouse model of fetal alcohol spectrum disorders (FASD). *J Behav Brain Sci* 3:85-99.

7. **Kleiber ML,** Laufer BI, Wright E, Diehl EJ, and Singh SM. (2012). Long-term alterations to the brain transcriptome in a maternal voluntary consumption model of fetal alcohol spectrum disorders. *Brain Res* 1458: 18-33. PMID: 22560501

8. **Kleiber ML**, Wright E, and Singh SM. (2011) Maternal voluntary drinking in C57BL/6J mice: Advancing a model for fetal alcohol spectrum disorders. *Behav Brain Res* 223:376-387. PMID: 21601595

9. Symons MN, Weng J, Diehl E, Heo E, **Kleiber ML**, and Singh SM. (2010) Delineation of the role of nicotinic acetylcholine receptor genes in alcohol preference in mice. *Behav Genet* 40:660-71. PMID: 20496163

10. **Kleiber ML**, Singh SM. (2009) Divergence of the vertebrate sp1A/ryanodine receptor domain and SOCS box-containing (*Spsb*) gene family and its expression and regulation within the mouse brain. *Genomics* 93:358-66. PMID: 19101625

11. Singh SM, Treadwell J, **Kleiber ML**, Harrison M, and Uddin RK. (2007) Analysis of behavior using genetical genomics in mice as a model: from alcohol preferences to gene expression differences. *Genome* 50:877-97. PMID: 18059552

POSTERS AND COLLOQUIA

1. Laufer BI, Kapalanga J, Diehl E, Mantha K, **Kleiber ML**, Chokroborty-Hoque A, Alberry BLJ, Koren G, Singh SM. Translating epigenetic alterations in a mouse model to humans. 14th Annual Meeting of the Fetal Alcohol Canadian Expertise (FACE) Research Association, St. John's, Newfoundland and Labrador, Canada. September 7, 2013. Poster presentation. J Popul Ther Clin Pharmacol 20(3): e243-e249.

2. **Kleiber ML,** Janus K, Stringer RL, and Singh SM. Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. Society of Biological Psychiatry, San Francisco, CA, USA. May 16-18, 2013. Poster presentation. Biol Psychiatry 73(9):1S-344S.

3. Diehl E, Laufer B, Mantha K, **Kleiber M**, and Singh S. Alterations in hippocampal gene expression and epigenetic methylation in a mouse model of fetal alcohol spectrum disorder: Towards understanding cognitive deficits. XXth World Congress of Psychiatric Genetics, Hamburg, Germany. October 14-18, 2012.

4. Laufer B, Mantha K, **Kleiber M**, Diehl E, Addison S, and Singh S. Alterations in genomically imprinted miRNA and snoRNA clusters in a mouse model of fetal alcohol spectrum disorders (FASD). XXth World Congress of Psychiatric Genetics, Hamburg, Germany. October 14-18, 2012.

5. Mantha K, **Kleiber M**, Laufer B, and Singh S. Heterogeneous behavioral manifestations in a mouse model of fetal alcohol spectrum disorders (FASD): Assessing the effects of gestational time and gene expression. XXth World Congress of Psychiatric Genetics, Hamburg, Germany. October 14-18, 2012.

6. **Kleiber, M.L.**, Laufer, B.I., Wright, E., Janus, K. and Singh, S.M. Alterations in neurogenomics programming resulting from maternal alcohol consumption in a mouse model of fetal alcohol spectrum disorders. International Congress of Human Genetics (The America Society of Human Genetics), Montreal, QC, CA. October 11-15, 2011. Poster presentation.

7. Laufer, B., Diehl, E., **Kleiber, M.L.**, Janus, K., Wright, E. and Singh, S.M. Disruption of imprinted regions in a mouse model of fetal alcohol spectrum disorders. International Congress of Human Genetics (The America Society of Human Genetics), Montreal, QC, CA. October 11-15, 2011. Poster presentation.

8. Janus, K., **Kleiber, M.L.**, Laufer, B., Chokroborty-Hoque, A., Diehl, E., Wright, E. and Singh, S.M. Mapping diagnostic heterogeneity in an animal model of fetal alcohol spectrum disorders. International Congress of Human Genetics (The America Society of Human Genetics), Montreal, QC, CA. October 11-15, 2011. Poster presentation.

9. **Kleiber, M.L.**, Janus, K., Wright, E. and Singh, S.M. Developmental alcohol exposure results in altered neurogenomic programming in a mouse model of fetal alcohol spectrum disorders. XIX World Congress of Psychiatric Genetics, Washington, DC, USA. September 10-14, 2011. Poster presentation.

10. **Kleiber, M.L.** and Singh, S.M. Transcriptome changes associated with alcohol exposure during synaptogenesis in a mouse model of fetal alcohol spectrum disorders. Behavior Genetics Association Conference, Newport RI. June 6-9, 2011. Poster presentation. Behavior Genet 41(6): 890-944.

11. **Kleiber, M.L.**, Janus, K., Wright, E., and Singh, S.M. Short and long-term alterations in neurotransmitter signalling pathways are associated with spatial learning deficits in a mouse model of Fetal Alcohol Spectrum Disorders. Biology Graduate Research Forum, London ON. October 2, 2010. Invited oral presentation.

12. **Kleiber, M.L.**, Janus, K., Wright, E., and Singh, S.M. Alterations in genes associated with learning in a mouse model of fetal alcohol spectrum disorders. Genetics Society of Canada, Hamilton, ON. June 17-20, 2010. Oral presentation.

13. Wright, E., Janus, K., **Kleiber, M.L.**, and Singh, S.M. Genetical genomics of a mouse model for Fetal Alcohol Spectrum Disorders (FASD). Genetics Society of Canada, Hamilton, ON. June 17-20, 2010. Poster presentation.

14. **Kleiber, M.L.** and Singh, S.M. Studies of developmental neurogenomics in mice as a model for Fetal Alcohol Spectrum Disorders. WorlDiscoveries Research Showcase, London ON. January 30, 2009. Poster presentation.

15. **Kleiber, M.L.**, and Singh, S.M. Studies of developmental neurogenomics in mice as a model for Fetal Alcohol Spectrum Disorders. Genetics Society of America Genetic Analysis: Models Organisms to Human Biology, San Diego CA. January 5-8, 2008. Poster presentation.

16. **Kleiber, M.L.**, and Singh, S.M. Dynamic expression of the SPRY-containing SOCS box protein *Spsb1* gene in the murine brain. Genetics Society of Canada, London ON. June 18-21, 2006. Poster presentation.

PROFESSIONAL AND DEPARTMENTAL SERVICE

2010-2013	Member, Research Committee, Department of Biology
2009-2013	Member, Bursaries and Subsidies Committee, Society of Graduate Students
2008-2012	Undergraduate Honours Thesis Advisor
2011	Chair, Biology Graduate Research Forum
2008-2010	Member, Graduate Education Committee, Department of Biology
2010	Biology Graduate Research Forum Development Committee
2009	Judge, Sanofi-Aventis Biotalent Challenge (Southwestern Ontario)
2006-2008	Biology Principal Representative, Society of Graduate Students
2006-2008	Volunteer, Biology Outreach Events
2002	Volunteer, Prevention and Early Intervention Program for Psychosis, London Health Science Centre

TEACHING AND EXPERIENCE

2012-2014	Invited Lecturer, Biology 2581b: Introductory Genetics "Gene and Genome Structure"
2009-2011	Invited Lecturer, Biology 1229: Nature of Biological Things "Alcohol and You: The effects of alcohol and genetics of alcohol abuse"
2011-2014	Tutorial Co-ordinator, University of Western Ontario Biology 2581b, Introductory Genetics. Class size: 1250.
2006-2014	Graduate Teaching Assistant, University of Western Ontario Biology 2581b: Introductory Genetics. Class size: 1250.
2006-2013	Graduate Teaching Assistant, University of Western Ontario Biology 3592a: Principles of Human Genetics. Class size: 150.

UNDERGRADUATE HONOURS THESIS ADVISORY COMMITTEE

2011-12	T. Hill: Involvement of Interleukin-22 in the control of β cell regenerative factor, *Reg*2, in the pancreas of the Non-Obese Diabetic Mouse following adjuvant immunotherapy. (*Supervisor: B Singh*)
2010-11	A. Cleminson: Genetic variation and mating strategy in the guppy (*Poecilia reticulata*). (*Supervisor: BD Neff*)
2009-10	S. Holmes: Nerve growth factor (Ngfβ) expression may affect anxiety phenotypes in Fetal Alcohol Spectrum Disorder (FASD). (*Supervisor: SM Singh*)
2008-09	R. Butler: NMDA (N-methyl-D-aspartate) receptor subunit expression during neurodevelopment and genetic susceptibility to ethanol exposure. (*Supervisor: SM Singh*)