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Amelioration of prenatal alcohol effects by environmental enrichment in a mouse model of FASD

Aniruddho Chokroborty-Hoque
The University of Western Ontario

Supervisor
DR. SHIVA SINGH
The University of Western Ontario

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ABSTRACT

Maternal alcohol consumption during pregnancy results in a spectrum of behavioural and cognitive deficits collectively known as Fetal Alcohol Spectrum Disorders (FASD). Currently, little is know about if and how the external environment may modulate these deficits. I have used C57BL/6 mice to study this interaction between prenatal alcohol exposure and the postnatal environment. Alcohol exposure during synaptogenesis produces high levels of anxiety-like traits and decreased memory performance. Alcohol-exposed mice (and matched unexposed controls) were put in 'environmentally-enriched' conditions of voluntary exercise, physical activities and cognitive stimulation to ascertain the effects of a positive postnatal environment. The results show that environmental enrichment ameliorates anxiety-like behaviour and memory deficits of alcohol-exposed mice. However this recovery is incomplete, indicative of the long-lasting, potentially permanent damage of prenatal alcohol exposure on the developing brain.

In follow-up studies, I have uncovered gene expression changes in the hippocampus that are associated with behavioural and cognitive amelioration. To accomplish this, I have used mouse hippocampal RNA for microarray and RNA-Seq. My results have identified several key genes and molecular pathways that are associated with synaptic and structural plasticity, neurogenesis, long-term potentiation and angiogenesis. The behavioural and molecular results of this

project represent a novel finding in the field of FASD research. The genes and pathways uncovered provide a possible explanation to understand FASD. They are also potential targets when formulating behavioural and pharmacological rehabilitative therapies.

KEYWORDS

Fetal Alcohol Spectrum Disorders, Mouse Model, Hippocampus, Synaptogenesis, Behaviour, Cognition, C57BL/6J, Environmental Enrichment, Neural plasticity, Prenatal alcohol exposure

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LIST OF ABBREVIATIONS

ADHD	Attention-Deficit Hyperactivity Disorder
AE	Alcohol Enriched
ANE	Alcohol Non-Enriched
ANOVA	Analysis of Variance
B6	C57BL/6J mouse
BAC	Blood Alcohol Concentration
BAM	Binary alignment map
BM	Barnes Maze
CA	*Corpus ammonis*
CE	Control Enriched
CNE	Control Non-Enriched
CNS	Central Nervous System
DEG	Differentially-Expressed Gene
DNA	Deoxyribonucleic Acid
EE	Environmental Enrichment
EPM	Elevated Plus Maze
F	F-statistic
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorders
FDR	False Discovery Rate
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDB	Light-Dark Box
LTP	Long Term potentiation
NOR	Novel Object Recognition
PAE	Prenatal Alcohol Exposure
PND	Post-Natal day
RNA	Ribonucleic Acid
SEM	Standard Error of Mean

LIST OF GENES AND PROTEINS

AD	Alcohol dehydrogenase
Agt	Angiotensinogen
ALDH	Aldehyde dehydrogenase
Ambn	Ameloblastin
Aoah	Acyloxyacyl hydrolase
Arhgap36	Rho GTPase activating protein 36
BCLAF1	BCL2 associated transcription factor 1
Camk2d	Calcium/Calmodulin dependent protein kinase II delta
Casr	Calcium sensing receptor
Cldn1	Claudin 1
Cldn11	Claudin 11
CLOCK	CLOCK circadian regulator
Col1a1	Collagen type I alpha 1 chain
Col3a1	Collagen type III alpha 1 chain
Col8a1	Collagen type VIII alpha 1 chain
Col4a5	Collagen Type IV alpha 5 chain
CYP2E1	Cytochrome P450 family 2 subfamily E member 1
Dcn	Decorin
E2F2	E2F2 transcription factor 2
EP300	E1A binding protein P300
ETS1	Ets avian erythroblastosis virus E26 oncogene homolog
GABA	Gamma-aminobutyric acid
Gabra6	Gamma-aminobutyric acid type A receptor alpha 6 subunit
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Gm129	Gene model 129
Hist1h3a	Histone cluster 1 H3A
Hist1h3f	Histone cluster 1 H3f
Hist4h4	Histone cluster 4 H4
Hspa1a	Heat shock protein family A (Hsp70) member 1A

Hspa1b	Heat shock protein family A (Hsp70) member 1B
Htr1b	5-Hydroxytryptamine receptor 1B
*Htr1f*5	Hydroxytryptamine receptor 1F
Icam1	Intercellular adhesion molecule 1
Il23a	Interleukin 23 Subunit Alpha
Kcnj13	Potassium channel inwardly rectifying subfamily J member
Met	MET Proto-oncogene, receptor tyrosine kinase
Mir669h	Mouse microRNA
mt-Tq	Mitochondrial encoded tRNA glutamine
NMDA	N-methyl-D-aspartate
Nr1d1	Nuclear receptor subfamily 1 group D member 1
Nr2f1	Nuclear receptor subfamily 2 group F member 1
Nr2f2	Nuclear receptor subfamily 2 group F member 2
Per1	Period circadian clock 1
Per3	Period circadian clock 3
Rab4b	RAB4B, member RAS oncogene family
Serpina3f	Serpin family A member 3
THRB	Thyroid receptor hormone beta
Vgll3	Vestigial like family member 3
Zfp879	Zinc finger protein 879

CHAPTER 1: INTRODUCTION

1.1 Prenatal alcohol exposure during neurodevelopment

The constellation of neurological, developmental and behavioural abnormalities resulting from maternal alcohol exposure during pregnancy is known as Fetal Alcohol Spectrum Disorders (FASD). The behavioural deficits associated with FASD are diverse, ranging from attention deficit, hyperactivity, impaired executive function, learning and memory deficits and poor social skills (Berman et al., 1996). Lemoine et al., in 1968, first described the birth defects in the children of alcoholic parents [reviewed in (Lemoine et al., 2003)]. Jones et al., in 1973, noted specific diagnostic criteria, which were, 1) characteristic facial dysmorphia (smooth philtrum, thin upper lip, almond shaped eyes) 2) impaired prenatal and/or postnatal growth, 3) central nervous system (CNS) or neurobehavioural disorders and 4) known exposure to alcohol (ethanol) *in-utero* (prenatal alcohol exposure).

The differentiation of FASD from other disorders like attention deficit hyperactivity disorder (ADHD) is challenging because while many of the behavioural deficits are present in individuals with FASD, not all of them are as a result of PAE (Jonsson et al., 2009). Also, not a single one of these behavioural deficits (nor any combination of them) is unique to FASD. Importantly, ADHD

also has very high comorbidity with FASD, further complicating accurate diagnoses (Manji et al., 2009).

1.2 Fetal Alcohol Spectrum Disorders (FASD): Statistics

As FASD is primarily distinguished by known maternal alcohol exposure, the stigma and shame associated with maternal drinking during pregnancy make self-reported metrics like these unreliable (Sokolowski, 2010). FASD is the most common cause of developmental and intellectual disability in the Western world (Stade et al., 2009). Incidence rates not only vary greatly but are also very highly community-specific with First Nations communities, for example, being at substantially elevated risk from 190 FASD cases per 1000 live births in a First Nations community in British Columbia to 55 to 100 FAS cases per 1000 live births in Manitoban First Nations communities (Malone and Koren, 2012). Despite efforts to raise awareness about the risks of drinking during pregnancy, approximately 14% of Canadian women consume alcohol while pregnant with the numbers being as high as 50% and 60% in isolated northern communities (Popova et al., 2016). In 2013, a study conducted by Popova et al., and published in 2015, conservatively estimated the cost of FASD in Canada to be between $1.3 billion and $2.3 billion (Popova et al., 2015) with the major contributing factors being the cost of productivity losses due to disability and premature mortality and the cost of health care.

1.3 Behavioural dysfunction associated with FASD

Children who have been exposed to alcohol prenatally mostly go unrecognized as being FASD-affected until challenged at school (Streissguth et al., 2004). Deficits in spatial learning and memory, for example, which increase in their severity with greater reported prenatal alcohol exposure have been shown to become more apparent with age and the onset of the encountering of classroom environments (Olson et al., 1998). Children with FASD have also been reported to have deficits in abstract thinking, cognitive flexibility, working memory, verbal learning and auditory memory (Mattson et al., 2011; Olson et al., 2009). Children diagnosed with FASD have been known to make decisions resulting in increased probability of delinquency, and criminal behaviour (Olson et al., 1998).

1.4 Ethanol metabolism

In adults, ethanol is metabolized in the liver by alcohol dehydrogenase (ADH) to acetaldehyde, which is then converted by aldehyde dehydrogenase (ALDH) into acetate. In the fetal liver, ADH is not expressed in sufficient levels to break down ethanol, which is instead carried out by CYP2E1 and other cytochromes but at much slower rates (Agarwal, 2001), implying that ethanol remains in the fetus much longer that in the maternal bloodstream. Importantly, these enzymes produce oxygen free radicals as part of their enzymatic action. These radicals are believed to contribute to ethanol teratogenicity. In mice, ALDH2 is produced by fetal liver, but this does not occur until the late gestation period (gestation days

15-20) (Ramchandani et al., 2001), implying that acetaldehyde is not readily metabolized and due to its instability, leads to free radical formation and cellular damage.

1.5 Role of genetic variation

Given the diversity of phenotypic outcomes from similar levels of ethanol exposure, it is necessary to speculate about the role of genetic factors in modulating the teratogenic effects of alcohol. For example, siblings of children with FAS are at a higher risk of FAS (170 per 1000 live births) among older siblings and 770 per 1000 live births in younger siblings (Zajac and Abel, 1992). Olson et al., found that monozygotic twins have a higher concordance rate for FAS diagnosis than dizygotic twins (Olson et al., 1998). In studies investigating the role of polymorphisms in ethanol metabolism enzymes, it has been found that ADH2*3, a non-synonymous polymorphism leads to a greatly increased ethanol turnover rate (80 times compared to other variants) (Agarwal, 2001). ADHD2*2, another enzyme variant, has been shown to be associated with a decreased FAS presence and is also associated with reduced alcohol consumption during pregnancy (Green et al., 2007; Kleiber et al., 2014a).

1.6 Animal models in FASD research

Currently, we do not have a clear mechanistic or etiological explanation for FASD, including clearly defining what dosage of alcohol at what particular neurodevelopmental state leads to which FASD behavioural deficit. Thus, current FASD research concerns itself with characterizing molecular and cellular changes associated with prenatal alcohol exposure. Animal models have greatly aided in FASD research, suggesting that the prenatal alcohol exposure results in the observed behavioural and cognitive deficits occur by altering critical cellular and molecular processes related to neurodevelopment (Hannigan, 1996).

Various animal studies which have attempted to explain the teratogenic properties of alcohol have shown that alcohol exposure during the brain growth spurt leads to reduced brain/body weight ratio, aberrant synaptogenesis, reactive gliosis, delayed myelination, and cell loss in the hippocampus and cerebellum (Berman and Hannigan, 2000; Hannigan and Berman, 2000; Helfer et al., 2009; Kelly et al., 2009). Heavy doses of binge-like alcohol exposure on post-natal day 7 have been shown to induce apoptosis with large reductions in neuronal populations in the cerebral cortex, hippocampal formation, anterior thalamus, mammillary bodies and cerebellum (Olney et al., 2002; Young and Olney, 2006). Binge-like alcohol exposure during the brain growth spurt has also shown to result in neurobehavioral deficits like spatial learning and memory deficits that

can persist into adulthood in rats (Hannigan and Berman, 2000; Olney et al., 2000a).

1.7 Human neurodevelopment: A short synopsis

Human neurodevelopment (and central nervous system development) begins at conception and continues into early adulthood. It proceeds non-linearly, progressing at a localized and region-specific manner, coinciding with functional maturation (Dobbing and Sands, 1979; Keverne and Curley, 2008; Nottebohm, 2002). In the process of neurodevelopment, the human brain becomes approximately 80% of its adult size by the age of 2 years (Boguski and Jones, 2004). While myelination begins *in-utero* and proceeds rapidly up to 2 years of age, it continues well into early adulthood. Synapse formation also proceeds at a rapid pace for the first two years of life and then plateaus over several years, during which time, neurons form complex dendritic trees (Singer, 2013). At about 5 years of age, neurodevelopment is characterized by cortical reorganization and neuronal growth. From the age of 5 to about 13, the experience-dependent pruning of inefficient cortical synapses occurs in a region specific manner, reducing synaptic density to about 60% of its maximum (Dobbing and Sands, 1979). All of these processes are vital for facilitating efficient neural transmission and the functional maturation of the brain. Importantly, the plateau phase of cortical thickening which begins around the age of 5 and lasts all through puberty is thought to reflect the need for consistently high synpatic density during the

critical formative years of a child's life when learning and experiences are relatively the most intense (Martin and Morris, 2002).

1.8 Brain growth spurt: A critical period in neurodevelopment

One of the most critical periods of neurodevelopment (and the focus of this project) is referred to as the 'brain growth spurt' that in humans corresponds to the third trimester of pregnancy (27 - 40 weeks), and in mice occurs from gestation day 18 to the post-natal day 9. During the brain growth spurt, apart from the brain's rapid weight gain, there is a massive proliferation of astroglia, oligodendrocytes and increased synaptogenesis (formation and proliferation of synapses) and dendritic arborization (increase in branching of dendrites in neurons) (Dobbing and Sands, 1979). Interestingly, while many neurodevelopmental processes are likely to be genetically programmed, countless studies have indicated that the variability in neurodevelopment across one's lifespan is partly due to environmental factors. A group of such factors are teratogens, whose exposure at different points of neurodevelopment alters downstream processes with long-lasting adverse effects (Lagali et al., 2010; Pickard et al., 1999). A cornerstone of teratology is that organ systems which are at their period of most rapid growth and development are the most vulnerable (Olney et al., 2000b).

1.9 The hippocampus

Critical to this project is the study of the hippocampus (Figure 1) in response to alcohol exposure. The structure of the hippocampus is conserved across mammals with analogous regions being present in other vertebrates (Maras and Baram, 2012). While the hippocampal structure is very similar between humans and rodents, the hippocampus occupies a much larger relative proportion of the mouse brain. The hippocampal complex consists of the hippocampus proper (which is divided into the CA1, CA2 and CA3 regions) and the dentate gyrus. The hippocampal formation includes the hippocampal complex, subiculum, presubiculum, parasubisculum, and entorhinal cortex (Lagali et al., 2010). While the hippocampus is similar to other cortical regions of the brain, in that it has large, pyramid-shaped projection neurons and small interneurons, its uniqueness comes largely from the organization of neurons into layers, largely unidirectional passage of information through intra hippocampal circuits and the highly distributed three-dimensional organization of its connections (Lagali et al., 2010). Highly processed sensory information from various brain regions travels from the entorhinal cortex via the subiculum into the hippocampus proper and then out through the dentate gyrus. The glutamatergic excitatory pyramidal neurons are the predominant hippocampal cell types and it is their individual morphology and transcriptional profiles that differentiates the CA1, CA2, and CA3 regions (Rice et al., 2000). The dentate gyrus has dentate granule neurons that project out of the

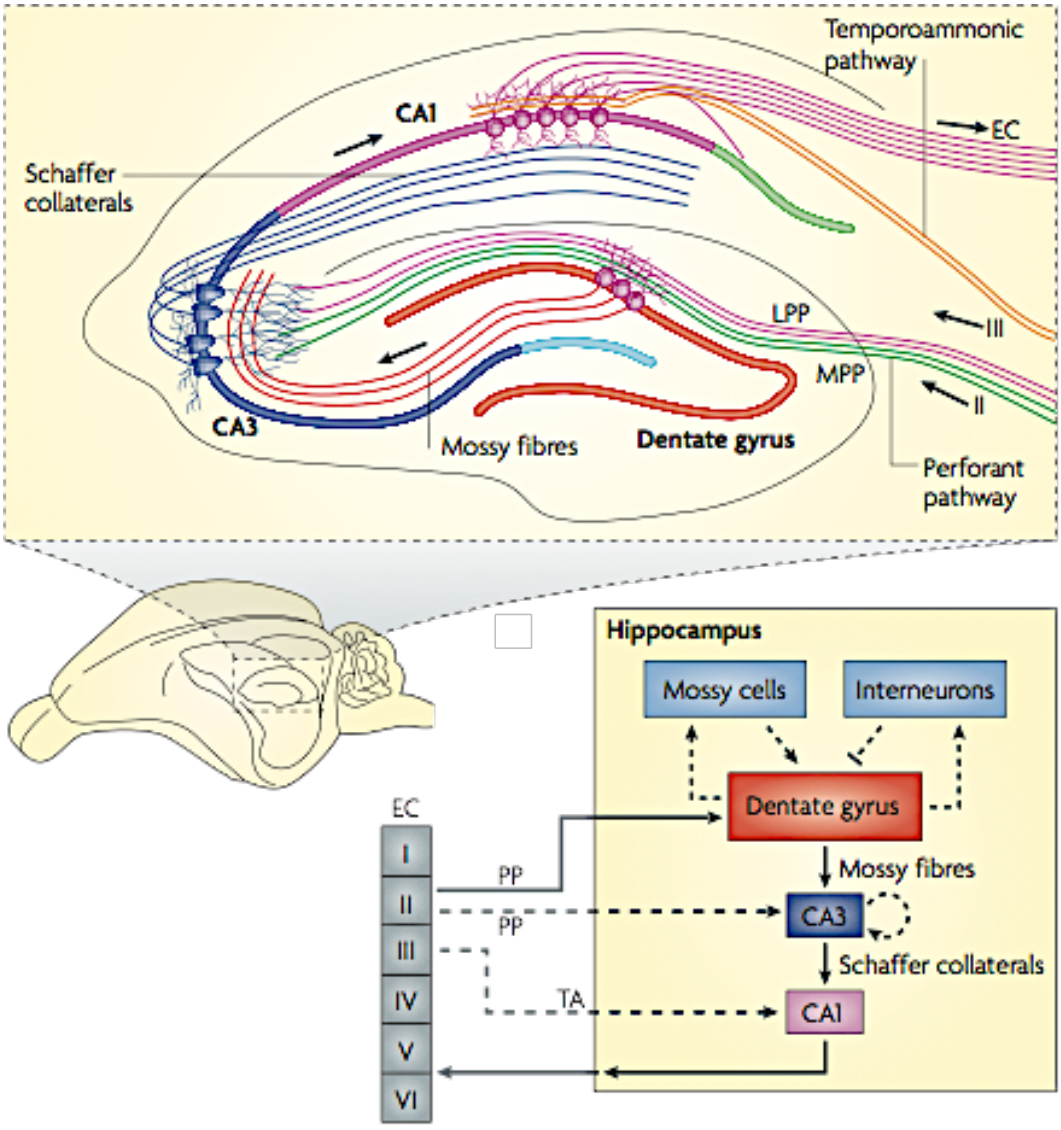


Figure 1: Neural circuitry of the rodent hippocampus. The trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is shown by solid arrows. *Figure adapted from 'New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?' Gage., F.H., et. al., (2010). Nature Reviews Neuroscience. 11:339-350*

hippocampus (Amaral et al., 2007). Both granule neurons and pyramidal neurons are laminated into distinct layers (Burger et al., 2007). The perforant pathway (PP) alongwith the lateral perforant pathway (LPP) and the medial perforant pathway (MPP) link axons of layer II neurons in the entorhinal cortex to the dentate gyrus (DG) and CA3. The DG projects to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons, via the Schaffer collaterals relay information to the CA1 pyramidal neurons, which also receive direct input from EC layer III neurons through the temporoammonic pathway (TA). CA1 pyramidal neurons send back projections to the deep-layered neurons of the EC, while the dentate granule neurons project to the mossy cells in the hilus and hilar interneurons. These interneurons send excitatory and inhibitory projections, respectively, back to the granule cells. During the 'brain growth spurt,' the hippocampus, specifically, has a significant increase in the proliferation of dentate gyrus granule cells, maturation of pyramidal cells of the CA1 and CA3 fields, as well as increases in synaptogenesis and mylenation, all of which are critical circuitry for learning and memory (Eichenbaum, 1999).

1.10 The effect of ethanol exposure during synaptogenesis: Research from animal models

Particularly important to this project is the effect of alcohol exposure during the brain growth spurt in mice, especially post-natal days 6 and 7. These days are the peak of synaptogenesis (Dobbing and Sands, 1979), which refers to the

establishment and maturation of synaptic connections in the brain. A critical feature of synaptogenesis is that more synaptic connections than are necessary are formed initially and during synaptic pruning, the necessary connections are reinforced while unnecessary connections are removed (Rice et al., 2000). Also critically important is the selective apoptosis of unnecessary neurons through the interaction of synaptic NMDA (N-methyl-D-aspartate) and GABA (gamma-aminobutyric acid) receptors (Olney et al., 2002) in which NMDA receptor activation promotes neuronal survival while GABA receptors promote apoptosis.

The Singh laboratory has established that the exposure of mice to a high dose of ethanol during this time (post-natal days 4 to 7) can be considered a model of binge drinking behaviour in humans (Kleiber et al., 2013, 2014b; Laufer et al., 2013; Mantha et al., 2014). The Singh lab has demonstrated that PND 4,7 mice exhibited delayed development, hyperactivity, and impaired learning and memory (Kleiber et al., 2012, 2014b).

Previous studies have shown that alcohol exposure during this time results not only in apoptotic neurodegeneration in the hippocampus and prefrontal cortex (via ethanol acting as both an NMDA antagonist and GABAA agonist) but also learning and memory impairment (Olney et al., 2000a; Titterness and Christie, 2012). It is the loss of these neurons in key brain regions like the hippocampus and prefrontal cortex, that is believed to account, in part, for the behavioural phenotypes associated with FASD (Gil-Mohapel et al., 2010; Guerri and Renau-

piqueras, 1997; Redila et al., 2006). Ethanol has also been shown to cause neurodegeneration via immune response activation in the brain (apart from apoptosis). Specifically the brain's immune system utilizes microglial cells, which remove damaged neurons and are important in guiding neuronal development by regulating glutamatergic receptors and maturation and synaptic transmission (Rice et al., 2000). Ethanol has been shown to trigger microglial activation (characterized by production of pro-inflammatory factors and reactive oxygen species), resulting in neuronal death (Ikonomidou, 2009; Olney et al., 2000a).

1.11 Ethanol exposure and the hippocampus

As the hippocampus is a critical region in the brain for learning and memory, it represents an important therapeutic target in FASD research, given that learning and memory deficits are a hallmark of third-trimester alcohol exposure. The hippocampus plays a major role in the formation of new memories (Eichenbaum, 1999). Damage to the hippocampus results in profound difficulties in the formation of new memories (File et al., 2000) particularly with spatial learning (Lynch et al., 2004). Long-term potentiation (LTP), which refers to the increase in strength of synaptic connections following neuronal activation, lasting for hours or days (Neves et al., 2008), is considered to be the physiological proxy by which memory formation is mediated in the hippocampus. It is believed that by changing synaptic strength, neuronal pathways can store information (memories) (Cramer et al., 2011) but the precise mechanisms by which this happens are

currently unknown. Changes in neuronal gene expression are believed to be, in part, responsible for LTP. In fact, the genetic impairment of LTP has been shown to result in impaired learning and memory in mice (Eichenbaum, 1999).

On post-natal days 6 and 7, the hippocampus is undergoing the peak of synaptogenesis, making it exceptionally vulnerable to the effects of ethanol exposure. For example, the levels of neuronal apoptosis due to ethanol exposure during synaptogenesis is particularly high in the hippocampus (Olney et al., 2002). Mice that are exposed to ethanol on post-natal days 1 to 6 display not only reduced neuronal numbers, but also reduced neurogenesis, synaptic efficacy, and dendritic spine density (Kimura et al., 2000). Mice that have been exposed to ethanol on post-natal day 7 exhibit spatial learning and memory deficits, similar to mice with hippocampal lesions (Mantha et al., 2014). Interestingly, the information on non-hippocampal dependent memory such as object and verbal memory are inconsistent between studies [reviewed in (Uecker and Nadel, 1996)].

1.12 Neurodevelopment, plasticity and the environment

This project revolves around a fundamental fact of neurodevelopment; namely, that human brain development is not complete at birth, rather it continues for decades. Neurodevelopment during this period is malleable and responsive to postnatal environment thus, providing targeted opportunities to direct postnatal brain development and alter the course of development of FASD. While the mechanisms behind post-natal alterations are still being understood, it is known

that variations in postnatal conditions promote hippocampal synaptogenesis and spatial learning and memory through systems known to mediate experience-dependent neural development, contributing to the lifelong changes in behaviors and mental abilities.

Hebb postulated that one's experiences and environment both can influence cognitive and neural development (Hebb, 1949). Classic experiments such as Hubel and Wiesel's [reviewed in (Wieloch and Nikolich, 2006)] were the first to demonstrate that the influence of the environment on neural plasticity and neurodevelopment in the visual system in cats. In the 1960s and 1970s, Rosensweig and colleagues [reviewed in (Rosenzweig, 1996)] examined the influence of environmental manipulations on brain weight, cortical thickness, dendrite structure, cognitive functioning, synaptogenesis, angiogenesis and gliogenesis.

1.13 Environmental enrichment

Environmental enrichment, as it stands now, involves making the animal's environment more cognitively and physically stimulating by the inclusion of toys, tunnels, bridges, nesting materials and running wheels. It also involves keeping animals in bigger groups to increase the number of social interactions. The plethora of objects enhance the sensory, physical and cognitive experiences of animals as well as their experience of novelty, as the objects within the cages or

the cages itself are moved around or changes frequently (Nithianantharajah and Hannan, 2006). Additionally, the inclusion of running has shown to increase the overall exploratory activity of animals (Fabel et al., 2009; Nithianantharajah and Hannan, 2006).

Studies in rodents have shown that environmental enrichment increases dendritic branching and spine number, synaptic density and neuronal cell size (Arai and Feig, 2011; Catlow et al., 2009; Toth et al., 2011). Environmental enrichment has also shown to enhance neurogenesis, long-term potentiation, neurotrophin levels, and nerve growth factor mRNA and *Creb* gene expression specifically in the dentate gyrus of the hippocampus (Ehninger and Kempermann, 2003; van Praag et al., 1999; Praag et al., 2002). Associated with the effects of environmental enrichment on neurodevelopment, studies have shown that environmental enrichment significantly improves performance on a wide variety of spatial and non-spatial memory tasks along-with decreasing stress- and anxiety levels in mice (Kronenberg et al., 2003; van Praag et al., 1999; Praag et al., 2002).

The impact of environmental enrichment on humans is far more limited and primarily centres on the principle of 'cognitive reserve' as related to Alzheimer's disease. Epidemiologic data has linked increased participation in intellectual and social activities in daily life and a slower cognitive decline in the elderly (Collins et al., 2009; Nithianantharajah and Hannan, 2011). Also, cognitive exercises have been shown to be an effective form of intervention for

slowing the trajectory of cognitive and functional decline that is associated with dementia (Kempermann, 2008). A number of studies have shown the protective benefits of environmental enrichment against the normal age-related decline of memory function and various neurological and psychological pathologies like depression, Huntington's disease and Alzheimer's disease in both humans and animal models (Hannan, 2014; Nithianantharajah and Hannan, 2011). In adults, specialized cognitive training has been shown to increase cortical brain activity (Collins et al., 2009; Milgram et al., 2006) and alter dopamine D1 receptor binding in both the prefrontal and parietal cortices (McNab et al., 2009).

The U.S. Bureau of Labour Statistics reported that only 6% of people spend their leisure time exercising with most people spending twice as much time watching television compared to participating in cognitive stimulating activities such as reading or socializing (U.S. Bureau of Labor Statistics, 2011). In fact, both cognitive and social stimulation have been shown to be crucial for normal development in childhood. For example, additional cognitive stimulation for children from lower socioeconomic backgrounds, either at home or in a preschool setting, can significantly improve their academic achievements (Carmichael and Lockhart, 2012; Petrosini et al., 2009).

1.14 Environmental enrichment and mouse models of FASD

Over the years, many treatments, pharmacological and behavioural, have been designed to ameliorate the behavioural and cognitive deficits that result from

prenatal alcohol exposure with some cognitive and behavioral interventions in schoolchildren with FASD showing promise. For example, Kable et al., used a directed math intervention to significantly improve scores on math (Kabel et al., 2007). Similarly, Peadon et al., used cognitive control therapy to not only increase student confidence and motivation but also academic performance (Peadon et al., 2009). Children with FAS who were taught rehearsal strategies had increased scores on the digit-span task (Hannigan and Berman, 2000).

Environmental enrichment, voluntary exercise and complex motor training are three behavioural interventions that have shown promise in the amelioration of the effects of fetal alcohol exposure in both mice and rats. Environmental enrichment has been shown to improve alcohol-induced spatial learning deficits and lower hyperactive behavior (Helfer et al., 2009; Waters et al., 1997). Similar to environmental enrichment, pure exercise paradigms such as voluntary running on wheels have also been shown to improve alcohol-induced spatial learning deficits and lower alcohol-induced hyperactivity and emotionality in the open field (Christie et al., 2005). However, whereas environmental enrichment failed to increase synaptic plasticity in alcohol-treated animals, wheel running has been shown to promote not only synaptic plasticity in alcohol-exposed rats but also increase the proliferation and survival of newly generated neurons in the dentate gyrus (Hamilton et al., 2014; Helfer et al., 2009), while some studies indicate that environmental enhancement alone is more effective for inducing neural changes

than exercise alone, others suggest that exercise, but not cognitive stimulation improves spatial memory (Schrijver et al., 2002; Toth et al., 2011).

1.15 High-throughput gene expression studies in FASD

The Singh laboratory's attempts to investigate PAE in B6 mice *in-vivo* have uncovered that trimester-specific FASD phenotypes have corresponding and associated gene expression changes. While the short-term effect initiates alterations in genes that primarily affect cellular structure and apoptosis, in the long term, PAE affected genes are different and involve various cellular functions including epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA regulation that may underlie long-term changes to gene expression patterns (Kleiber et al., 2012; Laufer, 2016; Mantha et al., 2014). Fundamentally, the identification and quantification of mRNA in any biological sample is an essential step in detecting differential gene expression.

I have used RNA sequencing for the high-throughput quantification of mRNA. Its advantages over conventional hybridization methods such as micro-arrays include a low amount of background noise, high levels of reproducibility and the ability to detect transcripts over a large range of expression levels (Rapaport et al., 2013). The raw data, once obtained from RNA-Sequencing (Illumina V4 chemistry), is processed using edgeR, a free to use digital expression software that detects differential gene expression after analyzing the number of

transcripts in replicated samples. edgeR uses an over-dispersed Poisson model to account for biological and technical variabilities, which are likely occurrences in experiments that have data from multiple biological replicates, resulting in the sample variance being greater than the sample mean (Chen et al., 2014a, 2014b) .

Following the analysis of RNA-Seq data with edgeR to determine differentially expressed genes (DEGs), a variety of software programs are used to identify important molecular pathways and gene networks that are associated with the observed phenotypes. This project uses the database and pathway analysis tool, ConsensusPathDB (Kamburov et al., 2013), for pathway analysis and gene annotation. ConsensusPathDB uses the hypergeometric distribution to calculate a p-value. This value is calculated by comparing the number of genes inputted by the user (separate up- and down-regulated genes) to the total number of genes that may be involved in particular pathways. A significant p-value is an over-representation of genes in a given pathway, containing more focus genes than expected by chance (Kamburov et al., 2013). In order to learn how the differentially expressed genes uncovered from this project interact with other molecules and also predict gene function, this project uses GeneMANIA, which uses a linear-regression algorithm to calculate and plot a functional association network of user-inputted genes with other genes in the GeneMANIA database that are known to interact with the input genes (Warde-Farley et al., 2010). Gene networks are plotted using Cytoscape ClusterMaker (‘GLay’ algorithm), an open-

source bioinformatics software (Su et al., 2010). Finally, using Enrichr (Chen et al., 2013), an open-source browser-based enrichment analysis tool, I uncovered various transcription factors for this project's list of differentially expressed genes. Ultimately, for a gene to be biologically relevant to FASD-related phenotypes, it must not only be differentially expressed via RNA-Seq analysis but also be implicated in behavioural, cognitive or neurodevelopmental pathways or networks. Finally, several biologically relevant genes are selected from the list of differentially-expressed genes obtained from RNA-Seq analyses and validated using real-time qPCR assays.

1.16 Hypothesis

Post-natal environmental enrichment ameliorates FASD-related behavioural and cognitive deficits in mice that have been exposed to alcohol during their third trimester of neurodevelopment, and these ameliorations are associated with hippocampal gene expression changes and alterations in specific molecular pathways and networks.

1.17 Specific objectives:

1. To establish that a binge-like prenatal ethanol exposure during the third trimester of mouse neurodevelopment results in quantifiable behavioural and cognitive deficits related to FASD.

2. To establish an environmental enrichment model of amelioration where

sustained environmental enrichment ameliorates FASD-related behavioural and cognitive changes in alcohol-exposed mice.

3. To establish that FASD-related behavioural and cognitive deficits are associated with hippocampal gene expression changes and alterations in molecular pathways and networks related to neurodevelopment, learning and memory.

4. To establish that amelioration of FASD-related deficits by environmental enrichment is related to environmental enrichment induced alterations in neurodevelopment, and learning and memory pathways in the hippocampus.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal care

The animal protocols conducted in this research were approved by the Animal Use Subcommittee at the University of Western Ontario (London, ON) (Appendix 1). The protocols 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). The Animal Care Facility at the University of Western Ontario was subsequently tasked with maintaining them. Prior to controlled breeding, mice were housed in same-sex colonies of two to four mice with *ad-libitum* access to water and food. All environmental factors (such as colony size, bedding, nestlets, cage type and size 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.

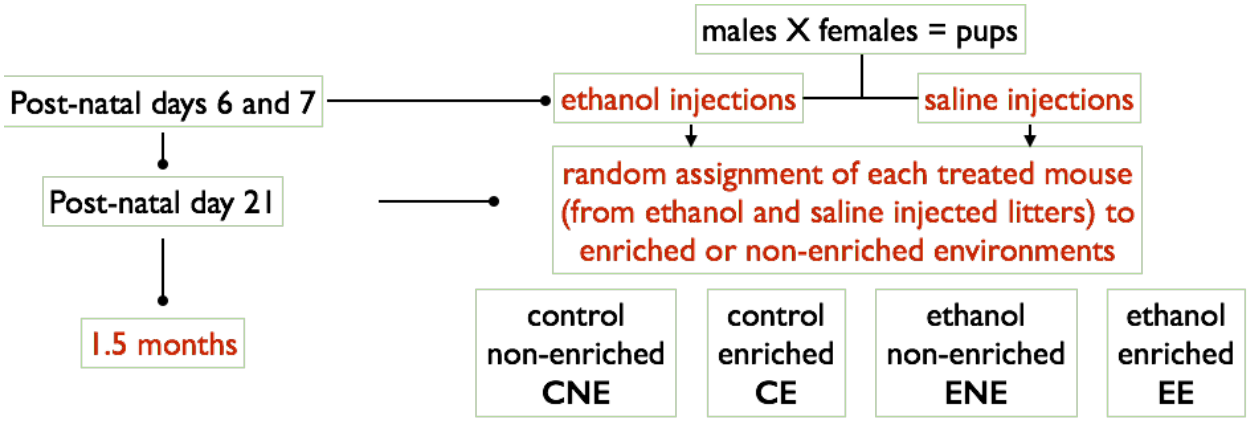
2.2 Breeding and ethanol/environment treatments

Approximately 8-week old nulliparous female B6 mice were individually housed and mated overnight with 8 to 12-week old B6 males. The subsequent morning, males were removed and during the gestation period, females were housed individually in standard caging. Towards gestational days 17-22 (the end of mouse gestation), cages were checked each evening at 5 pm and each morning at

10 am for the presence of pups with the day of birth being noted as postnatal day zero (P 0). Litters were culled to a maximum of 10 pups and litters containing less than two pairs of each sex were excluded from further study.

At P 0, all litters were assigned to either control (C) or alcohol (A) treatment. The alcohol treatment was designed to mimic a binge-like ethanol exposure matching the human third trimester-equivalent (T3) of neurodevelopment. This represented post-natal days P 6 and P 7 in mice (Dobbing and Sands, 1979). The alcohol treatment consisted of two subcutaneous injections of 2.5 g/kg ethanol in 0.15 M saline spaced two hours apart (0 h and 2 h), resulting in a peak blood alcohol concentration of over 0.3 g/dl for 4 to 5 hours following the initial injection. The acute ethanol injection model during the third trimester was chosen due to the model's propensity to result in more robust behavioural and cognitive phenotypes. The control mice were injected with an equivalent volume of 0.15 M saline only. Once the injections were completed, the tail of each pup was marked with non-toxic ink to allow for later identification and all the pups were returned to their respective cages where they remained with their dams until weaning at P 25. At P 25, each pup was randomly assigned to an environmental treatment of either non-enriched (NE) or enriched (E) cages, thus creating four total groups, control non-enriched (CNE), control-enriched (CE), alcohol non-enriched (ANE) and alcohol enriched (AE) groups (Figure. 2). The

environmental treatment was designed to closely approximate physical, cognitive and mental enrichment recommended for children affected with FASD (Hannigan, 1996; Hannigan et al., 2007). While non-enriched cages were standard shoe-box cages with bedding and housing, enriched cages were large colony-cages containing, (1) extra layers of bedding with food pellets hidden within them, (2) lots of nestlets, (3) running wheels (Med Associates Inc.), hoops and ladders. and (4) toys and objects of various shapes, colours and sizes (Figure 3). All hoops, ladders and toys were purchased from PetSmart. The respective positions of the objects inside enriched cages were changed every three days and the whole cage was changed ever week. The constant re-arrangement of objects and the environment itself are crucial to the novelty of the mice's environment along with providing new ways to engage and challenge their physical, mental and cognitive development. The total duration of enrichment was 1.5 months. After a battery of behavioural and cognitive tests over time, on P 85, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The hippocampus was dissected out from each mouse and used for gene-expression.



DAY	ENDOPHENOTYPE	TEST	SAMPLE SIZE
65	ANXIETY	ELEVATED PLUS MAZE	(males:10) = 10 X 4 groups total: 40
67	ANXIETY	LIGHT DARK BOX	(males:10) = 10 X 4 groups total: 40
69-71	RECOGNITION MEMORY	NOVEL OBJECT RECOGNITION	(males:10) = 10 X 4 groups total: 40
73-76	VISUO-SPATIAL LEARNING	BARNES MAZE	(males:10) = 10 X 4 groups total: 40
77	VISUO-SPATIAL MEMORY	SHORT TERM MEMORY	(males:10) = 10 X 4 groups total: 40
82	VISUO-SPATIAL MEMORY	LONG TERM MEMORY	(males:10) = 10 X 4 groups total: 40
85		HIPPOCAMPUS EXTRACTION	(males:10) = 10 X 4 groups total: 40

Figure 2: The behavioural pipeline of this project: Pups were produced after mating male and female mice. On post-natal days PND 6 and 7, each litter of pups was injected either with ethanol or saline solutions. On PND 21 (weaning), each mouse (from the ethanol and saline litters) was randomly assigned either an enriched or non-enriched cage setting, resulting in four mouse groups. After 1.5 months of environmental treatment, four behavioural and cognitive tests followed, after which mouse hippocampi were dissected.

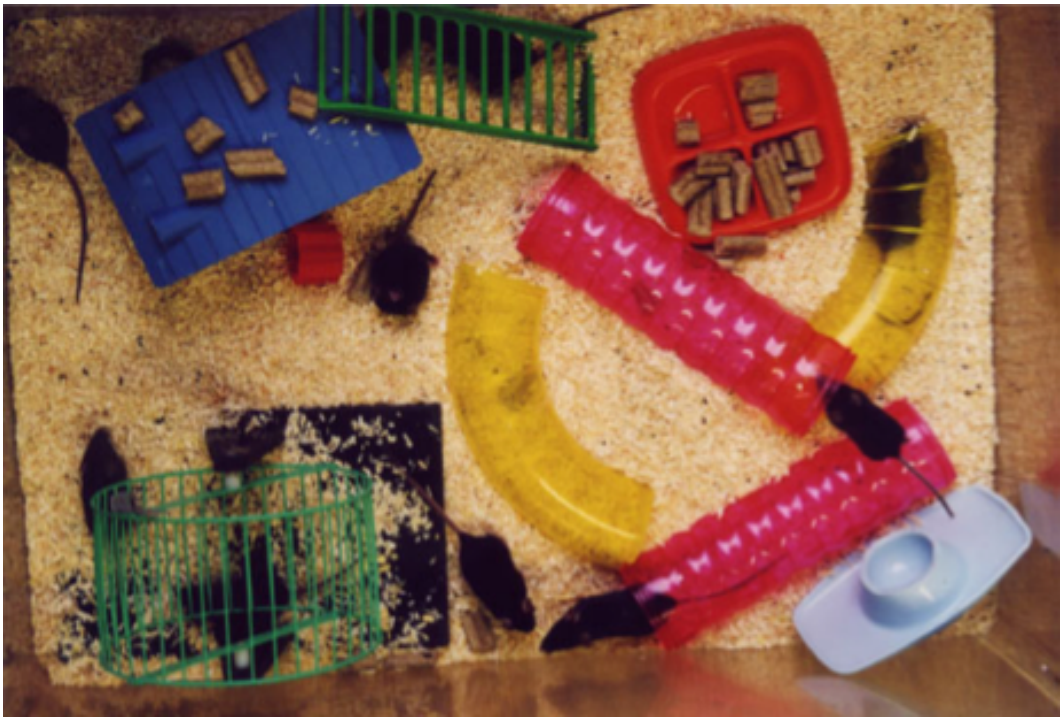


Figure 3: Environmental Enrichment (EE) setups for Ethanol-Enriched and Control-Enriched mice: Mice are put in groups of 2 (at the minimum) or more in large cages with access to running wheels, swings, ropes, tunnels and toys, all of which are in different colours, shapes and textures. Nestlets are also provided to encourage nest building. Hidden within the bedding of these cages are food pellets and toys to encourage the natural ethological tendency for mice to dig for food and objects. The relative positions of the objects within the cage are changed every three days. The enriched mice are moved to completely new cages (with new toys, setups and environments) every week.

2.3 Behavioural and cognitive measures

2.3.1 Anxiety-associated behavioural measures

Anxiety-related traits were assessed using two measures: an elevated plus maze assay, and a light-dark box assay.

Elevated Plus Maze

Mice were assessed for anxiety-related behaviours using the elevated plus maze (EPM) (Walf and Frye, 2007) at P 60 (Figure 4). The EPM consisted of 4 cardboard arms (with 2 open and 2 closed arms) 50 cm above ground. All arms were 50 cm (L) x 10 cm (W) with closed arms having an additional 40 cm (H) of cardboard. The 4 arms were connected in a plus formation to allow the mice to cross between arms. The EPM relies on the rodent's natural tendency to seek dark, sheltered areas, avoid bright (200 lux), exposed, and high-altitude areas. One hour prior to testing, mice were removed from the colony room and brought to the testing room for acclimatization. At the trial's beginning, a mouse was placed facing an open arm at the intersection of the apparatus and the mouse's movement was recorded for a single 5-minute period, which is considered sufficient to reveal differences in anxiety-related traits across mice. In between trials, the Plexiglas cover on the maze was wiped with 30% isopropanol. The mouse's movement was recorded by AnyMAZE Video Tracking software (San Diego Instruments, San Diego, CA).

Light-Dark box

The LDB (Figure 5) is used to assess of exploratory behaviour and anxiety-related behaviour in mice in a novel, illuminated environment (Bourin and Hascoe, 2003). The apparatus consisted of a big plastic container divided into two compartments, consisting of a 27 cm (L) x 27cm (W) x 27 cm (H) light arena and a 18 cm (L) x 27 cm (W) x 27 cm (H) dark arena with a 7.5 cm x 7.5 cm opening between the light and dark regions. On PD 62, one hour prior to testing, mice were removed from the colony room and brought to the testing room for acclimatization. At the trial's beginning, the mouse was placed in the light area facing the opening into the dark area. The mouse was allowed to freely explore both areas for 5 minutes, with an overhead light at 200 lux. Movement was recorded by AnyMAZE Video Tracking software. In between trials, the plastic arena was wiped with 30% isopropanol to get rid of odour cues from previous mice.

Figure 4: A photograph of the Elevated Plus Maze (EPM) apparatus used to assess stress- and anxiety- related endophenotypes in mice: A C57BL/6J mouse is shown in the top closed arm of the EPM. Plexiglas is used to cover the open arms of the maze. The EPM test measures the amount of time spent by a mouse in the open arms (relative to the time spent in closed arms), which is taken to be a proxy for the mouse's propensity to be explorative, and less stressed and anxious about new environments.

Figure 5: A photograph of the Light/Dark Box (LDB) used to assess stress- and anxiety-related endophenotypes in mice. A C57BL/6J mouse is shown in bottom right corner of the LDB. The LDB test measures the amount of time spent by a mouse in the light region (relative to the time spent in dark region). This measure is considered to quantify a mouse's explorative behaviour and can tell us something about the mouse's state of stress and anxiety.

2.3.2 Measures of cognition, memory and learning

Novel object recognition

The Novel Object Recognition test (Figure 6) is a three-day test and is used as a measure of recognition memory in a novel environment (Ennaceur, 2010). The apparatus consisted of a large plastic container measuring 45 cm (L) x 27cm (W) x 27 cm (H). On each testing day starting from PD 64 to PD 66, one hour prior to testing, mice were removed from the colony room and brought to the testing room for acclimatization. On day one, each mouse was placed in the middle of the container and allowed to roam and familiarize with the area for 15 minutes. On day two, the mouse was kept in the container and for a total duration of 5 minutes, was allowed to explore a pair of objects, each similar in shape, size, texture and colour. On day three, the mouse was kept in the container and for a total duration of 5 minutes, was allowed to explore a pair of objects, one 'familiar' object from day two's session and one 'novel' object of different shape, size, texture and colour. The time spent exploring the pair of objects on day two and three was recorded by AnyMAZE Video Tracking software. In between trials on all three days, the plastic container was wiped with 30% isopropanol and the bedding was changed completely.

Barnes Maze

From PD 66-78, the Barnes Maze (Figures 7 and 8) was used as a test of spatial learning, short-term recall, and long-term recall (Sunyer et al., 2007a). It consisted of a circular wood platform 105 cm above ground, 92 cm in diameter with 20 equally-spaced 5 cm diameter holes along the periphery of the platform with 7.5 cm between hole. While 19 holes were covered underneath with black cardboard sheets, one of the holes was the 'target hole' through which mice would be able to enter an escape box which was painted black to remain visually indistinguishable from other holes. Visual cues constructed by the experimenter were placed around the testing room, providing spatial cues and marks to help orient mice with respect to the target hole. A 150 W bright light and 85-decibel white noise (generated by the AnyMAZE software) were used as aversive stimuli. Mouse movement was tracked by the AnyMAZE software. Once the mouse entered the escape box, the aversive stimuli were turned off for a period of 1 minute to allow the mouse to acclimatize to the box. The platform and escape box were wiped with 30% isopropanol between trials.

a) Learning

Each mouse was given four trials over four 'acquisition days' (learning days) to learn the target hole's location. At the beginning of each trial, a mouse was placed in the centre of the maze inside a cylindrical chamber for 10 seconds. Upon removal of the chamber, recording by AnyMAZE commenced, the aversive

stimuli began and the mouse was allotted 3 minutes to explore the maze and locate the target-hole. Once the start-chamber was removed, the mouse was allotted 3 minutes to explore the maze and locate the target hole. If the mouse entered the target hole within the 3-minute period, the aversive stimuli were terminated. If the mouse failed to locate the target hole within 3 minutes, the mouse was guided to the escape box following the trial and allowed to remain inside the box for 1 minute. Each mouse was trained for four consecutive days, with each training day consisting of four 3-minute trials. There was a 15 minute interval between trials. The latency (seconds) to enter the escape box was measured using AnyMAZE software.

b) Memory

The fifth (short-term memory) and twelfth (long-term memory) days of Barnes Maze testing are referred to as "probe" trial (Sunyer et al., 2007b). A successful trial is defined as the selective search of the former location of the target hole. On the fifth and twelfth probe days, the escape box was removed, and the target hole was covered with black cardboard. Each mouse was given one 1-minute trial on each day to explore the maze. The number of explorations to each hole was recorded.

Figure 6: A photograph of the Novel Object Recognition test used to assess recognition memory in mice: The NOR test measures the amount of time spent by a mouse exploring the novel object. In a previous configuration of the above setup, two blue caps are the 'old objects' that the mouse spends time exploring during the first day of testing ('acclimatization'). On the second day of testing (above configuration), a 'novel object,' in this case a shiny bottle cap, replaces one blue cap and the NOR test measures how long the mouse spends exploring the shiny cap. A functioning, healthy recognition-memory performance is indicated by the longer time spent by the mouse exploring the novel object.

Figure 7: A photograph of the Barnes Maze used to assess spatial and learning memory in mice: Spatial cues, consisting of various shapes and colours, are displayed around the testing room. All holes, except the target hole, are covered with black cardboard cut-outs. The target hole (indicated with arrow) leads to a black escape box, positioned underneath the platform. Arrow points to the location of the target hole.

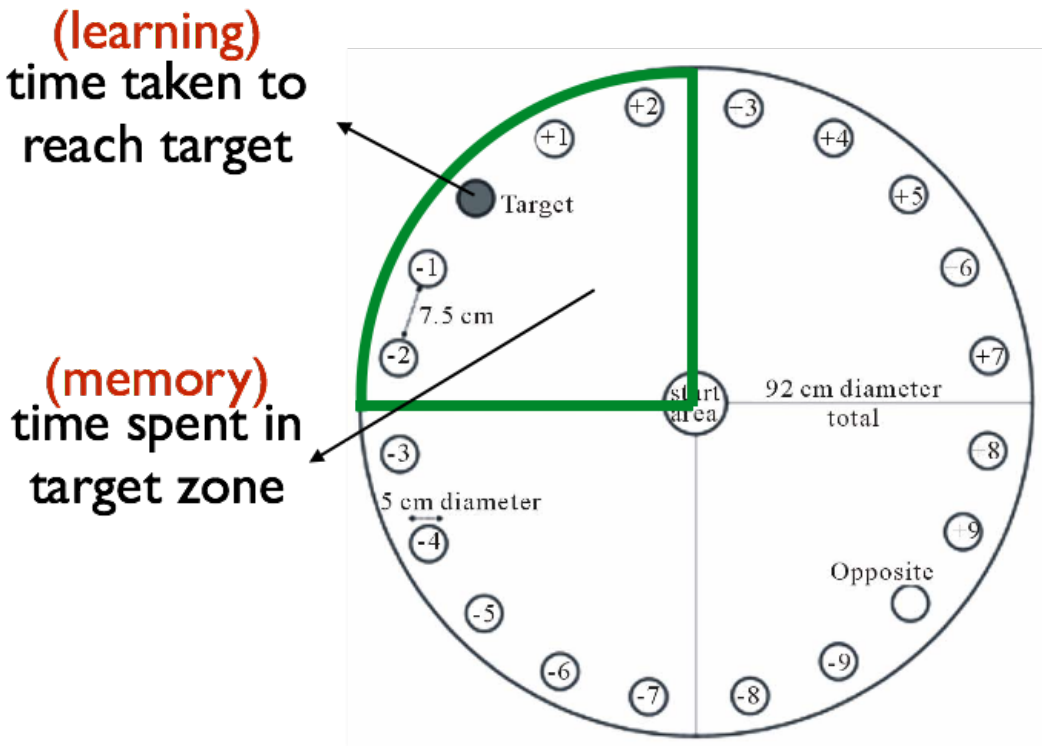


Figure 8: The BM test measures visuospatial learning and memory: The amount of time taken by a mouse to reach the target hole decreases over a four-day learning period. A mouse will take far less time reaching the target hole on the fourth day, compared to the time taken on the first day, indicating that the mouse has 'learnt' the task. Long-term and short-term memories are evaluated by first covering the target hole, and then measuring the amount of time a mouse spends in the target zone on days 5 and 12 respectively.

2.4 Statistical analysis of behavioural and cognitive data

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 16 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) methods were used for each test.

Regarding Barnes Maze analysis, when data were analyzed across days, repeated-measures ANOVA was used with 'treatment' and ‘environment’ as the between-subjects factors and 'day' (Barnes Maze) as within-subjects factor. In order to compare all four groups of mice for each day of the test, post-hoc univariate ANOVA analysis was conducted for each particular testing day. All data were reported as mean ± standard error of the mean (SEM) with a Bonferroni correction being applied for the Barnes Maze probe trials to correct for multiple testing ($p < 0.001$). The primary measure in this test was the latency to reach the target (a measure of escape time) for the four acquisition (learning) days. To evaluate short and long-term recall memory, the number of explorations to each hole when the location of the “escape” hole was blocked was measured. 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. After this transformation, the Box’s M test of homogeneity of covariance matrices and Mauchly’s sphericity test were performed to confirm that the within-group

covariance matrices of the latency data were not significantly different (p values > 0.05) and that a repeated-measures ANOVA could be applied appropriately.

2.5 Gene-expression measures

The hippocampal gene expression in all four groups of mice was assessed using RNA-Seq, micro-array and qPCR measures. Male mice were used exclusively to avoid the confounding factor of estrous cycle gene expression variation in females. The hippocampi from 40 male mice (10 mice in each of the ANE, AE, CNE, CE groups) were obtained. RNA from these hippocampi [twelve mice (3 biological replicates across 4 groups)] was used for RNA-Seq and microarray analysis, and qPCR confirmation (Figures. 9 and 10).

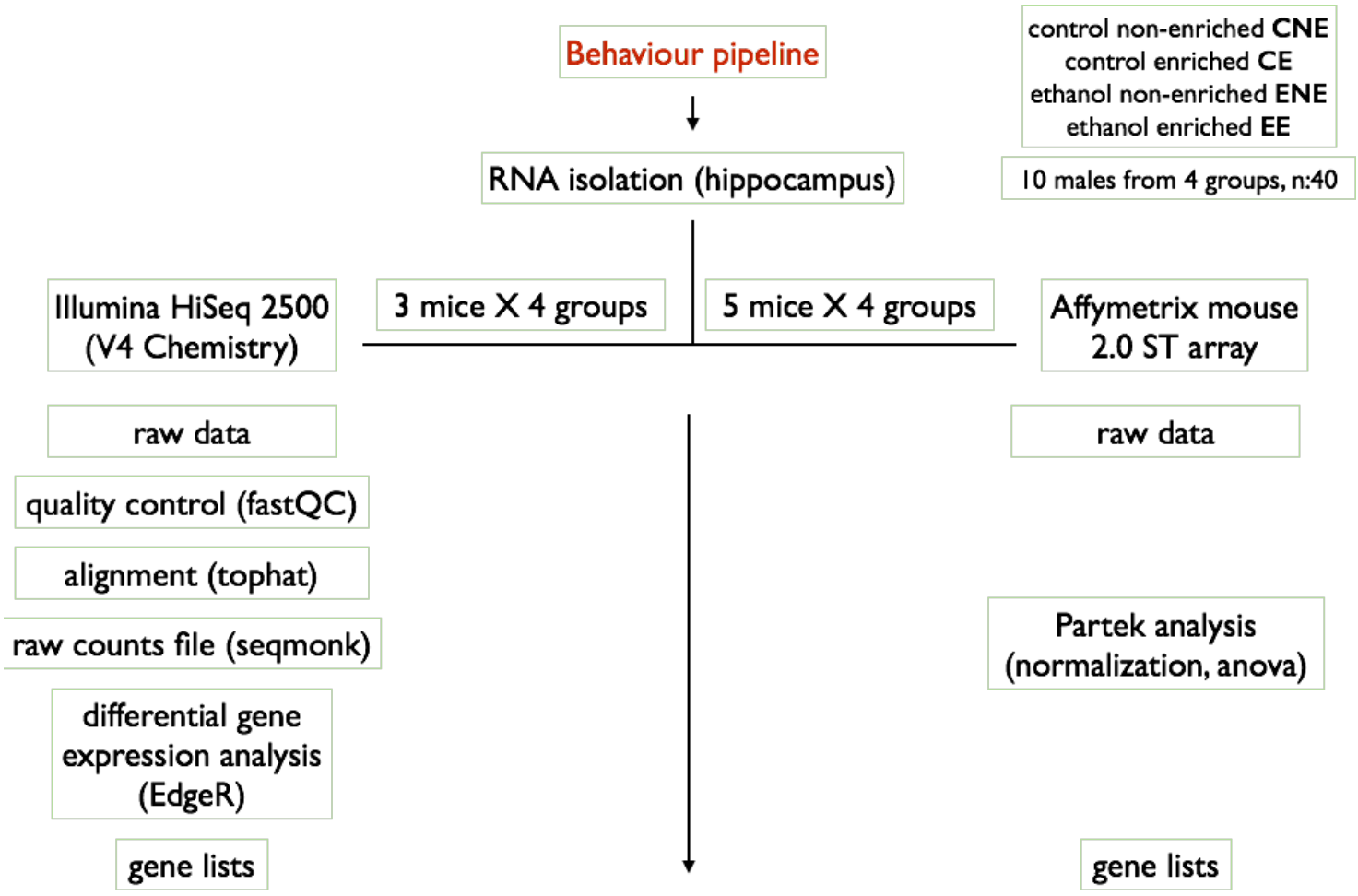


Figure 9: The molecular pipeline (part 1) of this project: RNA was isolated from mouse hippocampi. Microarray sequencing and RNA sequencing was performed on selected RNA samples. With Partek software, microarray data were analyzed and subsequent gene-lists. RNA-Seq data were analyzed using fastQC, Tophat and Seqmonk and the gene-lists were generated using EdgeR.

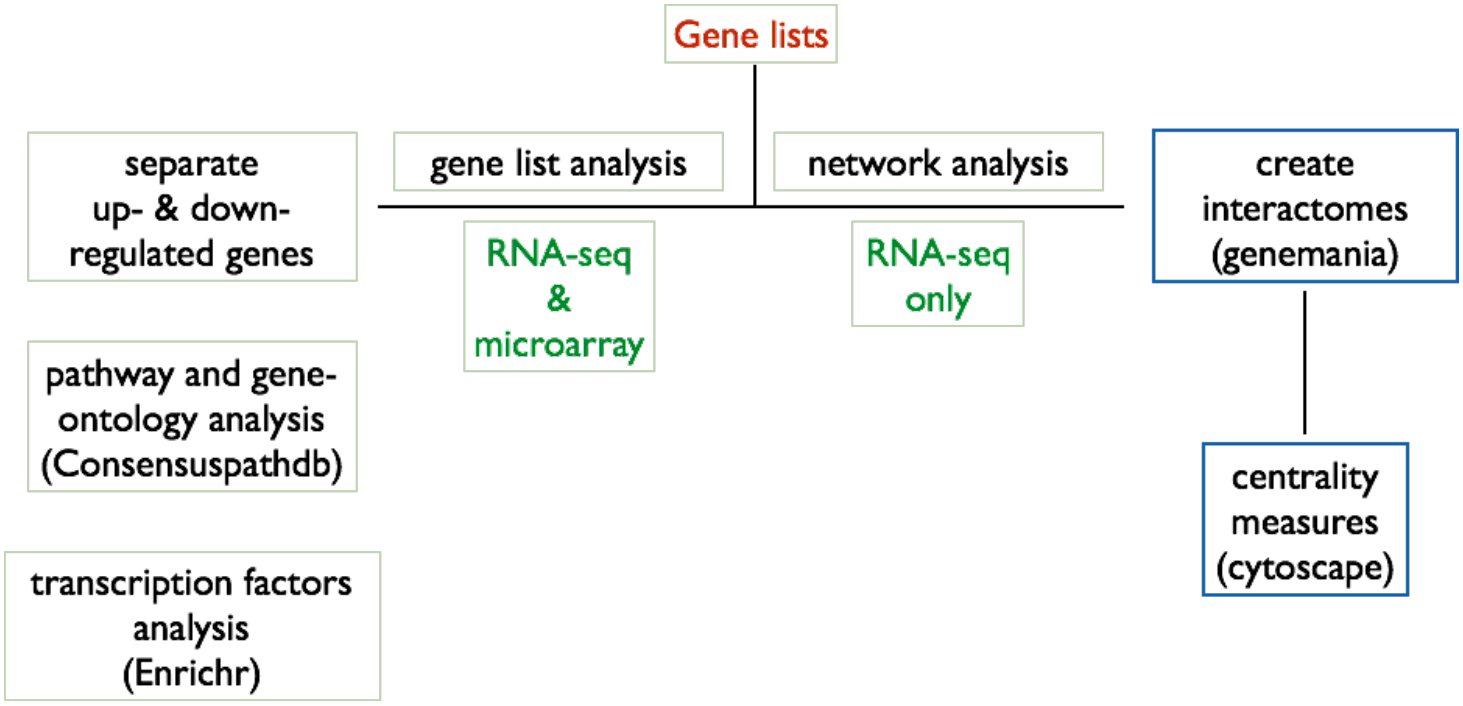


Figure 10: The molecular pipeline (part 2) of this project: All gene-lists were further analyzed in two different ways. Using Consensuspathdb and Enrichr, separate up- and down-regulated gene-lists were analyzed for pathway and gene-ontology analyses as well as transcription factor analysis. Gene-lists were also used to construct interactomes in Genemania and Cytsocape and network centrality measures were analyzed with Network Analyzer in Cytoscape.

2.5.1 Tissue collection and RNA isolation

Male mice at P79 were euthanized using CO_2 asphyxiation followed by cervical dislocation. After extracting the whole brain, the hippocampal tissue was isolated within 2 min of euthanization and snap-frozen in liquid nitrogen and stored at -80° C. Total RNA was isolated using the Qiagen AllPrep DNA/RNA Mini Kit (QIAGEN, Valencia, CA, USA). RNA quality and quantity were 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). All RNA samples that were used for analyses had an optical density $OD_{260/280}$ ratios of 2.0-2.1 and an RNA integrity number (RIN) of 8.0 – 10.0.

2.5.2 Microarray hybridization

Hippocampal RNA samples were checked for the appropriate 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 2.0 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 were converted to cell-based intensity calculations and exported as .CEL files.

2.5.3 Microarray data analysis

Using the Partek Genomics Suite software v.6.6 (Partek Inc., St. Louis, MO, USA), probe-level (.CEL) data were imported and summarized to gene-level data and subjected to quality-control analyses. Using the GeneChip®-Robust Multiarray Averaging (GC-RMA) algorithm, the data were background-corrected, quantile-normalized, summarized and $\log_2$-transformed, all the while taking into account probe sequence (GC content). Gene-level ANOVA p-values and fold changes were determined using the Partek Suite. Only those 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. Un-annotated 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.

2.5.4 RNA-Seq hybridization

Hippocampal RNA samples were sent to the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada). The quality of total RNA samples was checked on an Agilent Bioanalyzer 2100 RNA Nano chip following Agilent Technologies' recommendation. RNA library preparation was performed following the Illumina TruSeq® Stranded total RNA Library Preparation protocol. Briefly, 400 ng of total RNA was used as the input material and rRNA was depleted with RiboZero Gold. The rRNA-depleted RNA was fragmented for 4 minutes at 94°C and converted to double stranded cDNA, end-repaired and adenylated at the 3' to create an overhang A to allow for ligation of TruSeq adapters with an overhang T; library fragments were amplified under the following conditions: initial denaturation at 98°C for 10 seconds, followed by 13 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and finally, an extension step for 5 minutes at 72°C; at the amplification step, each sample was amplified with different barcoded adapters to allow for multiplex sequencing. One ul of the final RNA library was loaded on a Bioanalyzer 2100

DNA High Sensitivity chip (Agilent Technologies) to check for size; RNA libraries were quantified by qPCR using the KAPA Library Quantification Illumina/ABI Prism Kit protocol (KAPA Biosystems). Libraries were pooled in equimolar quantities and paired-end sequenced on an Illumina HiSeq 2500 platform using a High-Throughput Run Mode flowcell and the V4 sequencing chemistry following Illumina's recommended protocol to generate paired-end reads of 126-bases in length.

2.5.5 RNA-Seq data analysis

The RNA-Seq data analysis pipeline consisted of (i) performing quality control of all raw data files, (ii) aligning and annotating the sequencing reads to a reference genome to generate a BAM (Binary alignment map) file, (iii) generating uncorrected raw read counts to estimate transcript abundance and (iv) using EdgeR (Chen et al., 2014a), a statistical software package in the program R to identify differentially-expressed genes amongst all four groups (ANE, AE, CNE, CE). FastQC (Tan, 2013a), a quality control tool for high-throughput sequence data, was used to perform quality control checks on the raw sequence data obtained from The Centre for Applied Genomics. Tophat (Tan, 2013b), a fast splice junction mapper for RNA-Seq reads was used to align the reads to the mouse genome (mm10,Dec. 2011, Genome Reference Consortium GRCm38) using the ultra high-throughput short read aligner Bowtie to eventually generate 12 BAM files for each of the 3 biological replicates in the 4 groups. Following

this, Seqmonk (Andrews, 2012), a RNA-Seq quantitation pipeline program was used to generate uncorrected raw read counts which are the necessary input files for EdgeR which is designed for the analysis of replicated count-based expression data.

2.5.6 Gene set analysis of differentially-expressed genes

To examine the specific roles of genes that were identified to be affected by alcohol exposure and environmental enrichment, the gene-lists generated from EdgeR were analyzed using publicly available bioinformatic tools to assess over-represented Gene Ontology (GO) biological functions, molecular processes, biological pathway associations, and gene interaction networks. Gene ontology biological functions term enrichment was performed using ConsensusPathDB (Kamburov et al., 2013) which is a freely-available database that integrates different types of functional interactions from 30 public resources between genes, RNA, proteins, protein complexes and metabolites in order to assemble a more complete and a less biased picture of cellular biology. ConsensusPathDB analyzes user-specified lists of genes by over-representation analysis, where predefined lists of functionally-associated genes are tested for over-representation in the user-specified list based on the hypergeometric test. Cytoscape was used to perform network analysis of the input gene-lists and the networks were analyzed based on topological parameters like betweenness centrality (BC) and node degree using a Cytoscape plug-in called 'Network Analyzer.' Enrichr (Chen et al., 2013), a

publicly available gene-expression analysis tool was used for promoter analysis to identify relevant and significant transcription factors associated with these genes.

2.5.7 Confirmation of mRNA levels by qPCR

Quantitative reverse-transcription PCR (qRT-PCR) was performed to confirm the expression of select genes from hippocampal tissue identified by the RNA-Seq analyses. Using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), complementary DNA (cDNA) was synthesized from 2 µg of hippocampal RNA. PCR reactions were run using gene-specific TaqMan® Assay Reagents and TaqMan® Gene Expression Assay products in a StepOneTM Real Time PCR System cycler (Applied Biosystems, Foster City, CA, USA). Gene-specific primers 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 alcohol and environment treatment, as identified during gene-ontology analysis and pathway bioinformatic analyses. All reactions were multiplexed with Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) 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 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 CT (Schmittgen and Livak, 2008) method 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).

CHAPTER 3: BEHAVIOURAL RESULTS

The effect of third trimester alcohol exposure and post-natal environmental enrichment on FASD-relevant behaviours

This chapter details the results obtained from studying the effect of alcohol exposure and environmental enrichment on anxiety, learning and memory phenotypes for four groups of mice representing alcohol treated-non-enriched mice (ANE), alcohol treated-enriched mice (AE), control-non-enriched mice (CNE) and control-enriched mice (CE). The results from these analyses are outlined in the five main sections. The first two sub-sections detail the results of two anxiety tests, namely, the Elevated Plus Maze test and the Light-Dark Box test. The next three sections summarize the results of one test of learning (Barnes Maze) and two tests of memory (Novel Object Recognition and Barnes Maze).

3.1 Environmental enrichment improves PAE-related anxiety deficits

3.1.1 Anxiety measures in an Elevated Plus Maze assay

The results presented in Figure. 11 show that the post-natal environmental enrichment has a large effect on the anxiety measures as assessed in the Elevated Plus Maze. Mice, which were prenatally exposed to ethanol and subsequently underwent environment enrichment, spent a significantly longer time (~40 seconds) exploring the open arms compared to ethanol-exposed mice with no environmental enrichment (~10 seconds) ($p < 0.001$). The effect of environmental

enrichment on control mice (no alcohol) was even greater. For instance, control mice exposed to environmental enrichment spent six times as much time in the open arms (~60 seconds) compared to control non-enriched mice (~10 seconds) ($p < 0.001$). However, prenatal alcohol exposure used in this experiment did not affect the anxiety traits of non-enriched mice (as measured by this assay). There was no difference in the time spent in open-arms between alcohol-exposed mice and control mice. Overall, both groups of enriched mice (control and ethanol) spent more time in the open arms compared to non-enriched mice. The results argue that post-natal environmental enrichment lowers anxiety levels in mice, as measured by their performance in the Elevated Plus Maze assay.

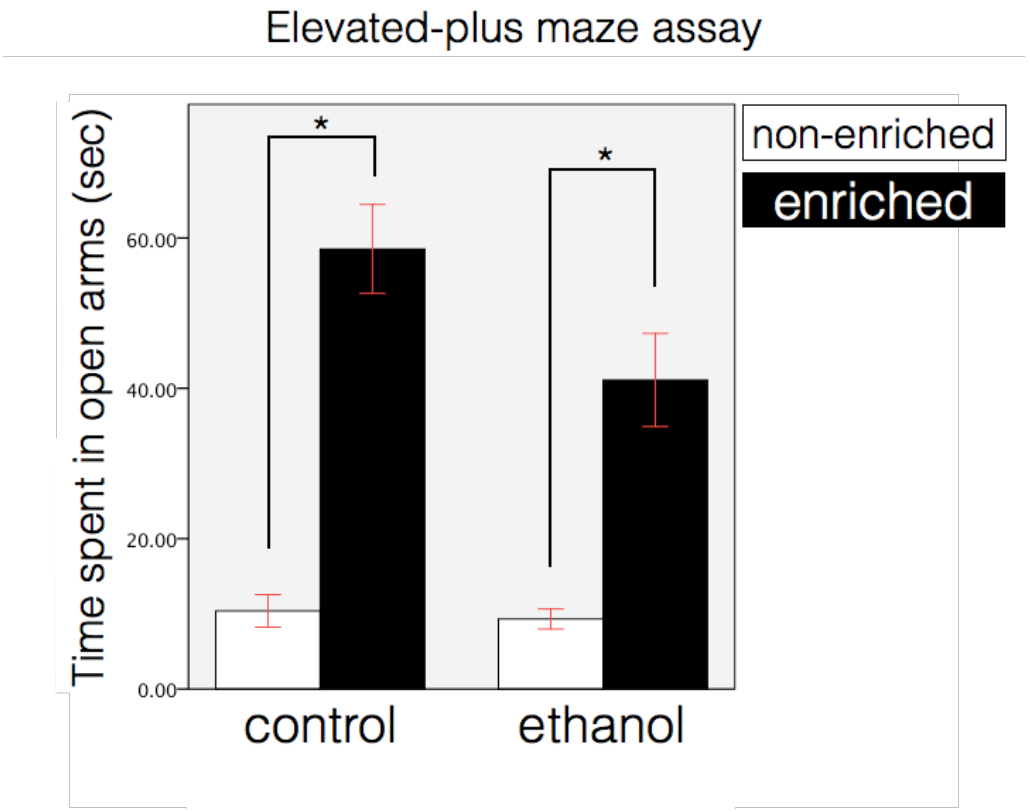


Figure 11: Elevated plus maze assay of anxiety-related phenotypes: Four groups of mice (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched) were tested over a 5 min period for time spent in open arms. Data presented represent mean ± SEM (n=10 mice per group). There was a significant main effect of enrichment ($F_{(3,76)} = 208.24$, $p < 0.0001$). Alcohol-enriched mice spent relatively more time in the open arms compared to alcohol non-enriched mice ($F_{(1,39)}=16.53$, $p < 0.001$), while control enriched mice spent relatively more time in the open arms compared to control-enriched mice ($F_{(1,39)}=16.53$, $p < 0.001$). (*, $p < 0.001$)

3.1.2 Anxiety measures in a Light-Dark Box assay

The results presented in Figure. 12 show that, similar to the results from the elevated plus maze assay, the post-natal environmental enrichment has a large effect in the anxiety measures as assessed in the Light-Dark Box. Mice prenatally exposed to ethanol and subsequently exposed to environmental enrichment, spent a significantly longer time (~160 seconds) exploring the light-region compared to ethanol-exposed mice with no environmental enrichment (~140 seconds) ($p < 0.001$). The effect of environmental enrichment on healthy mice was even greater. Control mice exposed to environmental enrichment spent 1.5 times as much time in the light-region (~200 seconds) compared to control, non-enriched mice (~130 seconds) ($p < 0.001$). As measured by this assay, prenatal alcohol exposure affected the anxiety traits of mice. Alcohol-exposed mice spent far less time in the light region of the box, as compared to control mice ($p < 0.05$). Overall, both groups of enriched mice (control and ethanol) spent more time in the light region compared to non-enriched mice (control and ethanol). Thus, post-natal environmental enrichment lowers anxiety levels in mice, as measured by their performance in the Light-Dark Box assay.

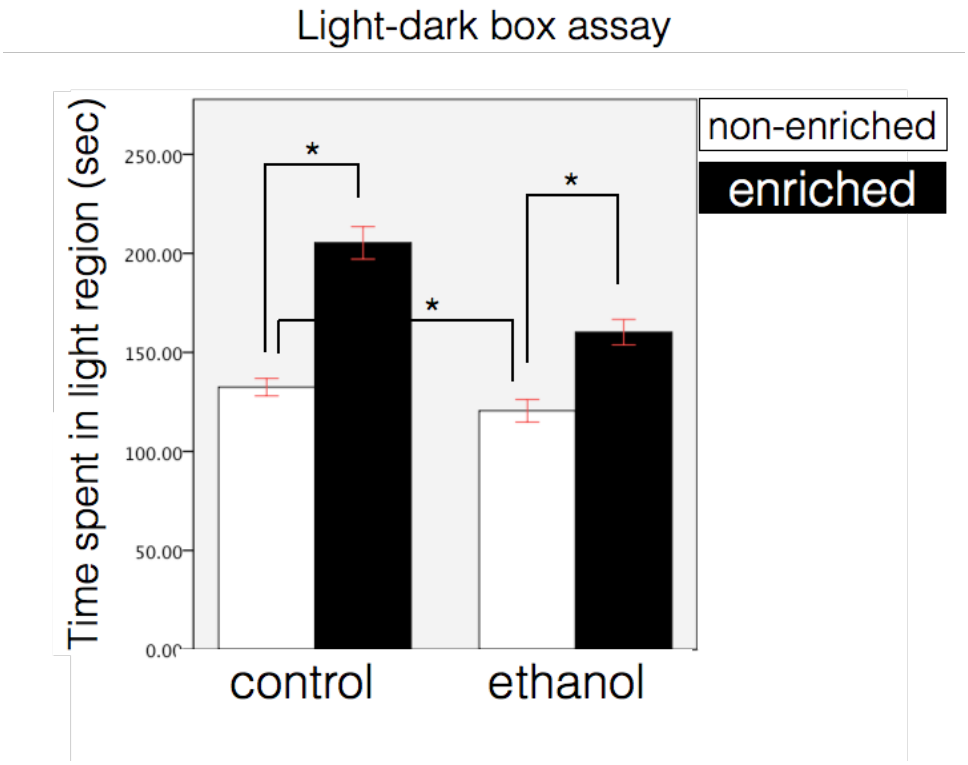


Figure 12: Light dark box assay of anxiety-related phenotypes: Four groups of mice (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched) were tested over a 5 min period for time spent in the lit region of the box. Data presented represent mean ± SEM (n=10 mice per group). There was a significant interaction between treatment and environment ($F_{(3,76)}$ = 27.43, p <0.001) with main effects of both alcohol ($F_{(3,76)}$ = 36.36, p <0.0001) and environment ($F_{(3,76)}$ = 158.07, p <0.0001). Alcohol enriched mice spent relatively more time in the lit region compared to alcohol non-enriched mice ($F_{(1,39)}$=16.53, p <0.001), while control enriched mice spent relatively more time in the light region compared to control enriched mice ($F_{(1,39)}$=16.53, p <0.001). (*, p <0.001)

3.2 Environmental enrichment improves FASD-related memory and learning deficits

3.2.1 Memory performance in Novel Object Recognition

The results presented in Figure. 13 show that post-natal enrichment has a large effect on recognition memory performance as assessed in the Novel Object Recognition assay. Mice prenatally exposed to ethanol and subsequently underwent environment enrichment, spent a significantly longer time (~60 seconds) exploring the novel object compared to ethanol-exposed mice with no environmental enrichment (~20 seconds) ($p < 0.001$). The effect of environmental enrichment on healthy mice was even greater. For instance, control mice exposed to environmental enrichment spent 3.5 times as much time exploring the novel object (~70 seconds) compared to control, non-enriched mice (~20 seconds) ($p < 0.001$). However, prenatal alcohol exposure did not affect the novel recognition memory performance of mice (as measured by this assay). There was no difference in the time spent in exploring the novel object, between alcohol-exposed mice and control mice. Overall, both groups of enriched mice (control and ethanol) spent more time exploring the novel object compared to non-enriched mice (control and ethanol). Thus, post-natal environmental enrichment increases recognition memory performance in mice, as measured by their performance in the Novel Object Recognition assay.

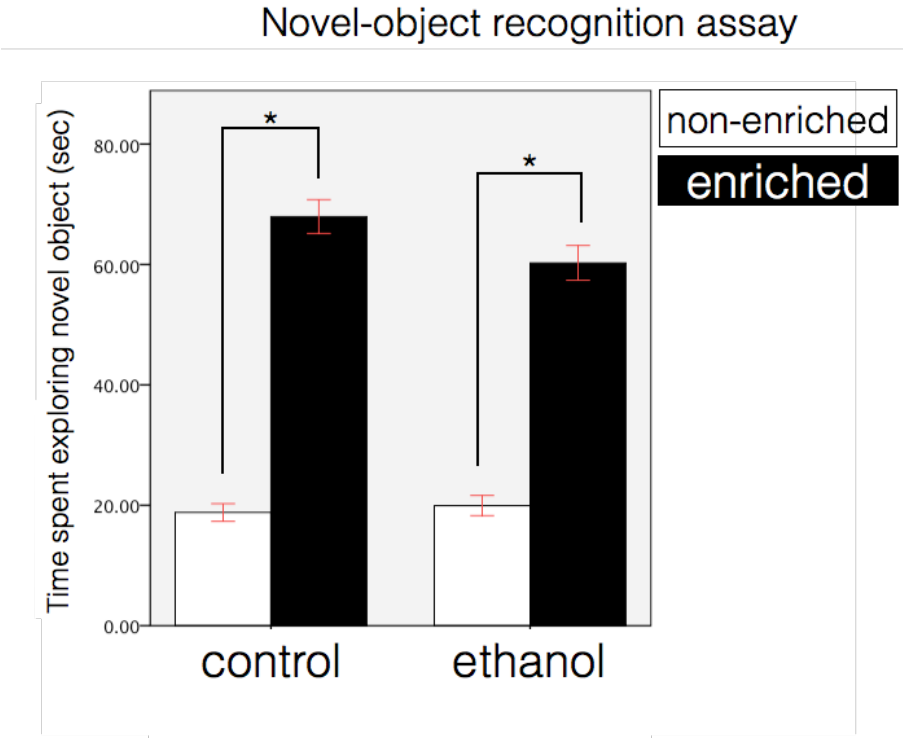


Figure 13: Novel object recognition assay of anxiety-related phenotypes. Four groups of mice (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched) were tested over a 2 min period for time spent exploring a novel object. Data presented represent mean ± SEM (n=10 mice per group). There was a significant main effect of enrichment ($F_{(1,39)}$= 98.38, p <0.001). Alcohol enriched mice spent relatively more time exploring the novel object compared to alcohol non-enriched mice ($F_{(1,39)}$=14.58, p <0.001), while control enriched mice spent relatively more time exploring the novel object compared to control enriched mice ($F_{(1,39)}$=14.58, p <0.001). (*, p <0.001)

3.2.2 Spatial learning performance in the Barnes Maze

All four groups of mice (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched) were assessed for spatial learning deficits in a mouse-oriented spatial learning task, the Barnes maze. The primary measure in this test was the latency to reach the target (a measure of escape time) for the four acquisition (learning) days.

The mean escape latencies of these mice across four acquisition trial days are shown in Figure. 14. Analysis indicated a significant effect for day of testing ($p < 0.0001$), treatment ($p < 0.0001$), environment ($p < 0.0001$) along with the interaction amongst all three ($F_{3,108} = 157.3$, $p < 0.0001$).

As shown by the Barnes Maze test, prenatal alcohol exposure reduces spatial learning performance in mice. Alcohol-exposed mice took a longer time (increased latency times) to reach the target hole, when compared to control mice. Escape latencies for the alcohol non-enriched groups ranged from ~180 seconds on the first learning day to ~ 30 seconds on the last training day (day 4), whereas escape latencies for the control non-enriched groups ranged from ~120 seconds on the first learning day to ~ 12 seconds on the last training day (day 4). However, environmental enrichment can rescue, to a certain extent, the spatial learning performance of alcohol exposed mice. Alcohol-enriched mice were faster in finding the target hole (reduced latency times) when compared to alcohol non-

enriched mice. Compared to the escape latencies of the alcohol non-enriched mice from day 1 to day 4, the escape latencies for the alcohol enriched groups ranged from ~148 seconds on the first learning day to ~ 20 seconds on the last training day (day 4). It is also important to note the potential of environmental enrichment to improve spatial learning performance of healthy mice as evidenced by the lower latency times of control enriched mice (from ~80 seconds on the first learning day to ~ 6 seconds on the last training day (day 4).

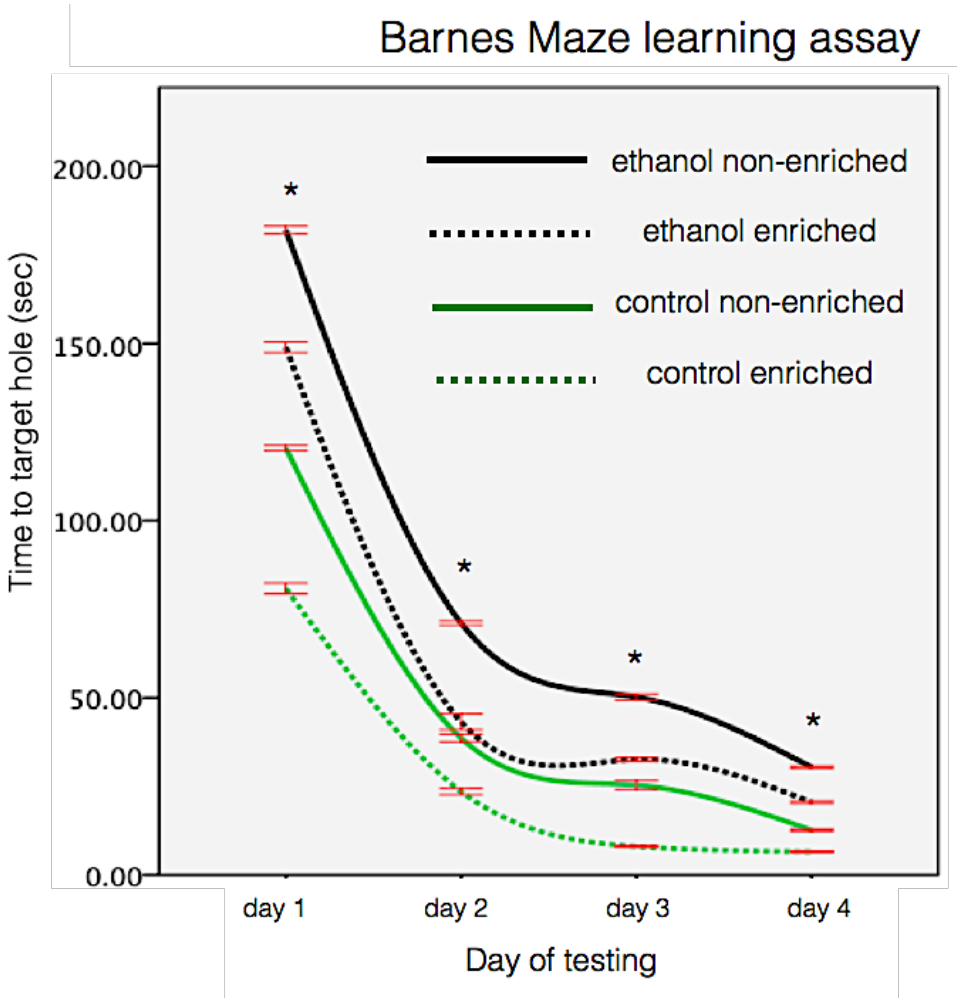


Figure 14: Latency to escape in the Barnes maze task for spatial learning: The mean (±SEM) time (seconds) to reach the target for four groups of mice (n = 10 mice per group) (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched) are displayed. The latency times represent the average of four trials per day of each animal across four consecutive acquisition (learning) days. (*, $p < 0.0001$)

3.2.3 Spatial memory performance in the Barnes Maze: Short-term recall

After assessing the learning ability of all four groups of mice during the acquisition learning phase of the Barnes maze assay, each mouse was tested for its 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).

The results of the short-term recall assay indicated the detrimental effects of third trimester alcohol exposure on short-term recall performance (Figure. 15). Alcohol non-enriched mice spent significantly less time exploring the target and surrounding holes (~ 2 seconds) compared to control non-enriched mice (~ 8 seconds) ($F_{1,76} = 4.2$, $p < 0.001$). However, the ameliorative effects of environmental enrichment on the spatial learning deficits due to prenatal alcohol exposure were visible given the increased time spent by alcohol-enriched mice exploring the target and surrounding holes (~5-6 seconds) ($F_{1,76} = 4.2$, $p < 0.001$). The results confirmed previous research; environmental enrichment improves the short-term recall performance as assayed by the Barnes Maze. Control-enriched mice spent (~ 12 seconds) exploring the target and surrounding holes ($p < 0.01$) when compared to control non-enriched mice (~ 8 seconds).

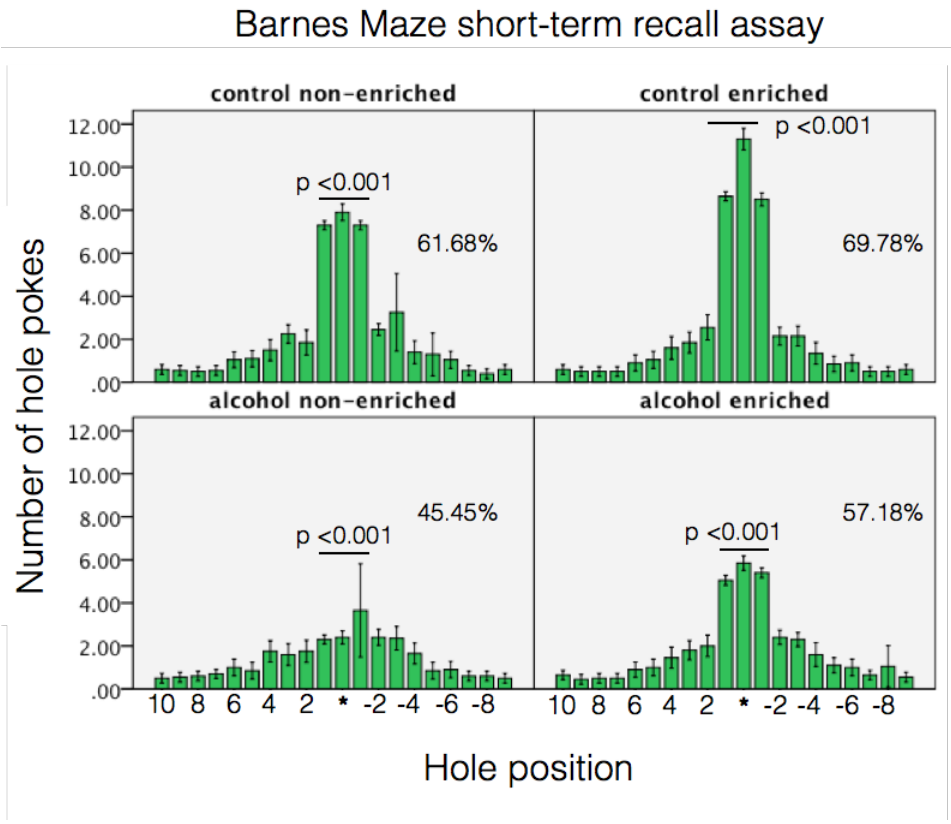


Figure 15: Number of explorations to each Barnes Maze hole during short-term recall memory trials: The mean number of explorations (±SEM) to each hole located around the periphery of the Barnes Maze is indicated for four groups of mice (n = 10 mice per group) (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched). Data represent short-term recall memory, tested on day 5 following four days of learning (acquisition) trials. (* represents p <0.001 in a two-way ANOVA). The number of times each group of mice poked the holes of the target quadrant compared to the holes of all remaining quadrants is denoted as a percentage (target quadrant pokes/all quadrant pokes).

3.2.4 Spatial memory performance in the Barnes Maze: Long-term recall

To assess the effect of 3rd trimester alcohol exposure and environmental enrichment in mice on long-term recall performance, I also assessed the number of explorations to each hole during a one minute testing period at testing day 12 (long-term recall memory) (Figure. 16).

The results of the long-term recall assay revealed the detrimental effects of third trimester alcohol exposure on long-term recall performance. Alcohol non-enriched mice spent significantly less time exploring the target and surrounding holes (~ 2 seconds) compared to control non-enriched mice (~ 4 seconds) ($F_{1,76}$ = 4.2, $p < 0.01$). The ameliorative effects of environmental enrichment on spatial learning deficits due to prenatal alcohol exposure were visible given the increased time spent by alcohol-enriched mice exploring the target and surrounding holes (~4 seconds) ($F_{1,76}$ = 4.2, $p < 0.001$). Interestingly, this further shows the ability for environmental enrichment to ameliorate long-term recall performance to such a degree that it 'catches up' to the performance of a normal, healthy non-enriched mouse (if the enrichment continues, there will be no significant difference between the alcohol-exposed enriched mice and the control non-enriched mice). As confirmed by previous research, environmental enrichment improves the long-term recall performance as assayed by the Barnes Maze. Control-enriched mice spent (~ 8 seconds) exploring the target and surrounding holes ($p < 0.01$) when compared to control non-enriched mice (~ 4 seconds).

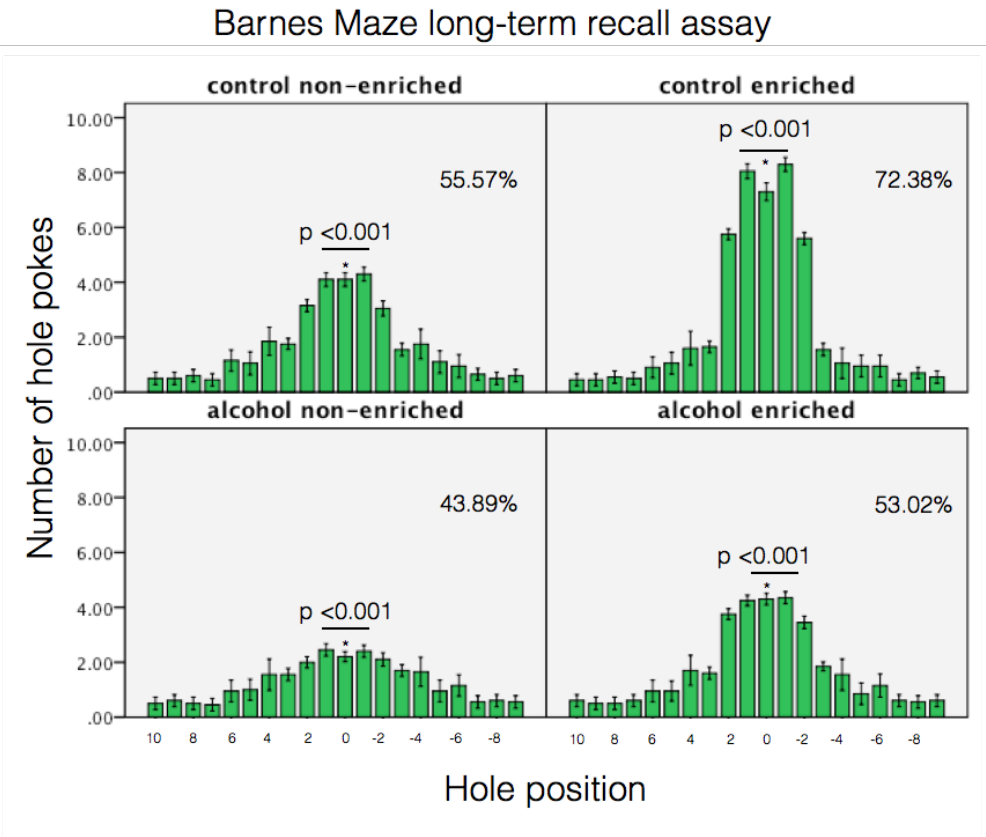


Figure 16: Number of explorations to each Barnes Maze hole during long-term recall memory trials: The mean number of explorations (±SEM) to each hole located around the periphery of the Barnes maze is indicated for four groups of mice (n = 10 mice per group) (Alcohol Non-Enriched, Alcohol Enriched, Control Non-Enriched, Control Enriched). Data represent short-term recall memory, tested on day 12 following four days of learning (acquisition) trials (* represents p <0.001 in a two-way ANOVA). The number of times each group of mice poked the holes of the target quadrant compared to the holes of all remaining quadrants is denoted as a percentage (target quadrant pokes/all quadrant pokes).

3.3 Summary of observations

The behavioural and cognitive results point to three important observations (Tables 1 and 2):

1. Third trimester alcohol exposure during mouse neurodevelopment results in anxiety (as found by the Light-Dark Box), learning and memory deficits. This is evidenced by the alcohol-exposed mouse's relative preference for staying the dark region of the light-dark box when compared to a control mouse. Cognitive deficits in the novel object memory test are evident because alcohol-exposed mice tend to spend equal times exploring old and novel objects when compared to control-mice, who explore the novel object for a longer time. In the Barnes Maze, compared to control mice, alcohol-exposed mice take a longer time to reach the target hole, indicating impaired learning capabilities and they also spend less time in the target-hole quadrant five and twelve days after the original test, when compared to control mice, indicating poor short-term and long-term memory.

2. Post-natal environmental enrichment, a combination of physical exercise, play, social, mental and cognitive stimulation ameliorates anxiety, learning and memory deficits in alcohol-exposed mice. This environmental paradigm also lowers anxiety and improves learning and memory performance in

control-mice. Behavioural improvements are evidenced by the enriched alcohol-exposed mouse's relative preference for staying within the open arms of the elevated plus-maze and light region of the light-dark box when compared to a non-treated alcohol-exposed mouse. Cognitive improvements in the novel object memory test are evident because enriched alcohol-exposed mice tend to spend more time exploring novel objects when compared to non-treated alcohol-exposed mice, who explore the novel object and the old object for an equal time. In the Barnes Maze, compared to non-treated alcohol-exposed mice, enriched alcohol-exposed mice reach the target hole faster, indicating an improvement in their learning capabilities. Enriched alcohol-exposed mice spend more time in the target-hole quadrant five and twelve days after the original test, when compared to non-treated alcohol-exposed mice indicating an improvement in short-term and long-term memory.

3. Most importantly, while environmental enrichment does ameliorate behavioural and cognitive deficits of alcohol-exposed mice, these mice are not able to fully recover to normal, baseline levels of visuospatial cognitive performance of non-enriched control mice. This indicates that alcohol exposure during synaptogenesis of mouse neurodevelopment results in permanent brain damage leading to cognitive deficits.

GROUP	FINDING	INTERPRETATION
	Anxiety: Elevated Plus Maze	Anxiety: Elevated Plus Maze
AN v CN	same time spent in open arms	prenatal alcohol exposure does not affect anxiety
CE v CN	more time in open arms	anxiety reduced by environmental enrichment
AE v AN	more time in open arms	anxiety reduced by environmental enrichment
	Anxiety: Light Dark Box	Anxiety: Light Dark Box
AN v CN	less time in light region	prenatal alcohol exposure increases anxiety
CE v CN	more time in light region	anxiety reduced by environmental enrichment
AE v AN	more time in light region	anxiety reduced by environmental enrichment

Table 1: Tabulated summary of all behavioural results: This table summarizes all the anxiety tests conducted on all four groups of mice. The (AN v CN) group compares the effect of prenatal alcohol exposure (AN) compared to control mice (CN). The (CE v CN) group compares control environmentally enriched mice (CE) to control non-enriched mice (CN) to delineate the effects of environmental enrichment on healthy mice. Lastly, the (AE v AN) group tells us the effect of post-natal environmental enrichment by comparing alcohol-exposed environmentally enriched mice (AE) to alcohol-exposed non-enriched mice (AN).

GROUP	FINDING	INTERPRETATION
	Recognition memory: Novel Object Recognition	*Recognition memory: Novel Object Recognition*
AN v CN	similar time spent with novel object	prenatal alcohol does not affect recognition memory
CE v CN	more time spent with novel object	recognition memory improved by environmental enrichment
AE v AN	more time spent with novel object	recognition memory deficit ameliorated by environmental enrichment
	Spatial learning: Barnes Maze	*Spatial learning: Barnes Maze*
AN	longest time out of all four groups to find target hole	prenatal alcohol decreases spatial learning
AE	less time than AN to find target hole	spatial learning performance deficit ameliorated by environmental enrichment
CN	less time than AN and AE to find target hole	prenatal alcohol decreases spatial learning compared to healthy mice
CE	shortest time out of all four groups to find target hole	spatial learning improved by environmental enrichment
	Spatial memory: Barnes Maze	*Spatial memory: Barnes Maze*
AN	shortest time out of all four groups in the target zone	prenatal alcohol decreases spatial memory
AE	shorter time in the target zone compared to AN	spatial memory deficit ameliorated by environmental enrichment
CN	longer time in the target zone compared to AN and AE	prenatal alcohol decreases spatial memory compared to healthy mice
CE	longest time out of all four groups in target zone	spatial memory improved by environmental enrichment

Table 2: Tabulated summary of all learning and memory results: This table summarizes all the learning and memory tests conducted on all four groups of mice. The (AN v CN) group compares the effect of prenatal alcohol exposure (AN) compared to control mice (CN). The (CE v CN) group compares control environmentally enriched mice (CE) to control non-enriched mice (CN) to

delineate the effects of environmental enrichment on healthy mice. Lastly, the (AE v AN) group tells us the effect of post-natal environmental enrichment by comparing alcohol-exposed environmentally enriched mice (AE) to alcohol-exposed non-enriched mice (AN).

CHAPTER 4: GENE EXPRESSION RESULTS

Gene expression changes following third trimester alcohol exposure and post-natal environmental enrichment

The results of this chapter concern detailed analysis of three lists of differentially-expressed genes, namely:

(i) Alcohol non-enriched (AN) versus control non-enriched (CN),

(ii) Control enriched (CE) versus control non-enriched (CN) mice and,

(iii) Alcohol enriched (AE) versus alcohol non-enriched (AN) mice

The chapter is broken into three main sections of gene-expression results. The first two sections outline the analyses by two different platforms, namely, (i) microarrays and (ii) RNA-Sequencing. The last section compares gene-expression results amongst three platforms and two methods of analysis, namely, microarray analysis done by Partek Genomics Suite, and RNA-Sequencing analysis done by edgeR and also the Partek Genomics Suite.

In each section, for the AN v CN group, I specifically examined gene expression in the mouse hippocampus at post-natal day 85 as a result of ethanol exposure during the peak of synaptogenesis on post-natal days 6 and 7. For the CE v CN group, I examined the gene-expression changes in the adult mouse hippocampus (post-natal day 85) as a result of environmental enrichment for 1.5 months with no exposure to alcohol during any time of its neurodevelopment.

Finally, for the AE v AN comparison, I examined the gene-expression changes in the adult mouse hippocampus (post-natal day 85) after it (i) had been exposed to ethanol on post-natal days 6 and 7 and then underwent environmental enrichment for 1.5 months.

Organization of Chapter Four: Gene Expression Results

Comparison		Type of Analysis	Microarray Method (Section 4.1)		RNA-Seq Method (Section 4.2)	
			Section no.	**Page no.**	**Section no.**	**Page no.**
1.	**Alcohol non-enriched v. Control non-enriched (effect of prenatal alcohol exposure)**	Gene Expression	4.1.1.1	70	4.2.1.1	100
		Gene Ontology	4.1.1.2	70	4.2.1.2	100
		Pathway Analysis	4.1.1.3	73	4.2.1.3	103
		Gene Network	4.1.1.4	76	4.2.1.4	107
		Transcription factor Analysis	4.1.1.5	77	4.2.1.5	109
2.	**Control enriched v. Control non-enriched (effect of post-natal environmental enrichment)**	Gene Expression	4.1.2.1	80	4.2.2.1	112
		Gene Ontology	4.1.2.2	80	4.2.2.2	112
		Pathway Analysis	4.1.2.3	83	4.2.2.3	114
		Gene Network	4.1.2.4	87	4.2.2.4	118
		Transcription factor Analysis	4.1.2.5	88	4.2.2.5	120
3.	**Alcohol enriched v. Alcohol non-enriched (effect of post-natal environmental enrichment and prenatal alcohol exposure)**	Gene Expression	4.1.3.1	91	4.2.3.1	123
		Gene Ontology	4.1.3.2	91	4.2.3.2	123
		Pathway Analysis	4.1.3.3	94	4.2.3.3	126
		Gene Network	4.1.3.4	96	4.2.3.4	130
		Transcription factor Analysis	4.1.3.5	97	4.2.3.5	132
Section 4.2.3.7: qPCR confirmation for genes from RNA-Seq analysis: Pages 135 Section 4.3: RNA-Seq & Microarray analysis methods comparison (Partek v edgeR v Microarray): Pages 137						

4.1 Gene-expression changes as uncovered by the microarray platform

4.1.1 The effect of prenatal alcohol exposure (AN v. CN)

4.1.1.1 Gene expression

For the alcohol non-enriched (AN) versus control non-enriched (CN) group, at a FDR corrected p-value cut-off of 0.05, 685 transcripts were significantly altered (393 up-regulated and 292 down-regulated) by alcohol treatment when compared to control mice. Changes to gene-expression were relatively subtle in both directions with 98% of genes being up- or down-regulated within the (-1.2 to +1.2) fold-change range. The top up-regulated gene was *Vgll3* (Vestigial like family member 3) (fold change: 2.46), which acts as specific co-activator for mammalian transcription enhancer factors and the top down-regulated gene was *mt-Tq* (Mitochondrially Encoded TRNA Glutamine) (fold change: -2.23), which is involved in tRNA aminoacylation.

4.1.1.2 Functional annotation

The total gene list (both up- and down-regulated transcripts) of the AN v CN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of biological processes related to nervous system development, cell adhesion, motility, growth, response to external stimulus. Molecular processes pertaining to extracellular matrix binding, response to external stimulus and myelin sheath structure were also uncovered

(Table 3). Most, if not all these annotations comprised a number of altered transcripts in the Collagen gene family (*Col4a5, Col1a1 and Col3a1*) and the Claudin gene family (*Cldn1, Cldn11*) all of which play structural roles in cellular re-arrangement and cell-to-cell interactions via signaling receptors.

Table 3: Gene ontology (GO) of up- and down-regulated genes in the hippocampus following prenatal ethanol exposure (AN v. CN)

GO name	**p-value**	**Up-regulated genes**
Nervous system development	6.43E-08	*Ugt8a; Cldn11; Rab26; Ift172; Dlk1; Col1a1*
Intrinsic component of membrane	7.29E-08	*Enpp6; Cyp51; Adra2a; Tspan9; Rab26; Col4a5*
Cell adhesion	6.25E-07	*Sdk2; Calr; Nfasc; Igfbp7; Col1a1; Cadm4*
Cell migration	1.65E-05	*Dpysl3; Calr; Paxip1; Gpc6; Col1a1; Plat*
Neurogenesis	0.0011	*Sox11; Grin3a; Nnat; Lamc1*
GO name	**p-value**	**Down-regulated genes**
Structural constituent of ribosome	9.94E-06	*Rps13; Col4a5; Rps15; Rps12; Gm6139*
Biosynthetic process	0.0080	*Bhlhe41; Col3a1; Rps12*
Gene expression	0.0413	*Lsm2; Zfp119b; Prdm5; Rpl21-ps4; Chrd*
Structural constituent of ribosome	9.94E-06	*Rps13; Col4a5; Rps15; Rps12; Gm6139*

4.1.1.3.Signaling pathways

Differentially expressed genes between alcohol non-enriched versus control non-enriched adults were also analyzed using ConsensusPathDB to uncover significantly up- and down-regulated pathways (Table 4). Pathways containing up-regulated genes such as axon guidance (Figure. 17) and extracellular matrix organization and L1CAM interactions were significantly altered in the mouse hippocampus in response to alcohol exposure during synaptogenesis. The top pathways containing significantly down-regulated genes pertained to meiotic synapsis, mRNA metabolism and RNA polymerase I promoter opening. Interestingly, a family of histone genes such as *Hist4h4, Hist1h3a* and *Hist1h3f,* were all found to be significantly involved in all of these pathways.

Table 4: Significantly altered pathways (consisting of up- and down-regulated genes) associated with prenatal alcohol exposure during synaptogenesis (AN v. CN)

Pathway name	**p-value**	**Up-regulated genes**
Axon guidance	0.0075	*Sema3c; Efnb1; Sema3d; Plxna3; Dpysl5*
Extracellular matrix organization	0.0198	*Dcn; Mmp15; Plod1; Ncan; Casp3; Adamts4*
L1CAM interactions	0.0198	*Ncan; Cd24a; Itga1; Nfasc; Dcx; L1cam*
Pathway name	**p-value**	**Down-regulated genes**
Metabolism of mRNA	0.0002	*Rps13; Rps12; Gm5481; Rps15; Lsm2; Exosc8; Hist1h3a; Hist4h4*
Meiotic Synapsis	0.0086	*Hist4h4; Hist1h3a; Hist1h3f; Hist1h2b*

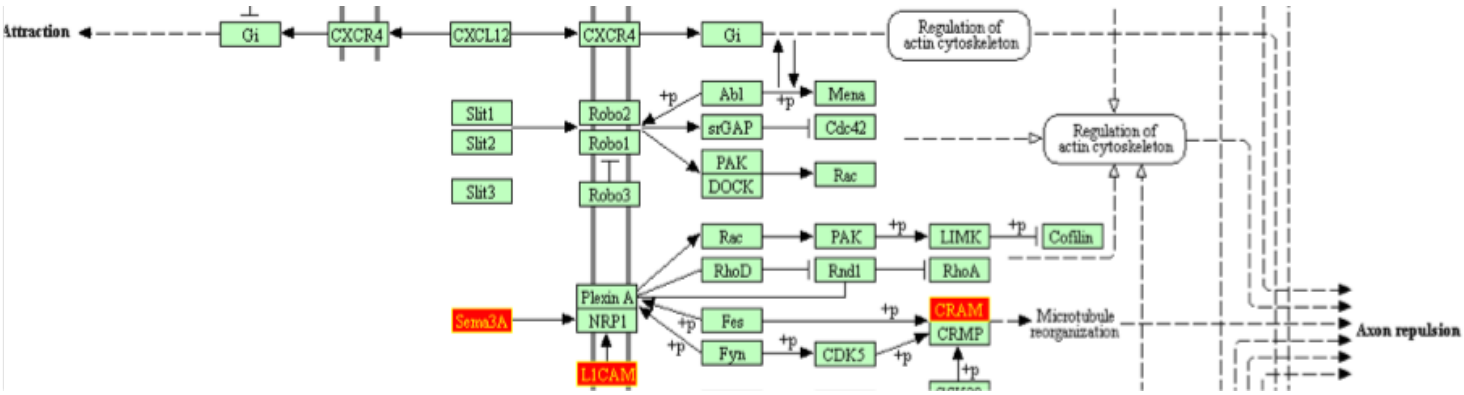


Figure 17: Axon guidance pathway (source: KEGG), one of the significantly altered pathways pathways (with up-regulated genes) in the adult hippocampus, in response to prenatal alcohol exposure. The genes in red represent the up-regulated, differentially-expressed genes.

4.1.1.4. Gene networks

Differentially-expressed genes in the adult mouse hippocampus (exposed to alcohol on post-natal days 6 and 7) were analyzed in a gene-network analysis tool, Genemania, to predict interacting molecular networks. Based on input genes, GeneMANIA constructed networks that include associated data from protein and genetic interactions, pathways, co-expression, co-localization and protein domain similarity. Nodes represent genes and edges connecting these nodes represent interactions genes. Nodes with large degree values (higher number of connections) represent the key genes. The AN v CN network was made up of 354 nodes with 3369 edges. The Endothelial Differentiation-Related Factor 1 (*Edf1*), a protein-coding gene involved in endothelial cell differentiation and lipid metabolism was the main hub gene in this network with a degree score of 64. Based on GENEMANIA's output, the top predicted pathways identified were involved in focal adhesion, extracellular matrix receptor interaction (with the major involvement of the collagen gene family) and ribosome structure.

4.1.1.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in alcohol non-enriched (AN) versus control non-enriched (CN) group (Table 5). The top transcription factor for this comparison group was BCLAF1 (BCL2 Associated Transcription Factor 1), which is involved in the regulation of apoptosis via histone modifications. The next two were BCL11B and GTF2B, which are involved in immune response processes and mRNA regulation, respectively.

Table 5: Top three transcription factors of all significantly altered genes associated with prenatal alcohol exposure during synaptogenesis (AN v. CN)

Transcription factor	Name of factor	Possible functions	p-value
BCLAF1	BCL2 Associated Transcription Factor 1	regulation of apoptosis	0.0023
BCL11B	B-Cell CLL/Lymphoma 11B	transcriptional repressor, immune response	0.0030
GTF2B	General Transcription Factor IIB	mRNA regulation	0.0034

4.1.1.6 Summary of results: the effect of prenatal alcohol on hippocampal gene expression

The effect of prenatal alcohol exposure has a subtle effect on a large number of genes (as analyzed by the microarray platform) in the adult hippocampus. The most up-regulated and down-regulated genes exhibit a fold-change of approximately 2.2 in either direction. Prenatal exposure affects a wide range of biological processes ranging from cell adhesion and cell migration (up-regulated genes) all the way to the structural regulation of the ribosome and gene-expression for the down-regulated genes. A majority of genes altered within these biological processes fall with the Collagen gene family, which is critical in cellular structure and development. Interestingly, the above results are also reflected in the effects of prenatal alcohol exposure on biological pathways in the hippocampus. The most highly altered pathways (with up-regulated genes) involve structural guidance of axons while the top down-regulated pathway is related to mRNA metabolism. Through gene network analysis, it is also clear that prenatal alcohol exposure majorly affects biological processes involved in extracellular matrix organization, focal adhesion and ribosome structure.

A possible interpretation of these observations may be that prenatal alcohol exposure leads to a massive alteration of biological processes related to the structural integrity and development of critical regions in the hippocampus. This may partially explain the behavioural deficits observed in alcohol-exposed mice.

4.1.2 The effect of environmental enrichment (CE v. CN)

4.1.2.1 Gene expression

For the control enriched (CE) versus control non-enriched (CN) group, at a FDR corrected p-value cut-off of 0.05, 351 genes were significantly altered (194 up-regulated and 157 down-regulated) by an enriched environment when compared to normal non-enriched, healthy mice. Only 13% of gene-expression changes were relatively subtle in both directions within the (-1.2 to +1.2) fold-change range. The top up-regulated gene was *Cldn1* (Claudin 1) (fold change: 2.72), which is an important component of epithelial tight-junctions and the top down-regulated gene was *Gm129* (gene model 129) (fold change: -1.74), which encodes a novel transcriptional repressor that modulates circadian gene expression.

4.1.2.2 Functional annotation

The complete gene list (both up- and down-regulated transcripts) of the CE v CN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of biological processes related to cell adhesion, circadian rhythm, membrane intrinsicity and cytoskeleton organization (Table 6). Like the previous gene-ontology analysis of alcohol-exposed enriched mice, the cell adhesion and cell motility processes showed the involvement of *Col3a1* and *Cldn1* genes, which are involved in cell adhesion and cytoskeleton motility whereas the genes involved in the circadian rhythm process

like the Period genes (*Per1* and *Per3*) also exhibited altered expression levels in response to environmental enrichment. Cellular processes like membrane intrinsicty were altered through the altered expression of genes like Calcium/Calmodulin Dependent Protein Kinase II Delta (*Camk2d*) gene and the Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit (*Gabra6*) gene, both of which play a vital role in downstream learning and memory processes in the hippocampus.

Table 6: Gene ontology (GO) of up- and down- genes in the hippocampus following environmental enrichment (CE v. CN)

GO name	**p-value**	**Up-regulated genes**
Intrinsic to membrane	5.11E-05	*Slc16a9; Kcnj2; Ntng1; Camk2d; Cdh18: Gabra6*
Neurotransmitter receptor activity	0.0002	*Sstr1; Drd1a; Chrna4; Camk2d*
Cell adhesion	0.0176	*Cxadr; Cldn1; Cyp1b1; Cdh18; Cpxm2*
GO name	**p-value**	**Down-regulated genes**
Circadian rhythm	0.0496	*Per3; Bhlhe41; Per2*
Response to hormone stimulus	0.0430	*Cacna1h; Rerg; Adipor2; Cd24a; Car2*
Cytoskeleton organization	0.0496	*Gfap; Pkp2; Dsp: Col3a1*

4.1.2.3 Signaling pathways

Differentially expressed genes between control-enriched versus control non-enriched adults were analyzed using ConsensusPathDB to uncover significantly up- and down-regulated pathways (Table 7). Two major pathways with up-regulated genes, namely, the Neuroactive ligand-receptor interaction pathway (Figure. 18) and the Calcium signaling pathway, showed the involvement of the Calcium/Calmodulin Dependent Protein Kinase II Delta (*Camk2d*) gene and the Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit (*Gabra6*) gene, similar to the gene-ontology analysis for the CE v CN group. The involvement of these genes, which are vital to learning and memory processes in the hippocampus, may help explain the improved cognitive performance of healthy-enriched mice. The most highly altered pathway containing down-regulated genes was the circadian rhythm pathway (Figure. 19). As previously uncovered in the gene ontology analysis, the period genes (*Per1* and *Per3*), involved in the rhythmic regulation of gene-expression, were involved in this pathway.

Table 7: Significantly altered pathways (consisting of all up- and down-regulated genes) associated with environmental enrichment (CE v. CN)

Pathway name	**p-value**	**Up-regulated genes**
Neuroactive ligand-receptor interaction	0.0001	*Chrna4; Drd1a; Rxfp1; Glra2; Grik3*
Circadian rhythm	0.0433	*Per3; Bhlhe41; Per2*
Pathway name	**p-value**	**Down-regulated genes**
Nitrogen metabolism	0.0596	*Car2; Glul*

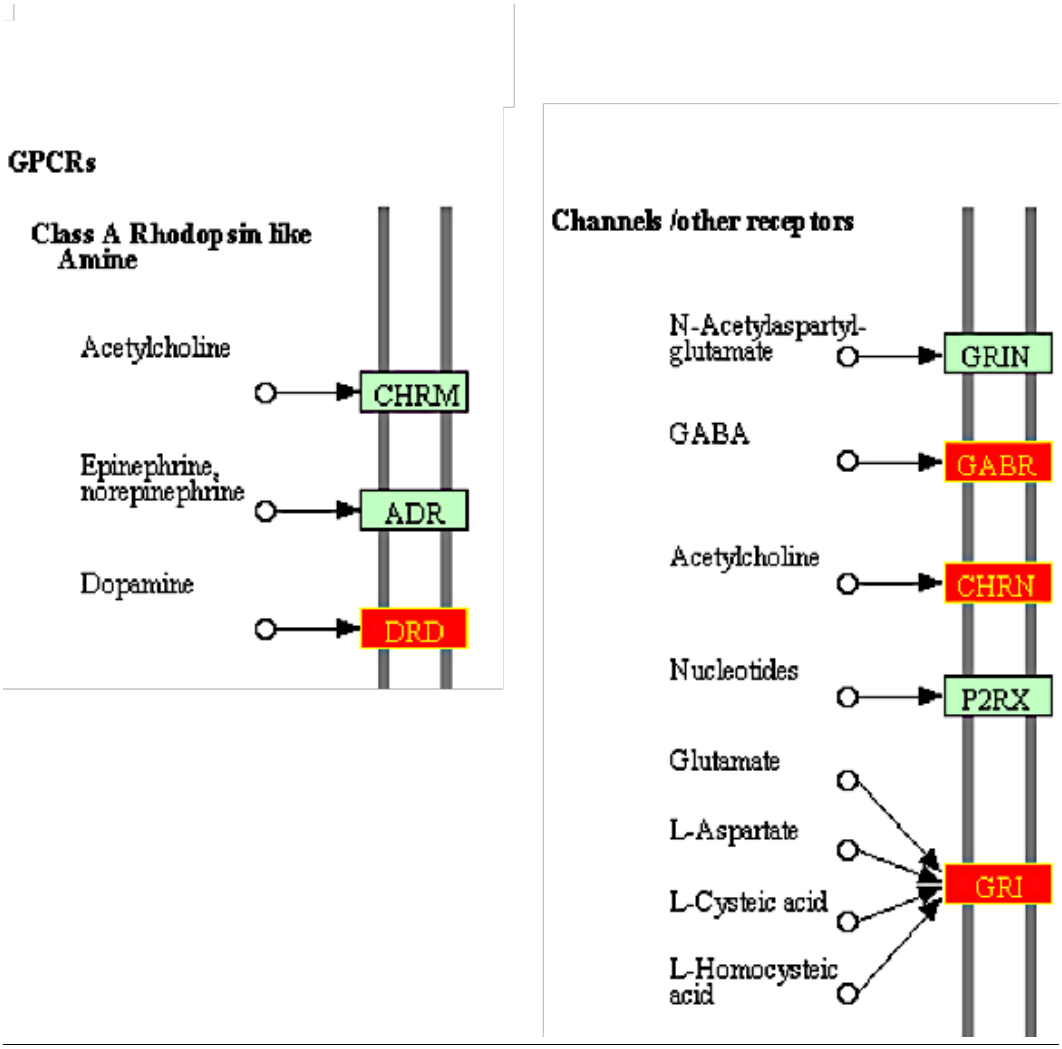


Figure 18: Neuroactive-ligand receptor activation pathway (source: KEGG), one of the top altered pathways (with up-regulated genes) in the adult hippocampus, in response to environmental enrichment. The genes in red represent the up-regulated, differentially-expressed genes.

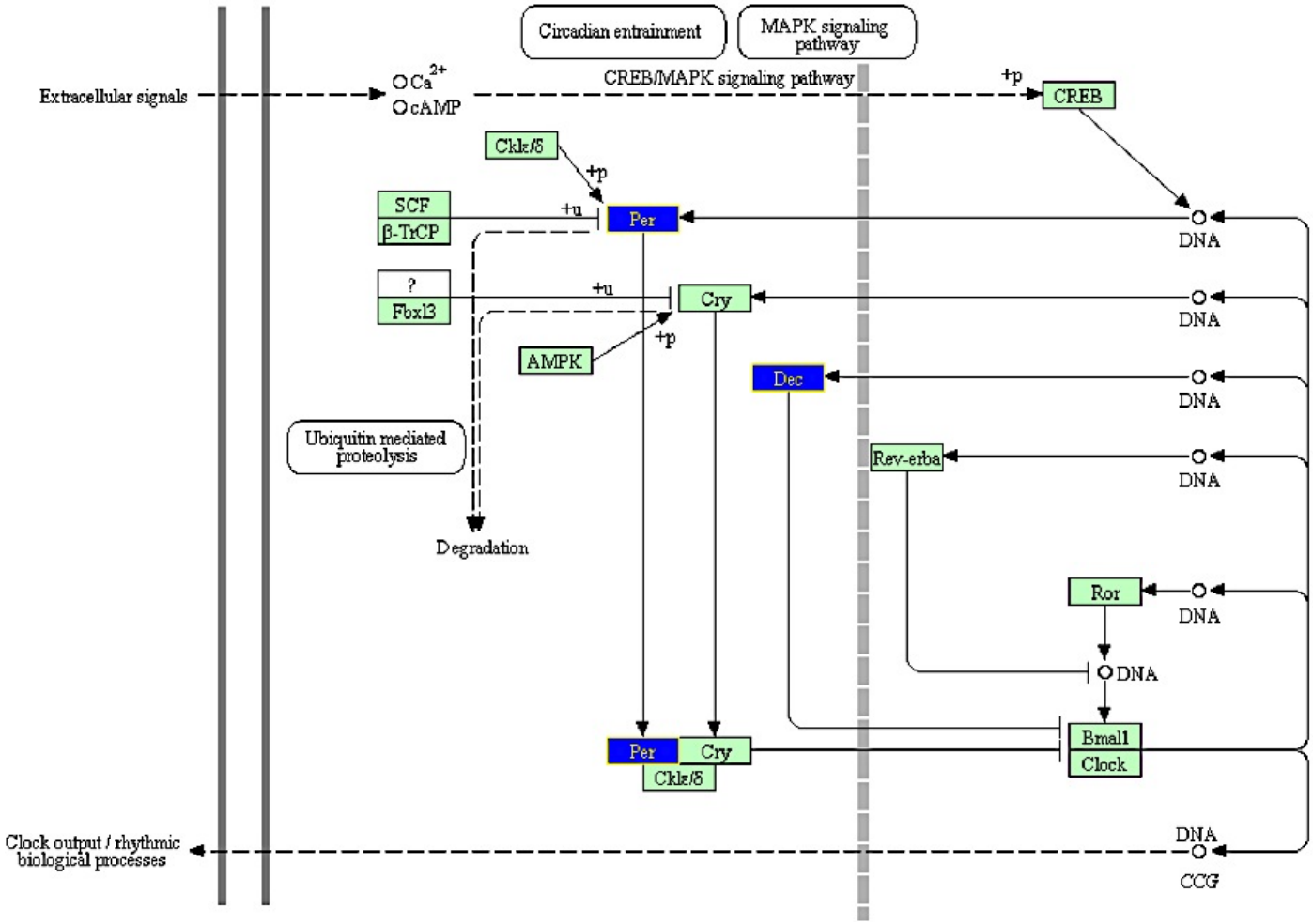


Figure 19: Circadian rhythm pathway, one of the top altered pathways (with down-regulated genes) (source: KEGG) in the adult hippocampus, in response to environmental enrichment. The genes in blue represent the down-regulated differentially expressed genes.

4.1.2.4 Gene networks

Differentially expressed genes in the adult mouse hippocampus exposed to alcohol followed by environmental enrichment were analyzed in Genemania, to predict interacting molecular networks. The CE v CN network was made up of 935 nodes with 29497 edges. *Rab4b*, a member of the RAS oncogene and involved in immune system function and endocytic trafficking of EGFR was the main hub gene in this network with a degree score of 121. Based on GENEMANIA's output, the top predicted pathways (in conjunction with previous analyses in gene-ontology and pathways) identified were involved in circadian rhythm, neuroactive ligand-receptor interaction and axon guidance.

4.1.2.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in control enriched (CE) versus control non-enriched (CN) group (Table 8). The top transcription factor for this comparison group was CLOCK (CLOCK circadian regulator), which is involved in the regulation of gene expression via a 24-hour cycle. The next two were RUNX and CEPB, which are involved hematopoiesis regulation and immune system response, respectively.

Table 8: Top three transcription factors of all up- and down-regulated genes associated with post-natal environmental enrichment (CE v. CN)

Transcription factor	Name of factor	Possible functions	p-Value
CLOCK	Clock Circadian Regulator	circadian rhythm regulator	0.0001
RUNX	Runt Related Transcription Factor 1	regulation of hematopoiesis	0.0034
CEPB	CCAAT/Enhancer Binding Protein Beta	involved in immune regulation and response in the hippocampus	0.095

4.1.2.6 Summary of results: the effect of environmental enrichment

The effect of the environmental enrichment used has a subtle effect and relatively few genes (as analyzed by the microarray platform) in the adult hippocampus with most of the effects being significantly large in both directions (above and below the log fold-change of -1.2). Environmental enrichment affects a wide range of biological processes ranging from the maintenance of membrane potential and neurotransmitter activity (up-regulated genes) all the way to the cytoskeleton organization and circadian rhythm regulation for the down-regulated genes. The most highly altered pathways (with up-regulated genes) involve neuroactive ligand receptor activation while the top pathway with down-regulated genes is related to the circadian rhythm. Gene network analysis also confirms pathway analysis results, in that environmental enrichment in healthy mice primarily affects signaling pathways involved in downstream cognition like the neuroactive ligand receptor activation pathway and the circadian rhythm, pathway.

These observations show that environmental enrichment in healthy mice affects pathways involved in the efficient processing of information while also altering pathways involved in synaptic and structural plasticity. This may be associated with the behavioural improvements observed in control-enriched mice.

4.1.3 The effect of prenatal alcohol exposure and post-natal environmental enrichment (AE v. AN)

4.1.3.1 Gene expression

For the alcohol enriched (AE) versus alcohol non-enriched (AN) group, at a FDR corrected p-value cut-off of 0.05, 443 transcripts were significantly altered (176 up-regulated and 267 down-regulated) by enriched environments in alcohol-exposed treatment when compared to non-enriched environments in alcohol-exposed mice. About 35% of gene-expression changes were relatively subtle (-1.2 to +1.2) with most of the changes being in the down-regulated direction. The top up-regulated gene was *Mir669h* (fold change: 2.92), a mouse micro-RNA and the top down-regulated gene was *Zfp879* (zinc finger protein 879) (fold change: -1.26), which is involved in DNA transcription. The complete list of genes meeting the stringency parameters are provided in Appendix 2 and this list was subsequently used for biological functions, pathways and gene-network analysis.

4.1.3.2 Functional annotation

The complete gene list (both up- and down-regulated transcripts) of the AE v AN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of molecular processes related to heterocyclic compound binding and RNA metabolism (Table 9). The nuclear receptor gene family (*Nr2f1; Nr1d1; Nr2f2*) was found to be involved specifically

in the steroid hormone receptor activity process.

Table 9: Gene ontology (GO) of up- and down-regulated genes in the hippocampus following ethanol exposure and environmental enrichment (AE v. AN)

GO name	**p-value**	**Up-regulated genes**
Heterocyclic compound binding	2.87E-06	*Zfp81; Nol8; Rel; Zfp759; Zbtb20; Plag1*
Macromolecule metabolic process	0.0003	*Acer2; Adam4; 1700049G17Rik; Chordc1*
RNA metabolic process	0.002	*Icam1; Zfp759; Zbtb20; Plag1; Zfp930*
GO name	**p-value**	**Down-regulated genes**
Steroid hormone receptor activity	0.0903	*Gpr30; Nr2f1; Nr1d1; Nr2f2*
Polyamine catabolic process	0.1940	*Sat2; Dhps*
Regulation of establishment of protein localization to plasma membrane	0.1964	*Gpr30; Nkd2; Rhog*

4.1.3.3.Signaling pathways

Differentially expressed genes between alcohol-enriched versus alcohol non-enriched adults were analyzed using ConsensusPathDB to uncover significantly up- and down-regulated pathways. Significantly altered pathways (containing up-regulated genes) such as mRNA metabolism and serotonin receptor pathways were altered in the mouse hippocampus in response to alcohol exposure during synaptogenesis (Table 10). While the former pathway included the involvement of heat shock protein genes such as *Hspa1a* and *Hspa1b*, the latter included various serotonin receptor genes such as *Htr1f* and *Htr1b*. The most significant pathways (containing down-regulated genes) pertained to adipogenesis (renin-angiotensinogen pathway) and gluconeogenesis. The angiotensinogen gene (*Agt*), which is involved in adipogenesis, is interesting in the context of environmental enrichment due to the role of AGT as a vasoconstrictor, possibly in response to increased angiogenesis during voluntary exercise (during environmental enrichment).

Table 10: Significantly altered pathways (consisting of up- and down-regulated genes) associated with prenatal alcohol exposure and environmental enrichment (AE v. AN)

Pathway name	**p-value**	**Genes**
Destabilization of mRNA by AUF1	0.015	*Hspa1b; Hspa1a*
Serotonin receptors	0.0166	*Htr1f; Htr1b*
Pathway name	**p-value**	**Genes**
Adipogenesis	0.3284	*Nr2f1; Hmga1; Dlk1; Agt; Gata2; Gdf10*

4.1.3.4. Gene networks

Differentially expressed genes in the adult mouse hippocampus exposed to alcohol followed by environmental enrichment were analyzed in Genemania, to predict interacting molecular networks. The AE v AN network was made up of 721 with 16601 edges. *Rab4b*, a member of the RAS oncogene, involved in immune system function and endocytic trafficking of EGFR was the main hub gene in this network with a degree score of 218. Based on GENEMANIA's output, the top predicted pathways identified were involved in ribosome pathway, arginine and proline metabolism and also glycine, serine and threonine metabolism.

4.1.3.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in alcohol enriched (AE) versus alcohol non-enriched (AN) group (Table 11). The top transcription factor for this comparison group was EP300 (E1A Binding Protein P300), a histone acetyltransferase involved in cell differentiation. The next two were HNF1B and PADI4, which are involved in pancreatic and macrophage development respectively.

Table 11: Top three transcription factors of all up- and down-regulated genes associated with prenatal alcohol exposure during synaptogenesis and post-natal environmental enrichment (AE v. AN)

Transcription factor	Name of factor	Possible functions	p-value
EP300	E1A Binding Protein P300	histone acetyltransferase, cell differentiation	0.0001
HNF1B	HNF1 Homeobox B	development of embryonic pancreas and nephrons	0.0088
PADI4	Peptidyl Arginine Deiminase 4	granulocyte and macrophage development	0.0015

4.1.3.6 Summary of results: the effects of prenatal alcohol exposure and post-natal environmental enrichment

The effect of environmental enrichment on prenatal alcohol exposure has a small subtle effect on the minority of genes (as analyzed by the microarray platform) in the adult hippocampus with most of the effects being major (above and below the log fold-change of -1.2) in both directions. The effects of environmental enrichment following prenatal alcohol exposure target a wide range of biological processes ranging from heterocyclic compound binding and RNA metabolism (up-regulated genes) to the protein localization and steroid hormone receptor activity for the down-regulated genes. One of the most highly altered pathways (with up-regulated genes) involved the serotonergic pathway while the top pathway with down-regulated genes is related to the adipogenesis/renin-angiostenin pathway. Gene network analysis, however, primarily implicates pathways involved in amino-acid metabolism.

These observations show that environmental enrichment in alcohol-exposed mice affects pathways involved in protein metabolism, the serotonergic pathway and the renin-angiostenin pathway. The involvement of various serotonin receptors and the angiostenin gene, for example, may explain the lowered anxiety levels in alcohol-enriched mice coupled with their improved performance in behavioural tests.

4.2 Gene-expression changes as uncovered by the RNA-Seq platform

4.2.1 The effect of prenatal alcohol exposure (AN v. CN)

4.2.1.1 Gene expression

For the alcohol non-enriched (AN) versus control non-enriched (CN) group, at a FDR corrected p-value cut-off of 0.05, 91 transcripts were significantly altered (65 up-regulated and 26 down-regulated) by alcohol treatment when compared to normal, healthy mice. Changes to gene-expression were relatively subtle in both directions with 78% of genes being up- or down-regulated within the (-1.2 to +1.2) fold-change range. The top up-regulated gene was *Il23a (Interleukin 23 Subunit Alpha)* (fold change: 6.29), which is involved in autoimmune inflammation and the top down-regulated gene was *Ambn (Ameloblastin)* (fold change: -8.12), which is important for enamel matrix formation and mineralization.

4.2.1.2 Functional annotation

The complete gene list (both up- and down-regulated transcripts) of the AN v CN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of biological processes related to multicellular organismal development, response to external stimulus, cell proliferation and cell adhesion (Table 12). Cellular processes related to collagen, basement membrane, neuron parts and cell projection were also identified.

Interestingly, these annotations comprised a number of altered transcripts in the Collagen gene family (*Col4a5, Col4a1, Col1a1* and *Col3a1*) which play structural roles in cells and contribute to cellular organization and tissue shape. By interacting with other cells via several receptor families, collagens regulate cellular proliferation, migration, and differentiation.

Table 12: Gene ontology (GO) of up- and down-regulated genes in the hippocampus following prenatal ethanol exposure (AN v. CN)

GO name	**p-value**	**Up-regulated genes**
Multicellular organismal development	5.34E-08	*Ick; Dnmt3a; Eln; Dpysl3; Tspan2*
Response to external stimulus	2.13E-05	*Dcn;; Col1a1; Il23a; Dpysl5; Wnt4; Cklf*
Cell adhesion	0.0006	*Cxadr; Nid1; Cldn11; Cd24a; Ambn; Dsp Col1a1; Col3a1; Wnt4*
GO name	**p-value**	**Down-regulated genes**
Immune response	0.0116	*Il20rb; Ifit3; Il33; C4b; Zc3hav1*
Sequestering of metal ion	0.0644	*Fth1; Slc30a3*

4.2.1.3 Signaling pathways

Differentially expressed genes between alcohol non-enriched versus control non-enriched adults were analyzed using ConsensusPathDB to uncover significantly altered pathways containing up- and down-regulated genes (Table 13). Consistent with gene-ontology analyses, collagen biosynthesis and formation alongwith extracellular matrix formation (Figure. 20) were identified as significant pathways (containing up-regulated genes) with altered expression in the *Col3a1* and *Col4a5* genes. The most significant pathways (containing down-regulated genes) pertained to the GABAergic synapse (Figure. 21), neurotransmitter receptor binding and downstream transmission in the postsynaptic cell and ion channel transport. The Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit (*Gabra6*) gene was involved in all three pathways indicating its involvement in signaling pathways pertaining to downstream cognitive processes and its involvement in the HPA-axis.

Table 13: Significantly altered pathways (consisting of up- and down-regulated genes) associated with prenatal alcohol exposure (AN v. CN)

Pathway name	**p-value**	**Up-regulated genes**
Collagen biosynthesis and modifying enzymes	0.004	*Col3a1; Col4a5*
Collagen formation	0.0054	*Col3a1; Col4a5*
ECM-receptor interaction	0.0061	*Col3a1; Col4a5*
Pathway name	**p-value**	**Down-regulated genes**
GABAergic synapse	0.0061	*Gabra6; Plcl1*
Neurotransmitter Receptor Binding	0.0132	*Camkk1; Gabra6*
Ion channel transport	0.0341	*Gabra6; Trpc6*

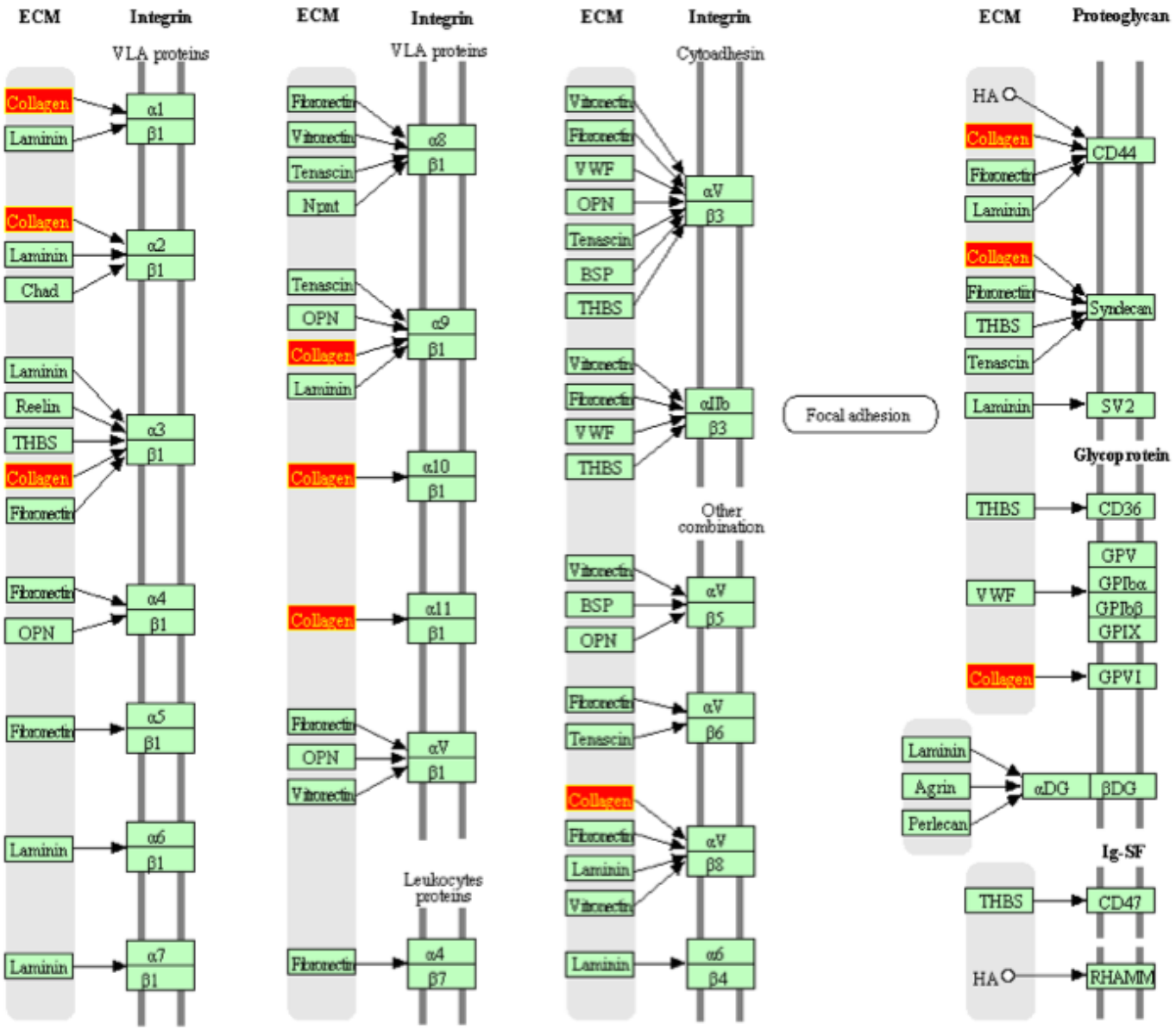


Figure 20: ECM-receptor pathway (source: KEGG), one of the top altered pathways in the adult hippocampus, in response to prenatal alcohol exposure. The genes in red represent the up-regulated differentially expressed genes.

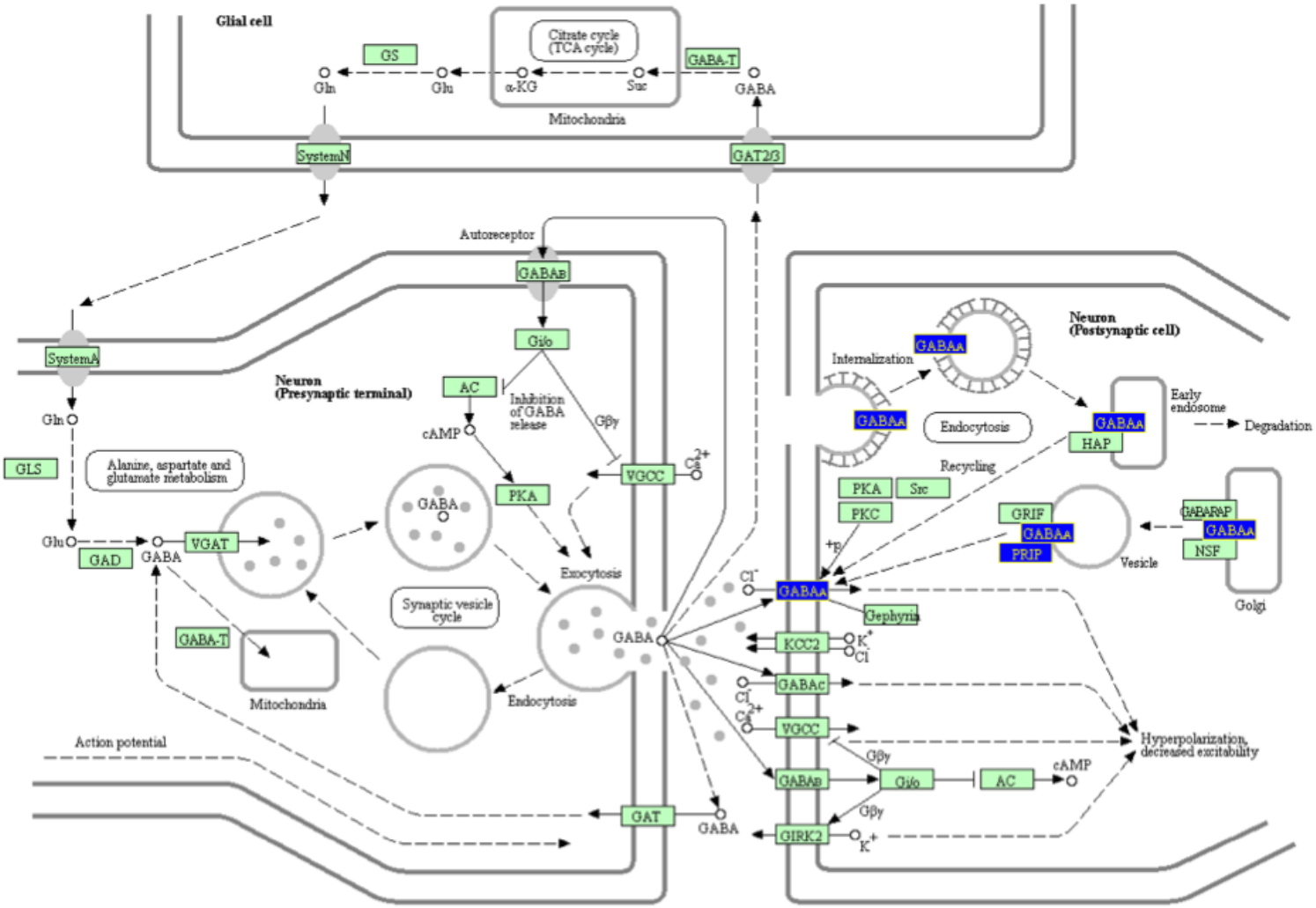


Figure 21: GABA-ergic synapse pathway (source: KEGG), one of the top down-regulated pathways in the adult hippocampus, in response to prenatal alcohol exposure. The genes in blue represent the down-regulated differentially expressed genes.

4.2.1.4 Gene networks

Differentially expressed genes in the adult mouse hippocampus (exposed to alcohol on post-natal days 6 and 7) were analyzed in a gene-network analysis tool, Genemania, to predict interacting molecular networks. Based on input genes, GeneMANIA constructed networks that include associated data from protein and genetic interactions, pathways, co-expression, co-localization and protein domain similarity. Nodes represent genes and edges connecting these nodes represent interactions genes. Nodes with large degree values (higher number of connections) represent the key genes. The AN v CN network was made up of 108 nodes (with 23 out of 108 genes being novel additions based on various GeneMANIA interaction data) with 1373 edges (Figure. 22). Decorin (*dcn*), a gene that codes for fibrillar collagen was the main hub gene in this network with a degree score of 85. Based on GENEMANIA's output, the top predicted pathways identified were involved in ECM-receptor interaction, protein digestion and absorption, focal adhesion and axon guidance with the major involvement of the collagen gene family.

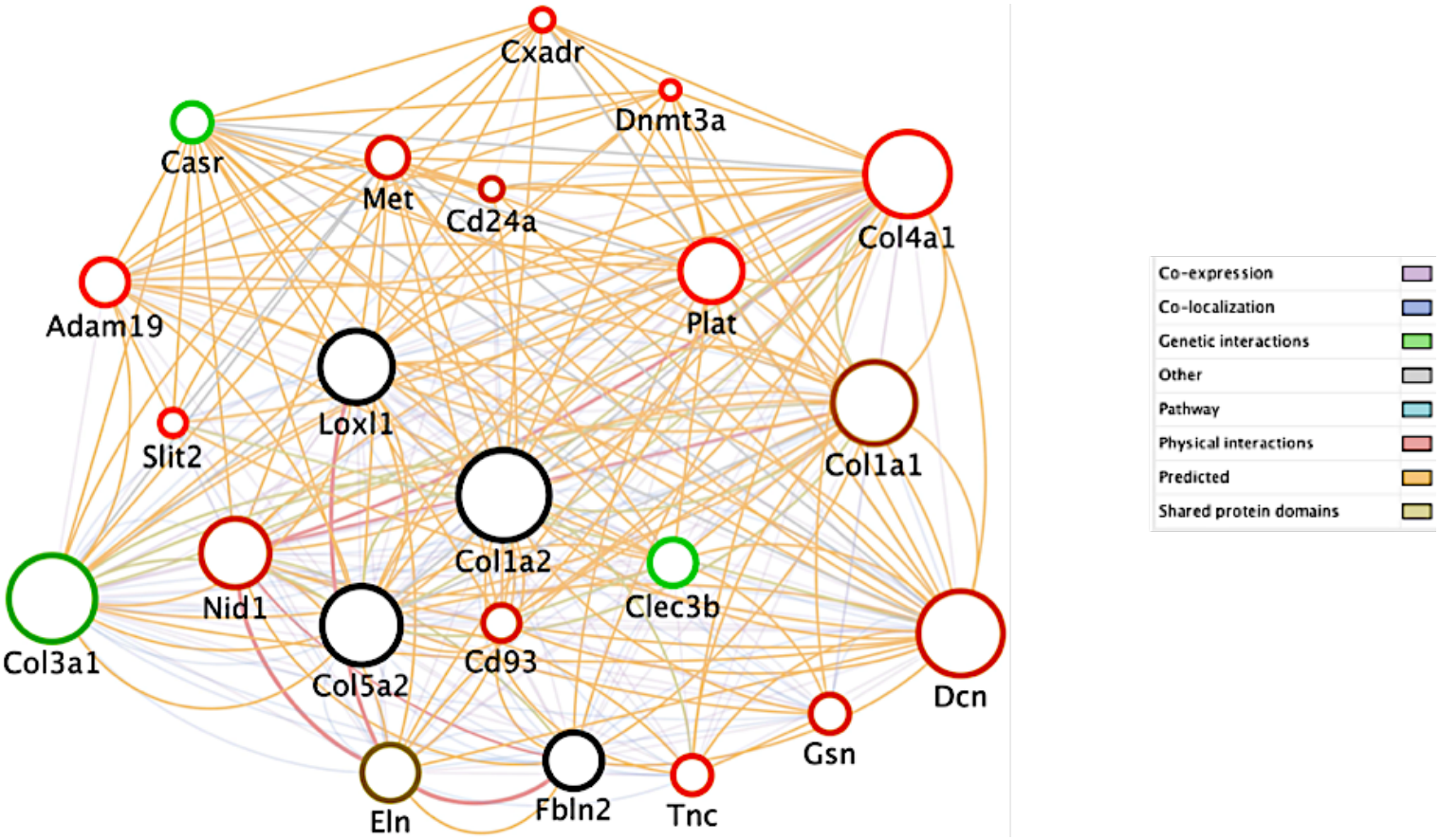


Figure 22: Gene network cluster for differentially expressed genes in response to prenatal alcohol exposure: The top clustered gene network showing differentially expressed genes from the alcohol non-enriched (AN) versus control non-enriched (CN) group comparison. Red nodes represent down-regulated genes. Green nodes represent up-regulated genes. Black nodes indicate genes from the GeneMANIA database. Network generated using GLay clustering algorithm and GeneMANIA. The size of the nodes (genes) indicates the degree of connectivity for each gene. Decorin (*Dcn*) is the main hub gene (gene with the highest connectivity) for this network.

4.2.1.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in alcohol non-enriched (AN) versus control non-enriched (CN) group (Table 14). The top transcription factor for this comparison group was E2F2 (E2F2 Transcription Factor 2) which is involved in development and maintenance of neural stem cells. The next two were AP3B1 and RELA, which are involved in cargo sorting in synaptic vesicles and apoptosis, respectively.

Table 14: Top three transcription factors of all up- and down-regulated genes associated with prenatal alcohol exposure during synaptogenesis (AN v. CN)

Transcription factor	Name of factor	Possible functions	p-value
E2F2	E2F Transcription Factor 2	maintenance and development of neural precursors and their differentiation	0.0041
AP3B1	Adaptor-Related Protein Complex 3, Beta 1 Subunit	cargo sorting in synaptic vesicles	0.084
RELA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A	apoptosis, immune system	0.0062

4.2.1.6 Summary of results: the effect of prenatal alcohol exposure

The effect of prenatal alcohol exposure has subtle effect on the majority (90%) of genes (as analyzed by the RNA-Seq platform) in the adult hippocampus. Prenatal exposure mostly affects a wide range of biological processes ranging from cell adhesion and organ development (up-regulated genes) all the way to the immune response and metal-ion sequestration for the down-regulated genes. The most highly altered pathways (containing up-regulated genes) involve collagen formation and synthesis, extracellular-matrix organization while the top altered pathways (containing down-regulated genes) relate to neurotransmitter receptor binding and ion-channel transport. Through gene network analysis, it is also clear that prenatal alcohol exposure majorly affects biological processes involved in extracellular matrix organization and focal adhesion with a major involvement of the collagen gene family.

These observations show that prenatal alcohol exposure leads to a massive detrimental alteration of biological processes related to the structural integrity and development of critical regions in the hippocampus, coupled with the down-regulation of pathways involved in information processing and downstream cognitive pathways. These results may partially explain the behavioural deficits observed in alcohol-expose mice.

4.2.2 The effect of environmental enrichment (CE v. CN)

4.2.2.1 Gene expression

For the control non-enriched (CE) versus control non-enriched (CN) group, at a FDR corrected p-value cut-off of 0.05, 109 genes were significantly altered (70 up-regulated and 39 down-regulated) by an enriched environment when compared to normal non-enriched, healthy mice. Most gene-expression changes were relatively subtle (94%) in both directions within the (-1.2 to +1.2) fold-change range. The top up-regulated gene was *Aoah (*Acyloxyacyl Hydrolase*)* (fold change: 5.33), which is involved in modulating host inflammatory response and the top down-regulated gene was *Ambn (*Ameloblastin*)* (fold change: -8.12), which is important for enamel matrix formation and mineralization.

4.2.2.2 Functional annotation

The complete gene list (both up- and down-regulated transcripts) of the CE v CN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of biological processes related to cell adhesion, circadian rhythm and cell motility (Table 15). The cell adhesion and cell motility processes showed the involvement of *Col3a1* and *Cldn1* genes, which are involved in cell adhesion and cytoskeleton motility, the genes involved in the circadian rhythm process like the Period genes (*Per1* and *Per3*) also exhibited altered expression levels in response to environmental enrichment.

Table 15: Gene ontology (GO) of up- and down-regulated genes in the hippocampus following environmental enrichment (CE v. CN)

GO name	**p-value**	**Genes**
Cell adhesion	0.0007	*Cxadr; Col8a1; Pecam1; Cdh18*
Neurotransmitter Receptor activity	0.0014	*Sstr1; Drd1a; Gabra2*
Ion channel complex	0.0080	*Camk2d; Glra3; Gabra2; Kcnf1; Wnt4*
GO name	**p-value**	**Genes**
Circadian rhythm	0.0116	*Il20rb; Ifit3; Il33; C4b; Zc3hav1*
Cadherin binding	0.0026	*Fth1; Slc30a3*
RNA polymerase II activity	0.002	*Ndrg1; Ctnna3*

4.2.2.3 Signaling pathways

Differentially expressed genes between control enriched versus control non-enriched adults were analyzed using ConsensusPathDB to uncover significantly up- and down-regulated pathways (Table 16). Two major pathways (containing up-regulated genes), namely, the extracellular matrix organization pathway (Figure. 23) and the collagen biosynthesis pathway were involved with the collagen genes *Col8a1* and *Col3a1* showing significant involvement. The most highly altered pathway containing down-regulated genes was the circadian rhythm pathway (Figure. 24). As previously uncovered in the gene ontology analysis, the period genes (*Per1* and *Per3*), involved in the rhythmic regulation of gene-expression, were involved in this pathway.

Table 16: Significantly altered pathways (consisting of up- and down-regulated genes) associated with environmental enrichment (CE v. CN)

Pathway name	**p-value**	**Up-regulated Genes**
Extracellular matrix organization	0.0039	*Ttr; Col8a1; Col3a1*
Collagen biosynthesis	0.0020	*Col3a1; Col8a1*
Pathway name	**p-value**	**Down-regulated Genes**
Circadian rhythm	0.0001	*Per3; Bhlhe41; Per1*

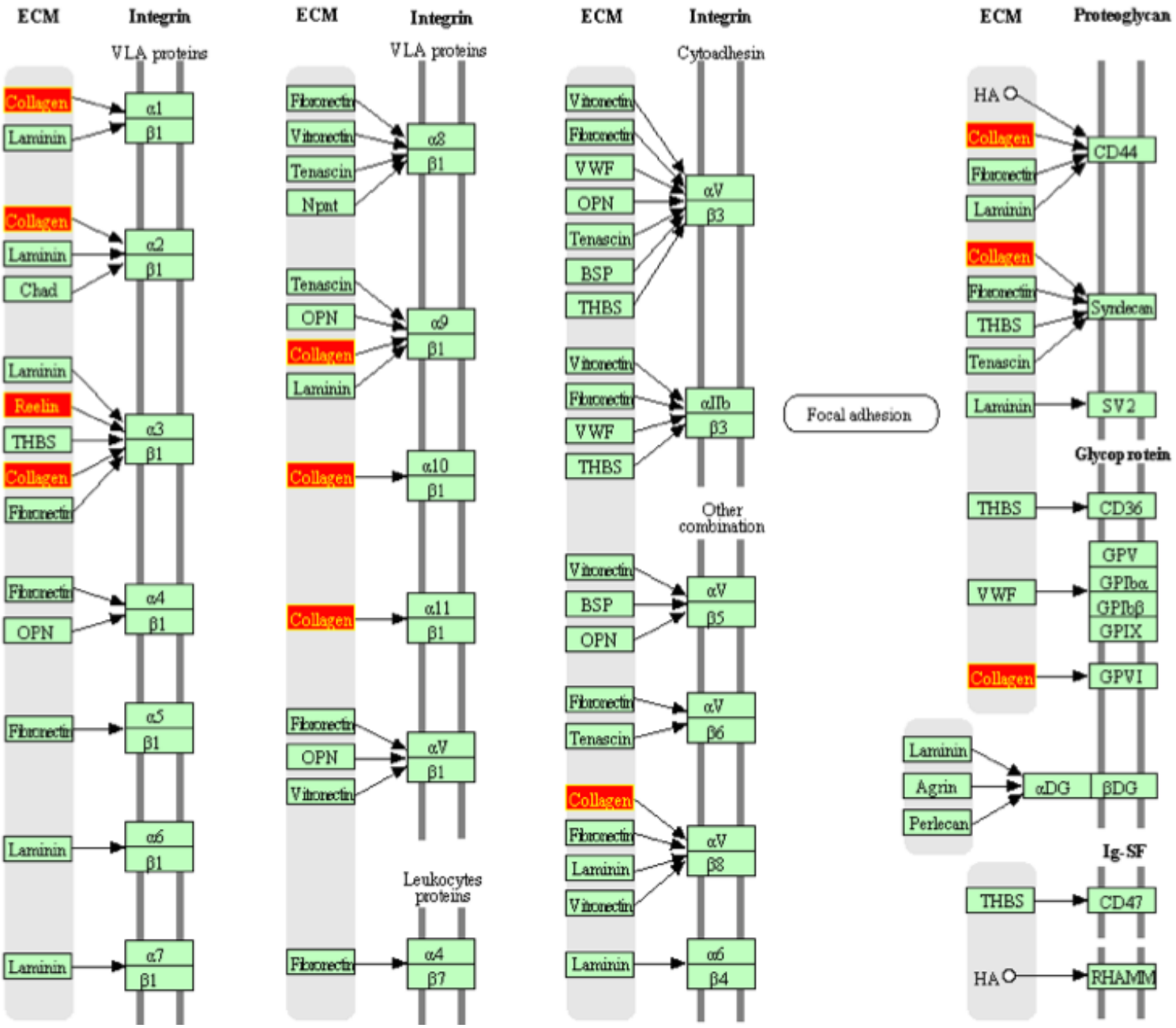


Figure 23: ECM-receptor pathway (source: KEGG), one of the top altered pathways in the adult hippocampus, in response to post-natal environmental enrichment. The genes in red represent the up-regulated differentially expressed genes.

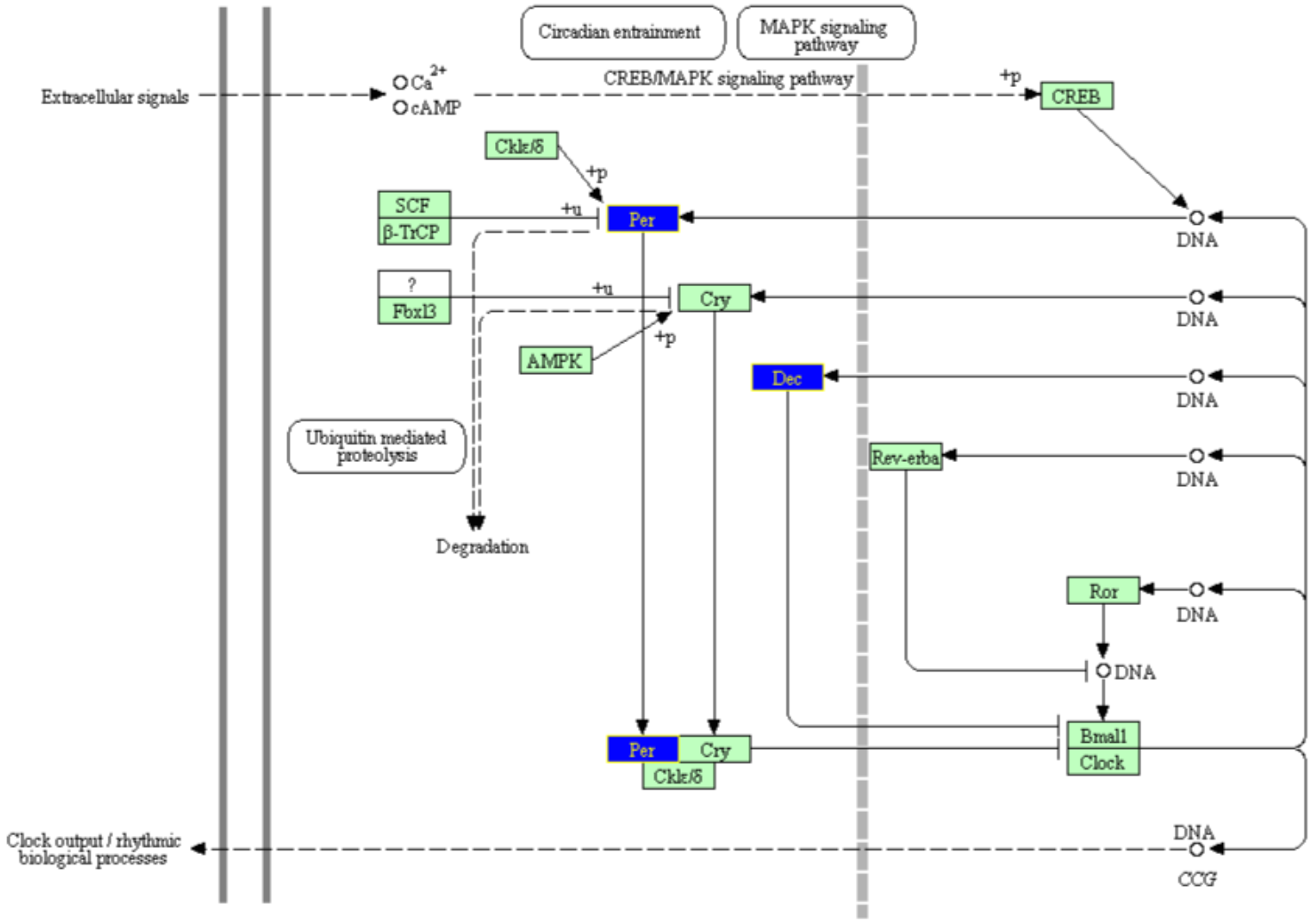


Figure 24: Circadian rhythm pathway (source: KEGG), one of the top down-regulated pathways in the adult hippocampus, in response to post-natal environmental enrichment. The genes in blue represent the down-regulated differentially expressed genes.

4.2.2.4 Gene networks

Differentially expressed genes in the adult mouse hippocampus exposed to alcohol followed by environmental enrichment were analyzed in Genemania, to predict interacting molecular networks. The CE v CN network was made up of 124 nodes (with 22 out of 124 genes being novel additions based on various GeneMANIA interaction data) with 1155 edges (Figure. 25). Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit (*Gabra6*), the major inhibitory neurotransmitter in the mammalian brain was the main hub gene in this network with a degree score of 51. Based on GENEMANIA's output, the top predicted pathways (in conjunction with previous analyses in gene-ontology and pathways) identified were involved in circadian rhythm and neuroactive ligand-receptor interaction.

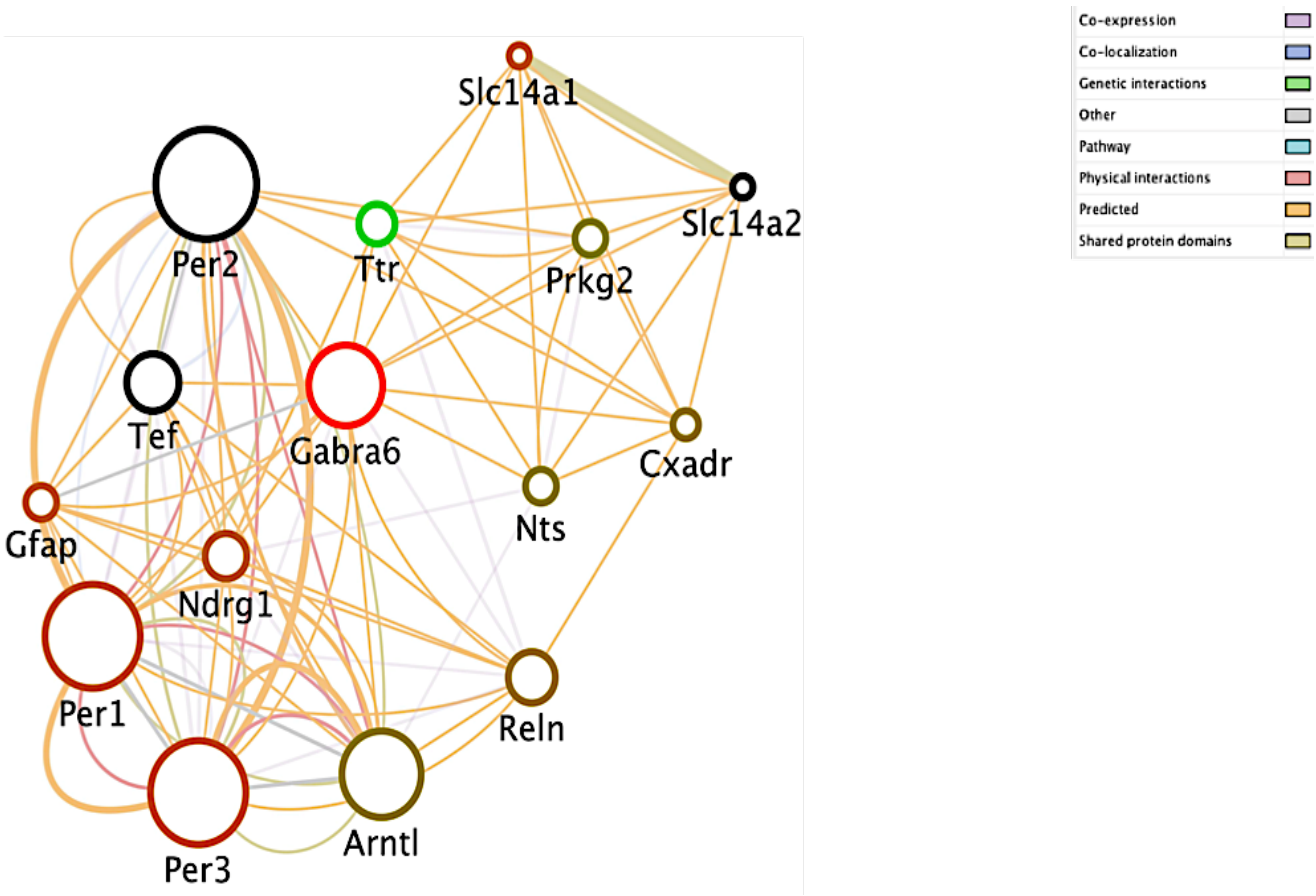


Figure 25: Gene network cluster for differentially expressed genes in response to post-natal environmental enrichment: The top clustered gene network showing differentially expressed genes from the control enriched (CE) versus control non-enriched (CN) group comparison. Red nodes represent down-regulated genes. Green nodes represent up-regulated genes. Black nodes indicate genes from the GeneMANIA database. Network generated using GLay clustering algorithm and GeneMANIA. Size of the nodes (genes) indicate the degree of connectivity for each gene. *Gabra6* is the main hub gene (gene with the highest connectivity) for this network.

4.2.2.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in control enriched (CE) versus control non-enriched (CN) group (Table 17). The top transcription factor for this comparison group was THRB (Thyroid receptor hormone beta), which is involved in migration and differentiation of neural stem cells, synaptogenesis, mylenation, cerebellar performance and development. The next two were CANX and MAFH, which are involved in protein folding in synaptic and non-synaptic vesicles, and neural cell differentiation, respectively.

Table 17: Top three transcription factors of all up- and down-regulated genes associated with post-natal environmental enrichment (CE v. CN)

Transcription factor	**Name of factor**	**Possible functions**	p-value
THRB	Thyroid Hormone Receptor, Beta	migration and differentiation of neural stem cells, synaptogenesis, mylenation, cerebellar performance and development	0.0041
CANX	Calnexin	protein folding chaperone in synaptic and non-synpatic vesicles	0.084
MAFH	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B	neural cell differentiation, microglial development	0.0062

4.2.2.6 Summary of results: the effect of post-natal environmental enrichment

The effect of environmental enrichment has a subtle effect on the majority of genes (as analyzed by the RNA-Seq platform) in the adult hippocampus with relatively few of the effects being major in both directions (above and below the log fold-change of -1.2). Environmental enrichment affects biological processes containing up-regulated genes involved in cell adhesion to neurotransmitter receptor activities and ion-channel complexes, and also containing down-regulated genes being involved in circadian rhythm and cadherin binding.

While the top pathways (containing up-regulated genes) are involved primarily in structural processes such as collagen synthesis and extracellular matrix organization, the top altered pathway (containing down-regulated genes) is the circadian rhythm pathway. Gene network analysis confirms pathway analysis results, in that environmental enrichment in healthy mice primarily affects neuroactive ligand receptor activation pathway and the circadian rhythm, pathway.

These observations show that environmental enrichment in healthy mice affects pathways involved in the efficient processing of information while also altering pathways involved in synaptic and structural plasticity. This may be associated with the behavioural improvements observed in control-enriched mice.

4.2.3 The effect of prenatal alcohol exposure and environmental enrichment (AE v. AN)

4.2.3.1 Gene expression

For the alcohol non-enriched (AE) versus alcohol non-enriched (AN) group, at a FDR corrected p-value cut-off of 0.05, 15 genes were significantly altered (9 up-regulated and 6 down-regulated) by enriched environments in alcohol-exposed treatment when compared to non-enriched environments in alcohol-exposed mice. 73% of changes to gene-expression were relatively subtle in both directions within the (-1.2 to +1.2) fold-change range. The top up-regulated gene was *Serpina3f (*Serpin Family A Member 3*)* (fold change: 7.82), which is a plasma protease inhibitor and the top down-regulated gene was *Arhgap36 (*Rho GTPase Activating Protein 36*)* (fold change: -1.89), which is heavily involved in GPCR signaling. The complete list of genes meeting the stringency parameters are provided in Appendix 3 and this list was subsequently used for biological functions, pathways and gene-network analysis.

4.2.3.2 Functional annotation

The complete gene list (both up- and down-regulated transcripts) of the AE v AN group was analyzed using ConsensusPathDB for over-represented gene ontology processes. ConsensusPathDB identified a number of biological processes related to muscle cell differentiation, muscle tissue development and lymphocyte

activation involved in immune response (Table 18). These annotations had a number of altered transcripts such as the Intercellular adhesion molecule 1 (*Icam1*) which is involved in endothelial cell adhesion and tight-junction regulation, and MET Proto-Oncogene, Receptor Tyrosine Kinase (*Met*), involved in cell proliferation and migration of neural precursors.

Table 18: Gene ontology (GO) of up- and down-regulated genes in the hippocampus following prenatal ethanol exposure and environmental enrichment (AE v. AN)

GO name	**p-value**	**Up-regulated genes**
Lymphocyte activation	0.0233	*Icam1; Bcl3*
Response to stress	0.0293	*Cxcl1; Hspa1b; Hspa1a; Icam1; Bcl3*
GO name	**p-value**	**Down-regulated genes**
Muscle cell differentiation	0.0007	*Met; Plagl1; AW551984*

4.2.3.3 Signaling pathways

Differentially expressed genes between alcohol enriched versus alcohol non-enriched adults were analyzed using ConsensusPathDB to uncover significantly up- and down-regulated pathways (Table 19). A major altered pathway included the destabilization of mRNA by AUF1 with the Heat Shock Protein gene family (*Hspa1b* and *Hspa1a*) being involved. The TNF signaling pathway (Figure. 26) was also one of the top pathways containing up-regulated genes and the involvement of the intercellular adhesion molecule 1 (*Icam1*) gene (consistent with gene ontology analysis) was also noted. The most highly altered pathways containing down-regulated genes were involved in Sema4D-mediated inhibition of cell attachment and migration, and adherens junction pathways (Figure. 27). As previously uncovered in the gene ontology analysis, the MET Proto-Oncogene, Receptor Tyrosine Kinase (*Met*) gene, involved in cell proliferation and migration of neural precursors, was also involved in both the down-regulated pathways.

Table 19: Significantly altered pathways (consisting of up- and down-regulated genes) associated with prenatal alcohol exposure and environmental enrichment (AE v. AN)

Pathway name	**p-value**	**Up-regulated genes**
Destabilization of mRNA by AUF1	5.35E-05	*Hspa1b; Hspa1a*
TNF signaling pathway	0.0001	*Cxcl1; Bcl3; Icam1*
Pathway name	**p-value**	**Down-regulated genes**
Sema4D-mediated inhibition of cell attachment and migration	0.0450	*Met*
Adherens junction	0.0780	*Met*

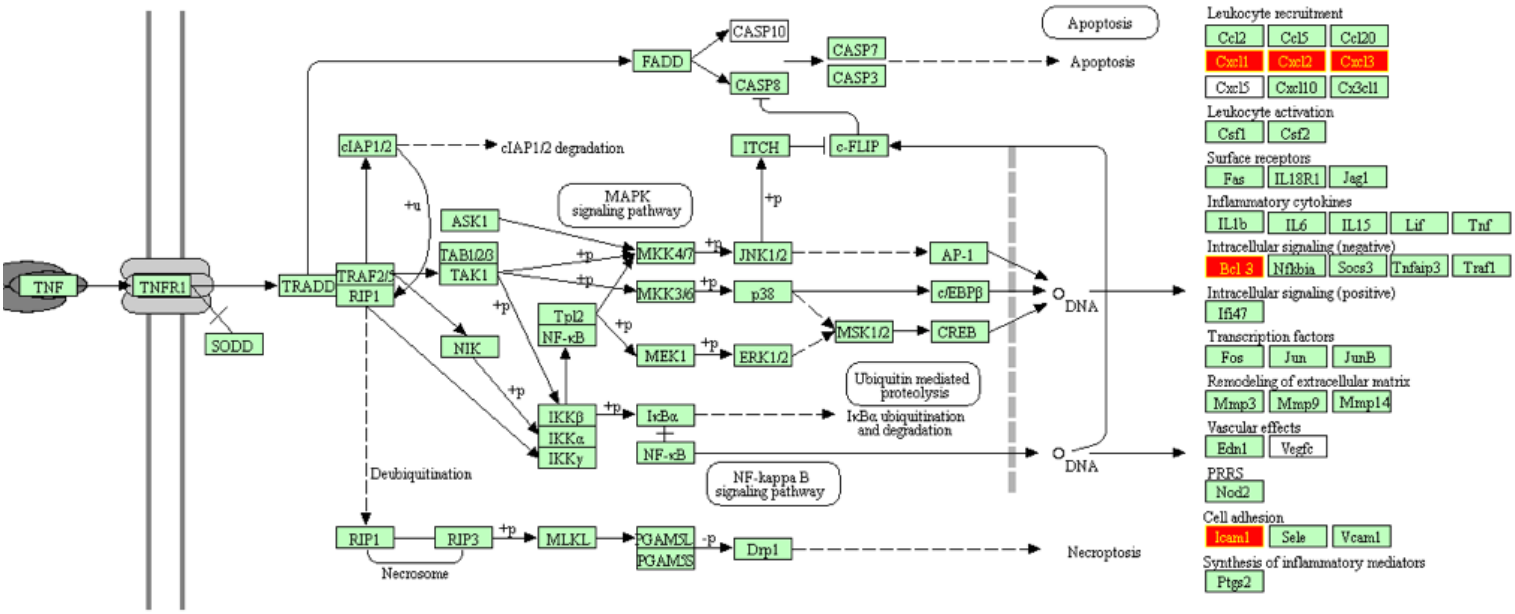


Figure 26: TNF-signaling pathway (source: KEGG), one of the top altered pathways in the adult hippocampus, in response to prenatal alcohol exposure and environmental enrichment. The genes in red represent the up-regulated differentially expressed genes.

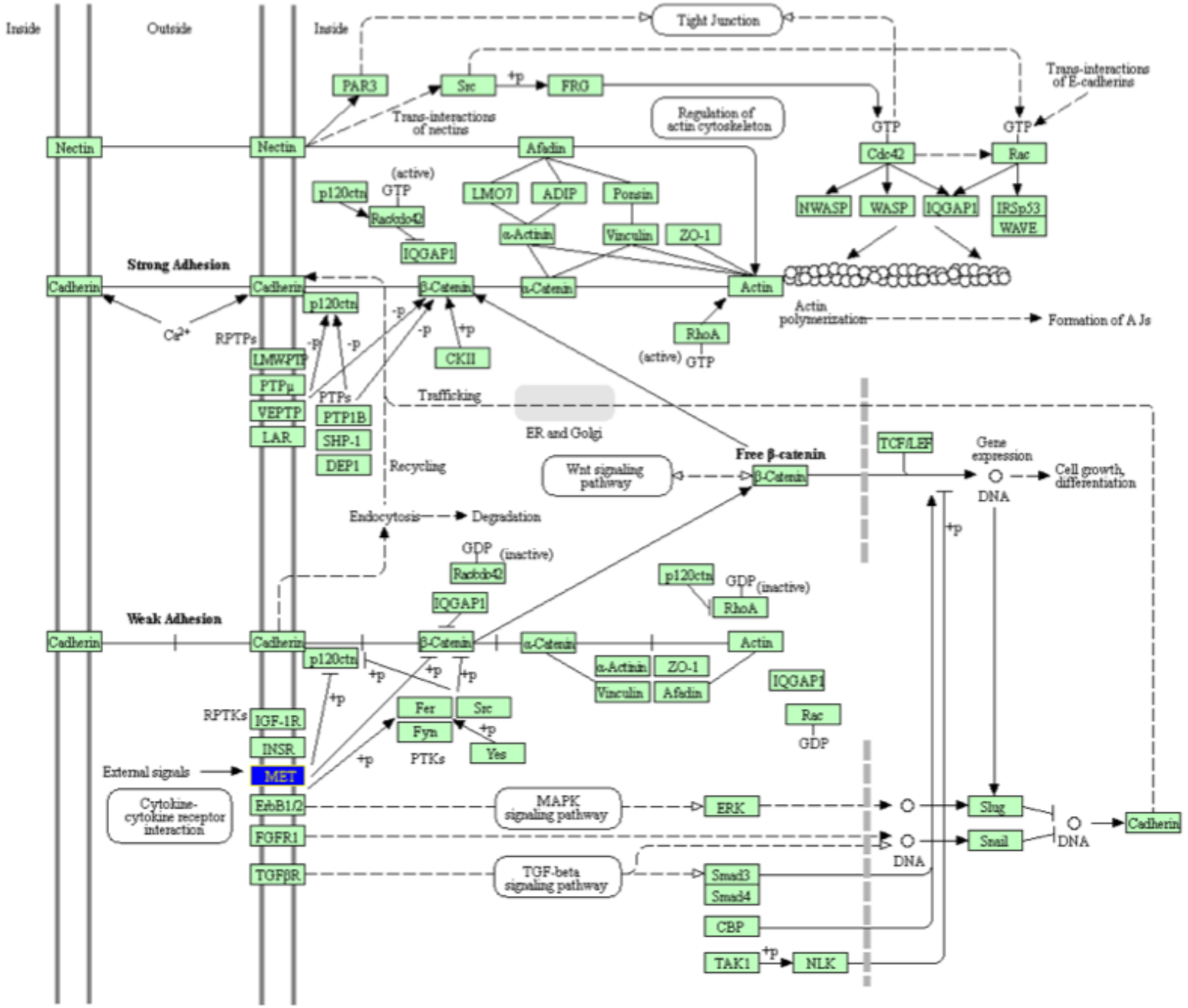


Figure 27: Adherens-junction pathway (source: KEGG), one of the top down-regulated pathways in the adult hippocampus, in response to prenatal alcohol exposure and environmental enrichment. The genes in blue represent the down-regulated differentially expressed genes.

4.2.3.4 Gene networks

Differentially expressed genes in the adult mouse hippocampus exposed to alcohol followed by environmental enrichment were analyzed in Genemania, to predict interacting molecular networks. The AE v AN network was made up of 35 nodes (with 20 out of 35 genes being novel additions based on various GeneMANIA interaction data) with 193 edges (Figure. 28). The Rho GTPase Activating Protein 36 (*Arhgap36*) gene, involved in p75 NTR receptor-mediated signaling was the main hub gene in this network with a degree score of 16. Based on GENEMANIA's output, the top predicted pathways identified were involved in TNF signaling, protein processing in the endoplasmic reticulum and the NOD-like receptor signaling pathway.

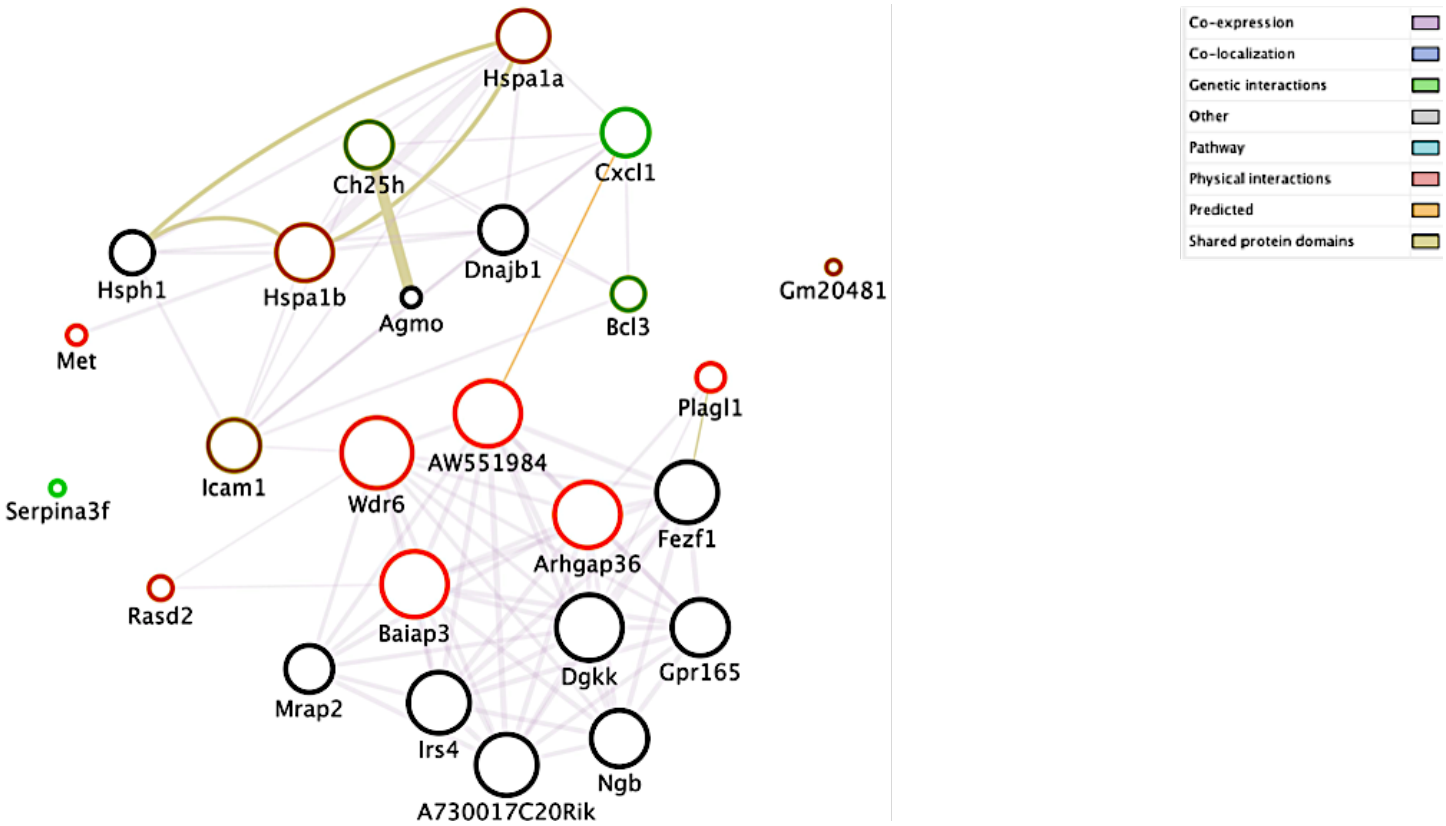


Figure 28: Gene network cluster for differentially expressed genes in response to prenatal alcohol exposure and environmental enrichment: The top clustered gene network showing differentially expressed genes from the alcohol enriched (AE) versus alcohol non-enriched (AN) group comparison. Red nodes represent down-regulated genes. Green nodes represent up-regulated genes. Black nodes indicate genes from the GeneMANIA database. The network was generated using GLay clustering algorithm and GeneMANIA. The size of the nodes (genes) indicates the degree of connectivity for each gene. The Rho GTPase Activating Protein 36 gene (*Arhgap36*) is the main hub gene (gene with the highest connectivity) for this network.

4.2.3.5 Transcription factor analysis

The online gene-expression analysis tool Enrichr was used to identify the top transcription factors for differentially expressed genes in alcohol enriched (AE) versus alcohol non-enriched (AN) group (Table 20). The top transcription factor for this comparison group was ETS1 (Avian Erythroblastosis Virus E26 Oncogene Homolog), which is involved in stem cell proliferation and neural development. The next two were STAT5A and ZC3H7A, which are involved in angiogenesis, neural cell specificity, and RNA regulation in nerve cells, respectively.

Table 20: Top three transcription factors of all up- and down-regulated genes associated with prenatal alcohol exposure during synaptogenesis and post-natal environmental enrichment (AE v. AN)

Transcription factor	Name of factor	Possible functions	p-value
ETS1	V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog	stem cell proliferation and neural development	0.0041
STAT5A	Signal Transducer And Activator Of Transcription 5A	angiogenesis, neural cell specifity	0.084
ZC3H7A	Zinc Finger CCCH-Type Containing 7A	regulating RNAs in nerve cells	0.0062

4.2.3.6 Summary of results: the effect of prenatal alcohol exposure and post-natal environmental enrichment

The effect of environmental enrichment on prenatal alcohol exposure has a small subtle (-1.2 to +1.2 fold-change range) effect on the majority (73%) of genes (as analyzed by the RNA-Seq platform) in the adult hippocampus. Interestingly, there was a wide range of fold-change values when comparing up-regulated and down-regulated genes. While the lowest down-regulated gene had a fold-change value of -1.89, the highest fold-change was 7.82. Altered biological processes included lymphocyte activation and stress response (up-regulated genes) while down-regulated genes were primarily involved in muscle-cell differentiation. One of the most highly altered pathways (with up-regulated genes) in response to environmental enrichment after alcohol-exposure involved the TNF-signaling pathway while the top pathway (with down-regulated genes) was related to the adherens-junction pathway. Gene network analysis implicates pathways involved in amino-acid metabolism, TNF-signaling and NOD-like receptor signaling.

These observations show that environmental enrichment in alcohol-exposed mice affects pathways involved in stress response but also cellular re-arrangement and structural development. While the former represents the biological response to alcohol exposure, the latter may point to the ameliorative effects of environmental enrichment. These may explain improved behavioural performance of alcohol exposed-enriched mice.

4.2.3.7 Validation of RNA-Seq selected results by quantitative RT-PCR

Six genes detected by RNA-Seq analysis were chosen for confirmation by real-time quantitative RT-PCR (qRT-PCR), selected based on their fold changes and prominence in biological functions and networks identified as altered following alcohol treatment and environmental enrichment (Figure. 29). All reactions were multiplexed with Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as an internal control. Genes chosen for qRT-PCR analysis were Heat shock protein 1 alpha (*Hsp1a*), Intercellular cell-adhesion molecule 1 (*Icam1*), Calcium sensing receptor (*Casr*), Collagen type III alpha 1 (*Col3a1*), Claudin 1 (*Cldn1*), Potassium channel inwardly rectifying subfamily J member 13 (*Kcnj13*). Fold-changes were calculated from deltaCt values. All genes confirmed as significantly different from controls (Student's t-test, $p < 0.05$).

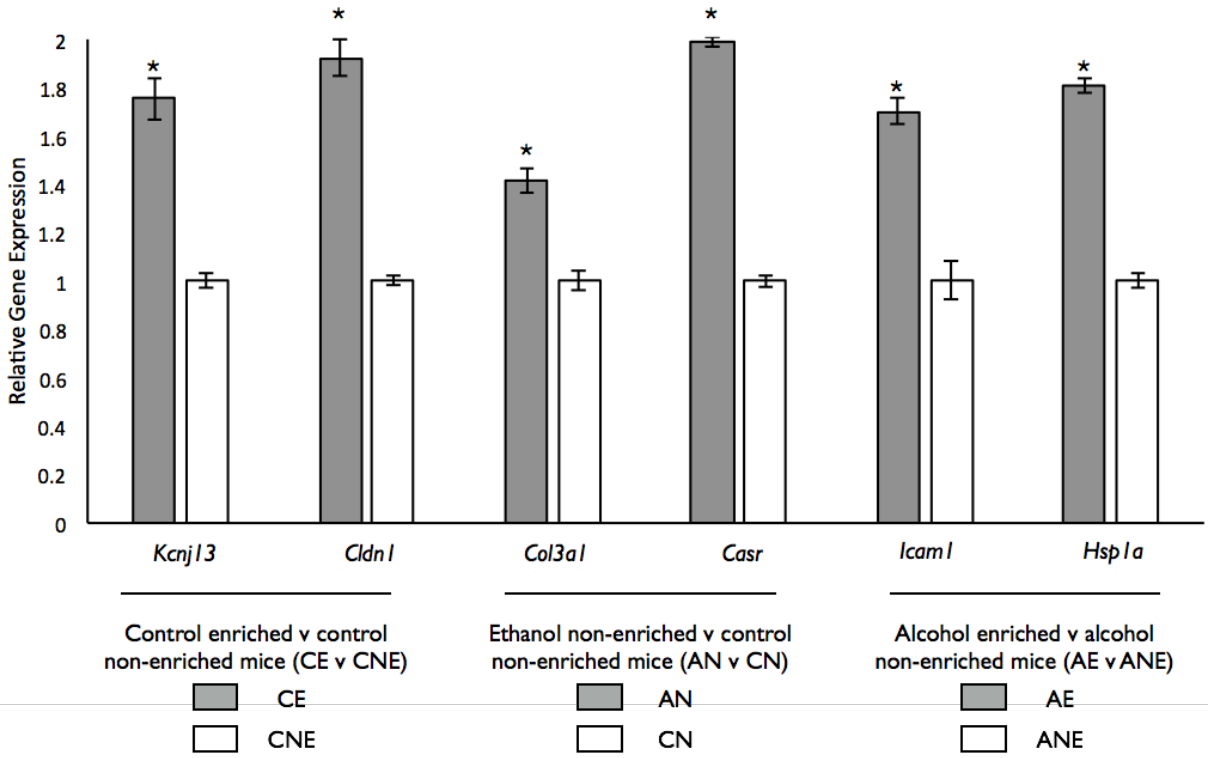


Figure 29: Quantitative RT-PCR validation of RNA-Seq identified changes in mRNA levels in the adult brains of alcohol-exposed enriched mice relative to the control group of alcohol-exposed non-enriched mice. Data are shown as mean (±SEM) average delta CT. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) is used as an internal control. Genes listed are calcium sensing receptor (*Casr*), claudin 1 (*Cldn1*), collagen type III alpha 1 (*Col3a1*), heat shock protein 1 alpha (*Hsp1a*), intercellular cell-adhesion molecule 1 (*Icam1*), and potassium channel inwardly rectifying subfamily J, member 13 (*Kcnj13*). Expression of all genes was significantly different from controls (specific comparisons wherever indicated) (Students' t-test, $p < 0.05$).

4.3 A comparison of gene-expression data as obtained and analyzed by different platforms and software

The results of this section will compare gene-expression results obtained and analyzed by my colleague, Celeste Cote and myself. Specifically, this section will be comparing results from:

(i) RNA-Seq (RE): RNA-Seq data analyzed on edgeR (my analysis)

(ii) RNA-Seq (RP): RNA-Seq data analyzed on Partek (analysis by Celeste)

(iii) Microarray (M) date analyzed on Partek (my analysis)

As I did, Celeste analyzed three main lists of differentially-expressed genes, namely, alcohol non-enriched (AN) versus control non-enriched (CN) mice, alcohol enriched (AE) versus alcohol non-enriched (AN) mice and control enriched (CE) versus control non-enriched (CN) mice. For the AN v CN group, she specifically examined gene-expression in the mouse hippocampus at post-natal day 85 after ethanol exposure during the peak of synaptogenesis on post-natal days 6 and 7. For the AE v AN group, she examined the gene-expression changes in the adult mouse hippocampus (post-natal day 85) after the mouse (i) had been exposed to ethanol on post-natal days 6 and 7 and then underwent environmental enrichment for 1.5 months. Finally, for the CE v CN group, she examined the gene-expression changes in the adult mouse hippocampus (post-

natal day 85) after the mouse underwent environmental enrichment for 1.5 months with no exposure to alcohol during any time of its neurodevelopment. The contents of this section compare the molecular results obtained from all three methods of analyses in terms of gene-expression data and pathway analysis.

4.3.1 Gene-expression data

Both the RNA-Seq Partek (RP) and Microarray (M) analyses generated a significantly larger number of differentially-expressed genes (DEG) compared to those generated from RNA-Seq edgeR (RE) analysis. However, while the DEG lists produced from RE and M were corrected for multiple comparisons (FDR corrected), the DEG list obtained from RP did yield less than three genes when corrected for multiple comparisons.

Fifty-three genes were found to be common between the RE, RP and M lists, all of which were differentially expressed in response to alcohol exposure during synaptogenesis (Figure. 30). Table 21 specifically lists the top five up- and down-regulated genes that were not only significantly altered but also found amongst all three platforms during prenatal alcohol exposure. The Insulin-Like Growth Factor Binding Protein 1 (*Igfbp 13*) was the top up-regulated gene and is involved in modulating the interaction of insulin-growth factors with cell-surface receptors and promoting cell migration. The Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit (*Gabra6*) gene was the top down-regulated gene and is critical for learning and memory processes in the hippocampus.

Among healthy mice, there were forty-nine genes, which were found to be common between the RE, RP and M lists that responded to environmental enrichment (Figure. 31). Table 22 specifically lists the top five up- and down-regulated genes that were not only significantly altered but also found amongst all three platforms during post-natal environmental enrichment. The top up-regulated gene was *Cldn1* (Claudin 1), which is an important component of epithelial tight-junctions. *Sntn* (Sentan, Cilia Apical Structure Protein) was the top down-regulated gene and is involved in bridging ciliary membranes and peripheral singlet microtubules.

In response to environmental enrichment after alcohol exposure, only eight genes were found to be common for the RE, RP and M lists (Figure. 32). Table 23 specifically lists the top up- and down-regulated genes that were not only significantly altered but also found amongst all three platforms during post-natal environmental enrichment and prenatal alcohol-exposure (Appendix 4 tabulates all genes common amongst the three platforms). *Ch25h* (Cholesterol 25-Hydroxylase), the top up-regulated gene is involved in lipid and bile metabolism, whereas *Balp3* (BAI1 Associated Protein 3), the top down-regulated gene is a brain-specific angiogenesis inhibitor.

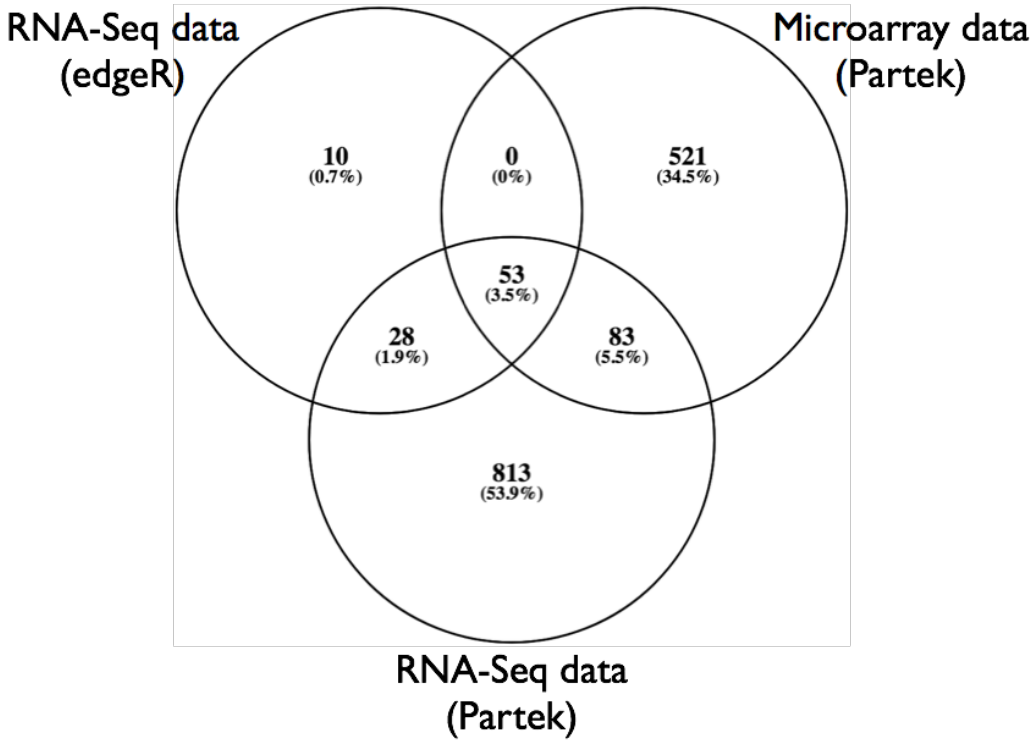


Figure 30: Venn diagram comparing the number of differentially expressed genes in response to prenatal alcohol exposure: A total of 53 genes were found to be common between all 3 different generated lists of differentially expressed genes in the adult hippocampus, in response to prenatal alcohol exposure. RNA-Seq data was analyzed using two different methods, edgeR and Partek whereas the RNA itself was processed using two different technologies, microarray and RNA-Seq.

Table 21. The top five up- and down-regulated differentially-expressed genes in the adult hippocampus (common to all three platforms) in response to prenatal alcohol exposure. RNA-Seq data was analyzed using edgeR and Partek, whereas the RNA itself was processed using microarrays or RNA-Seq technology.

		RNA-Seq (edgeR)		**RNA-Seq (Partek)**		**Microarray**	
Gene symbol	**Gene name**	**Fold change**	**p-value**	**Fold change**	**p-value**	**Fold change**	**p-value**
Igfbpl1	Insulin Like Growth Factor Binding Protein	1.58	0.02	3.01	0.03	1.32	0.02
Bace2	Beta-Site APP-Cleaving Enzyme 2	1.55	0.02	1.50	0.00	2.82	0.00
Gnb4	G Protein Subunit Beta 4	1.48	0.00	1.58	0.00	1.54	0.00
Eln	Elastin	1.34	0.00	1.21	0.01	2.27	0.00
Col1a1	Collagen Type I Alpha 1 Chain	1.13	0.00	1.41	0.00	2.18	0.00
Zc3hav1	Zinc Finger CCCH-Type Containing, Antiviral 1	-0.64	0.03	-1.24	0.03	-1.52	0.00
Plk5	Polo Like Kinase 5	-1.04	0.00	-1.46	0.05	-2.14	0.01
Il16	Interleukin 16	-1.24	0.05	-1.51	0.02	-0.85	0.00
Gkn3	Gastrokine 3	-1.35	0.03	-1.26	0.00	-2.26	0.01
Gabra6	Gamma-Aminobutyric Acid (GABA) A Receptor, Alpha 6	-2.69	0.04	-8.41	0.04	-1.52	0.02

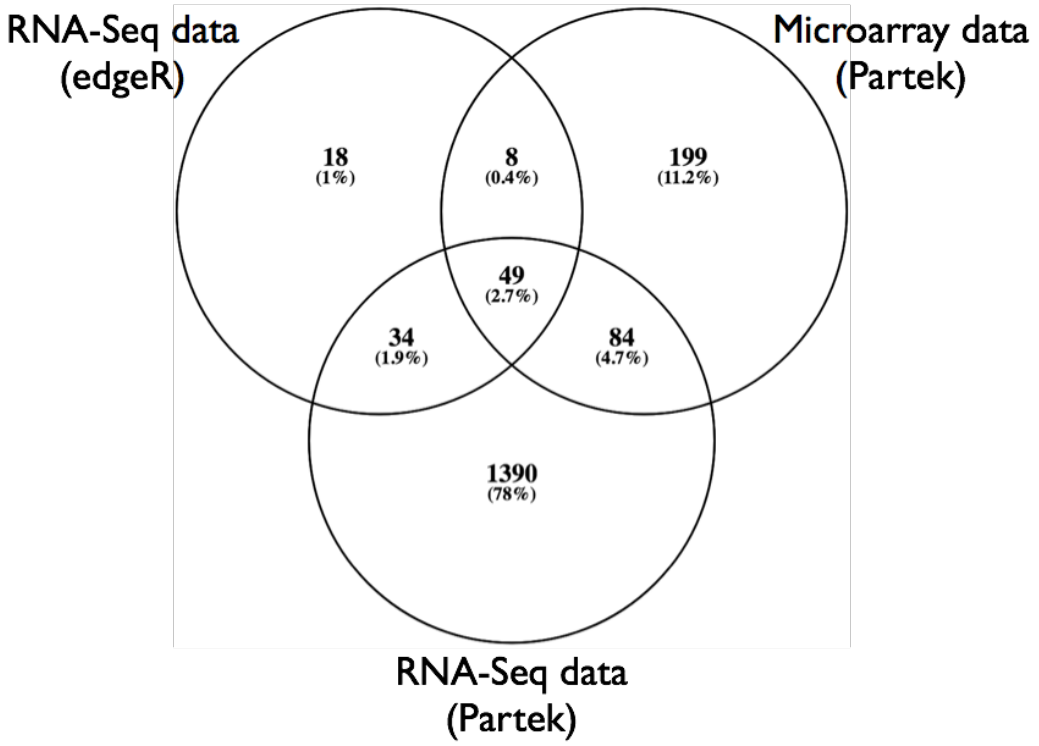


Figure 31: Venn diagram comparing the number of differentially expressed genes in response to post-natal environmental enrichment: A total of 49 genes were found to be common between all 3 different generated lists of differentially expressed genes in the adult hippocampus, in response to post-natal environmental enrichment. RNA-Seq data was analyzed using two different methods, edgeR and Partek whereas the RNA itself was processed using two different technologies, microarray and RNA-Seq.

Table 22. The top five up- and down-regulated differentially expressed genes in the adult hippocampus (common to all three platforms) in response to postnatal environmental enrichment. RNA-Seq data was analyzed using edgeR and Partek, whereas the RNA itself was processed using microarrays or RNA-Seq technology.

		RNA-Seq (edgeR)		**RNA-Seq (Partek)**		**Microarray**	
Gene symbol	**Gene name**	**Fold change**	**p-value**	**Fold change**	**p-value**	**Fold change**	**p-value**
Cldn1	Claudin 1	1.68	0.01	3.37	0.01	2.73	0.01
Bmp3	Bone Morphogenetic Protein 3	1.27	0.01	2.46	0.00	1.83	0.00
Glra3	Glycine Receptor Alpha 3	1.05	0.03	1.60	0.01	2.06	0.00
Cbln4	Cerebellin 4 Precursor	0.97	0.01	2.09	0.00	1.70	0.01
Gm14169	Predicted gene 14169	0.96	0.02	1.91	0.01	1.29	0.01
C4b	Complement C4B (Chido Blood Group)	-0.56	0.03	-1.46	0.02	-1.39	0.03
St18	ST18, C2H2C-Type Zinc Finger	-0.65	0.00	-1.40	0.00	-1.45	0.02
Tnfaip6	TNF Alpha Induced Protein 6	-0.78	0.01	-1.63	0.02	-1.52	0.01
Sntn	Sentan, Cilia Apical Structure Protein	-2.21	0.05	-3.94	0.01	-1.57	0.04

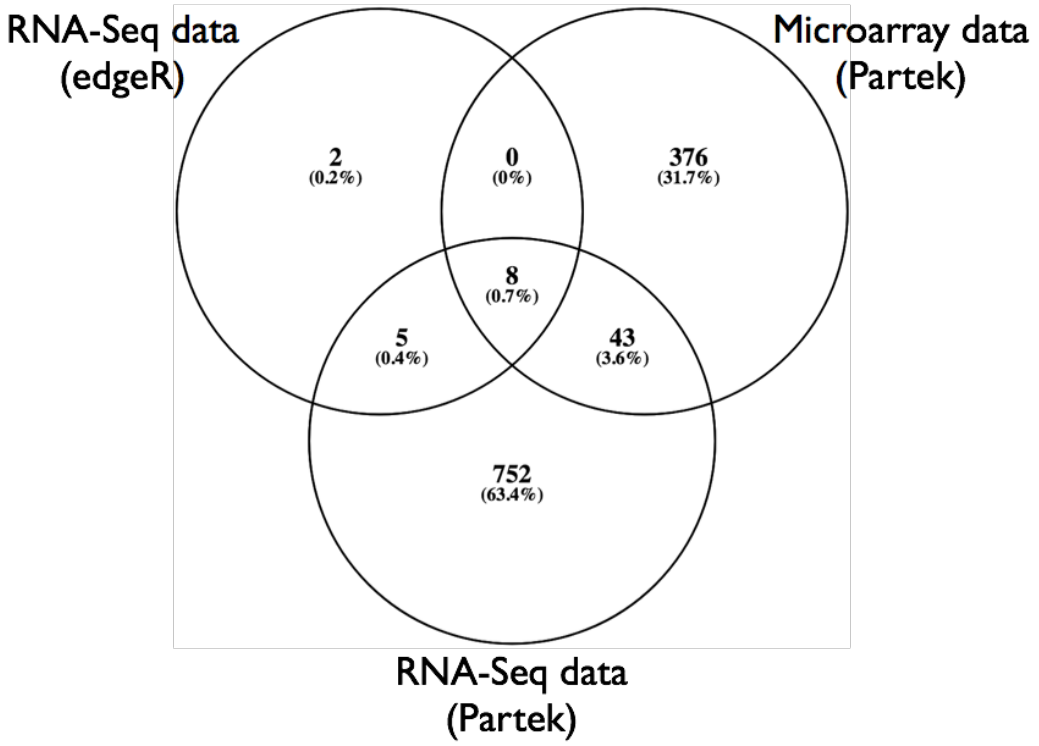


Figure 32: Venn diagram comparing the number of differentially expressed genes in response to prenatal alcohol exposure and post-natal environmental enrichment: A total of 8 genes were found to be common between all 3 different generated lists of differentially expressed genes in the adult hippocampus, in response to prenatal alcohol exposure and post-natal environmental enrichment. RNA-Seq data was analyzed using two different methods, edgeR and Partek whereas the RNA itself was processed using two different technologies, microarray and RNA-Seq.

Table 23. The top up- and down-regulated differentially expressed genes in the adult hippocampus (common to all three platforms) in response to prenatal alcohol exposure and postnatal environmental enrichment. RNA-Seq data was analyzed using edgeR and Partek, whereas the RNA itself was processed using microarrays or RNA-Seq technology.

		RNA-Seq (edgeR)		**RNA-Seq (Partek)**		**Microarray**	
GENE NAME		**FOLD CHANGE**	**p-value**	**FOLD CHANGE**	**p-value**	**FOLD CHANGE**	**p-value**
Ch25h	Cholesterol 25-Hydroxylase	3.303	0.009	10.054	0.023	1.841	0.041
Icam1	Intercellular Adhesion Molecule 1	2.297	0.001	5.144	0.020	2.420	0.025
AW551984	Expressed sequence AW551984	-1.023	0.001	-1.998	0.000	-1.397	0.026
Baiap3	BAI1 Associated Protein 3	-1.145	0.000	-1.678	0.030	-1.237	0.003

4.3.2 Pathway-analysis data

All differentially expressed genes obtained from RNA-Seq Partek (RP), RNA-Seq edgeR (RE) and Microarray (M) lists were analyzed for uncovering gene ontologies and significant biological pathways using ConsensusPathDB.

In response to prenatal alcohol exposure, ConsensusPathDB identified a number of biological processes related to multicellular organismal development, response to external stimulus, and cell adhesion (Table 24). Interestingly, most of these annotations comprised the *Col1a1* and *Dcn* genes, both of which play structural roles in cells and contribute to cellular organization and tissue shape. In continuation of previously uncovered 'themes' of structural remodeling of hippocampal neuro-anatomy in response to prenatal alcohol exposure (my analysis as done in section 4.2), pathway analysis uncovered a number of pathways involved in Semaphorin and L1CAM signaling, and extracellular matrix organization (Table 25). In fact, as Table 26 shows, extracellular matrix organization was the only pathway that was common across all three platforms, further confirming the critical role of *Col1a1* and *Dcn* genes in response to prenatal alcohol exposure.

In response to post-natal environmental enrichment, ConsensusPathDB identified (Tables 27 and 28) biological processes related to structural plasticity and remodeling, (namely, the cell adhesion process) and the cell-to-cell junction organization pathway with the involvement of the Claudin family of genes

(*Cldn1*, *Cldn5*, and *Cldn18*). However, given the effect of environmental enrichment, apart from structural plasticity, there was also evidence of functional plasticity with the ion channel complex and neuromuscular junction processes being involved. Genes such as *Kcnj13*, *Gabra6* and *Camk2d* (previously described in sections 4.2.1 to 4.2.3.6) are critical in learning and cognition processes. In fact, the ligand-gated ion-channel transport was a significantly altered molecular pathway (involving the *Gabra2* gene) that was found when analyzing all forty-nine genes common to the CE v CN group amongst all three platforms. The Circadian Rhythm pathway not only came up in the gene-ontology and pathway analysis, but it was the only significantly altered pathway (containing down-regulated genes) for all three platforms in response to environmental enrichment.

While there was no common pathway (Table 32) among all three platforms in response to prenatal alcohol exposure and post-natal environmental enrichment, the regulation of mRNA stability was found to be the most significantly pathway (containing up-regulated genes) common among all three platforms with genes coding for heat shock proteins being altered.

Table 24: Gene ontology (GO) of the fifty-three genes that were common to all three platforms in the adult hippocampus following prenatal ethanol exposure (AN v. CN)

GO name	p-value	Up-regulated genes
Extracellular matrix binding	0.0001	*Dcn; Nid1; Eln; Slit2*
Multicellular organismal development	0.0001	*Dcn; Cldn11; Tnc; Col1a1*
Response to external stimulus	0.0007	*Dcn; Col1a1*
Leukocyte migration	0.0089	*Cxadr; Il33; Slit2; Cd24a*
Collagen synthesis	0.0169	*Dcn; C1ql2; Col1a1*

Table 25: Pathways uncovered for those differentially expressed genes in the adult hippocampus following prenatal alcohol exposure that are common among all three platforms (AN v CN)

Pathway source	**Pathway name**	**p-value**	**Up-regulated genes**
Reactome	Axon guidance	0.0045	*Dpysl5; Dpysl3; Nfasc; Slit2; Dcx; Cd24a*
Reactome	CRMPs in Sema3A signaling	0.0139	*Dpysl5; Dpysl3*
Reactome	L1CAM interactions	0.0200	*Nfasc; Cd24a; Dcx*
Reactome	Extracellular matrix organization	0.0512	*Dcn; Nid1; Tnc; Col1a1*

Table 26. Pathways uncovered by each of the three platforms for differentially expressed genes in the adult hippocampus following prenatal ethanol exposure that are common among all three platforms (AN v CN)

Effect of prenatal alcohol exposure				
	Direction of gene regulation	**Pathway name**	**p-value**	**Genes**
RNA-Seq (edgeR)	Up	Collagen biosynthesis and modifying enzymes	0.004	*Col3a1; Col4a5*
	Up	ECM-receptor interaction	0.0061	*Col1a1, Dcn*
	Down	GABAergic synapse	0.0061	*Gabra6*
RNA-Seq (Partek)	Up	Collagen biosynthesis and modifying enzymes	0.000	*Col3a1; Col4a5*
	Up	ECM-receptor interaction	0.005	*Col1a1, Dcn*
	Down	GABAergic synapse	0.116	*Gabra6*
Microarray	Up	ECM-receptor interaction	0.0198	*Col1a1, Dcn*

Table 27: Gene ontology (GO) of the forty-nine common genes that were common to all three platforms in the adult hippocampus following post-natal environmental enrichment (CE v. CN)

GO name	p-value	Up-regulated genes
Ion channel complex	0.00158	*Camk2d; Glra3; Gabra2; Kcnf1;*
Circadian rhythm	0.0027	*Arntl; Dbp; Per3; Bhlhe41*
Cell adhesion	0.0035	*Cxadr; Cdh18; Tnfaip6; Cntnap3; Cldn1; Arhgap6; Angpt1; Cldn5*
Neuromuscular junction	0.0343	*Camk2d; Cxadr*

Table 28: Pathways uncovered for those differentially expressed genes in the adult hippocampus following post-natal environmental enrichment that are common among all three platforms (CE v CN)

Pathway source	Pathway name	p-value	Up-regulated genes
Reactome	Ligand-gated ion channel transport	0.000	*Glra3; Glra2; Gabra2*
KEGG	Circadian rhythm	0.002	*Per3; Bhlhe41; Arntl*
Reactome	Cell-cell junction organization	0.029	*Cdh18; Cldn1*

Table 29: Pathways uncovered by each of the three platforms for differentially expressed genes in the adult hippocampus following post-natal environmental enrichment that are common among all three platforms (CE v CN)

Effect of post-natal environmental enrichment				
	Direction of gene regulation	**Pathway name**	**p-value**	**Genes**
RNA-Seq (edgeR)	Up	Neuroactive ligand-receptor interaction	0.023	*Gabra2*
	Up	Ligand-gated ion channel transport	0.001	*Gabra2*
	Down	Circadian rhythm	0.0001	*Per1, Per3*
RNA-Seq (Partek)	Down	Circadian rhythm	0.000	*Per1, Per3*
Microarray	Up	Neuroactive ligand-receptor interaction	0.001	*Gabra2*
	Up	Ligand-gated ion channel transport	0.010	*Gabra2*
	Down	Circadian rhythm	0.0433	*Per1, Per3*

Table 30: Gene ontology (GO) of the eight common genes that were common to all three platforms in the adult hippocampus following prenatal ethanol exposure and post-natal environmental enrichment (AE v. AN)

GO name	p-value	Up-regulated genes
Muscle cell differentiation	0.1087	*Plagl1; AW551984*

Table 31: Pathways uncovered for those differentially expressed genes in the adult hippocampus following prenatal alcohol exposure and post-natal environmental enrichment that are common among all three platforms (AE v AN)

Pathway name	**p-value**	**Up-regulated genes**
Destabilization of mRNA by AUF1	0.0001	*Hspa1b; Hspa1a*

Table 32: Pathways uncovered by each of the three platforms for differentially expressed genes in the adult hippocampus following prenatal ethanol exposure and post-natal environmental enrichment that are common among all three platforms (AE v AN)

Effect of prenatal alcohol and post-natal environmental enrichment				
	Direction of gene regulation	**Pathway name**	**p-value**	**Genes**
RNA-Seq (edgeR)	Up	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements	5.35E-05	*Hspa1a, Hspa1b*
RNA-Seq (Partek)	Up	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements	0.004	*Hspa1a, Hspa1b*
Microarray	Up	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements	0.015	*Hspa1a, Hspa1b*

CHAPTER 5: DISCUSSION

This project is based on the central hypothesis that (i) post-natal environmental enrichment ameliorates FASD-related behavioural deficits that arise due to prenatal alcohol exposure and that (ii) the amelioration of these deficits is potentially associated with and characterized by specific changes in hippocampal gene expression and associated molecular pathways.

Specifically, this project recapitulated previously established behavioural and cognitive deficits in a mouse model of prenatal alcohol exposure during synaptogenesis (Kleiber et al., 2012, 2014b; Mantha, 2013; Mantha et al., 2014). Through high-throughput RNA-sequencing analyses, this project also showed that these deficits are associated with altered gene-expression patterns and molecular pathways in the hippocampus. Also, this project demonstrated that a sustained rehabilitative strategy of post-natal environmental enrichment could ameliorate some, if not all aspects of behavioural deficits that have been brought about by prenatal alcohol exposure. It also showed the limitation of environmental enrichment to fully reverse anxiety deficits and visuospatial-cognitive deficits (that arise due to prenatal alcohol exposure) thereby demonstrating the long-term, permanent effects of alcohol exposure during neurodevelopment. Lastly, this project demonstrated that environmental enrichment improves behavioural performance in healthy mice as well, and uncovered hippocampal-specific, gene-expression patterns and molecular pathways associated with such improvements.

This chapter is divided into multiple sections:

1. Sections 5.1 to 5.3 deal with a discussion of behavioural results.

2. Sections 5.4 and 5.5 deal with a discussion of molecular results.

5.1 The effect of post-natal environmental enrichment on FASD-related anxiety deficits

In this thesis, the Light-Dark Box (LDB) test and the Elevated-Plus Maze (EPM) test were used to characterize anxiety phenotypes in mice. To briefly recap the results, the EPM test showed no difference in anxiety behaviour between alcohol-exposed non-enriched mice and control non-enriched mice (Figure. 11). On the other hand, as shown in the LDB test, there was a significant deterioration in anxiety behaviour, with alcohol-exposed non-enriched mice being considerably more anxious compared to control non-enriched mice (Figure. 12). The difference in behavioural observations for the same behavioural measure as conducted by two different tests points to the differential sensitivity of the multiple methods testing of stress- and anxiety-like endophenotypes in animal models (Crawley, 2000; Lathe, 2004; van der Staay et al., 2009). An integration of both of these tests under a single test-apparatus has been previously suggested (Chadman et al., 2009; Davies, 2010; Lipp et al., 2005; Wahlsten et al., 2003).

Two possible explanations for the lack of difference the anxiety behaviour between control non-enriched mice and alcohol-exposed non-enriched mice in the

EPM test may come from previous studies that have pointed out (i) the modulation of impulsive or risk-taking behaviour in the alcohol-exposed mice (Pohorecky, 1991), and (ii) the anxiolytic effects of alcohol exposure in general (Frye et al., 1981). Children with FASD have also been known to show impulsive and risk-taking behaviour (Olson et al., 1998; Streissguth et al., 2004). A further dissection of the differences between anxiety-like, and impulsive and risk-taking behaviour via a more comprehensive behavioural testing might allow for a more satisfactory explanation.

Maternal alcohol consumption during pregnancy has been shown to confer risk for adult anxiety and depressive disorders in FASD-individuals (Kelly et al., 2000; Streissguth et al., 2004). Also, previous studies that link maternal binge drinking during pregnancy to anxiety and depressive behaviours in alcohol-exposed individuals implicate the aberrant functioning of the hypothalamic-pituitary-adrenal (HPA) axis either via the alteration of cortisol or adrenocorticotropin (ACTH) levels, or the aberrant alteration of HPA-axis specific regulator genes either at the basal resting level or under stressed conditions (Horst et al., 2013; Jacobson et al., 2011). Interestingly, anxiety behaviour in alcohol-exposed non-enriched mice also appears to be dependent on the timing of alcohol exposure. For example, previous studies from the Singh lab have shown no differences between alcohol-exposed non-enriched and control non-enriched mice in either EPM or LDB tests in a first-trimester-equivalent

exposure but have revealed such differences in a second-trimester equivalent exposure paradigm (Mantha, 2013).

In this project, both groups of enriched mice (alcohol-exposed enriched mice and control enriched mice) showed ameliorated levels of anxiety behaviours in the EPM and LDB tests, when compared to the alcohol-exposed non-enriched mice and control non-enriched mice respectively. At the time of writing this thesis, there are currently no studies involving pre- and post-natal alcohol-exposure rodent models explaining the ameliorative effects of environmental enrichment on anxiety behaviour in cellular, molecular or physiological terms either in the whole-brain or the hippocampus specifically. So far, only one study has demonstrated the ameliorative effect of voluntary exercise (wheel running) on anxiety deficits in a third-trimester alcohol exposure mouse model (Thomas et al., 2008). Given that the environmental enrichment model used in this project used running wheels (and voluntary exercise) as a part of the rehabilitative paradigm rather than treating it as a separate entity, it is difficult to quantify the contribution of voluntary exercise alone in either the amelioration of the anxiety in alcohol-exposed enriched mice. However, the general observation that environmental enrichment lowers anxiety levels in both healthy mice and mice models of complex disorders is re-iterated by numerous previous studies (Cymerblit-Sabba et al., 2013; Ilin and Richter-Levin, 2009; Kovesdi et al., 2011; Nithianantharajah and Hannan, 2006; Pietropaolo et al., 2006; Schrijver et al., 2002; Vivinetto et al.,

2013).

The primary hippocampal circuits involved in anxiety behaviours in pre- and post-natal alcohol-exposure rodent models include the altered modulation of NR1 and NR2A subunits of N-methyl-D-aspartate receptors (NMDAR) and aberrant development of the cholinergic and serotonergic termini in the CA1 and CA3 regions of the hippocampus (Hellemans et al., 2008, 2010; Olney et al., 2000a; Redila et al., 2006). Previous studies have shown that environmental enrichment alters the gene expression of not only glucocorticoid, cholinergic and serotonergic receptors but also neurotrophins and cell-adhesion molecules that are regulated via the glucocorticoid pathway (Fox et al., 2006; Hamilton et al., 2014; Laviola et al., 2008). It is, thus, probable that by specifically targeting hippocampal circuitry and the HPA-axis in alcohol-exposed-enriched mice, environmental enrichment can lower anxiety deficits that are characteristic of prenatal alcohol exposure.

The results of this project reiterate the importance of social, physical and cognitive stimulation in reducing anxiety deficits for children who have been prenatally exposed to alcohol. A number of studies have shown the positive effects of specialized group-classes, counseling sessions, directed play-time and extended parent-child interaction to nurture social and communication skills in prenatally alcohol-exposed children (Bertrand, 2009; Hannigan, 1996;

Kodituwakku, 2010; Kodituwakku and Kodituwakku, 2011; Paley and O'Connor, 2009).

5.2 The effect of post-natal environmental enrichment on FASD-related cognitive deficits

5.2.1 Recognition memory

As found in this thesis, there was no difference in recognition-memory performance between alcohol-exposed non-enriched mice and control non-enriched mice (Figure. 13). In other words, prenatal alcohol exposure used did not affect recognition memory performance in mice. Previous studies of prenatal alcohol exposure in both, children (Astley et al., 2009; Hamilton et al., 2003; Manji et al., 2009; Uecker and Nadel, 1996; Willford et al., 2004) and rodents (Cippitelli et al., 2010; Popović et al., 2006; Ryabinin et al., 2002), have shown that recognition memory remains intact in-spite of prenatal alcohol exposure during neurodevelopment.

A possible explanation for these observations (including results from this project) is that prenatal alcohol exposure, while severely damaging brain areas involved in spatial cognitive tasks (as evidenced by the results of the Barnes Maze test), spares those areas that underlie object-recognition tasks. Furthermore, lesion studies, for example, implicate the perirhinal cortex as a one of the most critical brain regions involved in recognition memory, but not spatial memory (Barker

and Warburton, 2011; Ennaceur et al., 1989; Winters et al., 2008), yet it is the hippocampus proper, rather than the perirhinal cortex, that has been shown to be one of the most detrimentally affected brain regions due to the teratogenic effects of alcohol, especially during synaptogenesis (Crews et al., 2003; Olney et al., 2000a; Young and Olney, 2006).

Another piece of evidence posits the possible neuro-modulatory role of the medial prefrontal cortex (mPFC), a critical brain region involved in cognition, working memory and executive function (Eichenbaum, 1999; Eichenbaum and Cohen, 2014; Singer, 2013). In a study published by Cippitelli et. al., in 2012, prenatal alcohol exposure did not cause any neurodegeneration in the mPFC (Cippitelli et al., 2010), thereby strengthening the hypothesis that the mPFC may also be involved in keeping recognition memory intact in alcohol-exposed mice. In healthy mice, hippocampal lesion studies indicate that the interaction of the hippocampus with the perirhinal or prefrontal cortex is crucial for object recognition. Importantly, the 24-hr delayed NOR test (like the one done in this project) shows no deficit in recognition memory performance even with severe hippocampal lesions, instead implicating the crucial function of the perirhinal and prefrontal cortices in successful object recognition (Broadbent et al., 2010; Cohen and Stackman Jr., 2014; Dere et al., 2007).

In this project, enriched groups of mice (both alcohol-exposed enriched

mice and control-enriched mice) showed a significant improvement in recognition memory performance, confirming the observations of previous studies in both healthy (Ilin and Richter-Levin, 2009; Laviola et al., 2008; Marashi et al., 2003) and diseased rodents (Christie et al., 2005; Nithianantharajah and Hannan, 2006; Patten et al., 2013; Rampon and Tsien, 2000). As shown in previous studies, environmental enrichment has been shown to up-regulate genes involved in molecular pathways related to synaptic and structural plasticity (Kempermann, 2002; Nithianantharajah and Hannan, 2006; Rampon et al., 2000). Thus, it is possible that environmental enrichment induces the formation of synapses and facilitates neurotransmitter receptivity between the hippocampus and the perirhinal and prefrontal cortices in both alcohol-exposed enriched mice and control-enriched mice. Thus, the results of the NOR test indicate that prenatal alcohol exposure does not negatively impact recognition memory, but the impact of environmental enrichment is suitably great to improve the recognition memory performance in both alcohol-exposed and healthy mice.

5.2.2 Spatial learning and memory

In this project (Figure. 14), the Barnes Maze test demonstrated the permanently damaging effects of prenatal alcohol exposure on neurodevelopment as demonstrated by the consistently poor performance of alcohol-exposed non-enriched mice in the Barnes Maze test. Specifically, the latency to reach the target hole and time spent in the target-hole quadrant was, respectively, greater and

lesser for alcohol-exposed non-enriched mice, when compared to control non-enriched mice. These results are supported by previous literature, which demonstrate the long-term detrimental effects of prenatal alcohol exposure on learning and memory, particularly with hippocampal-dependent learning circuitry involved in visuospatial memory and performance (Gabriel et al., 2002; Savage et al., 2002; Zou et al., 2009).

Broadly speaking, the hippocampus is thought to play a critical role in spatial cognition (Burgess et al., 2002; Eichenbaum, 1999). Its sensitivity to the teratogenic and apoptotic effects of ethanol, particularly on hippocampal circuitry such as decreases in cell volume within CA1 and CA3 pyramidal cells and dentate gyrus neurons (Crews et al., 2003; Olney et al., 2000a; Young and Olney, 2006), all of which are critical in spatial navigation (D'Hooge and De Deyn, 2001; Fossella and Casey, 2006; Swanson and Bota, 2010; Vaillend et al., 2002) may explain the deficits in spatial cognitive performance as demonstrated by alcohol-exposed non-enriched mice.

Both the short- and long-term memory recall trials on day 5 and day 12 exposed the visuospatial learning deficits reflected during the acquisition trails (Figures 15 and 16). The results of this project indicate that ethanol exposure impairs learning during task acquisition and also the ability of the mouse to recall visuospatial relationships between the maze layouts, visual clues on the wall and

the target-hole itself. These results are a continued confirmation of previous studies of the effects of prenatal alcohol exposure on spatial cognition in both rodents (Becker et al., 1996; Redila et al., 2006; Savage et al., 2002) and children (Hamilton et al., 2003; Olson et al., 1998; Uecker and Nadel, 1996; Willford et al., 2004). Particularly relevant to this project is the 'stepping stone' task that has been shown to be very sensitive to the effects of prenatal alcohol exposure. In this task, the subject has to navigate a collection of stones to find an invisible path (both in the long and short versions). Adolescents with FAS, for example, exhibit deficits in both short-term recall and spatial learning. Also, children who have been exposed to alcohol gestationally require more trials to reach their first successful task completion while also making more errors as compared to healthy controls (Olson et al., 1998).

This project also demonstrated the possibility of partial amelioration of these spatial cognition deficits via environmental enrichment, as shown by the improved cognitive performance by alcohol-exposed enriched mice. Previous research has shown that a combination of social, cognitive and motor stimuli, or simply, extensive voluntary exercise can successfully ameliorate spatial navigation deficits in alcohol-exposed mice (Berman et al., 1996; Christie et al., 2005; Hannigan et al., 2007; O'Leary-Moore et al., 2006; Wainwright et al., 1993). In children who have been prenatally exposed to alcohol, a variety of

activities designed to stimulate cognitive processes within enriched social, academic, physical and personal settings have been shown to improve not only spatial memory performance but various aspects of executive functioning and working memory (Bertrand, 2009; Kodituwakku, 2010; Koren, 2011; Manji et al., 2009; Paley and O'Connor, 2009; Peadon et al., 2009; Rasmussen et al., 2009).

The improvement in visuospatial cognitive performance after environmental enrichment in both alcohol-exposed enriched mice and control-enriched mice can be attributed to a variety of factors such as a significant increase in neurotrophic factors that stimulate the growth of neurons, synapses and glial cells, increased neurogenesis, enhanced long-term potentiation and stimulation of dendritic branching (Helfer et al., 2009; Kempermann et al., 1997; Kuhn et al., 1996; Nithianantharajah and Hannan, 2011; Warraich and Kleim, 2010). Voluntary exercise in particular, has been shown to have a strong effect of dendritic cell volume, BDNF up-regulation and hippocampal angiogenesis, all of which have been associated with improvements in spatial cognition (Fabel et al., 2009; Schrijver et al., 2002; Wolf et al., 2006).

5.3 Summary of behavioural results

This project used a prenatal alcohol-exposure mouse model that mimicked maternal alcohol exposure during the third trimester of pregnancy, specifically

during the initiation and peak of synaptogenesis (Tables 1 and 2). The purpose of using this model was threefold:

1. The model was intended to recapitulate in mice (which it did), most, if not all the behavioural (anxiety) and cognitive (learning and memory) deficits typical of FASD-related abnormalities. As evident from the results of the Elevated Plus Maze test and the Light Dark Box test, anxiety-related deficits were not captured in a definitive, convincing manner. This failure to capture one of the most commonly diagnosed behavioural deficits in FASD individuals, namely, elevated and abnormal stress and anxiety reactivity, is a telling sign of the fallibility of mouse behavioural modeling in general. However, the battery of four phenotyping tests performed represents one of the very few comprehensive assessments of both behaviour and cognition in a mouse model of prenatal alcohol exposure anywhere in literature.
2. This model was used to examine the effects of environmental enrichment on behavioural and cognitive deficits due to prenatal alcohol exposure. As found in this project, alcohol-exposed mice that underwent environmental enrichment not only exhibited lower levels of anxiety and higher levels of exploratory behaviour, but they were also better at learning and memorizing new information. These findings represent a novel addition to the field of FASD research pertaining to exploring various rehabilitative

avenues to ameliorate FASD-related behavioural and cognitive abnormalities. Overall, these results were critical in (i) establishing environmental enrichment as a potential rehabilitative tool and (ii) justifying the pursuit of ascertaining the molecular correlates of behavioural and cognitive amelioration due to environmental enrichment in this model of prenatal alcohol exposure.

3. Lastly, environmental enrichment failed to completely reverse FASD-related behaviour. The fact that alcohol-exposed enriched mice did not catch up to the same level as control-enriched mice were indicative of the long-lasting damage by alcohol. Long-term environmental enrichment protocols may be needed to establish if alcohol-specific deficits could be fully ameliorated.

Figure 33: Summary of behavioural and molecular results of post-natal environmental enrichment in a mouse model of prenatal alcohol exposure

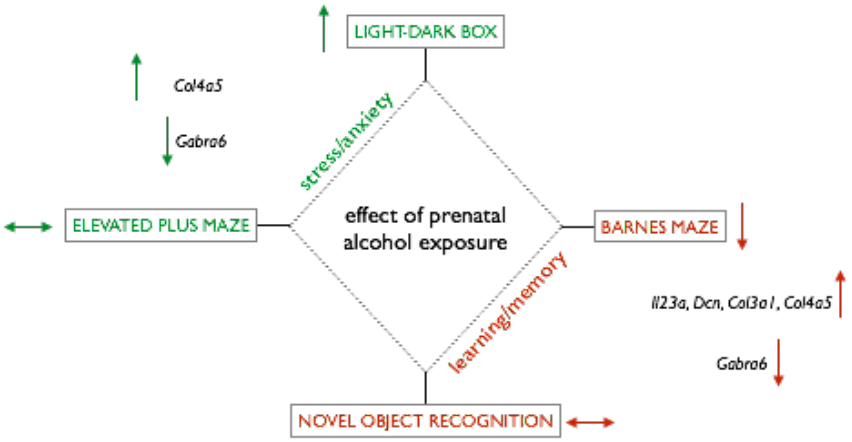


FIGURE 33 (A)

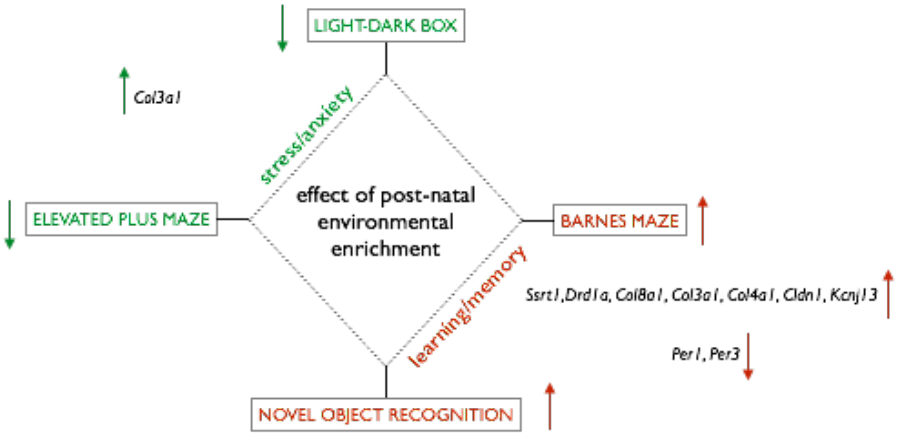


FIGURE 33 (B)

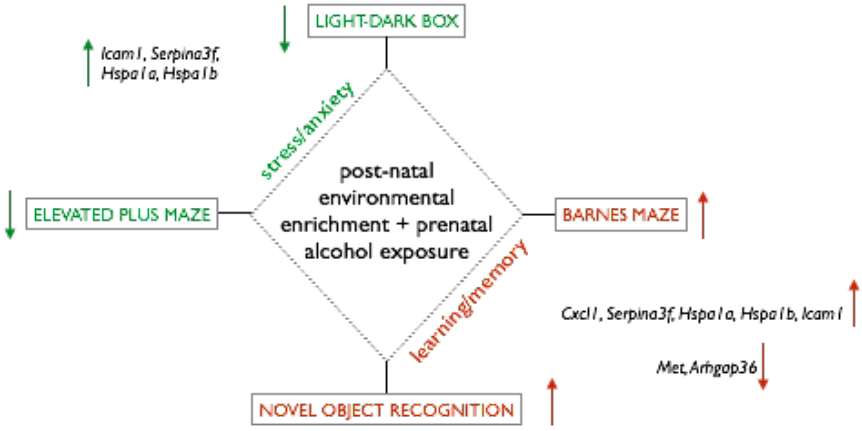


FIGURE 33 (C)

5.4 The effects of prenatal alcohol exposure on gene expression patterns in mice

The following discussions specifically deal with data (and subsequent analysis) obtained from the RNA-Seq platform, analyzed by edgeR only. A complete discussion comparing and contrasting (i) RNA-Seq versus microarray technology, and (ii) edgeR versus Partek Genomics Suite is detailed in chapter 6, sections 6.2.2 and 6.2.3 respectively. Chapter 6 section 6.2.4 discusses the comparison of results (wherever applicable) obtained using all three platforms (RNA-Seq data analyzed by edgeR, RNA-Seq data analyzed by Partek, Microarray data analyzed by Partek).

Previous literature has established the (i) teratogenic effects of ethanol on neurodevelopment, (ii) the long lasting behavioural abnormalities typical of FASD and (iii) the correlation of such behavioural deficits in aberrant gene expression patterns and molecular pathways (Kleiber et al., 2014b; Laufer, 2016; Laufer et al., 2013; Mantha et al., 2014). The novelty of this project lays in (i) establishing a working model of environmental enrichment to examine its effects on behavioural measures of alcohol-exposed mice, (ii) correlating such measures with high throughput gene-expression studies in mice hippocampi, and (iii) identifying biologically relevant molecular mechanisms associated with behavioural amelioration in alcohol-exposed enriched mice for further studies in order to design more effective rehabilitative (behavioural and pharmacological)

therapies for FASD-affected individuals.

5.4.1 The effect of prenatal alcohol exposure on gene-expression is extensive yet subtle

In order to discern the effects of alcohol exposure on neurodevelopment, gene-expression data (obtained from RNA-Seq) was first analyzed by edgeR to obtain the list of differentially expressed genes (DEG). This list was then further analyzed for gene-annotation, pathway and transcription factor analysis, and network analysis using ConsensusPathDB, GeneMania and Cytoscape. Thus, the first list of differentially expressed genes was obtained after analyzing alcohol non-enriched mice versus control non-enriched mice (AN v CN) to ascertain how alcohol alters hippocampal gene-expression patterns.

This project found that the extent of gene-expression alterations due to alcohol exposure are significant yet subtle for the majority of hippocampal genes. A total of 78% of genes showed significant alterations in the ± 1.2 fold change range. This is in line with previous studies from our laboratory, with the interesting exception being that the gene-expression results obtained previously were done on the whole brain and using microarrays (Kleiber, 2015; Laufer et al., 2013; Mantha et al., 2014). Previous literature implicates ethanol-induced apoptosis of various cell types that subsequently changes not only the overall gene expression patterns but also the cellular composition of the brain (Gil-Mohapel et

al., 2010; Olney et al., 2000a; Redila et al., 2006; Young and Olney, 2006). For example, developmental processes such as cell-growth, proliferation and migration, all dependent on external cues, are disrupted due to ethanol exposure.

The *Il23a* (Interleukin 23 subunit alpha) gene was the most highly up-regulated gene (6.29 fold-change). Interleukins are critical cytokines regulating immune and inflammatory responses. Also, the dysregulation of inflammatory cytokines has been shown to impair cognitive processes in mice (Cottrell and Seckl, 2009; Mehler, 2008; Perera et al., 2008; Titterness and Christie, 2012). Our laboratory has consistently shown alcohol exposure results in the significant alteration of various interleukin genes (Kleiber et al., 2014b; Laufer et al., 2013; Mantha et al., 2014).

5.4.2 Prenatal alcohol exposure results in massive dysregulation of structural integrity during neurodevelopment

Gene-ontology analysis for this project uncovered the dysregulation of critical genes in the hippocampus specifically related to collagen synthesis, extracellular-matrix formation, and cell adhesion (Table 12). This indicated that the bulk of alcohol's effects were involved in major structural disruptions that began during neurodevelopment, but whose consequences in terms of behavioural deficits continued into adulthood. Similarly, pathway analysis of up-regulated genes also implicated the same critical pathways, namely, collagen biosynthesis and

extracellular matrix organization (Table 13).

Within these pathways, the *Col3a1* and *Col4a5* genes were found to be the main players. *Col3a1* (Collagen Type III Alpha 1 Chain) is involved in the development of cortices and the regulation of neuronal migration during neurodevelopment (Lee et al., 2013). *Col4a5* (Collagen Type IV Alpha 5 Chain) is a major structural component of glomerular basement membranes and is critical for forming the matrix supporting structure of neurons and glial cells (Akula et al., 2014).

Multiple studies, both from our lab and others, have indicated that the neurodevelopmental deficits due to prenatal alcohol exposure may result from aberrant dysregulation of cell-adhesion related genes with downstream interference in cell proliferation, cell migration alterations in cytoskeletal elements or extracellular matrix components (Clark et al., 2000; Kleiber et al., 2014b; Laufer et al., 2013; Mantha et al., 2014; Nowoslawski et al., 2005; Nuñez and Mccarthy, 2004). In humans, studies have shown reduced hippocampal volumes in ethanol-exposed children, which itself is associated with a severely reduced ability to properly carry out behavioural and cognitive functions (Green et al., 2009; Jacobson et al., 2011).

Gene-network analysis of the (AN v CN) DEG list identified *Dcn* (Decorin) as the hub gene (Figure 22). *Decorin*, an extracellular matrix protein, is involved

in the organization of collagens, cell-growth, division, and adhesion. Given the up-regulation of extracellular matrix and collagen synthesis pathways, both of which are vital for neuronal migration and cortical development (Kooy, 2010; Swanson and Bota, 2010; Vaillend et al., 2002), it is possible that *Decorin* plays an important role in modulating and regulating the trajectory of neurodevelopmental response by the hippocampus to the effects of ethanol.

5.4.3 Prenatal alcohol exposure alters critical information processing and cellular development pathways that are partially responsible for behavioural deficits in stress and memory

This project uncovered several biological pathways that contained significantly down-regulated genes in response to prenatal alcohol exposure, namely, the GABAergic synapse, neurotransmitter receptor binding and ion channel transport (Table 13).

Gabra6 (Gamma-Aminobutyric Acid Type A Receptor Alpha6 Subunit) was the sole gene common to all the three pathways. Incidentally, it was one of the most significantly down-regulated genes in the (AN v CN) DEG list (-2.9 fold change). It is the major inhibitory neurotransmitter in the vertebrate brain (Ben-Ari, 2002). Our lab has also previously found *Gabra6* as one of the significantly altered genes as a result of prenatal alcohol exposure (Kleiber et al., 2014b). *Gabra6* is critically important in cognition pathways (by modulating chloride

channels that are important downstream components) (Singer, 2013; Vaillend et al., 2002) and the HPA axis (Represa and Ben-Ari, 2005). As previously noted, an upregulation of *Gabra6* due to prenatal alcohol exposure has been associated with neuronal cell death (Ikonomidou, 2009; Olney et al., 2000a) and major loss of hippocampal volume is one of the correlative hallmarks of behavioural deficits in human FASD patients (Guerri and Renau-piqueras, 1997; Jacobson et al., 2011; Norman et al., 2009). It has also been shown to be an important modulator of neurogenesis (Covic et al., 2010), a process considered vital in learning and memory (Johnston et al., 2016; Lledo et al., 2006; Paizanis et al., 2007).

Related to neurogenesis and neural cell death, transcription factor analysis uncovered that *E2f2* (E2F Transcription Factor 2) was the most significantly affected transcription factor involved in the hippocampal response to prenatal alcohol exposure (Table 14). The deregulation of E2F expression has been shown to lead to p53-mediated apoptosis in both control (Qin et al., 1994) and alcohol-exposed mice (Lossie et al., 2014). *E2f2* is also involved in the maintenance and development of neural precursors and their differentiation (Hinoi et al., 2002; MacDonald and Roskams, 2009; Mehler, 2008), and neural plasticity (Lledo et al., 2006; Lyons and West, 2011) further supporting the hypothesis that ethanol exposure during synaptogenesis elicits a neuroprotective response centered on maintaining structural and cellular integrity.

5.4.4 Summary

Prenatal alcohol exposure results in changes in the expression of specific genes. It may lead to processes related to structural formation, regulation and maintenance of the hippocampus. It also disrupts the proper functioning of information processing pathways that are critical for learning and memory. These results may partially explain the behavioural deficits observed in alcohol-exposed mice [(Figures 33 (a) and 35 (a)]. It follows that structural aberrations of the hippocampus (resulting from prenatal alcohol exposure) not only lead to poor executive functioning in FASD individuals (Hard et al., 2005; Lossie et al., 2014; Muralidharan et al., 2013) but also have an indirect effect on stress and anxiety (Berman and Hannigan, 2000; Streissguth et al., 2004). The results of this project further confirm previous studies from our laboratory related to the behavioural and molecular effects of prenatal alcohol exposure during synaptogenesis (Chater-diehl, 2017; Kleiber et al., 2013).

5.5 The molecular effects of post-natal environmental enrichment on healthy mice

Environmental enrichment has been shown to increase learning and memory performance in mice while also lowering stress and anxiety levels (Nithianantharajah and Hannan, 2006). The discussion of this section is based on the results obtained hippocampi from mice subjected to post-natal enrichment as compared to controls.

5.5.1 Post-natal environmental enrichment may modulate structural plasticity in the healthy adult hippocampus

The results included under this section show that environmental enrichment modulates the expression of a selected set of genes. The gene-ontology analysis and pathway analysis using these genes have identified biological processes such as extracellular matrix organization, collagen biosynthesis and cell adhesion as being significantly altered due to post-natal environmental enrichment (Tables 15 and 16). It is interesting to note that both prenatal alcohol exposure and post-natal environmental enrichment altered the same pathways (extracellular matrix organization, collagen biosynthesis and cell adhesion) but in slightly different ways [namely, the involvement of *Col4a5* in the (AN v CN) group but *Col8a1* in CE v CN]. *Col3a1* turned up in both comparisons.

As previously noted, *Col3a1* (Collagen Type III Alpha 1 Chain) aids in the development of cortices and regulates neuronal migration. The neurons of the central nervous system, including the hippocampus are embedded in the glycoprotein scaffolding known as the extracellular matrix, which is constantly being degraded and rebuilt for structural rearrangements such as extension of synaptic connections in response to external cues such as environmental enrichment (Kempermann et al., 2010; Lista and Sorrentino, 2010; Trachtenberg et al., 2002). Environmental enrichment has been shown to be vital for neurogenesis in mice (Kempermann et al., 1997; Kuhn et al., 1996;

Nithianantharajah and Hannan, 2006, 2011; Schafer and Gage, 2016). Critically, the migration of new neurons via the rostral migratory stream and in the olfactory bulb is regulated by cell-cell and cell-extracellular matrix interactions involving genes such as *Col3a* (Abrous et al., 2005; An et al., 2013; Lledo et al., 2006; Ming and Song, 2011; Pozniak and Pleasure, 2006). *Col8a1* (Collagen Type VIII Alpha 1 Chain) is a critical component of the endothelia of blood vessels (Cherepanova et al., 2009; Lopes et al., 2013).

The involvement of *Col8a1* confirms from previous studies the effects of environmental enrichment on angiogenesis and increased vasculature in the brain due to increased physical activities and exercise (Fabel et al., 2009; Kobilo et al., 2011; van Praag et al., 2005). Interestingly, gene network analysis of the (CE v CN) DEG list identified *Col4a1* (Collagen Type IV Alpha 1 Chain) as the hub gene (Figure. 25). The *Col4a1* gene is specifically involved in the inhibition of endothelial cell proliferation, migration and tube formation, including blood vessels (Choi, 2015; Trouillet et al., 2017). Its upregulation in the (CE v CN) DEG list points to the modulation of blood-vessel formation and maintenance with possible antagonistic properties towards *Col8a1*.

The *Cldn1* (Claudin 1) gene (found to be up-regulated in response to environmental enrichment) was confirmed by qPCR (Figure. 29). The *Claudin* family of genes plays a major role in the regulation and maintenance of tight-

junctions (Hagen, 2017; Reinhold and Rittner, 2017), which in turn are crucial in structural and cellular re-arrangements of synaptic junctions during environmental enrichment.

Several hypotheses linking neurodevelopment and neuroplasticity have emerged over the past few years. For example, the large-scale tendency for children and young adults to exhibit neuroplasticity has been attributed to the overabundance of neuronal cells and synaptic connections during early post-natal development, all of which undergo programmed pruning later in life (Benfenati, 2007; Kovas and Plomin, 2006; Lista and Sorrentino, 2010; Martin and Morris, 2002; West and Greenberg, 2011). Various other neurodevelopmental events in general, such as extracellular matrix processes and mylenation, each with their specific mechanisms of plasticity may also contribute to better understanding neuroplasticity and neurodevelopment (Chen and Tonegawa, 1997; Sossin and Lacaille, 2010).

5.5.2 Post-natal environmental enrichment significantly alters the circadian rhythm pathway in healthy mice

Another significantly altered pathway in the (CE v CN) group list was the circadian rhythm pathway (Table. 16). Within this pathway, the Period Circadian Clock 1 and 3 genes (*Per1* and *Per3* respectively) are regulated by external environmental cues and regulate locomotor activity, metabolism, and arousal of

the central nervous system. The circadian clock in general has been previously implicated in downstream learning and memory pathways (Lyons and West, 2011; Schnell et al., 2014; Xia and Storm, 2017). For example, studies have shown that chronic disruption or phase-shifting of circadian rhythm impairs long-term memory and working memory in rodents and humans (Feng et al., 2007; McClung and Nestler, 2008). The circadian rhythm has also been implicated in the regulation of activity-induced neurogenesis, which in turn is associated with improved cognitive performance (Holmes et al., 2004; Mahar et al., 2014; Schnell et al., 2014).

5.5.3 Post-natal environmental enrichment modulates the expression of genes involved in cognition in healthy mice

This project found that post-natal environmental enrichment alters the expression of genes involved in information- and signal- processing (biological processes like neurotransmitter receptor activities and ion channel transport) and emotion regulation (Table 16). For example, one of the genes (up-regulated in the CE v CN comparison) confirmed by qPCR was the *Kcnj13* (Potassium channel inwardly rectifying subfamily J, member 13) gene (Figure. 29). The potassium channel family of genes has been shown to be crucial in modulating cell-membrane potential (Wang and Fawcett, 2012), that, in turn, is critical for long-term potentiation, a cellular and physiological proxy for learning and memory processes. They also help facilitate the movement of proteins crucial for

neurotransmitter release, possibly affecting synaptic signaling (Faissner et al., 2010; Larimore et al., 2017).

Transcription factor analysis for this group uncovered *Thrb* (Thyroid Hormone Receptor, Beta) as the most significantly altered transcription factor. *Thrb* is involved in the neural stem cell migration and proliferation, synaptogenesis and mylenation (Ookubo and Sadamatsu, 2015) (Table 17). The Thyroid Hormone Receptor is a part of a large family of hormone receptors involved in physiological responses, immunosuppression and inflammation that have been implicated in the facilitation or supression of spatial learning and memory (Cheng et al., 2010; Gauthier et al., 2001). Given the improved cognitive performance of healthy mice when exposed to environmental enrichment, one can postulate the correlative participation of *Thrb* as a possible contributing factor.

Importantly, post-natal environmental enrichment also alters the expression of genes like *Sstr1* (Somatostatin receptor 1) and *Drd1a* (Dopamine D1-like receptors), both of which are involved in neurotransmitter receptor activity, a biological process containing significantly up-regulated genes (as found in this project) in adult hippocampi after post-natal environmental enrichment. *Sstr1*, for example, is involved in excitatory glutamatergic transmission and regulates synaptic plasticity (Cammalleri et al., 2009). Previous studies have implicated *Drd1a* in regulation of information flow through hippocampal circuits involved in

spatial learning (Tonegawa and Sari, 2016) and synaptic plasticity (Aira et al., 2016; Chiken et al., 2015).

5.5.4 Summary

My results show that post-natal environmental enrichment has altered the expression of a set of genes that are involved in the regulation of biological processes related to neural migration, regulation and maintenance of tight-junctions in the hippocampal synapses and angiogenesis in mice [(Figures 33 (b) and 35 (b)]. Also, control-enriched mice were shown to have significantly affected circadian rhythm pathways and biological processes related to information processing (which are, in turn, critical for learning and memory). These results may partially explain and (also confirm from previous studies) the behavioural improvements observed in control-exposed mice of this project. For example, previous studies have shown that structural alterations of mouse hippocampi, resulting from post-natal environmental enrichment, lead to improved performances in spatial learning and memory with decreased levels of stress and anxiety (Kobilo et al., 2011; Nithianantharajah and Hannan, 2006). Environmental enrichment has been shown to modulate those genes in the brain that contribute to structural and synaptic plasticity (Benfenati, 2007; Lyons and West, 2011; Morris et al., 2003; Sossin and Lacaille, 2010) and neurogenesis (Fabel et al., 2009; Zhao et al., 2008). While the exact role of new neurons in the hippocampus is still under debate (Kempermann, 2002), as indicated above, there

is overwhelming evidence that neurogenesis and increased neural plasticity due to physical and mental stimulation is associated with improved learning and memory performance.

5.6 The molecular effects of post-natal environmental enrichment on mice treated with prenatal alcohol

In this section, the discussion will be based on the differentially expressed genes (DEG) obtained from the alcohol-enriched mice versus alcohol non-enriched mice (AE v AN). The objective is to evaluate if environmental enrichment affects hippocampal gene-expression patterns in mice that have been exposed to alcohol during neurodevelopment. The results of this section represent, for the first time in FASD literature, the molecular underpinnings of post-natal environmental enrichment in a mouse model of FASD at a high-throughput transcriptomic scale.

5.6.1 Post-natal environmental enrichment alters hippocampal gene-expression patterns in alcohol-exposed mice, a model for FASD

In this project, 73% of all significantly affected genes in the adult hippocampi of alcohol-exposed enriched mice had a relatively subtle yet significant change in gene-expression levels (within the ± 1.2 fold change range). A complete list of all genes up- and down-regulated in the adult hippocampus due to prenatal alcohol exposure is found in Appendix 3.

The top up-regulated gene in this analysis was *Serpina3f* (Serpin Family A Member 3) with a fold change of 7.82. *Serpina3f* is a plasma protease inhibitor involved in apoptosis, Alzheimer's disease, inflammation and complement activation (Law et al., 2006). It is also involved in the mobilization of hematopoietic progenitor cells (Winkler et al., 2005). Given the apoptotic effects of prenatal alcohol exposure and the subsequent immune response by the hippocampus to alcohol's teratogenic effects, *Serpina3f* may play an important role modulating the immune response. However, given the increased vasculature and angiogenesis in the brain due to voluntary exercise (Hertzog et al., 2009; Voss et al., 2013) (a part of the environmental enrichment paradigm used in this project), *Serpina3f's* role may also extend to regulate hematopoiesis.

Previous literature has examined the role of voluntary exercise and environmental enrichment in alcohol-exposed mice and found that these rehabilitative paradigms increase neurogenesis, cortical spine density, long-term potentiation and neurotrophin levels (Christie et al., 2005; Parks et al., 2008; Redila et al., 2006; Weinberg et al., 1995). While the up-regulation of genes involved in pathways related to these molecular processes have been previously shown to be associated with improvement in behavioural and cognitive outcomes in various studies of environmental enrichment of mouse disease models (Hannan, 2014; McOmish and Hannan, 2007; Nithianantharajah and Hannan, 2006), it is critical to note in this project that (i) the phenotypic amelioration is

incomplete, indicating permanent damage of ethanol exposure and (ii) the involvement of completely new molecular pathways and gene-expression patterns rather than a reversal of previously implicated ethanol-affected genes or pathways is noted.

5.6.2 Post-natal environmental enrichment may modulate behavioural amelioration by altering the expression of heat shock proteins

Gene-ontology and pathway analysis of differentially expressed genes in the AE v AN group has uncovered the effect of post-natal environmental enrichment in alcohol-exposed mice on biological processes such as stress response and destabilization of mRNA by AUF1 (Tables 18 and 19). Importantly, the *Hspa1b* (Heat Shock Protein Family A Member 1B) gene and *Hspa1a* (Heat Shock Protein Family A Member 1A) gene were involved in these processes. Previous research in our lab has uncovered the possible contribution of heat shock proteins in behavioural deficits typical of alcohol-exposed mice (Kleiber et al., 2014b; Laufer et al., 2013; Mantha et al., 2014). In support of these results, studies have demonstrated the upregulation of heat shock proteins during short-term exercise in rats (Hu et al., 2009; Krause and Rodrigues-Krause, 2011) and long term environmental enrichment in mouse models of autism (Hu et al., 2016), both of which are associated with improvement in visuospatial memory and lowered stress- and anxiety-like endophenotypes. Importantly, heat shock proteins are involved in the regulation of synaptic plasticity, neurogenesis, and memory

consolidation (Alberini, 2009; Grote and Hannan, 2007; Hooper et al., 2016). As shown in this project, alcohol-exposed enriched mice showed improved learning and memory performance in the Barnes Maze and Novel Object Recognition tests. It is possible to speculate that heat shock proteins, via their actions during stress response, learning and cognition, play an important role in the beneficial effects of environmental enrichment.

5.6.3 The ameliorative effects of post-natal environmental enrichment in alcohol-exposed mice is partially associated with its effects on hippocampal immunomodulation

One of the most significantly altered pathways in alcohol-exposed and enriched mice was the TNF signaling pathway (Table 19). Tumor necrosis factor (TNF) is a ligand that promotes inflammatory signaling, mediates immune response towards neuronal damage and recently, has been shown to be involved in synaptic scaling and neurogenesis (Dellarole et al., 2014; Mccoy and Tansey, 2008; Yirmiya and Goshen, 2011). The action of immune signaling molecules, such as the one found in this project's TNF signaling pathway, namely, *Cxcl1* (Chemokine (C-X-C motif) ligand 1), has been specifically shown to be involved in activity-dependent synapse formation (Kolodziej et al., 2008).

There is growing evidence that neuro-immune interactions may underlie important mechanisms by which neural plasticity and vulnerability co-exist within

the hippocampus (Yirmiya and Goshen, 2011). As stated above, immune mechanisms are activated by environmental enrichment to regulate the remodeling of neural circuitry, promote long-term potentiation and neurogenesis (Grote and Hannan, 2007; Nithianantharajah and Hannan, 2006). These mechanisms are modulated by complex interactions between chemokines and cytokines with not only neurons (old and new) but also individually with, neurotrophins (e.g., BDNF) and also hormones (e.g. glucocorticoids), all of which are important targets of environmental enrichment. However, as previously shown, the teratogenic effects of alcohol also activate a massive neuro-immune response (Ikonomidou, 2009; Kleiber et al., 2014; Laufer et al., 2013; Mantha et al., 2014; Olney et al., 2000) and an overproduction of pro-inflammatory cytokines and chemokines, thereby upsetting the delicate beneficial interaction and balance with detrimental effects on neural plasticity and as observed in this project, deficits in anxiety response, and cognition. Thus, the amelioration in behavioural and cognitive performance in alcohol-exposed enriched mice through hippocampal immunomodulation remains a prime target for further investigation and a potential target for future therapies.

5.6.4 The ameliorative effects of post-natal environmental enrichment in alcohol-exposed mice are associated with its effects on neural plasticity and angiogenesis

One of the common themes during the investigation of the effects of prenatal alcohol exposure and post-natal environmental enrichment (as done in the

previous two sections) has been the alterations of genes and biological processes related to structural remodeling and plasticity (Table 19). This theme continues when investigating the effects of post-natal environmental enrichment in alcohol-exposed enriched mice. For example, the *Icam1* (Intercellular Adhesion Molecule 1) gene, a member of the TNF signaling pathway (as discussed above), is involved in cell adhesion and was found to be significantly altered in the (AE v AN) group. Importantly, previous research from our lab has implicated the *Icam1* gene as being significantly altered in various mouse models of prenatal alcohol exposure (Kleiber et al., 2014; Mantha et al., 2014). However, cellular adhesion molecules have also been implicated in the formation of dendritic spines and functional synapses (Alberini, 2009; French and Pavlidis, 2011; Lledo et al., 2006). Specifically, they have been implicated in cell interactions during neurodevelopment (during which time it promotes neurite outgrowth, axon pathfinding and myelination), synaptic plasticity and memory formation but are also susceptible to alterations due to stress-related disorders and depression (Tanti and Belzung, 2013; Tanti et al., 2013). As of now, this is the first report of *Icam1* up-regulation in response to environmental enrichment in prenatally alcohol-exposed mice and its possible correlation with decreased stress- and anxiety-like phenotypes and improved visuospatial learning and memory performance.

The major significantly altered pathway with down-regulated genes in the (AE v AN) group was the Sema4D mediated inhibition of cell attachment and

migration, within which the *Met* (MET Proto-oncogene, Receptor Tyrosine Kinase) gene was to be significantly altered. *Met* is a member of the Semaphorin family, which consists of a large number of secreted and/or membrane molecules that play various roles in axon guidance, synaptogenesis, synapse formation and stability (Lopez-Atalaya et al., 2011; Shen and Cowan, 2010; Soleman et al., 2013). Within this family, the *Met* gene is a receptor tyrosine kinase that has been shown to promote spine morphogenesis, complexity and clustering (Qiu et al., 2014; Tyndall and Walikonis, 2006). The MET protein has also been implicated in regulation of cortical bone osteogenesis via its interactions with bone morphogenetic proteins (BMPs) (Abed et al., 2015; Shibasaki et al., 2015). Importantly, studies have shown that an up-regulation of genes involved in BMP signaling is significantly associated with impairments in neurogenesis and declining cognitive performance whereas the down-regulation of genes involved in BMP signaling results in increased neurogenesis and improved cognitive performance (Encinas et al., 2013; Gobeske et al., 2009; Meyers et al., 2017).

Gene-network analysis of the (AE v AN) DEG list identified *Arhgap36* (Rho GTPase Activating Protein 36) as the main hub gene (Figure. 28). *Arhgap36* is critically involved as a positive regulator of the Sonic Hedgehog pathway (Rack et al., 2014). The Sonic Hedgehog pathway has been shown to not only control the patterning of progenitor cells, and their neuronal and glial progeny, during neurodevelopment, but also mediate activity-dependent and injury-induced

hippocampal neurogenesis and long-term potentiation (Hung et al., 2015; Yao et al., 2016). Thus, the indirect involvement of *Arhgap36* via its modulation of the Sonic Hedgehog pathway may be able to partially explain the amelioration of behavioural deficits in alcohol-exposed enriched mice.

Transcription factor analysis uncovered *Ets1* (V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 1) gene (Table 20). *Ets1* and its family of transcription factors are critical activators and repressors of angiogenesis (Lelie et al., 2001). Given the increase in angiogenesis due to voluntary exercise (and environmental enrichment) and the correlation with improved cognitive performance, its downstream involvement in various learning and memory processes, as indicated by previous research, may also explain the reversal of behavioural deficits in alcohol-exposed enriched mice.

5.6.5 Summary

Post-natal environmental enrichment in prenatally alcohol-exposed mice results in the alteration in gene expression of those genes that regulate biological processes such as structural synpatic plasticity and angiogenesis (in response to exercise and physical activities). These results may partially explain and (also confirm previous studies) the behavioural improvements observed in alcohol-exposed mice of this project [(Figures 33 (c) and 35 (c)]. For example, previous studies have shown that environmental enrichment, voluntary exercise and handling of alcohol-exposed mice when they are still pups leads to improved performances in

spatial learning and memory with decreased levels of stress and anxiety (Berman et al., 1996; Christie et al., 2005; Hannigan et al., 2007; Weinberg et al., 1995). The results of this project are novel, in terms of delineating the transcriptomic signature of adult hippocampi in alcohol-exposed mice in order to explain behavioural ameliorations in them. As this project found, it is a combination of genes and molecular pathways that directly target cellular and synaptic plasticity and angiogenesis and indirectly target the up-regulation of long-term potentiation, neurogenesis and neural cell survival that results in the amelioration of typical FASD-specific behavioural deficits.

5.7 Network centrality measures

A novel avenue of investigation and clarification in this project refers to network centrality analysis. In terms of network metrics, the connectivity of a gene is a one-dimensional metric, namely, its degree. This project measured another general two-dimensional centrality measure: 'betweenness'. Betweenness is based on the frequency with which a node lies on the shortest path between all other nodes (Hu et al., 2016). Genes with high-betweenness not only control the flow of information across a network, but because of this, are important in minimizing response times within a cell (Cantini et al., 2015; Didier et al., 2015; Hahn and Kern, 2004). As uncovered in this project, the gene-network (of all DEGs) of environmentally-enriched healthy mice had the highest betweenness score whereas the gene-network (of all DEGs) of non-enriched alcohol-exposed mice

had the lowest (Figure. 34). One can interpret this finding by concluding that the ease of information flow (for example, immune response, structural re-arrangements, neurotransmitter release, protein synthesis) is much more pronounced in healthy mice who have undergone environmental enrichment and lowest in mice who have been exposed to alcohol during synaptogenesis. This observation from a global gene-network perspective may not only account for the increased cognitive performance of healthy enriched mice but the behavioural and cognitive deficits of alcohol-exposed mice. Topological analysis of gene- and protein- networks is a relatively new field of study (Didier et al., 2015; Khatri et al., 2012). Specifically, a combination of global centrality measures like 'betweenness' and local centrality measures like 'degree' is increasingly being utilized to assess the biological significance of genes and proteins. For example network nodes with high degrees are defined as hub genes and nodes with high betweenness are defined as bottleneck genes, both of which are pivotal in networks (Ambedkar et al., 2015; Cantini et al., 2015; Khuri and Wuchty, 2015). Further research needs to be done by utilizing these genes as potential candidate genes as targets for pharmaceutical therapies or simply to provide a better explanation for observed phenotypes.

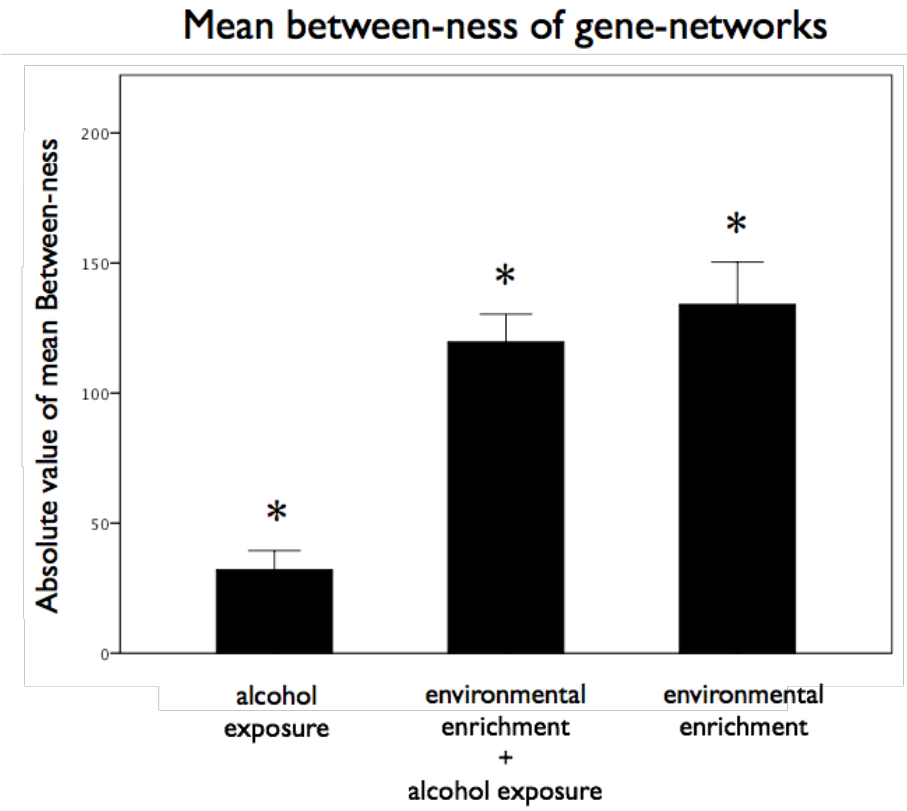


Figure 34: Mean between-ness of gene-networks. After gene-networks for the (AN v CN), (CE v CN) and (AE v AN) groups were created using Genemania and Cytoscape, Network Analyzer was used to calculate the mean-between-ness for each network and SPSS was used to statistically compare the mean values between each network. Data are shown as mean (±SEM). Healthy mice that were environmentally-enriched had the highest mean-between-ness of all three groups. Post-natal environmental enrichment improves the mean-between-ness score for all significantly altered genes in alcohol-exposed mice that have undergone environmental enrichment. (* represents $p < 0.001$ in one-way ANOVA).

CHAPTER 6: PROJECT CAVEATS AND LIMITATIONS

6.1 Project caveats regarding behavioural modeling in mice

6.1.1 The mouse model as an experimental paradigm

The modeling of complex disorders using animal models is limited by various factors, the first of which is face validity (Fernando and Robbins, 2011; Nestler and Hyman, 2010), a concept that signifies the extent of similarity between the behaviour exhibited by the animal model and specific symptom as shown by the human. In other words, the translation of results from mouse behavioural phenotyping to human subjects should be treated with caution, because the face validity of a complex disorder is almost impossible to establish, due to the subjective nature of deciding how to characterize specific symptoms of human subjects in mice (van der Staay et al., 2009).

Another limitation of using animal models is predictive validity, which involves identifying potential treatments or drug therapies for ameliorating or potentially reversing behavioural, cognitive, physical and developmental abnormalities as established by that animal model (van der Staay et al., 2009). This thesis represents a novel foray into exploring a drug-free avenue of rehabilitation, namely, environmental enrichment, for FASD-related deficits. However, more independent work is currently needed to establish its predictive validity.

Thirdly, the construct validity (Markou et al., 2009; Toth et al., 2011) refers to the ability of a test to measure that which it is intended to measure. Given our current nebulous explanation and understanding of complex disorders in human subjects, not to mention the difficulty of successfully and accurately modeling it in mice, the construct validity is rarely established in most animal models (van der Staay et al., 2009). A case in point in this thesis is the diverging results of the anxiety-like endophenotypes of all four mouse groups as measured by two different tests, namely, the Elevated Plus Maze test and Light Dark Box (Figures 11 and 12). As both tests of anxiety give different results, the conclusion regarding the effect of prenatal alcohol exposure and post-natal environmental enrichment should be approached with caution.

A plethora of other factors such as (i) experimenter's influence (e.g., odour cues), (ii) mouse strain variations, (iii) influences from previous behavioural testing, and (iv) varying behavioural and cognitive results across different labs, are all important factors when considering the interpretation of mouse behavioural testing (Crawley, 2000, 2007; Lathe, 2004; van der Staay et al., 2009). Also, the behavioural (and molecular) analyses were restricted to male mice (i.e. due to female fighting). Critically, the hippocampal tissues analyzed were from male mice that had undergone behavioral testing and this might be a potentially confounding issue. While behavioural testing in this project was conducted

according to strict guidelines and protocols to ensure minimum variability in testing conditions, a certain amount of technical and human error should be taken into account.

6.1.2 Technical considerations in mouse behavioural testing

An important point to consider when interpreting the behavioural results of this project is the fact that cardboard material was used in the construction of the LDB box, EP maze and the Barnes maze. While cardboard material has been used as construction material by previous behavioural researchers, it is possible that the odour cues that were left by mice remained on the apparatuses could have influenced testing results as odour cues are used as scent marks for communication purposes (Bourin and Hascoe, 2003; Leary and Brown, 2008; Sharma et al., 2010; Walf and Frye, 2007).

Secondly, the inconsistent results of stress- and anxiety-like endophenotypes (when comparing alcohol-exposed mice to healthy mice) may be the result of either (i) the inadequacy of the testing apparatuses (or the testing paradigm itself) to reliably measure stress- and anxiety-like endophenotypes or (ii) the mouse's familiarization to stress and anxiety-related experiments over time.

Thirdly, BACs in this project were not measured. However, the amount of ethanol injected was based on previous studies in mice where BAC had been

measured (Ikonomidou, 2009; Olney et al., 2000a; Young and Olney, 2006) and given the amount of ethanol injected, one can safely concluded that the BAC in alcohol-exposed mice would have been above the critical threshold for neurodegeneration. Nevertheless, this caveat should be duly noted.

Finally, it is important to note the dearth of longitudinal rehabilitative studies (including drug therapies) in human FASD subjects. So far, previous literature indicates the constant need by affected FASD children to undergo rehabilitation as the ameliorative effects are not long-lasting in humans (Floyd et al., 2009; Kodituwakku, 2010; Peadon et al., 2009) and rodents (Gabriel et al., 2002; Hannigan et al., 2007; Schreiber et al., 2013). While this project does demonstrate the ameliorative effects of environmental enrichment, it is imperative to carry out longitudinal studies with and without continued environmental enrichment to assess the long-term effects of drug-free rehabilitative therapies.

6.2 Caveats and limitations for molecular studies on FASD

6.2.1 Animal models in FASD research

The use of animal models (e.g. mice) to dissect the molecular underpinnings of complex disorders such as FASD, especially through large-scale transcriptomic studies, is not without its limitations and any conclusions drawn must be in the context and awareness of such limitations.

For example, this RNA-sequencing (RNA-Seq) project consisted of three

biological replicates for each of the four experimental groups. Thee biological replicates are considered for drawing statistically relevant conclusions in the context of RNA-Seq studies (Gao et al., 2010; Marioni et al., 2008; Sims et al., 2014). Previous studies have suggested that biological replicates are a key factor for differential gene-expression analysis in RNA-Seq studies (Liu et al., 2014; Sims et al., 2014). Thus, future studies in the context of this field of research would certainly benefit from a higher number of biological replicates to ensure a greater reliability of the results. Future work should certainly take into account the need for a greater number of biological replicates.

It is a standard technical practice to follow up RNA-Seq with single-gene confirmation studies with qPCR (Liu et al., 2014; Marioni et al., 2008; Sims et al., 2014). To ensure that the statistical reliability of this project's gene confirmation studies, I ensured that the qPCR experiments for this project's selected gene-list (chosen from RNA-Seq analysis) had five biological replicates, along-with the required three technical replicates (Bustin et al., 2009; Radonić et al., 2004) for each experimental group.

6.2.2 Choice of high-throughput platform (microarray v. RNA-Seq)

The use of two different high-throughput gene-expression platforms in this project (microarray and RNA-Seq) necessitated a decision regarding the selection of one of those platforms and its results for further discussion and final conclusions.

Ultimately, the RNA-Seq platform was chosen to lead the analyses for this project.

In this project, all the discussion and conclusions regarding the molecular underpinnings of post-natal environmental enrichment in a mouse model of prenatal alcohol exposure is based on the gene-expression, pathway analysis and gene-network analysis specifically from the RNA-Seq data. RNA-Seq as a technological platform offers a number of advantages over the micro-array platform. RNA-Seq allows for a greater dynamic range of detection than microarrays with a greater ability to detect transcripts of low abundance (given a sufficient depth of sequencing) (Sims et al., 2014). Importantly, RNA-Seq has no limit to detect maximal expression levels. This allows for a better differentiation among transcripts that are highly expressed. In microarrays, the signal intensity plateaus for highly expressed transcripts making accurate differentiation and detection impossible (Black et al., 2014). As microarray technology is a hybridization-based technique, it is not only limited to transcripts bound to array slides, but also limited to the quality and availability of bioinformatics data for that organism (genome and transcriptome) (Stefano, 2014). While RNA-Seq not only detects annotated transcripts, it can also detect novel sequences, splice variants, exon junctions and non-coding RNA for unknown organisms (Dewey, 2013; Marioni et al., 2008; Sims et al., 2014).

Currently, one way to directly compare microarray data with RNA-Seq data is to transform microarray gene-expression data via z-score processing and to ensure that the signature genes of that particular organism developed for one platform are directly transferrable to the RNA-Seq platform (Chavan et al., 2013; Fumagalli et al., 2014).

6.2.3 Choice of analysis method (edgeR) for RNA-Seq analysis

One technical point that merits a comprehensive discussion is the gene-expression analysis of the RNA-Seq data. While edgeR is considered to be a robust, well-documented and well-accepted statistical package for analyzing RNA-Seq data to perform gene-expression analysis (Anders et al., 2013; Chen et al., 2014a), there does not currently exist an industry gold-standard for RNA-Seq data analytics (Guo et al., 2013; Rapaport et al., 2013; Seyednasrollah et al., 2015; Sims et al., 2014). Currently, there are numerous software packages (both commercial and free), each of them comes with their own sets of limitations, making a standardized comparison difficult to interpret. The popularity of a commercial software like Partek for high-throughput gene-expression analysis rests on its incredible ease of use, graphic user interface and accessibility for non-bioinformaticians (Harris et al., 2010; Zhang et al., 2014). In fact, numerous studies, including many from our own lab have relied on the consistency, ease of use and accuracy of Partek for analyzing micro-array data for gene-expression analysis (Chater-Diehl, 2017; Kleiber et al., 2013, 2014b; Laufer et al., 2013;

Mantha et al., 2014). However, the same cannot be said for its applicability in RNA-Seq analysis.

For example, in Partek, the alignment transformation of RNA-Seq raw data is based on FPKM (Fragments Per Kilobase of gene model per Million fragments mapped) values rather than raw count values (Xing et al., 2006). It will then perform statistical tests based on a beta-negative binomial model that in turn is assumed to reflect the underlying distribution of the FPKM. However, a number of studies have shown that FPKM may not be the most appropriate way to normalize RNA-Seq data, because, for example, it reduces sample variability when compared to raw counts and may also cause conservative bias (Conesa et al., 2016). Recent studies have shown that normalization methods like FPKM that are based on gene-length bias total read counts of targeted genes (Anders et al., 2013; Rapaport et al., 2013).

edgeR is a statistical software program in the R Bioconductor package with a steep-learning curve as it requires an intermediate knowledge of computer coding for its implementation. However, currently, it is considered to be the best performer of RNA-Seq differential gene expression analysis when compared to a variety of other software packages including Partek (Anders et al., 2013; Guo et al., 2013; Rapaport et al., 2013; Sims et al., 2014). Due to the expensive cost of RNA-Seq, scientists have to contend with a small number of biological replicates.

This leads to one of the most common challenges in RNA-Seq analysis, namely, the problem of overdispersion, which occurs when the Poisson distribution underestimates the variation seen in the data and subsequently fails to control type-I error (Liu et al., 2014). The negative binomial distribution model has been shown to successfully take into account overdispersion in order to best fit the distribution of read counts across biological replicates (Chen et al., 2014a; Rapaport et al., 2013).

Another problem in RNA-Seq is the possibility of unequal sequencing depth between groups since the difficulty in the titration and multiplexing of Illumina RNA-Seq libraries results in unbalanced library sizes or low sequencing depths for some samples. edgeR is an extremely flexible statistical package which handles raw count data irrespective of whether the data is over-dispersed (in which case it uses the negative binomial model) or not (in which case it uses the Poisson distribution model). Rather than using FPKM normalization, it uses a quantile-adjusted method to standardise total read counts (library sizes) across samples. In the case of over-dispersed data, edgeR employs an exact test for the negative binomial distribution based on the normalised data (Chen et al., 2014b; Zhang et al., 2014). edgeR has also shown to be more accurate in terms of its ability to uncover true positives with the default 0.05 FDR setting. A potential danger to this is an overestimation of differentially-expressed genes that may turn out to be false positives (Anders et al., 2013; Guo et al., 2013). Thus, further

confirmation with qPCR remains a necessary and good practice.

6.2.4 Commentary on gene-expression results across all three platforms

As mentioned in Chapter 2 (Materials & methods) and Chapter 4 (Molecular results), hippocampal RNA was processed using two different technologies [microarray (Affymetrix GeneChip® Whole Transcript WT Expression Arrays) and RNA-Seq(Illumina V4)]. The data obtained from microarrays was analyzed using the Partek Genomics Suite and the RNA-Seq data was analyzed using the Partek Genomics Suite and edgeR. To summarize, the molecular results deal with:

1. RNA-Seq (RE): RNA-Seq data analyzed on edgeR (analysed by me)
2. RNA-Seq (RP): RNA-Seq data analyzed on Partek (analysed by Celeste Cote, my colleague)
3. Microarray (M) date analyzed on Partek (analysed by me)

Even though, as mentioned before, the conclusions in this chapter are solely and specifically based on the RNA-Seq data as analyzed by edgeR (RE), it is worth commenting on certain observations.

The number of genes that were common between all three platforms should be interpreted carefully because, while the differentially expressed genes from the RE and M lists were FDR-corrected, the RP list was not. In fact, after FDR-correction, Partek eliminated almost 96-99% of genes from all three comparisons

(AN v CN, CE v CN and AE v AN). However, as previous research from our lab has demonstrated, genes that are not FDR-corrected (as analyzed by Partek) have not only been used for further downstream analysis (gene ontology, pathway analysis, gene-networks) but have also been confirmed by qPCR (Kleiber et al., 2012, 2014b; Laufer et al., 2013). This lends strong support to the hypothesis that the effects of prenatal alcohol exposure during neurodevelopment are biologically significant yet subtle to detect (Kleiber et al., 2014b). Thus, in future studies, non-FDR corrected differentially expressed genes as uncovered by the Partek analysis of RNA-Seq data should be further investigated in order to obtain potentially novel insights into the molecular correlates of post-natal environmental enrichment and prenatal alcohol exposure. The differences in the magnitudes of fold-changes for each gene among all three platforms (Tables 21, 22 and 23) is consistent with previous studies which have demonstrated the discrepancy in fold-changes values for gene-expression data when compared across microarrays and RNA-Seq platforms (Rapaport et al., 2013; Sims et al., 2014) and also among various gene-expression analysis methods (Chen et al., 2014a; Zhang et al., 2015).

It is, however, interesting to note that, notwithstanding various technical and statistical differences amongst all three platforms, there were certain molecular pathways that were significantly altered among all three platforms (Table 32). For example, the ECM-receptor interaction pathway was significantly altered in the adult hippocampus, in response to prenatal alcohol exposure. The Circadian

rhythm pathway was also significantly altered in response to environmental enrichment and this was confirmed through all three platforms. Lastly, all three platforms found that the regulation of mRNA stability was significantly altered in response to post-natal environmental enrichment after prenatal alcohol exposure.

Critically, all these results were arrived at, independent of one another [independent of choice of data analysis (edgeR or Partek) or technology (microarray or RNA-Seq)]. These results, then, may possibly point to the importance of these molecular pathways in the context of prenatal alcohol exposure and/or post-natal environmental enrichment and thus, are strong candidates for future studies. While the accuracy and depth of detection using RNA-Seq + edgeR (RE) analysis is relatively strict compared to the RNA-Seq + Partek (RP) and microarray analyses (M), the results of the RP and M analyses can be considered, to a certain extent, to be independent validation and support for the RE results.

CHAPTER 7: CONCLUSION

7.1 Conclusion

Post-natal environmental enrichment ameliorates behavioural and cognitive deficits in a mouse model of prenatal alcohol exposure and the amelioration is associated with specific transcriptomic alterations in the hippocampus.

In this thesis, I was able to examine the effects of physical, cognitive and social enrichment on both alcohol-exposed and healthy groups of mice. Two salient observations from the thesis are:

1. Post-natal environmental enrichment is able to ameliorate behavioural and cognitive deficits that arise due to prenatal alcohol exposure. That this amelioration is incomplete may be indicative of the permanent, long-term damaging effects of prenatal alcohol exposure (Figures 10 to 16, Tables 1 and 2) and/or the nature of enrichment used.
2. The amelioration of behavioural and cognitive deficits in alcohol-exposed enriched mice and the improvement of behavioural measures in control-enriched mice are associated with specific gene-expression patterns and activation of molecular pathways in the adult hippocampus (Tables 12 to 32, Figures 20 to 29).

Importantly, the various molecular processes in the adult hippocampus that have shown to be associated with behavioural and cognitive amelioration in alcohol-

exposed enriched mice represent a novel finding in FASD literature (Figure. 35). These processes are an important resource for future studies and should be further investigated in order to design effective and long-term rehabilitative therapies (behavioural and pharmacological) for FASD affected individuals.

Figure 35: Molecular correlates of post-natal environmental enrichment in a mouse model of prenatal alcohol exposure

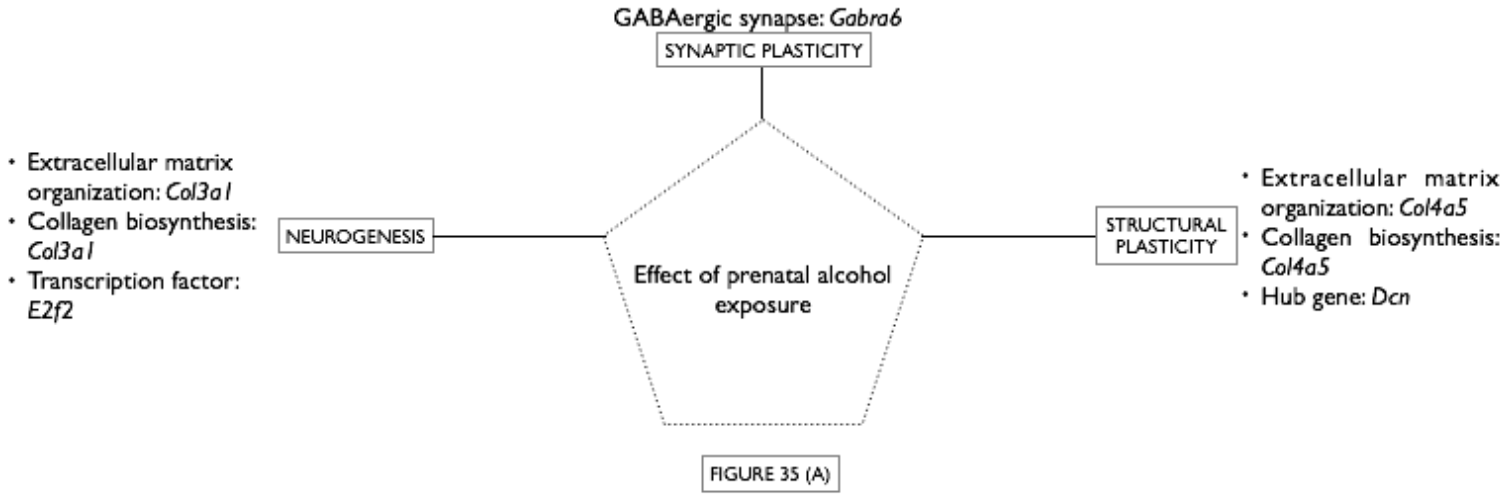


FIGURE 35 (A)

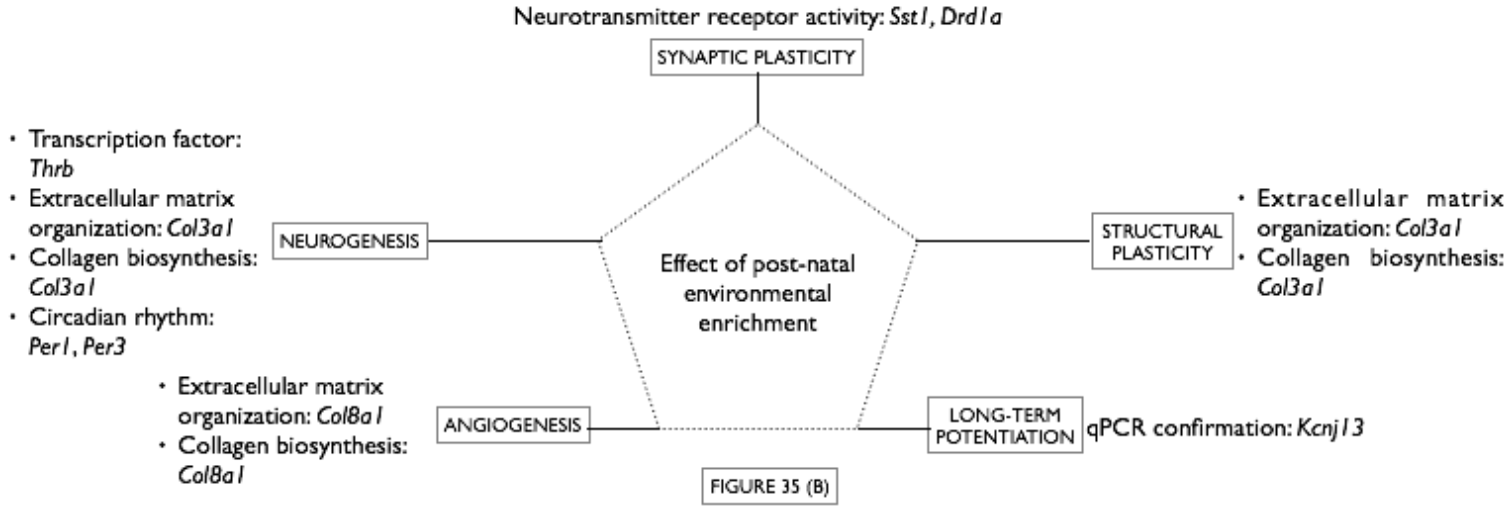


FIGURE 35 (B)

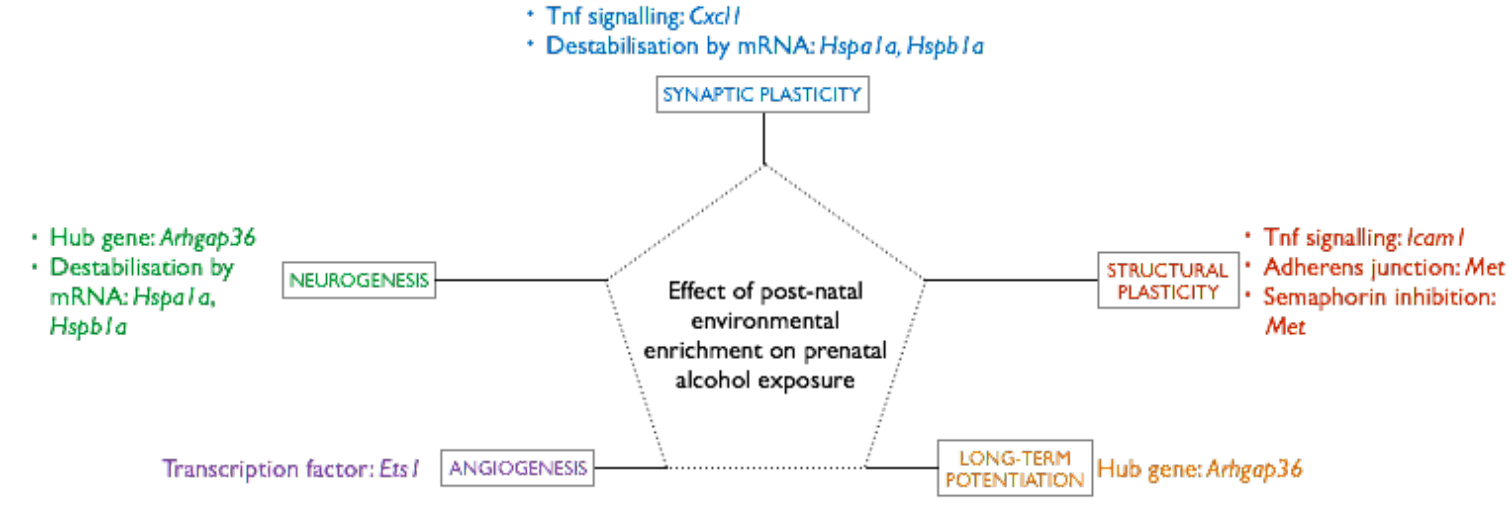


FIGURE 35 (C)

Figure 35: (i) Every gene in the figure was significantly altered (up- or down-regulated) as found in the RNA-Seq edgeR analysis. The function of each gene (within its pathway) is associated either directly or indirectly with biological processes such as synaptic and structural plasticity, neurogenesis, angiogenesis, and long-term potentiation (as described from sections 5.4 to 5.6.5). **(ii)** Even though the molecular pathways were significantly altered in response to the various treatments (prenatal alcohol exposure, post-natal environmental enrichment or a combination of both), the ultimate behavioural and cognitive phenotype was associated with treatment-specific transcriptomic. For example, extracellular matrix organization was a significantly altered pathway both in the effect of prenatal alcohol exposure and the effect of post-natal environmental enrichment. However, while in the former, *Col4a5* was the significantly altered gene, in the latter, it was *Col3a1*. **(iii)** The additional effects of post-natal environmental enrichment, be it in control mice or alcohol-exposed enriched mice, are associated with molecular processes aiding in angiogenesis and long-term potentiation. As found in this project, these processes are exclusively the effects of increased physical activities and voluntary exercise resulting from environmental. **(iv)** While this project specifically deals with the hippocampus, prenatal alcohol exposure also damages other areas of the developing brain (Brocardo et al., 2011; Guerri, 2001; Minana et al., 2000; Olney, 2004; Olson et al., 1998). Thus, while altered genes and pathways may be able to affect some

structural and functional changes in the hippocampus that may translate into ameliorated behavioural and cognitive improvement, their effects are effective only to a certain extent and/or post-natal environmental enrichment fails to elicit molecular changes in various other damaged regions of the adult mouse brain to bring about a complete recovery. **(v)** As previously pointed out in section 5.2, several of the genes significantly altered in response to prenatal alcohol exposure have been found to be altered in previous studies conducted in the Singh laboratory (Kleiber et al., 2012; Laufer et al., 2013; Mantha et al., 2014).

7.2 Future directions

A multidisciplinary approach that combines biological strategies (targeting various molecular, cellular, physiological and metabolic processes) along-with improved maternal care, accurate prognosis and environmental enrichment seems to be the most effective strategy to combat FASD (Kodituwakku, 2010; Paley and O'Connor, 2009; Peadon et al., 2009; Rasmussen et al., 2009). However, these approaches should be tailored to the cognitive and behavioural profile of FASD specifically. For example, even though children who are diagnosed with FASD and ADHD are often characterized by one of the primary deficits being a lack of attention, these two groups have distinct cognitive profiles (Koren et al., 2014) and hence, intervention strategies for one will not work for the other.

An important part of this project is the delineation of the neuro-molecular basis of developmental disorders and brain plasticity in the context of prenatal alcohol exposure and post-natal environmental enrichment. It is, thus, critical that this 'dynamic reciprocal interaction' (Kodituwakku, 2007) between the environment and the nervous system be taken into account when creating individual neurocognitive profiles in children with FASD and creating tailor-made interventions for them (Kodituwakku, 2010). For example, a number of studies in rodents have demonstrated that a guided intervention of various motor experiences can influence the plasticity of specific brain regions in alcohol-

exposed rats (Hamilton et al., 2014; Klintsova et al., 2002; Schreiber et al., 2013). However, so far, the results of this project are novel, in terms of explaining the effects of the environment on the hippocampus of alcohol-exposed mice in terms of genes and molecular pathways.

It is also possible, in fact, inevitable, that the molecular 'benefits' of environmental enrichment may be harnessed to develop a new class of drugs that target those molecules that mediate and facilitate the beneficial effects of environmental enrichment (McOmish and Hannan, 2007). Specifically, these drugs would be able to bypass the varied individual response to environmental conditions to ensure a more targeted and consistent response. The specific molecular pathways (uncovered by this project) responsible for downstream neural plasticity and behavioural recovery provide a good starting point for future drug discovery studies.

The results of this thesis point to two important observations:

1. The amelioration of FASD-related behavioural deficits through environmental enrichment has specific molecular signatures in terms of gene expression patterns and molecular pathways.
2. This project's results are an essential starting point to explore future molecular studies to establish causal relationships between the observed phenotype and gene-expression data. The hippocampal tissues generated from the project can be further utilized in various ways, e.g., hippocampal

DNA can be used for MeDIP sequencing to generate methylomic data. Laser dissection microscopy can be used to isolate specific cell/neuron types in the hippocampus, and single-cell transcriptomics for the various hippocampal sub-regions can be used to pinpoint region- and cell-specific gene-expression changes. Also, hippocampal tissues can be analyzed using immunohistochemistry to evaluate changes in cell composition, synaptic density and neurogenesis.

3. The various genes and molecular pathways uncovered in this project can be used as a starting point for drug-discovery studies that directly exploit these molecular pathways in order to design more effective and efficacious rehabilitative therapies for FASD-related abnormalities.

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APPENDICES

Appendix 1. Animal use protocol approvals from Animal Care and Veterinary Services at the University of Western Ontario.

Appendix K – Ethical Approval

Mouse

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
PH: 519-661-2111 ext. 86768 • FL 519-661-2028

Appendix 2. List of differentially-expressed genes obtained from microarray analysis of AE v AN group

Gene	Gene name	Fold-change	p-value
9330185C12Rik	RIKEN cDNA 9330185C12 gene	1.731	0.009
mt-Ti	mitochondrially encoded tRNA isoleucine	1.506	0.005
Mirlet7a-1	microRNA let7a-1	1.427	0.028
Bmp3	bone morphogenetic protein 3	1.418	0.007
Dsc3	desmocollin 3	1.414	0.006
Hist1h2bn	histone cluster 1, H2bn	1.387	0.045
Cbln4	cerebellin 4 precursor protein	1.372	0.028
Gpr52	G protein-coupled receptor 52	1.359	0.010
Trav6d-4	T cell receptor alpha variable 6D-4	1.355	0.019
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	1.323	0.022
Arntl	aryl hydrocarbon receptor nuclear translocator-like	1.304	0.000
Gm7551	predicted gene 7551	1.302	0.013
Hist1h3a	histone cluster 1, H3a	1.293	0.039
A130040M12Rik	RIKEN cDNA A130040M12 gene	1.289	0.044
Gabra2	gamma-aminobutyric acid A receptor, subunit alpha 2	1.289	0.027
Gm20419	predicted gene 20419	1.288	0.017
Gm14322	predicted gene 14322	1.282	0.020
Camk2d	calcium/calmodulin-dependent protein kinase II, delta	1.272	0.034
Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	1.269	0.029
Ptger3	prostaglandin E receptor 3 (subtype EP3)	1.267	0.026
Gm5643	heterogeneous nuclear ribonucleoprotein A1 pseudogene	1.258	0.022
Gm20634	predicted gene 20634	1.258	0.022
Angpt1	angiopoietin 1	1.254	0.013
Hist1h1e	histone cluster 1, H1e	1.253	0.020
BC080695	cDNA sequence BC080695	1.253	0.020
Ceacam1	carcinoembryonic antigen-related cell adhesion molecule 1	1.246	0.011
Cdh18	cadherin 18	1.243	0.019
Traj58	T cell receptor alpha joining 58	1.243	0.020
Nr4a2	nuclear receptor subfamily 4, group A, member 2	1.230	0.021
Rel	reticuloendotheliosis oncogene	1.222	0.026
AU023762	expressed sequence AU023762	1.222	0.008
Fmod	fibromodulin	1.222	0.017
Ascl1	achaete-scute complex homolog 1	1.222	0.004
Ggt5	gamma-glutamyltransferase 5	1.221	0.001
Nmbr	neuromedin B receptor	1.221	0.016
Zbtb10	zinc finger and BTB domain containing 10	1.219	0.009
Plag1	pleiomorphic adenoma gene 1	1.219	0.002
Arhgap29	Rho GTPase activating protein 29	1.216	0.043
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	1.215	0.003
9430021M05Rik	RIKEN cDNA 9430021M05 gene	1.206	0.037
A330102I10Rik	RIKEN cDNA A330102I10 gene	1.201	0.009
Fam83d	family with sequence similarity 83, member D	-1.200	0.012
Speer4d	spermatogenesis associated glutamate (E)-rich protein 4d	-1.201	0.035
Tmem186	transmembrane protein 186	-1.204	0.024
Epb4.1l4a	erythrocyte protein band 4.1-like 4a	-1.205	0.039
Bhlhe41	basic helix-loop-helix family, member e41	-1.207	0.007
St18	suppression of tumorigenicity 18	-1.209	0.009

Bace2	beta-site APP-cleaving enzyme 2	-1.209	0.021
Ppp1r36	protein phosphatase 1, regulatory subunit 36	-1.213	0.046
Erbb3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	-1.215	0.035
Grp	gastrin releasing peptide	-1.218	0.030
n-R5s26	nuclear encoded rRNA 5S 26	-1.219	0.018
Rnu3a	U3A small nuclear RNA	-1.219	0.016
Ppap2c	phosphatidic acid phosphatase type 2C	-1.219	0.030
Gdf10	growth differentiation factor 10	-1.222	0.000
Ppp1r14a	protein phosphatase 1, regulatory (inhibitor) subunit 14A	-1.223	0.039
Fbl	fibrillarin	-1.223	0.001
Rhoa	ras homolog gene family, member A	-1.226	0.028
Scn7a	sodium channel, voltage-gated, type VII, alpha	-1.227	0.036
LOC100862618	protein tweety homolog 2-like	-1.227	0.016
Plxnb3	plexin B3	-1.233	0.029
Enpp6	ectonucleotide pyrophosphatase	-1.234	0.023
Mag	myelin-associated glycoprotein	-1.235	0.042
Rhog	ras homolog gene family, member G	-1.238	0.006
Gm3227	predicted gene 3227	-1.240	0.002
Gm10462	predicted gene 10462	-1.242	0.037
Cpne2	copine II	-1.243	0.045
7630403G23Rik	RIKEN cDNA 7630403G23 gene	-1.244	0.024
Lect1	leukocyte cell derived chemotaxin 1	-1.246	0.009
Plagl1	pleiomorphic adenoma gene-like 1	-1.247	0.001
Rps6ka4	ribosomal protein S6 kinase, polypeptide 4	-1.247	0.001
Mthfd1l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	-1.248	0.000
Mrpl32	mitochondrial ribosomal protein L32	-1.249	0.010
Agt	angiotensinogen (serpin peptidase inhibitor, clade A, member 8	-1.258	0.004
LOC100503055	60S ribosomal protein L29-like	-1.259	0.004
Rpl13-ps3	ribosomal protein L13, pseudogene 3	-1.264	0.015
Tnfaip6	tumor necrosis factor alpha induced protein 6	-1.264	0.021
Prr5	proline rich 5 (renal)	-1.274	0.008
Per2	period circadian clock 2	-1.277	0.036
Cd24a	CD24a antigen	-1.281	0.000
Ninj2	ninjurin 2	-1.282	0.017
Amigo2	adhesion molecule with Ig like domain 2	-1.288	0.037
Mir505	microRNA 505	-1.298	0.044
Aspa	aspartoacylase	-1.305	0.024
Calb2	calbindin 2	-1.312	0.029
Gm11681	predicted gene 11681	-1.330	0.015
Cxcr4	chemokine (C-X-C motif) receptor 4	-1.342	0.004
Fam216b	family with sequence similarity 216, member B	-1.349	0.013
Sntn	sentan, cilia apical structure protein	-1.370	0.040
Nkd2	naked cuticle 2 homolog	-1.374	0.005
Cdhr3	cadherin-related family member 3	-1.376	0.006
Rarres2	retinoic acid receptor responder (tazarotene induced) 2	-1.418	0.002
Dbp	D site albumin promoter binding protein	-1.449	0.000
Gm129	predicted gene 129	-1.524	0.001
Tmem212	transmembrane protein 212	-1.626	0.025
Vgll3	vestigial like 3	-1.635	0.008

Appendix 3. List of differentially-expressed genes obtained from RNA-Seq analysis of AE v AN group

Gene	Gene name	Fold-change	p-value
Arhgap36	rho GTPase Activating Protein 36	-1.899	0.014
Baiap3	BAI1 Associated Protein 3	-1.145	0.000
AW551984	expressed sequence AW551984	-1.023	0.001
Plagl1	PLAG1 Like Zinc Finger 1	-0.716	0.010
Met	MET proto-oncogene, receptor tyrosine kinase	-0.559	0.032
Wdr6	WD Repeat Domain 6	-0.418	0.032
Rasd2	RASD Family Member 2	0.440	0.057
Hspa1b	heat shock protein Family A (Hsp70) Member 1B	1.464	0.016
Hspa1a	heat shock protein Family A (Hsp70) Member 1A	1.548	0.019
Gm20481	predicted gene 20481	1.702	0.044
Icam1	intercellular Adhesion Molecule 1	2.297	0.001
Ch25h	cholesterol 25-Hydroxylase	3.303	0.009
Bcl3	B-Cell CLL/Lymphoma 3	3.912	0.014
Cxcl1	C-X-C Motif Chemokine Ligand 1	5.564	0.012
Serpina3f	serine peptidase inhibitor, clade A, member 3F	7.840	0.014

Appendix 4. List of differentially-expressed genes common across all three platforms for the AE v AN group

Gene	Gene name	Fold-change	p-value	Platform
AW551984	expressed sequence AW551984	-1.023	0.001	RNA-Seq (EdgeR)
		-1.397	0.026	RNA-Seq (Partek)
		-1.998	0.000	Microarray
Baiap3	BAI1 Associated Protein 3	-1.145	0.000	RNA-Seq (EdgeR)
		-1.237	0.003	RNA-Seq (Partek)
		-1.678	0.030	Microarray
Ch25h	cholesterol 25-Hydroxylase	3.303	0.009	RNA-Seq (EdgeR)
		1.841	0.041	RNA-Seq (Partek)
		10.054	0.023	Microarray
Icam1	intercellular adhesion molecule 1	2.297	0.001	RNA-Seq (EdgeR)
		2.420	0.025	RNA-Seq (Partek)
		5.144	0.020	Microarray
Plagl1	PLAG1 Like Zinc Finger 1	-0.716	0.010	RNA-Seq (EdgeR)
		-1.398	0.001	RNA-Seq (Partek)
		-1.608	0.003	Microarray
Wdr6	WD Repeat Domain 6	-0.418	0.032	RNA-Seq (EdgeR)
		-1.301	0.006	RNA-Seq (Partek)
		-1.312	0.004	Microarray

CURRICULUM VITAE

ANIRUDDHO CHOKROBORTY-HOQUE

EDUCATION

Doctoral Candidate (Biology) Western University, London, Canada	**2011 - 2017**
M.Sc. (Biology) Western University, London, Canada	**2008 - 2011**
Research Fellow (Biology) Jawaharlal Nehru University, India	**2007 - 2008**
M.Sc. (Biology) Amity University, Noida, India	**2005 - 2007**
B.Sc. (Chemistry) University of Delhi, India	**2002 - 2005**

ACADEMIC PUBLICATIONS

- Chokroborty-Hoque A, Singh SM. 2017. *Hippocampal gene expression changes following environmental enrichment in a mouse model of fetal alcohol spectrum disorders* Genes, Brain and Behaviour *(in preparation)*
- Chokroborty-Hoque A, Singh SM. 2017. *Amelioration of behavioural and cognitive deficits via environmental enrichment in a mouse model of fetal alcohol spectrum disorders* Behavioral Brain Research *(in preparation)*
- Chokroborty-Hoque A, Alberry B, Singh SM. 2014. *Exploring the complexity of intellectual disability in fetal alcohol spectrum disorders.* Experimental models of early exposure to alcohol: a way to unravel the neurobiology of mental retardation. Vol. 2:90, 30 - 38.
- 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.* Frontiers in Genetics. Vol. 5:61, 1-12

CONFERENCE PRESENTATIONS

Seventh International Conference on FASD, Vancouver	**MAR 2017**
Fetal Alcohol Canadian Expertise conference, Ottawa	**OCT 2015**
World Congress of Psychiatric Genetics, Toronto	**SEP 2015**
Western Graduate Research Forum, Western University	**AUG 2014**
McGill Biomedical Graduate Conference, McGill University	**MAR 2014**

POSTER PRESENTATIONS

American Society of Human Genetics, Baltimore	**AUG 2015**
Society of Neuroscience, Western University	**APR 2014**