Replenishing what is Lost: Using Supplementation to Enhance Hippocampal Function in Fetal Alcohol Spectrum Disorders

by

Anna Ruth Patten
Bachelor of Science, University of Otago, 2006

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Supervisory Committee

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Anna Ruth Patten
Bachelor of Science (Honours), University of Otago, Dunedin, New Zealand

Supervisory Committee

Dr. Brian R. Christie (Division of Medical Sciences, Department of Biology)
Supervisor

Dr. Leigh Anne Swayne (Division of Medical Sciences, Department of Biology)
Departmental Member

Dr. Francis Choy (Department of Biology)
Departmental Member

Dr. Robert Burke (Department of Biochemistry and Microbiology)
Outside Member

Abstract

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Outside Member

Fetal Alcohol Spectrum Disorders (**FASD**) are the most common cause of cognitive impairment in the United States (Sokol et al., 2003). In young school children in North America and some Western European countries, recent reports have estimated the prevalence of FASD to be as high as 2-5% (May et al., 2009). Currently there are no widely accepted treatment options for FASD, mainly due to the fact that the underlying neurological deficits that occur with prenatal ethanol exposure (**PNEE**) are still largely unknown. This thesis examines the long-lasting changes that occur in the hippocampus following PNEE using biochemical and electrophysiological techniques. We find that PNEE produces a reduction of the endogenous antioxidant glutathione (**GSH**), resulting in an increase in oxidative stress that is accompanied by long-lasting reductions in long-term potentiation (**LTP**) of synaptic efficacy. Interestingly, males exhibited greater deficits in synaptic plasticity than females, despite similar reductions in GSH in both sexes. By depleting GSH in control animals we determined that LTP in the DG of female animals is more resistant to changes in GSH, which may explain the sexual dichotomy observed in these studies of PNEE. Based on these findings, ethanol-exposed animals received postnatal dietary supplementation with either a precursor of GSH, N-

Acetylcysteine (**NAC**) or Omega-3 fatty acids. These supplements helped to counteract the effects of PNEE and improved hippocampal function. The findings in this thesis support the hypothesis that increasing antioxidant capacity can enhance hippocampal function, which in turn may improve learning and memory in FASD, providing a therapeutic avenue for children suffering with these disorders.

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4HHE	4-hydroxyhexenal
4HNE	4-hydroxynonenal
AA	Arachidonic acid
ADH	Alcohol dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropoionic acid
ANOVA	Analysis of variance
AP-1	Activator protein-1
APV	2-amino-5-phosphonopentanoic acid
ARBD	Alcohol-related birth defects
ARND	Alcohol-related neurological disorders
ATP	Adenosine triphosphate
BAC	Blood alcohol concentration
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
CA	*Cornu Ammonis*
CaMKII	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CNS	Central nervous system
CPP	3(2-carboxypiprazin-4-yl)-propyl-1-phosphonic acid
CRE	cAMP response element
CREB	cAMP response element binding protein
CYP2E1	Cytochrome P450 enzyme 2E1
DEM	Diethyl maleate
DG	Dentate gyrus
DGC	Dentate granule cell
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-dithiobis(2-nitrobenzoic) acid
EAAT	Excitatory amino acid transporter
EC	Entorhinal cortex
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
fEPSP	Field excitatory postsynaptic potential
fMRI	functional magnetic resonance imaging
G6PH	Glucose-6-phosphate-dehydrogenase
GABA	Gamma aminobutyric acid
GCS	γ-glutamylcysteinyl synthetase
GD	Gestation day
GGT	Gamma-glutamyl transpeptidase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSH-t	Total levels of glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HO·	Hydroxyl radical
HPA	Hypothalamus-pituitary-adrenal
IGF-1	Insulin-like growth factor-1
i.p.	Intraperitoneal

IQ	Intelligence quotient
L-BOAA	β-*N*-oxalyl amino-L-alanine
LPP	Lateral perforant path
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
mGluR	metabotropic glutamate receptor
MPP	Medial perforant path
MRI	Magnetic resonance imaging
NAC	N-Acetyl cysteine
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADP-ICH	NADP-linked isocitrate dehydrogenase
NFκB	Nuclear factor kappa B
NMDA	*N*-methyl-D-aspartate
NOS	Nitric oxide synthase
NPD1	Neuroprotectin D1
O_2-·	Superoxide
$ONOO^-$	Peroxynitrite
OVX	Ovariectomy
PI3K-Akt	Phosphoinositide 3-kinase-protein kinase B
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PND	Postnatal day
PNEE	Prenatal ethanol exposure
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphotidylserine
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SEM	Standard error of the mean
SOD	Superoxide dismutase
t-BOOH	*tert*-butyl hydroperoxide
TBARS	Thiobarbituric acid reactive substances
TBS	Theta burst stimulation
TNB	5-thio-2-nitrobenzoic acid
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
γGlu-Cys	Gamma-glutamyl-cysteine
·NO	Nitric oxide

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Dedication

I dedicate this work to Mark and my family for their love and support

1. Introduction

This Chapter is based in part on the following Book Chapter:

Anna Patten, Patricia Brocardo, Joana Gil-Mohapel and Brian Christie (2013) Oxidative Stress in Fetal Alcohol Spectrum Disorders: Insights for the Development of Antioxidant-Based Therapies. In "Systems Biology of Free Radicals and Anti-Oxidants". *Springer-Verlag (Germany). (In Press).*

1.1 Fetal Alcohol Spectrum Disorders

Ethanol is lipid and water soluble, and when it is consumed by pregnant females it rapidly affects the fetus through the placental membrane (Idanpaan-Heikkila et al., 1972). Ethanol can cause significant damage to the fetus (Jones, 1975, Sokol et al., 2003). The most severe disorder that results from prenatal ethanol exposure (**PNEE**) is Fetal Alcohol Syndrome (**FAS**). FAS is a disorder characterised by facial dysmorphologies such as midfacial hypoplasia, wide spaced eyes and a smooth philtrum, growth retardation and CNS dysfunction resulting in cognitive, motor and behavioural problems (Sokol et al., 2003). Since FAS was first defined in the 1970's (Jones and Smith, 1973, Jones, 1975) it has been realised that the extent of the damage caused by ethanol can vary due to the timing, frequency and volume of ethanol consumed, as well as the genetics and metabolism of the mother, leading to a wide variability in the severity and symptoms associated with PNEE. The disorders that result from PNEE are now grouped under the umbrella term Fetal Alcohol Spectrum Disorders (**FASD**), which encompasses children who show various forms of central nervous system (**CNS**) dysfunction including alcohol-related birth defects (**ARBD**) and alcohol-related neurological disorders (**ARND**) that result from PNEE but often lack the facial dysmorphology needed to meet the diagnostic criteria for FAS (Burd and Martsolf, 1989, Sokol et al., 2003).

Although it has been recognized since the 1970's that ethanol is a teratogen, there are still large numbers of children affected by PNEE (May et al., 2009). In part, this is because many women do not realize they are pregnant in the first trimester and continue binge drinking (O'Leary et al., 2010a, O'Leary et al., 2010b). Furthermore, in many countries a significant percentage of pregnant women continue to consume ethanol throughout pregnancy – 10-20% in the USA, 40% in Uruguay and 50% in some parts of Italy (Ceccanti et al., 2007, Prevention, 2009, Hutson et al., 2010). In the United States the lifetime cost for an individual suffering from FAS may be as high as $2 million. The majority of these costs are required for special education, medical, and mental health treatment (Lupton et al., 2004). Currently in Canada the annual cost of health care problems associated with PNEE is over $5 billion (Stade et al., 2009). Therapeutic options for children and families affected with FASD include behavioural and psychological support, but there are currently no pharmacological therapies for treating the underlying neurobiological consequences of FASD (Kalberg and Buckley, 2007, Bertrand, 2009).

1.1.1 Cognitive symptoms

A commonality that occurs throughout the FASD spectrum is CNS dysfunction in infancy and adolescence that manifests as cognitive and behavioural problems that can last into adulthood (Jones and Smith, 1973, Streissguth and LaDue, 1987, Streissguth et al., 1990, Streissguth et al., 1991, Streissguth et al., 1994, Kerns et al., 1997). Children with FASD display a multitude of neuropsychological issues including deficits in mathematical ability, verbal fluency, memory, attention, learning capabilities, executive function, fine motor control and social interaction, with the number of issues and the

extent of damage varying from child to child (Streissguth et al., 1990, Streissguth et al., 1994, Kerns et al., 1997, Alfonso-Loeches and Guerri, 2011). To be diagnosed with an intellectual disability generally a child must have an intelligence quotient (**IQ**) score below 70, and scores between 71 and 85 are considered to represent borderline intellectual function (DSM IV, 2000). Children with FAS generally have IQs estimated in the low 70s but the range can be anywhere between 20 and 120 (Streissguth et al., 1991, Olson et al., 1998). Children without the complete FAS diagnosis also generally have low IQs with averages in the low 80s (Mattson et al., 1998).

1.1.2 Rodent models of FASD

To further understand the mechanism of the toxic effects of ethanol on the developing brain, and in order to develop and test potential therapies to combat these effects, rodent models are often utilized in the laboratory. Rodents provide a simple and easy to control model due to their short lifespan, and the ability to manipulate social and behavioural contexts. For example, it is possible to control the pattern of ethanol exposure (chronic or acute), timing of exposure (1^{st}, 2^{nd} or 3^{rd} trimester equivalents), the amount of ethanol the fetus is exposed to, and the level of stress that the mother experiences during the pregnancy (reviewed by (Gil-Mohapel et al., 2010).

A drawback to using rats or mice is that the 3^{rd} trimester equivalent of brain development that encompasses the 'brain growth spurt' (Dobbing and Sands, 1973); see section 1.1.3) occurs postnatally (from postnatal day (**PND**) 1-10; (West, 1987). This creates an issue, because in order to expose the brain to alcohol through all three trimester equivalents, alcohol must be administered to neonate pups, and the mechanisms of exposure, absorption and elimination of this substance are significantly different during

the prenatal and postnatal periods. While the gavage model is often used in the laboratory to expose animals to alcohol during the third trimester equivalent (Thomas et al., 1996, Helfer et al., 2009, Boehme et al., 2011, Gil-Mohapel et al., 2011, Brocardo et al., 2012), in this thesis the majority of the experiments are conducted using the liquid diet model. This model does not include alcohol exposure during the third trimester but still produces reliable deficits in neurological function similar to those observed in humans (reviewed by (Gil-Mohapel et al., 2010). The liquid diet model is a widely used model of moderate FASD (reviewed by (Gil-Mohapel et al., 2010). Throughout gestation, food is provided to pregnant dams as a liquid diet in which 35.5% of the calories are derived from ethanol (6.61% v/v).

The Canadian legal intoxication limit corresponds to a blood alcohol concentration (**BAC**) of 80 mg/dl. Most animal studies use a dosage of alcohol exposure that produces a BAC in the range of 100-400 mg/dl (i.e. moderate to binge-like levels of exposure). The liquid diet utilized in this thesis produces BACs between 80-150 mg/dl (Christie et al., 2005, Lan et al., 2006, Lan et al., 2009, Patten et al., 2012, Titterness and Christie, 2012).

1.1.3 Ethanol and the developing brain

The mammalian brain develops in six major phases, commencing with neural cell genesis, followed by neuronal migration, glial cell proliferation, axon and dendrite proliferation, synaptogenesis and finally myelination of the axons (Erecinska et al., 2004). These steps occur in all regions of the brain but different regions develop at different times depending on their caudal or rostral location.

Human brain development begins in embryogenesis during a process called neurulation. On approximately gestation day (**GD**) 18, the plate invaginates and begins to

fold, with the neural tube beginning to form at approximately GD 21 (Rice and Barone, 2000). A population of cells, known as neural crest cells, separate from the apex of the neural tube and these will eventually develop into sensory ganglia for the spinal and cranial nerves, Schwann cells and the meninges (Rice and Barone, 2000). The neural tube is complete by GDs 26 – 28 in humans and this corresponds roughly to GDs 10.5 – 11 in rats. Next the forebrain, the midbrain and the hindbrain begin to form through processes of proliferation, differentiation, migration, synaptogenesis, apoptosis and myelination (Rice and Barone, 2000). While the majority of the processes are completed by birth, synaptogenesis and myelination continue to occur throughout childhood and adolescence, and neurogenesis can occur into adulthood in specific areas of the brain (the subventricular zone and the subgranular zone of the dentate gyrus (**DG**) subregion of the hippocampus (Altman and Das, 1965). The majority of developmental neurogenesis however, is complete by 22 weeks of gestation in humans, and just prior to birth in rodents (Erecinska et al., 2004).

Ethanol can cause irreversible structural damage to the developing brain where it acts as a positive allosteric modulator of the γ-aminobutyric acid (**GABA**)$_A$ receptor and an N-methyl-D-aspartate (**NMDA**) receptor antagonist (reviewed by (Grant, 1994), but also causes many damaging effects due to the products produced due to its metabolism (see section 1.2). Molecular and neurochemical events can be disrupted by ethanol, altering gene expression, cell-cell interactions and growth factor response (reviewed by (Alfonso-Loeches and Guerri, 2011), and different brain structures can be affected to greater or lesser extent depending on the developmental timing of ethanol exposure (Guerri et al., 2009). During the embryonic stage of gastrulation (which corresponds to weeks three and

four of human gestation; 1st trimester) ethanol exposure can interfere with neural tube development and cause microencephaly (Miller, 1996a) and the facial dysmorphology that characterizes FAS (Sulik et al., 1981, Sulik, 2005). During the 2nd trimester of development (7 – 20 weeks in humans; GDs 12 – 21 in rodents) cell proliferation and migration are occurring profusely. Ethanol can disrupt these processes by altering migration, impairing the timing of cell proliferation and reducing neuron and glial cell numbers in many areas of the brain including the neocortex, hippocampus and sensory nucleus (Gressens et al., 1992, Rubert et al., 2006, Suzuki, 2007). Indirectly, ethanol can alter the expression of neurotrophic factors such as transforming growth factor β (Luo and Miller, 1998, Miller and Luo, 2002, Siegenthaler and Miller, 2005), and affect the migration of cortical neurons and glia during this time period (Miller and Robertson, 1993, Siegenthaler and Miller, 2005). Ethanol exposure also causes devastating effects during the 3rd trimester of pregnancy (weeks 28 – 40; PNDs 1 – 10 in rats and mice), when the 'brain growth spurt' occurs (Dobbing and Sands, 1979). Neurons are very susceptible to the apoptotic effects of ethanol during this period (Ikonomidou et al., 2000) and excessive cell death may lead to long-term deficits in learning and memory processes (Wozniak et al., 2004).

Autopsies of patients affected with FASD show that damage occurs throughout the brain and that microencephaly is particularly apparent in many cases, along with errors in migration, and anomalies in the cerebellum and brainstem (Jones and Smith, 1973). Further studies have shown that the CNS is disorganised, and deformities occur in the basal ganglia, hippocampus and pituitary gland (Jones, 1975, Clarren et al., 1978). Since the development of magnetic resonance imaging (**MRI**) more specific deficits have been

identified. For example, the cranial, cerebral and cerebellar vaults show reductions in volume in FASD subjects (Swayze et al., 1997), the cerebellum can also be reduced in volume and surface area (Mattson et al., 1994, Autti-Ramo et al., 2002) and the basal ganglia are often much smaller in size (Mattson et al., 1994), possibly explaining the motor deficits often observed in patients with FASD (Guerri et al., 2009). In this thesis we have chosen to focus on the hippocampus due to its role in learning and memory processes, and the impact of PNEE in this region (see section 1.3). The specific effects of PNEE on the structure and function of the hippocampus, are discussed in detail in section 1.3.1.4.

1.1.4 Underlying mechanisms of PNEE damage

Because of the variety of deficits that occur with FASD it can be hard to pinpoint exactly what occurs in the developing CNS to produce these disorders. Many different brain regions are involved, and the areas and extent of damage depend on the amount and timing of ethanol ingestion. Many molecular mechanisms may play a role, and these may be activated at different stages of development or at different dose thresholds of exposure (reviewed by (Goodlett et al., 2005, Gil-Mohapel et al., 2010). These include: disrupted cell energetics (Miller and Dow-Edwards, 1988, Snyder and Singh, 1989, Snyder et al., 1992, Shibley and Pennington, 1997, Fattoretti et al., 2003); cell cycle interference, and a deregulation of developmental timing (Phillips, 1989, Miller and Robertson, 1993, Miller, 1996b, Liesi, 1997, Lindsley et al., 2003); alterations in retinoic acid signaling (Deltour et al., 1996); interference with cell and growth factor signaling (Luo and Miller, 1996, Zhang et al., 1998, Ge et al., 2004), and apoptosis (Bhave and Hoffman, 1997, Zhang et al., 1998, Ikonomidou et al., 2000). Furthermore, many neurotransmitters,

adhesive molecules, transcription factors and trophic factors can be either up- or down-regulated by PNEE, making FASD a very complex syndrome (reviewed by (Goodlett et al., 2005).

While the underlying causes of FASD abnormalities are multifaceted, a clear relationship between PNEE and oxidative stress in the brain has also been established (Reyes et al., 1993, Guerri et al., 1994, Henderson et al., 1995, Ramachandran et al., 2001, Heaton et al., 2002, Ramachandran et al., 2003, Siler-Marsiglio et al., 2005, Dembele et al., 2006, Brocardo et al., 2012, Patten et al., 2012), for review see (Guerri et al., 1994, Brocardo et al., 2011). This relationship will be further investigated in this thesis.

1.2 Oxidative stress and PNEE

Oxidative stress results when there is an imbalance between the production of reactive oxygen species (**ROS**) and/or reactive nitrogen species (**RNS**) and the endogenous ability to detoxify these species or repair the resulting damage (reviewed by (Sies, 1991, Dringen, 2000). Oxidative stress is upregulated by disease, stress and exposure to toxins and can lead to cell death through apoptosis and necrosis (Ratan et al., 1994, Tan et al., 1998, Ott et al., 2007).

1.2.1 Reactive oxygen species and reactive nitrogen species

ROS/RNS are highly reactive and the majority contain an unpaired electron (in this case forming a free radical). The most common ROS/RNS found in the CNS are hydroxyl radicals (**HO·**), superoxide (**O_2-·**) and nitric oxide (**·NO**). Physiologically, basal levels of ROS/RNS are produced intracellularly by normal cellular mechanisms,

including mitochondrial energy metabolism as well as the xanthine oxidase, nitric oxide synthase (**NOS**), and reduced nicotinamide adenosine dinucleotide phosphate (**NADPH**) oxidase pathways in the cytoplasm (reviewed by (Halliwell, 1991, Finkel and Holbrook, 2000). ROS/RNS are not always damaging and can be produced in large quantities by inflammatory cells to help kill invading microorganisms (Swindle and Metcalfe, 2007). ROS/RNS are also involved in cell-cell interactions and may be important mediators of cell growth and differentiation due to the redox-controlled nature of some transcription factors such as activator protein-1 (**AP-1**) and nuclear factor kappa B (**NFκB**) (Dean et al., 1997). These transcriptional factors can be regulated by redox state due to the presence of highly conserved cysteine residues in the deoxyribonucleic acid (**DNA**) binding domains of the proteins (Sen and Packer, 1996, Sun and Oberley, 1996). In general, reducing environments increase DNA binding of redox controlled transcription factors whereas an oxidised environment inhibits binding (Sun and Oberley, 1996). Damaging levels of ROS/RNS can occur through exposure to external sources of ROS/RNS such as cigarette smoke, pollution, radiation and chemical agents such as alcohol (Zadak et al., 2009).

About 2-4% of oxygen used by the mitochondria is converted to O_2-· (Chance et al., 1979) which is possibly the most abundant ROS in cells. O_2-· can be converted to hydrogen peroxide (**H_2O_2**) by the action of superoxide dismutase (**SOD**, reaction **1**). SODs are a class of metalloprotein enzymes responsible for inactivating O_2-· and different types of SOD are found depending on cellular location. For example, a SOD that contains copper and zinc is found in the cytosol as well as in between mitochondrial

membranes (CuZnSOD), however, inside the mitochondria, a manganese containing SOD (MnSOD) is more predominant (Fridovich, 1997).

$$2O_2\text{-}\cdot + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \textbf{(1)}$$

H_2O_2 can be converted to water and molecular oxygen by the action of glutathione peroxidase (**GPx**) or catalase (**CAT**), thus preventing lipid peroxidation (see section 1.2.2). GPx is a selenocysteine containing antioxidant enzyme that utilizes glutathione (**GSH**), and when H_2O_2 is reduced by GPx, GSH acts as an electron donor in the reaction and is converted to glutathione disulfide (**GSSG;** reaction **2**) (Aoyama et al., 2008).

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O \quad \textbf{(2)}$$

Glutathione reductase (**GR**) is a homodimeric flavoprotein that plays an important role in regenerating GSH from GSSG and preventing oxidative damage due to a lack of GSH. GR utilises the co-factor NADPH to reduce oxidised GSSG back to GSH (reaction **3**). In this process an electron is transferred from the reduced form of NADPH to GSSG thereby creating GSH and NADP+ (Dringen, 2000).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad \textbf{(3)}$$

CAT is an important heme-containing enzyme found in peroxisomes that also prevents H_2O_2 build up in the cell (reaction **4**). CAT allows H_2O_2-producing cellular reactions to

occur without causing damage to the cell, by degrading any H_2O_2 produced. CAT is also an important enzyme involved in the metabolism of ethanol, particularly in the fetal brain (Hamby-Mason et al., 1997).

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \quad \textbf{(4)}$$

If H_2O_2 is not detoxified by CAT or GPx and instead reacts with iron via the Fenton/Haber-Weiss reaction, HO· is produced (reaction **5** (Aoyama et al., 2008). HO· is particularly dangerous when it is produced near membranes as it can cause lipid peroxidation which leads to the propagation of a free radical chain reaction and damage to the membrane (Forman et al., 2009).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO\text{-}$ (Fenton Reaction)

$Fe^{3+} + O_2\text{-}\cdot \rightarrow Fe^{2+} + O_2$ (Haber-Weiss Reaction)

$$O_2\text{-}\cdot + H_2O_2 \xrightarrow{Fe^{2+/3+}} HO\cdot + HO\text{-} + O_2 \quad \text{(Sum)} \quad \textbf{(5)}$$

O_2-· can also cause damage to lipids and proteins, particularly if it reacts with ·NO. ·NO is produced by **NOS**, during the conversion of L-arginine to L-citrulline (reaction **6**).

$$\text{L-arginine} \xrightarrow{NOS} \text{L-citrulline} + \cdot NO \quad \textbf{(6)}$$

The reaction between ·NO and O_2-· produces peroxynitrite ($ONOO^-$) which can diffuse 10,000x faster than O_2-· or HO· (reaction **7** (Beckman, 1994). $ONOO^-$ can oxidise

proteins, lipids and DNA and can also cause the nitration of amino acids and inactivate mitochondrial enzymes (Pacher et al., 2007).

$$O_2\text{-}\cdot + \cdot NO \rightarrow ONOO^- \quad \textbf{(7)}$$

1.2.2 Lipid peroxidation

Lipid peroxides are formed when ROS/RNS react with polyunsaturated fatty acids (**PUFA**s). PUFAs are particularly vulnerable to peroxidation due to the presence of one or more methylene groups between *cis* double bonds (Marnett, 1999). There are three stages to lipid peroxidation; initiation, propagation and termination. Initiation occurs when a ROS such as HO· attacks a fatty acid and a fatty acid radical is formed by removal of a hydrogen atom from the methylene group (reaction **8** (Marnett, 1999).

$$PUFA + HO\cdot \rightarrow \underset{\textit{Fatty acid radical}}{PUFA\cdot} + H_2O \quad \textbf{(8)}$$

Fatty acid radicals are extremely unstable and they readily react with molecular oxygen to create a peroxyl-fatty acid radical (reaction **9**).

$$PUFA\cdot + O_2 \rightarrow \underset{\textit{Peroxy-fatty acid radical}}{PUFAO_2\cdot} \quad \textbf{(9)}$$

Peroxy-fatty acid radicals are also unstable and can react with another fatty acid to create a lipid peroxide and a new fatty acid radical (reaction **10**).

$$PUFAO_2\cdot + PUFA \rightarrow \underset{\textit{Lipid Peroxide}}{PUFAO_2H} + \underset{\textit{Fatty acid radical}}{PUFA\cdot} \quad \textbf{(10)}$$

This process can be further amplified, which is referred to as the propagation stage of lipid peroxidation. Termination only occurs when two fatty acid radicals react with each other or a fatty acid radical reacts with an antioxidant such as vitamin C to produce a non-radical product.

The peroxides are unstable and when decomposed they form a complex series of compounds, including reactive carbonyl products such as malondialdehyde (**MDA**) and 4-hydroxyalkenals. 4-hydroxynonenal (**4HNE**) is mainly formed from peroxidation of omega-6 fatty acids, whereas 4-hydroxyhexenal (**4HHE**) is mainly formed from peroxidation of omega-3 fatty acids (Esterbauer et al., 1991). These compounds cause alterations in the physical properties of the membrane affecting its fluidity and may inactivate or modulate receptors or enzymes associated with the membrane leading to cellular damage (Montuschi et al., 2004). MDA produced during the lipid peroxidation cascade can form covalent protein adducts and DNA adducts, which can be toxic or mutagenic (Marnett, 1999). MDA is considered the most mutagenic product of lipid peroxidation, whereas 4HNE is thought to be the most toxic (Esterbauer et al., 1990). While ROS/RNS are highly reactive and short-lived, the toxic aldehyde products of lipid peroxidation can be more damaging as they are long-lived and can diffuse from the site of origin to attack intracellular and extracellular targets (Esterbauer et al., 1991).

1.2.3 Protein oxidation

Protein oxidation occurs when proteins are covalently modified by ROS (direct) or by-products of oxidative stress, such as MDA (indirect) (Stadtman and Levine, 2000). Protein carbonyls are the most common product of protein oxidation; these can be

derivatives of amino acids such as Proline, Arginine, Lysine and Threonine. The carbonyls form when redox cycling cations such as iron (Fe^{+2}) or copper (Cu^{+2}) bind to proteins and modify side chains on amino acids with the help of ROS such as H_2O_2 and O_2-· (Stadtman and Oliver, 1991). Toxic carbonyls can also form when amino acid residue side-chain hydroxyls are oxidized to form ketone and aldehyde derivatives (Berlett and Stadtman, 1997). Protein oxidation can occur under physiological conditions due to electron leakage from the mitochondria, metal-ion dependant reactions or autoxidation (Dean et al., 1997). MDA production, which occurs due to lipid peroxidation (section 1.2.2), can also result in protein aggregation, altered phosphorylation and inactivation of enzymes (Mattson, 1998). Cells can cope with low levels of carbonyls and either detoxify or destroy them by proteolysis (Dean et al., 1997). High levels of carbonyls, such as those produced during oxidative stress, can overcome the protective cellular mechanisms and can accumulate leading to cellular damage and neurodegeneration. When protein carbonyls accumulate in the cell they can alter cellular function, due to decreases in catalytic activity or signalling interruptions, ultimately leading to cell death (Stadtman and Levine, 2000).

1.2.4 Antioxidants

Antioxidants are substances that can prevent the formation of ROS/RNS and/or promote the removal of ROS/RNS and their precursors (Halliwell and Gutteridge, 1995); reviewed by (Halliwell, 2006, Brocardo et al., 2011). Antioxidants can be classified as either exogenous or endogenous, and endogenous antioxidants can be further classified as enzymatic or non-enzymatic (reviewed by (Halliwell, 2006, Brocardo et al., 2011). The most common endogenous non-enzymatic antioxidant is GSH. Endogenous enzymatic

antioxidants include CAT, SOD, GPx, GR, glutathione-S-transferase (**GST**), thioredoxins, peroxiredoxins, glutaredoxins and glucose-6-phosphate-dehydrogenase (**G6PDH**) (Brocardo et al., 2011).

1.2.4.1 Glutathione (GSH)

GSH is a low molecular weight thiol compound (Forman et al., 2009) that is found in the cytosol of most cells (Meister, 1988a). It is a tripeptide formed of three amino acids - glutamate, cysteine and glycine (Anderson, 1998). The major role of GSH in the cell is to act as a non-enzymatic antioxidant and as a co-factor in several other antioxidant reactions (Forman et al., 2009). This makes GSH a very important molecule in the cellular environment, and because of this, its synthesis and maintenance are tightly regulated. The functional importance of GSH is due to the thiol group located on its cysteinyl residue, which enables it to act as a powerful reductant (Lash, 2006).

GSH can exist in a reduced form, with a free thiol group (GSH) or in an oxidised form with a disulfide bond between two molecules (GSSG). At basal cellular conditions only 1% of total glutathione (**GSH-t**) is represented by GSSG (Lenton et al., 1999), and GSH is only converted to GSSG when a cell experiences oxidative insult (Anderson, 1985). For this reason and for simplicity purposes, in this thesis the term GSH is used to represent reduced glutathione and GSH-t is used when referring to both the reduced and oxidized forms of glutathione.

1.2.4.1.1 Synthesis and metabolism of GSH

GSH is synthesised in a two step process. First, γ-glutamylcysteinyl synthetase (**GCS**) catalyses the adenosine triphosphate (**ATP**)-dependent binding of glutamate and cysteine, creating γ-glutamyl-cysteine (**γGlu-Cys**). This is the rate-limiting step in GSH synthesis,

as it depends on the availability of cysteine, which is generally taken by the cell when needed via the sodium-dependent excitatory amino-acid transporter (**EAAT**) (Aoyama et al., 2008). In neurons, the majority of the cysteine needed for GSH synthesis comes from astrocytes (Aoyama et al., 2008). The second step in GSH synthesis occurs when glycine is added to the γGlu-Cys molecule by glutathione synthase to form GSH (Anderson, 1998). GSH regulates its own synthesis via feedback inhibition of the GCS enzyme (Richman and Meister, 1975). See Figure 1-1. In the brain GSH is synthesised in astrocytes and neurons and the two cell types play a role in detoxifying ROS/RNS (Dringen, 2000).

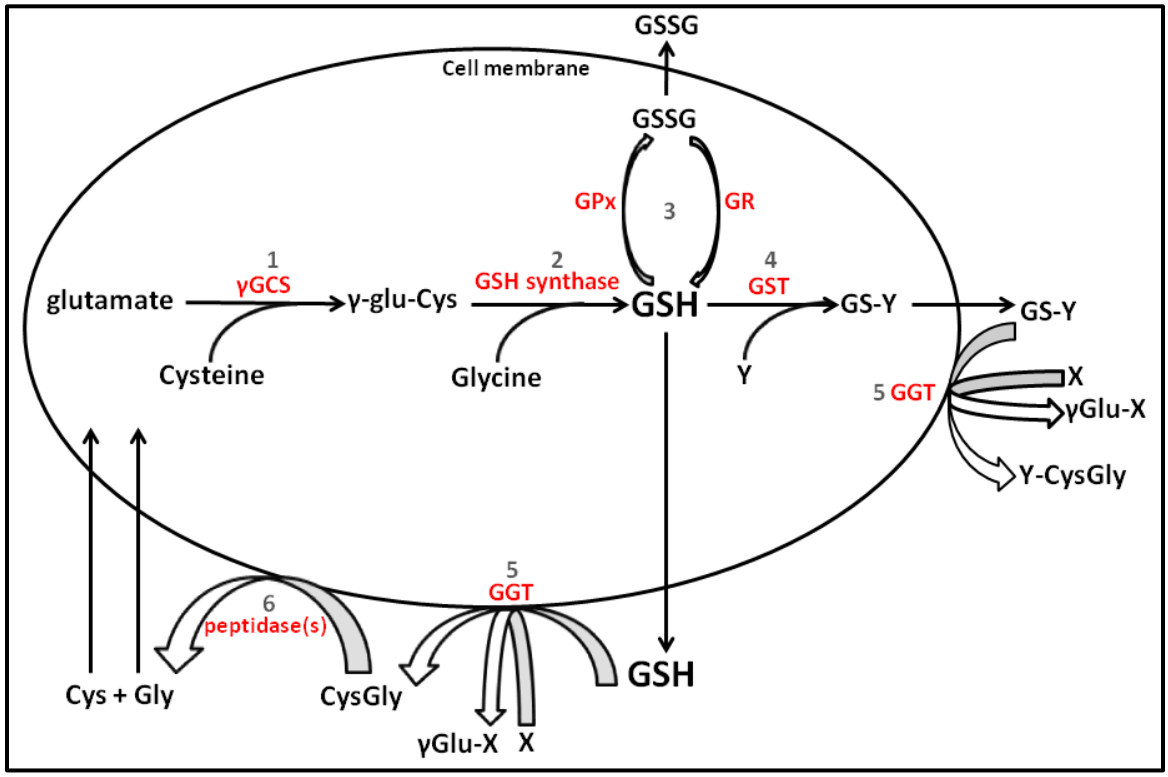

Figure 1-1 GSH synthesis and metabolism.
GSH is a tripeptide composed of glutamate, cysteine and glycine, where cysteine constitutes the rate limiting factor in the synthesis of GSH. This is a two step process: (1) Firstly, γ-glu-cys is formed from glutamate and cysteine by the action of GCS, and then (2) glycine is added by glutathione synthase. GSH can cycle between oxidized (GSSG) and reduced (GSH) states by the actions of GPx and GR (3). GSH can also be utilized by GST in the detoxification of toxins (Y; 4). GSH is recycled by GGT. GGT produces γ-CysGly and γGlu-X, where X is an acceptor of the

γ-glutamyl moiety (5). The cys-gly is converted back into cysteine and glycine by dipeptidases (6). (Modified from (Dringen, 2000). **Abbreviations:** γglutamyl-cysteine synthase (GCS); γglutamyl transpeptidase (GGT); Glutathione peroxidase (GPx); Glutathione reductase (GR); Glutathione-S-transferase (GST).

Gamma-glutamyl transpeptidase (**GGT**) is an enzyme located in the plasma membrane that metabolises excreted GSH as well as GSH metabolites/adducts. This enzyme works in conjunction with a dipeptidase that regenerates glycine and cysteine from the metabolised GSH (Peuchen et al., 1997). See Figure 1-1.

1.2.4.1.2 GSH as an antioxidant

In its reduced form, GSH acts as an antioxidant that protects cells from oxidative stress. It does this non-enzymatically by donating an electron that can reduce disulfide bonds in certain ROS such as hydroperoxides, which are then reduced to their respective alcohols. The most important detoxification carried out non-enzymatically by GSH is that of HO·, as none of the endogenous antioxidant enzymes are able to destroy this radical (reaction **11** (Bains and Shaw, 1997). In the process of donating an electron, GSH itself becomes reactive, but it readily binds to another reactive GSH, originating GSSG. GR converts GSSG back to GSH using NADPH as a co-factor (Figure 1-2; reaction **3**).

$$2GSH + 2HO\cdot \rightarrow GSSG + 2H_2O \qquad \textbf{(11)}$$

GSH also functions as a co-substrate in the metabolism of xenobiotics and can act as a co-factor for many metabolic enzymes. When peroxides such as H_2O_2 are reduced by GPx (Figure 1-2; reaction **2**), GSH acts as an electron donor in the reaction (Aoyama et al., 2008) and is then recycled back to GSH by the action of GR (Figure 1-2; reaction **3**). Furthermore, GST, an enzyme found predominantly in astrocytes (Peuchen et al., 1997),

uses GSH as a co-factor to form mixed disulfides with various endogenous and xenobiotic compounds that are then exported out of the cell (Aoyama et al., 2008); Figure 1-2). GST is also the enzyme responsible for converting the toxic products of lipid peroxidation such as 4HNE to the GSH-HNE adduct reducing its damaging ability (Xie et al., 1998). In contrast to reactions catalysed by GR and GPx (reactions **2** & **3**, respectively), when GST utilises GSH, this is not recycled and is instead excreted while bound to the xenobiotic that was detoxified (Dringen, 2000); Figure 1-2).

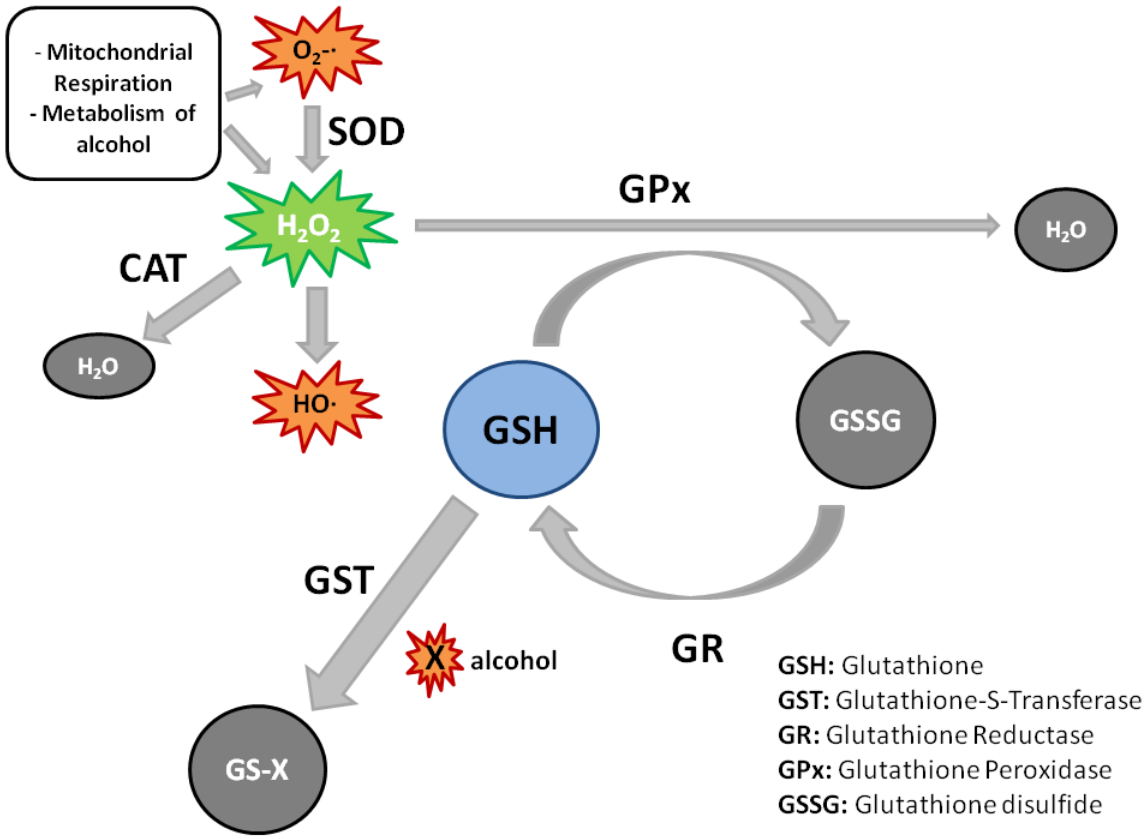

Figure 1-2 The role of GSH in detoxification of ROS.
GSH can non-enzymatically detoxify ROS. However, it is also utilised by other enzymes as a co-factor to aid in detoxification. For example, if the body is exposed to a toxin such as alcohol then GST can bind GSH to the toxin and create a detoxified compound that can then be excreted from the system. The cellular production of H_2O_2 also increases with exposure to certain toxins. H_2O_2 can easily form HO·, which is extremely reactive and toxic. One of the enzymes that detoxify H_2O_2 is GPx. It converts H_2O_2 into water using GSH as a co-factor. GR is responsible for converting oxidised GSSG back to its reduced form, thus recycling GSH. **Abbreviations:**

Catalase (CAT); Hydrogen peroxide (H_2O_2); Hydroxyl radical ($HO\cdot$); Superoxide ($O_2\text{-}\cdot$); Superoxide dismutase (SOD); Water (H_2O).

1.2.4.1.3 The role of GSH in other cellular processes

GSH can have various other roles aside from decreasing oxidative stress. In the mitochondria GSH-t plays a major role in regulating apoptosis by maintaining the redox state of the mitochondrial permeability transition pore (Yuan and Kaplowitz, 2009), and in the nucleus it aids in regulating cell division by altering the redox potential of the nuclear machinery (Pallardo et al., 2009). GSH is also an important cellular storage of cysteine; cysteine in its free form can lead to excitotoxicity through overactivation of the NMDA receptor and cell damage through free radical generation (Janaky et al., 2000), however, when incorporated into GSH, its toxic capabilities are rendered.

GSH can also maintain intracellular sulfhydryl containing proteins in their active form by thiol-disulfide exchange reactions (Lash, 2006). This is particularly important for the mitochondria, which contain many enzymes that need to be kept in their reduced form in order to function (Lash, 2006). The GSH/GSSG redox couple can also interact with other redox couples in the cytosol and maintain the appropriate intracellular redox balance that is necessary to regulate protein folding and conformation, membrane transport and enzyme activity as well as receptor dynamics (Beck et al., 2001). By influencing the redox state of the cell, GSH-t can also regulate transcriptional activation (Sun and Oberley, 1996, Jang and Surh, 2003) and various post-transcriptional processes (Diaz Vivancos et al., 2010, Markovic et al., 2010).

1.2.5 Oxidative stress and antioxidants in the brain

In the brain physiological levels of ROS/RNS can play a role in signal transduction mechanisms and can help to maintain homeostasis (Swindle and Metcalfe, 2007). Indeed,

·NO plays an important role in synaptic plasticity, and is essential for the induction of long-term potentiation (**LTP**; see section 1.4.3) (Bon and Garthwaite, 2003, Hopper and Garthwaite, 2006). However, a large amount of ROS/RNS can be generated in the brain by many mechanisms including the activation of phospholipases, NOS, xanthine oxidase and the Fenton and Haber-Weiss reactions (reaction **5**; (Lewen et al., 2000). This is due to the high rate of oxygen consumption by this organ (Sokoloff, 1999), its comparatively low levels of antioxidants including SOD, CAT and GPx (Henderson et al., 1999, Dringen, 2000), the high concentration of PUFAs (the targets of lipid peroxidation), and the high concentration of metals that catalyze ROS/RNS formation. Furthermore, several neurotransmitters, including dopamine, serotonin and norepinephrine are autoxidizable (i.e. they can spontaneously react with molecular oxygen) and this can generate O_2-· or quinones and semiquinones that deplete GSH (Spencer et al., 1998).

Excessive amount of oxidative stress in the brain can lead to neurodegeneration and cell death when cellular integrity is breached due to lipid, protein and DNA damage (Halliwell, 2007).

1.2.6 PNEE and oxidative stress

Ethanol can increase the generation of ROS/RNS by activating mitochondrial respiration and the consequent formation of O_2-·, HO·, H_2O_2, or ·NO, or via its oxidation by enzymes such as cytochrome P450 enzyme 2E1 (**CYP 2E1**), which generate hydroxyethyl radicals (Montoliu et al., 1995, Haorah et al., 2008). See Figure 1-3.

Cell damage is induced when ROS/RNS produced by the metabolism of ethanol accumulate and self perpetuate over time, interacting with carbohydrates, proteins, lipids and nucleic acids which causes cell damage and death (Haorah et al., 2008). Of particular

importance for FASD, during prenatal and early postnatal development, levels of antioxidants are much lower than in mature cells, making developing neurons considerably more susceptible to oxidative damage (Henderson et al., 1999, Bergamini et al., 2004).

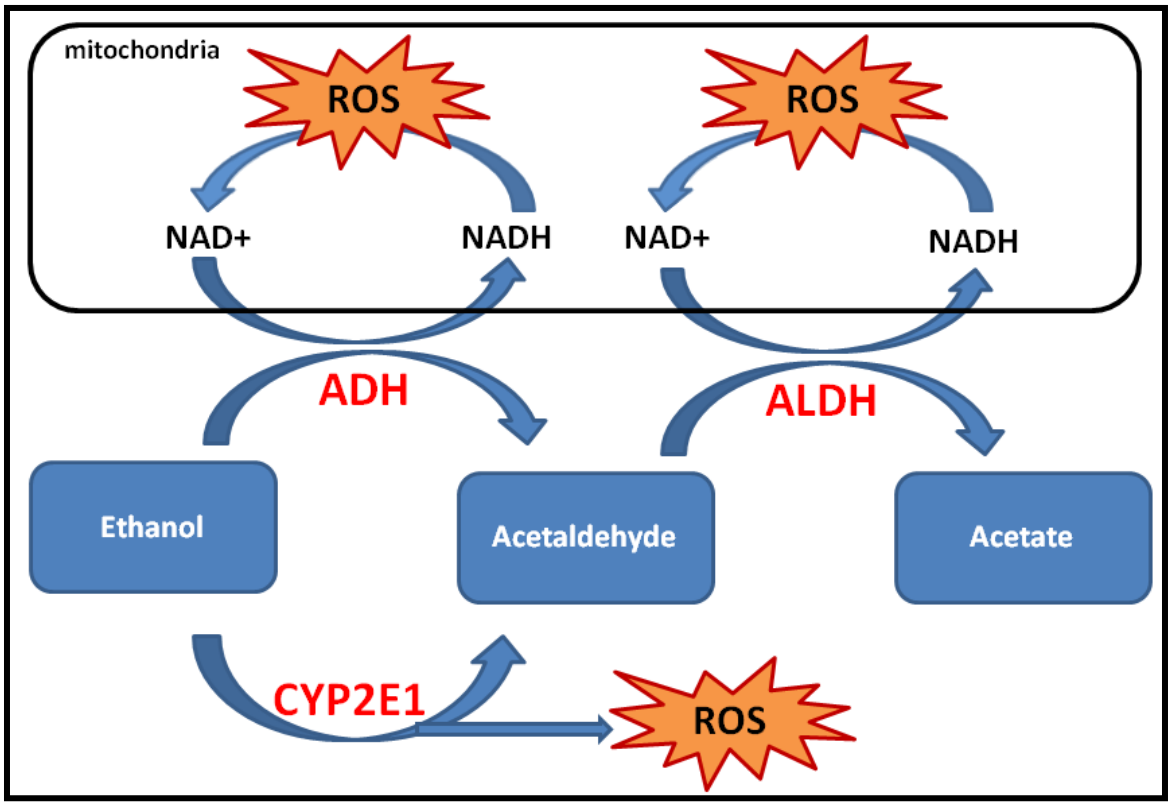

Figure 1-3 The metabolism of ethanol.
Ethanol is first metabolised by ADH or CYP2E1 to acetaldehyde. Acetaldehyde is then metabolised to acetate by ALDH. These processes produce high amounts of ROS in both the mitochondria and cytoplasm. (Modified from (Brocardo et al., 2011). **Abbreviations:** Alcohol dehydrogenase (ADH); Aldehyde dehydrogenase (ALDH); Cytochrome P450 2E1 (CYP2E1); Nicotinamide adenine dinucleotide (NAD^+); reduced nicotinamide adenine dinucleotide (NADH); Reactive oxygen species (ROS).

Many studies have shown that ethanol increases oxidative stress in the developing and juvenile brain (Reyes et al., 1993, Henderson et al., 1995, Henderson et al., 1999, Heaton et al., 2003, Ramachandran et al., 2003). In addition, the effects of this increased

oxidative stress can be long-lasting (Dembele et al., 2006, Brocardo et al., 2012, Patten et al., 2012). This may arise as a result of a decrease in antioxidants and an increase in stable lipid peroxidation products, protein carbonyl formation, and DNA mutations. Together these will influence cell function and lead to the accumulation of toxic products (Figure 1-4).

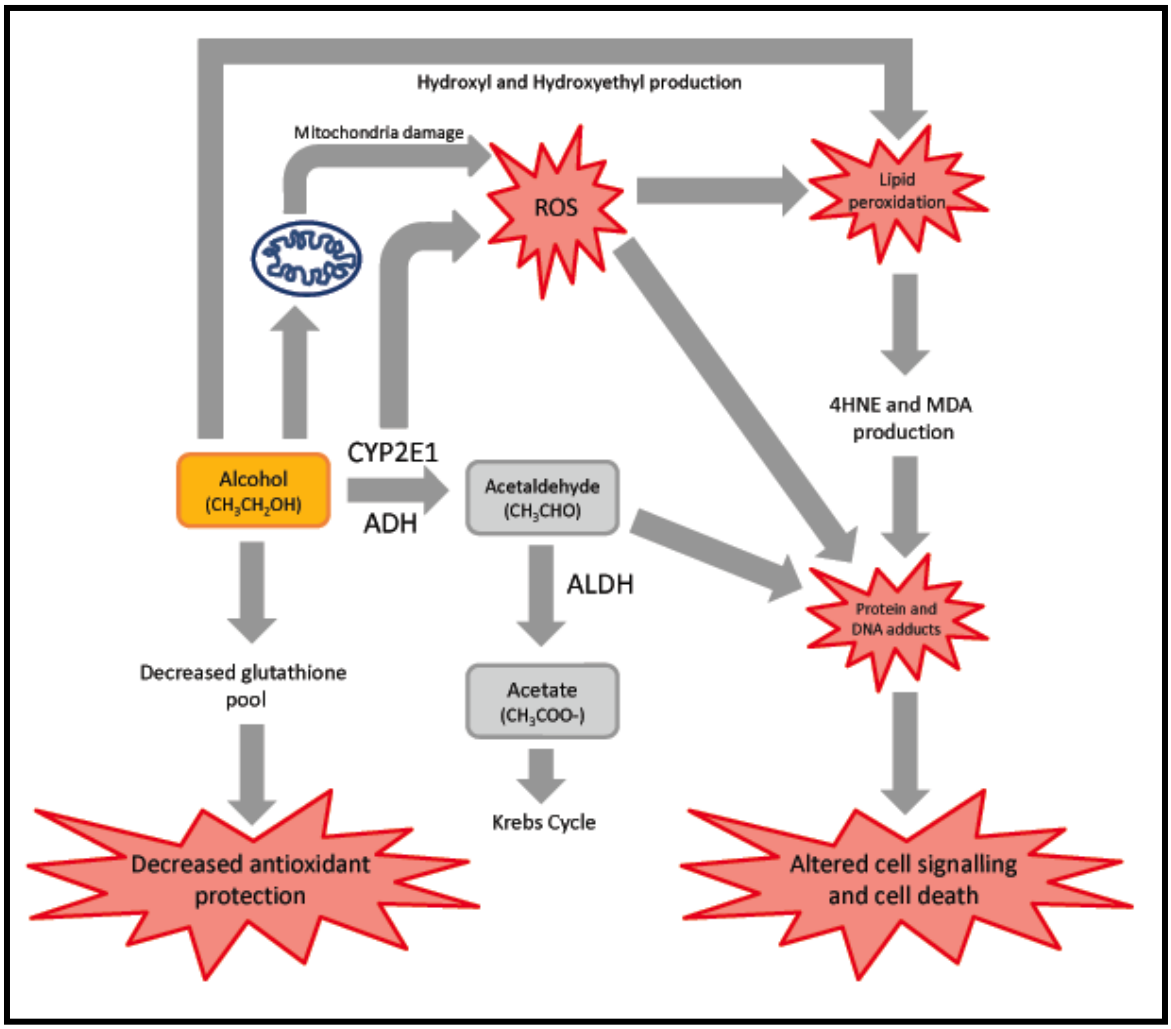

Figure 1-4 FASD and oxidative stress.
Ethanol can cause oxidative damage through direct and indirect pathways. Alcohol can spontaneously produce ROS such as HO· and hydroxyethyl radicals, which cause lipid peroxidation. Alcohol can also cause mitochondrial membrane dysfunction leading to ROS/RNS production. Alcohol metabolism to acetaldehyde by CYP2E1 also generates ROS, which cause lipid peroxidation leading to protein and DNA oxidation and adduct formation. Acetaldehyde, the intermediate of alcohol metabolism, is also able to form protein and DNA adducts. Indirectly, alcohol can increase oxidative stress by decreasing the GSH pool. GSH is one of the major co-

factors used by endogenous enzymatic antioxidants to protect the cell against oxidative damage. **Abbreviations:** Alcohol Dehydrogenase (ADH); Aldehyde dehydrogenase (ALDH); Cytochrome P450 Enzyme 2E1 (CYP2E1). 4-Hydroxynonenal (4HNE); Glutathione (GSH); Malondialdehyde (MDA); Reactive Oxygen species (ROS). (Modified from (Haorah et al., 2008).

There is a lack of data examining the long-term cognitive effects of the oxidative damage produced by PNEE. In this thesis the role of oxidative stress in synaptic plasticity is examined in the hippocampus of ethanol-exposed offspring.

1.3 Learning and memory deficits following PNEE

1.3.1 The hippocampal formation

The hippocampal formation, or hippocampus, is a bilateral structure that is found in the medial temporal lobe in the mammalian brain and is considered part of the limbic system. The hippocampal formation consists of the dentate gyrus (**DG**), the *Cornu Ammonis* (**CA**) 1 and CA3 regions (also known as the hippocampus proper) and the subiculum (reviewed in (Blumenfeld, 2010, Krebs et al., 2011). The hippocampal formation is the focus of intensive research as it is generally recognized as playing an important role in learning and memory, particularly that associated with declarative (i.e., explicit) memory (see section 1.3.2 (Andersen, 2006b), and it is one of the areas of the brain most affected by PNEE (Berman and Hannigan, 2000).

1.3.1.1 Anatomy of the DG

The main focus of this thesis is the effects of PNEE on the DG subregion of the hippocampus. The DG is a three layered cortical region that has an identifiable V shape formed by three distinct areas: the suprapyramidal blade (located between CA3 and CA1), the infrapyramidal blade (opposite to the suprapyramidal blade) and the crest (where the two blades join; Figure 1-5). Each area has three cortical layers: the molecular

cell layer, the granule cell layer, and the polymorphic layer (also referred to as the hilus; Figure 1-5). Each layer of the DG differs in both its cellular content and its connectivity. The DG contains a variety of cell types including excitatory principal neurons (dentate granule cells, **DGC**s), inhibitory interneurons, glial cells and precursor cells.

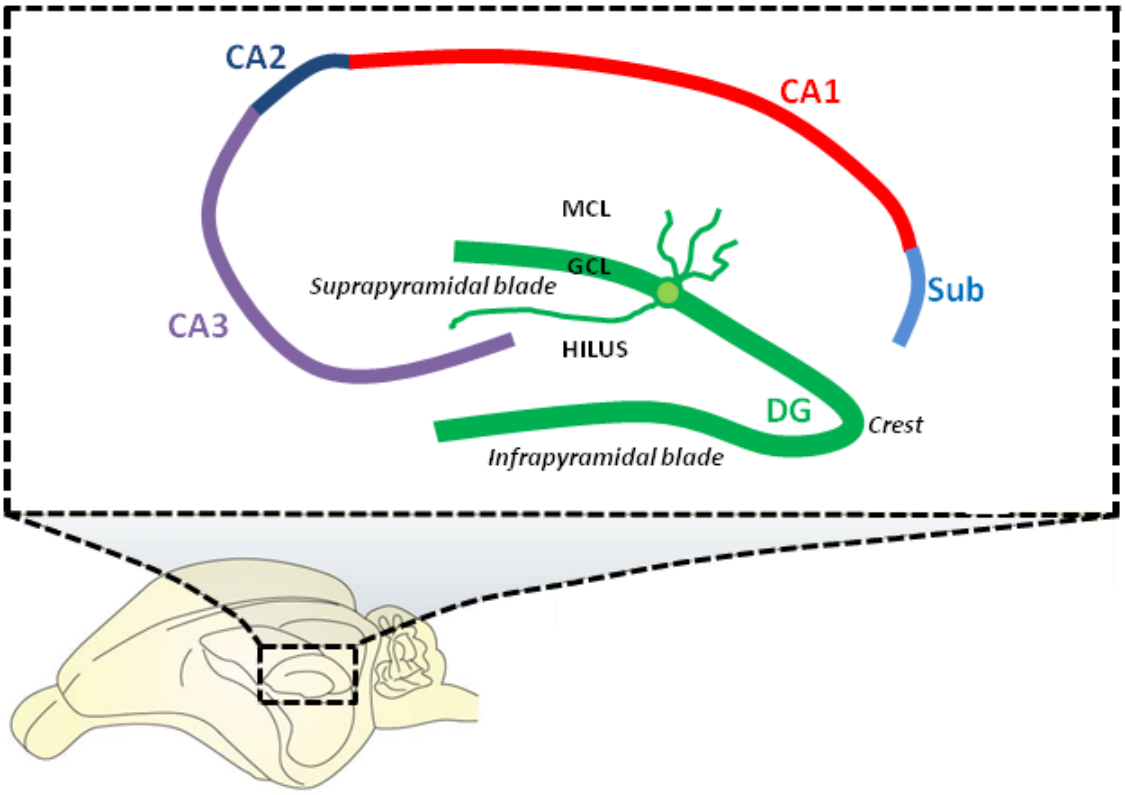

Figure 1-5 Anatomy of the hippocampus.
The hippocampus consists of the DG, CA1-3 and the Sub. The DG consists of a suprapyramidal blade, an infrapyramidal blade and the crest where the two blades join. There are three layers to the DG, the MCL, the GCL and the hilus. (Modified from (Deng et al., 2010). **Abbreviations:** *Cornu Ammonis* regions (CA1-3); Dentate gyrus (DG); Granule cell layer (GCL); Molecular cell layer (MCL); Subiculum (Sub).

Pyramidal basket cells and a heterogenous population of interneurons are found in the molecular cell layer and are mainly involved in inhibitory control. The granule cell layer contains the cell bodies of the DGCs. The hilus, which lies between the suprapyramidal and infrapyramidal blades of the granule cell layer, contains mainly mossy cells and

interneurons. DGCs are the major excitatory neurons in the DG. DGCs are tightly packed in the granule cell layer and have characteristic small round soma (Amaral, 2006). Each DGC has multiple primary dendrites that extend into the adjacent molecular cell layer (Desmond and Levy, 1985).

1.3.1.2 Information flow in the hippocampus

Information flow in the hippocampus is generally unidirectional and is known as the trisynaptic circuit (Anderson et al., 1971) (Figure 1-6). The first connection in the trisynaptic circuit originates from Layer II of the entorhinal cortex (**EC**) and projects to the DG via a group of fibres known as the perforant path. The fibres synapse on the dendrites of the DGCs located in the molecular cell layer. There are two subdivisions of the perforant path: medial (MPP) and lateral (LPP). The MPP originates in the medial EC and projects to the middle one-third of the molecular cell layer, whereas the LPP originates in the lateral EC and projects to the outermost third of the molecular cell layer. Both the MPP and LPP provide excitatory input onto the DGCs but are physiologically distinct and have different short-term and long-term plasticity properties (McNaughton, 1980, Bramham et al., 1991, Colino and Malenka, 1993). The MPP and LPP are the major inputs of cortical information into the hippocampus and the DGCs play an important role in processing and filtering information before it is sent to other regions of the hippocampus via the trisynaptic circuit (Anderson et al., 1971).

From the DG, DGC axons project onto pyramidal cells in the CA3 region of the hippocampus (Figure 1-6). This pathway is unique in that the bundles of axons are unmyelinated and are therefore known as the mossy fibres. Interestingly, the connections

between DGCs and pyramidal cells of the CA3 are rather sparse, but this characteristic is in line with several of the proposed functions of the DG (discussed in section 1.3.2).

The third component of the trisynaptic circuit is the CA3 to CA1 projections known as the Schaffer Collaterals. Pyramidal cells of the CA3 send axons into the stratum radiatum and stratum oriens of the CA1 and innervate apical and basal dendrites of the CA1 pyramidal cells (reviewed by Anderson 2006). The CA3-CA1 connection is one of the most studied in the CNS and much of the knowledge regarding synaptic transmission and plasticity has come from studying this pathway.

The CA1 then projects information to the subiculum and both the CA1 and the subiculum project fibres back to Layers V and VI of the EC (Naber et al., 2001) to complete the cortical information loop.

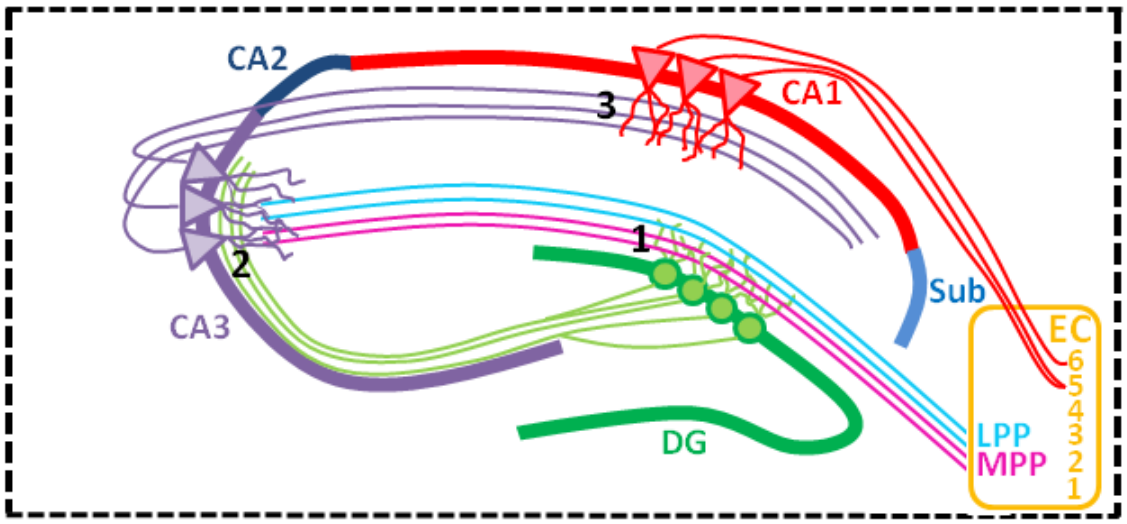

Figure 1-6 The hippocampal trisynaptic circuit.
The first synapse in the trisynaptic circuit arises from LPP (blue) and MPP (pink) fibres from Layers 2 and 3 of the EC (yellow) projecting onto the DGCs (green) of the DG (1). The second synapse is formed by the DGC axons that project onto CA3 pyramidal cells (purple; 2). These axons are also known as the mossy fibres. CA3 pyramidal cell axons project onto pyramidal cells of the CA3 (red), via the Schaffer collaterals (3). CA1 neurons send projections to the Sub (not shown) and out of the hippocampal formation back to layers 5 and 6 of the EC. (Modified from (Deng et al., 2010). **Abbreviations:** *Cornu Ammonis* regions (CA1-3); Dentate granule cells (DGCs); Dentate gyrus (DG); Entorhinal cortex (EC); Lateral perforant path (LPP); Medial perforant path (MPP); Subiculum (Sub).

Inhibitory GABAergic interneurons in the hippocampus control the activity of excitatory cells through $GABA_A$ receptor activation, which hyperpolarise cells due to chloride influx, thus preventing the propagation of action potentials. Blockade of $GABA_A$ receptors leads to epileptiform activity in the hippocampus, highlighting the importance of interneuron modulation of synaptic activity (Danglot et al., 2006). Interneurons are found in the molecular cell layer and in the hilus region of the DG, where they control activity of the perforant path and the mossy fibres.

1.3.1.3 Hippocampal development

During embryonic development neural stem cells originate in the walls of the lateral ventricles and migrate to form the CA1 and CA3, followed by the DG. In humans, development of the hippocampus begins during the second half of the 1st trimester and continues throughout the 3rd trimester (Bayer, 1980, Seress, 2007). This is in contrast to the rat brain, where hippocampal development begins during the second half of the gestation period (GD 15) and continues until approximately PND 14 (Bayer and Altman, 1975, Altman and Bayer, 1990, Rice and Barone, 2000).

In the rat brain, the generation of DGCs starts at approximately GD 17 and continues throughout development and into adult life. During the developmental period, DGCs migrate to form the suprapyramidal blade, and later the infrapyramidal blade, with the aid of glial-guided migration (Frotscher et al., 2007). DGCs show a very distinct bipolar orientation, in that their dendrites branch into the molecular cell layer, and their axons (the mossy fibres) extend into the hilus region (Frotscher et al., 2007) and terminate onto interneurons and pyramidal cells of the CA3 region. Fibres from the EC begin to reach

the DG during the 2nd trimester in humans (GD 18/19 in the rat) and will eventually terminate on the outer two thirds of the molecular layer.

In the rat brain, interneurons develop prenatally between GDs 13 – 18 (i.e., around the same time as CA pyramidal cells and prior to DGCs) (reviewed by (Danglot et al., 2006). GABAergic synaptogenesis tends to occur postnatally. At birth, the rodent hippocampus contains very few synapses and synaptogenesis increases considerably during the first 10 days of postnatal life (Pokorny and Yamamoto, 1981, Steward and Falk, 1991). Conversely, in humans the period of intense synaptogenesis corresponds to the 3rd trimester and this process continues during the first two years of life (Dobbing and Sands, 1979).

1.3.1.4 PNEE and the hippocampus

It is unclear whether PNEE causes a reduction in cell number in the hippocampus, with some studies reporting decreased cell numbers in the CA and DG regions (Barnes and Walker, 1981, Wigal and Amsel, 1990, Perez et al., 1991, Livy et al., 2003) and others reporting no changes (Livy et al., 2003, Tran and Kelly, 2003) but the overall consensus is that there is a negative effect of ethanol on the development of the hippocampal formation (reviewed by Gil-Mohapel et al., 2010).

The pyramidal cells of the CA regions and the DGCs of the DG region may develop abnormally due to PNEE. This includes a reduction in dendritic length and arborisation in the CA1 (Davies and Smith, 1981, Smith and Davies, 1990, Yanni and Lindsley, 2000) that is accompanied by decreased spine density and synapse numbers (Ferrer et al., 1988, Kuge et al., 1993, Clamp and Lindsley, 1998, Lindsley and Clarke, 2004). The axons of DGCs (i.e. the mossy fibres) have also been reported to have abnormal branching in

response to PNEE (West and Hamre, 1985) and appear to form fewer synapses onto CA3 neurons (Tanaka et al., 1991). These deficits in cell number and synapse formation suggest that PNEE may interfere with the appropriate neuronal circuitry needed for the proper functioning of the hippocampus.

Various studies conducted on individuals affected with FASD have also supported the idea that the hippocampus is affected by PNEE. MRI analysis in children exposed to ethanol *in utero* revealed that during a verbal learning task there was less activation in the medial and posterior temporal regions, suggesting dysfunction in the temporal lobe memory system, which includes the hippocampus (Sowell et al., 2007). Further studies have revealed a decrease in the concentration of choline compounds in the hippocampal formation and decreases in overall hippocampal volume (Astley et al., 2009b). Asymmetry in hippocampal morphology is also common, with reductions in the left hippocampus often observed (Riikonen et al., 1999, Riikonen et al., 2005, Willoughby et al., 2008). These structural deficits have also been correlated with deficits in learning and memory (Willoughby et al., 2008).

1.3.2 The role of the hippocampus in learning and memory

Originally, it was believed that the hippocampus was involved in olfaction (reviewed by (Brodal, 1947, Compston, 2010) or emotion (reviewed by (Papez, 1995). In the fifties, Dr. William Scoville performed a bilateral limbic surgery on patient Henry Molasion (commonly known as H.M.), giving the first real insight into the function of the hippocampus (Scoville and Milner, 1957). In order to treat H.M.'s recurrent seizures, the area of the brain where the seizures were being generated, which included the hippocampus, amygdala, collateral sulcus, perihinal cortex, EC and the medial

mammillary nucleus, was removed (Corkin et al., 1997). Following surgery, H.M. no longer presented with seizures, but he was afflicted with severe memory deficits; he lacked the ability to form and retain long-term memories of new facts (semantic memory) and events (episodic memory). However, his intellect, perception and working memory were not affected. This was the first case where a strong link was made between the hippocampus and semantic and episodic memory (collectively referred to as declarative or explicit memory).

Numerous studies have now refined the role of the hippocampus in the formation and maintenance of explicit (i.e. intentional and conscious) spatial memory (Vargha-Khadem et al., 1997, O'Reilly and Norman, 2002). In the 1980's, Dr Richard Morris created the Morris water maze test with the purpose of training rodents to learn the spatial location of a hidden platform submerged in cloudy water as a test for hippocampal function (Morris et al., 1982). He observed that hippocampal lesions significantly impaired performance in this task (Morris et al., 1982).

Other studies have identified place cells in the hippocampus, which has lead to the cognitive map theory of hippocampal function (O'Keefe and Dostrovsky, 1971, O'Keefe and Conway, 1978). These are cells that become active when a rodent is in a specific location in a specific environment (O'Keefe and Dostrovsky, 1971) and such cells have also been detected in humans (Ekstrom et al., 2003). The presence of place cells within the hippocampus provides evidence that this structure organizes and stores stimuli with respect to a spatial framework (i.e. a cognitive map) and may be used for spatial navigation (Taube et al., 1990, Fyhn et al., 2004). Indeed, recent studies have suggested that the hippocampal formation acts as a network that encodes events and their contexts,

with each distinct region playing a specific but complementary role in the processing of spatial information.

Within this scenario, the DG is believed to play a unique role in spatial pattern separation (reviewed by (Kesner, 2013). Spatial pattern separation is the enhancement of contrast between two spatial patterns or events. At the neuronal level this is thought to occur either through change in neuronal firing rate or through the firing of different neuronal sets (Leutgeb et al., 2007, Treves et al., 2008). The DGCs display sparse firing characteristics and there are very few connections between DGCs and CA3 pyramidal cells (Galimberti et al., 2006), which make the DG optimally structured for spatial pattern separation. Furthermore, the existence of populations of DGCs that fire only when a rodent is in a given environment indicate that the DG contains place fields that are important for pattern separation (Muller and Kubie, 1987, Eichenbaum et al., 1989, Jung and McNaughton, 1993).

Recent evidence suggests that there is a heterogeneous distribution of function along the septotemporal axis of the hippocampus (reviewed by (Bannerman et al., 2004). While the more dorsal portions of the hippocampus (which receive septal input) play a role in declarative memory and sensory information processing, it is believed that the ventral hippocampal formation is more vital for emotional information processing and stress response regulation (Hunsaker et al., 2007, Fanselow and Dong, 2010). Indeed, recent findings have indicated that as well as spatial pattern separation, the dorsal DG plays a role in context-pattern separation for geometry and colour of the environment whereas the ventral portion of the DG may be involved in odor pattern separation (Kesner, 2013).

Although the DG is the focus of this thesis, it is relevant to describe some of the functions of the other regions of the hippocampus. The CA3 region has been implicated in spatial pattern completion – the capacity to retrieve previously stored information when presented with partial or incomplete inputs (Kesner, 2007). Evidence for this comes from studies where mice that lacked *N*-methyl-D-aspartate (**NMDA**) receptor expression in the CA3 were trained on the Morris water maze (see section 1.4.2 for a discussion on NMDA receptors). While these mice were able to perform the task as well as control mice, if the external environment was altered deficits in performance were observed (Nakazawa et al., 2002). These results indicate that the ability to use partial spatial input was compromised and implicate the CA3 in spatial pattern completion.

The CA1 region of the hippocampus has been implicated in the processing of the temporal order of spatial patterns or events. This function is important to ensure that spatial events separated by time are encoded with minimum overlap (Kesner et al., 2004).

Together, the three major regions of the hippocampus – the DG, CA3 and CA1 encode spatial memory, but each region has its own distinct role within this function. The DG is associated with pattern separation, the CA3 with spatial pattern completion, and the CA1 with temporal order processing. The three regions work in concert to encode spatial memory.

1.3.3 PNEE and learning and memory

There is a multitude of data indicating that learning and memory, particularly spatial, reference and working memory, are impaired in animals that were exposed to ethanol during development. Deficits in Morris water maze tasks where animals show impairments in spatial acquisition, reference and place memory are observed in animals

when ethanol exposure occurs both prenatally (Blanchard et al., 1987, Gianoulakis, 1990, Westergren et al., 1996, Kim et al., 1997, Matthews and Simson, 1998, Gabriel et al., 2002, Richardson et al., 2002, Iqbal et al., 2004, Christie et al., 2005, Incerti et al., 2010) and postnatally (Goodlett and Peterson, 1995, Pauli et al., 1995, Tomlinson et al., 1998, Johnson and Goodlett, 2002, Wozniak et al., 2004, Thomas et al., 2008, Thomas et al., 2010). Further, PNEE causes increases in the number of errors in the T-maze task during retention testing (Lochry and Riley, 1980, Nagahara and Handa, 1997) or reversal learning (Riley et al., 1979, Wainwright et al., 1990, Lee and Rabe, 1999), and deficits in spontaneous alternation in the T-maze (Riley et al., 1979, Abel, 1982, Zimmerberg et al., 1989, Thomas et al., 1996, Incerti et al., 2010, Thomas et al., 2010), consistent with hippocampal damage (Berman and Hannigan, 2000). In a study of more complex learning and memory behaviours, spatial/object discrimination, spatial orientation, simple object recognition and complex object recognition were examined in a prenatal and/or pre-weaning model of ethanol exposure using four different conditions of the Can Test (Popovic et al., 2006). Ethanol exposed animals performed poorly on most tasks compared to controls and cognitive function impairments became increasingly evident during the more complex tasks (Popovic et al., 2006). Greater impairments were observed in animals that received ethanol in the early neonatal period (3^{rd} trimester equivalent) (Popovic et al., 2006).

Radial Arm Maze deficits are also observed in ethanol-exposed rats (Reyes et al., 1989b, Stone et al., 1996, Wozniak et al., 2004) and mice (Pick et al., 1993). While most ethanol-exposed animals were able to learn the task, they required significantly more training trials compared to control animals (Reyes et al., 1989b, Pick et al., 1993, Stone et

al., 1996, Wozniak et al., 2004). Stone et al., (1996) hypothesized that spatial performance may be more sensitive to the effects of PNEE than other learning tasks, as no deficits in passive avoidance tasks were observed in ethanol-exposed animals that had shown prior deficits in the radial arm maze. This is in agreement with other studies reporting that PNEE does not cause deficits in non-spatial learning and memory tasks such as the delayed non-matching to sample test (Kim et al., 1997), active avoidance tasks (Molina et al., 1984) or taste discrimination (Becker et al., 1988). However, some non-spatial tasks that involve the hippocampus such as fear conditioning and delayed eye blink conditioning are also disrupted by PNEE (Brown et al., 2007, Murawski and Stanton, 2010, Hamilton et al., 2011).

Learning and memory and executive function are also impaired in individuals with FASD (Streissguth et al., 1989, Uecker and Nadel, 1996, 1998, Mattson et al., 1999, Mattson and Riley, 1999, Hamilton et al., 2003). Functional brain abnormalities can be examined in human populations using functional MRI (**fMRI**) technology. fMRI has been used to examine spatial working memory in adults and children with FASD, and impairments compared to control subjects are commonly observed (Malisza et al., 2005, Spadoni et al., 2009). fMRI has also indicated that subjects with FASD exhibit abnormal patterns of brain activation during tasks involving verbal learning (Sowell et al., 2007), verbal working memory (O'Hare et al., 2009), and visual working memory (Astley et al., 2009a). These abnormal patterns of brain activation, often observed in the frontal lobe, may indicate an increased reliance on frontal memory systems in order to compensate for medial temporal lobe memory system dysfunction (Sowell et al., 2007). Alternatively,

subjects with FASD may recruit a more extensive network of brain regions to accomplish learning and memory tasks (O'Hare et al., 2009).

A theory that has been posed by numerous researchers in the FASD field is that the growth retardation and neurobehavioural deficits observed with PNEE may reflect a delayed maturation of the CNS (Abel, 1982) reviewed by (Berman and Hannigan, 2000). Indeed, in some cases it appears that ethanol-exposed animals are able to eventually perform well in some tasks (Riley and Vorhees, 1986, Wozniak et al., 2004, O'Leary-Moore et al., 2006), or perform better in adulthood than at juvenile ages (Gianoulakis, 1990, Nagahara and Handa, 1997, Gabriel et al., 2002). While many studies have shown that the effects of PNEE are long lasting and continue into adulthood (Riley, 1990, West and Goodlett, 1990, Zimmerberg et al., 1991, Stone et al., 1996, Westergren et al., 1996, Kim et al., 1997, Matthews and Simson, 1998, Lee and Rabe, 1999, Iqbal et al., 2004, Christie et al., 2005, Brocardo et al., 2012), the idea that some behaviours or effects of ethanol may be transient opens an opportunity for modification by different treatment approaches.

1.4 Synaptic plasticity in the hippocampus

Because the hippocampus plays a crucial role in spatial memory, it has been widely examined for potential neuronal correlates for learning and memory. Numerous studies have revealed that the hippocampus is a highly plastic region and the following sections will further describe the mechanisms of synaptic plasticity that occur in this region and their importance for learning and memory.

1.4.1 Synaptic plasticity

The brain is not a static organ, and it can change both physically and functionally depending on environment and experience. It is now widely accepted that memory formation is closely dependent on the capacity of the brain to regulate long-lasting changes in neuronal communication (Martin et al., 2000, Lisman and McIntyre, 2001). Synaptic plasticity is defined as the brain's capacity to alter the way individual neurons communicate with each other to store new information, and was first described by the Polish neurophysiologist Jerzy Konorski in 1948 (Konorski, 1948). This idea was refined by the Canadian neuropsychologist Donald Hebb, in the book "The Organisation of Behaviour", which states:

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both of the cells such that A's efficiency, as one of the cells firing B, is increased"

- (Hebb, 1949)

Experimental evidence supporting the notion of synaptic plasticity didn't arise until 1973, when Bliss and Lomo discovered that applying a brief amount of electrical stimulation at high frequency could induce a long-lasting increase in synaptic efficacy in the rabbit DG (Bliss and Lomo, 1973). It has since been discovered that two major forms of synaptic plasticity exist – LTP, which is an increase in synaptic efficacy in response to high frequency stimulation, and long-term depression (**LTD**), which is a decrease in synaptic efficacy due to low frequency stimulation (Bliss and Collingridge, 1993, Christie et al., 1994).

1.4.2 The NMDA receptor

The NMDA receptor is a ligand activated excitatory receptor that is controlled by the neurotransmitter glutamate and is closely associated with LTP and learning and memory. The NMDA receptor is composed of four subunits, each of which has four functional domains. The N-terminal domain is an extracellular region that contains a leucine/isoleucine/valine-binding segment that acts as the binding site for many non-competitive NMDA receptor antagonists (Perin-Dureau et al., 2002). The agonist binding domain corresponds to the second extracellular domain and binds either glutamate or glycine. The transmembrane domain is formed by three transmembrane alpha helices and a re-entry loop. The re-entry loop forms the channel pore, with a site at the apex that determines the receptor's cation selectivity. The final domain is the C-terminal domain that can bind postsynaptic density proteins (Paoletti and Neyton, 2007).

There are three subtypes of subunits that can form the NMDA receptor: GluN1, GluN2 and GluN3. The NMDA receptor is a heterotetramer complex and is composed of two obligatory GluN1 subunits and two regulatory GluN2 or GluN3 subunits. The GluN1 subunit is important for assembly and trafficking of the NMDA receptor to the membrane (Cull-Candy and Leszkiewicz, 2004) and contains an agonist binding domain that binds glycine/D-serine (Paoletti and Neyton, 2007). There are four subtypes of the GluN2 subunit (A-D), and these subunits are responsible for glutamate binding (Cull-Candy and Leszkiewicz, 2004). The GluN3 subunit is less well characterised, but like GluN1 subunits, glycine /D-serine also binds at the agonist binding domain of these subunits (Cavara and Hollmann, 2008).

The NMDA receptor is unique in that it requires concurrent binding of glutamate and glycine (or D-serine in the brain) and membrane depolarization to become activated.

Upon glutamate and glycine/D-serine binding, a conformational change in the transmembrane domain results in the opening of the channel pore. The voltage dependent nature of the NMDA receptor however, does not allow ions to enter the cell, until sufficient depolarization occurs, and the magnesium ion that blocks the channel is removed. Once the channel is open, the receptor is primarily permeable to calcium and to a lesser extent, sodium and potassium (Sharma and Stevens, 1996).

1.4.3 Long-term potentiation

LTP is a model of activity dependent synaptic plasticity that is used to study synaptic efficacy as a neurobiological model of learning and memory. LTP is characterized by an increase in the size of an evoked postsynaptic potential or current in response to the same stimulus (Bliss and Collingridge, 1993) due to a persistent increase in the strength of synaptic transmission (Matsuzaki et al., 2004). There are various forms of LTP that exist in the hippocampus, but the most well characterized requires activation of two excitatory ionotropic glutamate receptors – the α-amino-3-hydroxy-5-methyl-4-isoxazolepropoionic acid (**AMPA**) receptor and the NMDA receptor (Collingridge et al., 1983, Bliss and Collingridge, 1993). It is postulated that LTP, and its counterpart LTD, work together to refine and sculpt memories.

The most studied form of LTP is induced by the activation of the NMDA receptor, which results in an influx of calcium ions. This leads to the activation or inhibition of a cascade of intracellular chemical signalling pathways that influence pre- and postsynaptic mechanisms and ultimately result in an increase in synaptic strength (reviewed by (Bliss and Collingridge, 1993).

1.4.3.1 Mechanism of LTP

Excitatory synaptic transmission occurs predominantly through AMPA receptors, which are permeable to sodium and potassium. When presynaptic glutamate is released onto non-potentiated synapses it will bind to postsynaptic AMPA receptors and cause a temporary depolarization of the postsynaptic neuron due to a net influx of positive ions into the cell and the consequent generation of an excitatory postsynaptic potential (**EPSP**). This depolarization is not sufficient to induce LTP, as the NMDA receptor must also be activated (Bliss and Collingridge, 1993, Malenka and Nicoll, 1999).

LTP induction requires a strong afferent stimulation that sufficiently depolarizes the membrane of the postsynaptic neuron (through AMPA receptor activation) and unblocks the NMDA receptor. Depolarization concurrent with glutamate release allows the NMDA receptor to open causing a spike in intracellular calcium (Yang et al., 1999). It is the increase in intracellular calcium that activates multiple kinases important for the maintenance of LTP (Lynch et al., 1983, Malenka et al., 1989). Calcium can activate calmodulin, which in turn activates the calcium/calmodulin dependent protein kinase II (**CaMKII**). CaMKII can autophosphorylate, thus remaining activated long after calcium levels have decreased to baseline. CaMKII can maintain LTP by phosphorylating AMPA and NMDA receptors. This increases their activity and their channel opening probability, resulting in a further depolarization of the cellular membrane. CaMKII can also aid in trafficking more AMPA receptors to the membrane. Other molecules such as protein kinase C (**PKC**), protein kinase A (**PKA**), mitogen-activated protein kinase (**MAPK**) and cyclic adenosine monophosphate (**cAMP**) have also been implicated in LTP and may work alongside CaMKII to enhance NMDA and AMPA receptor activity and deactivate

phosphatases (reviewed by (Malenka and Nicoll, 1999). See Figure 1-7 for a diagram of LTP generation and maintenance.

Late phase LTP is dependent on changes in gene expression and increased protein synthesis (Frey et al., 1996, Nguyen and Kandel, 1996). cAMP response element binding protein (**CREB**) is a target for most intracellular cascades involved in synaptic plasticity and can be phosphorylated and activated by CaMKII and extracellular signal-regulated kinases (**ERK**; which are activated by PKA and PKC) (Sweatt, 2001). CREB is a transcription factor that binds to cAMP response elements (**CRE**) located in specific genes (Lonze and Ginty, 2002). This initiates the transcription of numerous genes related to synaptic plasticity processes (Dragunow, 1996, Davis et al., 2000). The generation of late LTP is known as synaptic consolidation (Bramham, 2007). See Figure 1-7.

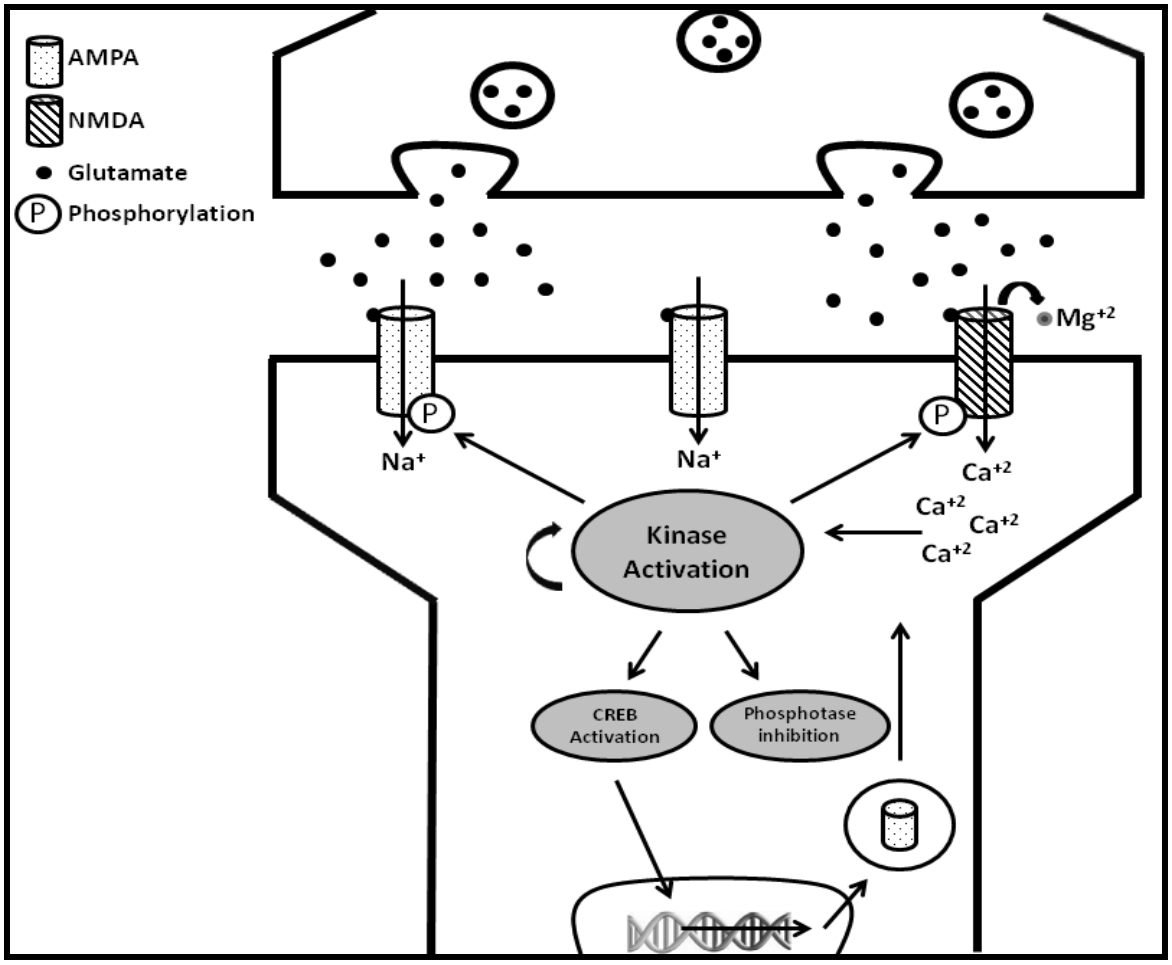

Figure 1-7 Mechanism of LTP.
When glutamate is released from the presynaptic terminal it binds to AMPA and NMDA receptors. Depolarization due to sodium influx through AMPA receptors relieves the magnesium block from the NMDA receptors so that calcium can flow into the cell. Increased intracellular calcium activates several kinases including PKA, PKC, CaMKII and MAPK. Kinases can phosphorylate AMPA and NMDA receptors. AMPA receptor phosphorylation increases channel opening probability, whereas NMDA receptor phosphorylation increases calcium conductance. Kinases can also inhibit phosphotases to prevent receptor dephosphorylation. Finally, kinases can activate the transcription factor CREB, which translocates to the nucleus and binds to CRE on specific DNA sequences. This increases transcription of synaptic plasticity related genes, and can increase AMPA receptor production. New AMPA receptors can be transported to the membrane. Phosphorylation of receptors, insertion of new AMPA receptors, and phosphatase inhibition increase synaptic efficacy in the postsynaptic cell. When glutamate is released at a later time from the presynatpic terminal the response of the postsynaptic cell will be potentiated – and hence long-term potentiation occurs. **Abbreviations:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropoionic acid (AMPA); cAMP response element (CRE); cAMP response element binding protein (CREB); Calcium (Ca^{2+}); Calcium-calmodulin dependent kinase (CaMKII); Magnesium (Mg^{2+}); mitogen-activated protein kinase (MAPK); *N*-methyl-D-aspartate (NMDA); Protein kinase A (PKA); Protein kinase C (PKC); Sodium (Na^{+}).

LTP is characterized by cooperativity, associativity and input-specificity (Bliss and Collingridge, 1993). Cooperativity is the need of a specific threshold of intensity to be reached in order for LTP to be induced, i.e., the tetani applied to the tissue must be sufficiently strong to activate enough fibres to evoke a response (McNaughton et al., 1978). Associativity occurs if a weak input is potentiated when it occurs concurrently with a strong tetanus that is applied at a separate but convergent input (Levy and Steward, 1979). Finally, LTP is also input specific since other inputs that are not active at the time of tetanic stimulation are not involved in the potentiation induced in the tetanised pathway (Andersen et al., 1977).

1.4.3.2 Experimental induction of LTP

LTP can be induced by a number of mechanisms, the most widely utilised being the delivery of tetanic stimuli (which is usually a train of 50 – 100 stimuli at 100Hz or more) to the pathway of interest (Bliss and Lomo, 1973, Bliss and Collingridge, 1993). This repeated stimulation results in repetitive presynaptic glutamate release and allows for sufficient depolarization of the postsynaptic cell and removal of the magnesium block in the NMDA receptor (Coan and Collingridge, 1985). Recordings can be taken intra- or extracellularly, depending on what is to be measured and the manipulations that are being undertaken. Other methods of LTP induction include theta-burst stimulation (**TBS**), which consists of bursts of stimuli with various frequencies delivered 200ms apart (Larson et al., 1986); and primed-burst stimulation (a single initial stimulus - the primer - followed 200ms later by a single burst of 4 shocks at 100Hz) (Rose and Dunwiddie, 1986), which are thought to more closely represent the activity pattern that occurs during normal learning activity in the brain. While the 200 ms interval between bursts provides

time for the postsynaptic membrane to return to resting potential, it is believed that temporal summation is still taking place (Sweatt, 2003). The summation results from an increase in disinhibition at the synapse mediated through presynaptic $GABA_B$ receptor activation which is highest 200 ms after glutamate release (Brucato et al., 1996, Staubli and Scafidi, 1999). This disinhibition allows increased excitability, which activates NMDA receptors and allows calcium to flow into the postsynaptic cell and LTP to be induced (Sweatt, 2003). TBS was the method of LTP induction utilized in this thesis, as it more closely represents the electrical activity that occurs during the learning process.

1.4.3.3 LTP in the DG

LTP can be measured in the DG by stimulating the MPP or LPP that project from the EC to the DG (see Figure 1-6). EC pyramidal cell axons synapse onto the dendrites of the DGCs and excite the DGCs by the release of the neurotransmitter glutamate. This form of synaptic plasticity is NMDA-dependent and requires signalling cascades that involve CaMKII and PKA (Wu et al., 2006), as explained above (section 1.4.3.1).

TBS is a common method of LTP induction in the DG because this region appears to be particularly tuned to theta-like oscillations (Vanderwolf, 1969, Winson, 1974) that are of 5-10 Hz frequency (Andersen et al., 1966) and are known to occur during learning or exploration of a new environment (Winson, 1978, Buzsaki, 2002, Klausberger et al., 2003). The TBS protocol that delivers bursts of stimuli 200 ms apart (i.e. 5 Hz) is commonly used both *in vitro* and *in vivo* (Bawin et al., 1984, Larson et al., 1986, Greenstein et al., 1988, Pavlides et al., 1988).

1.4.4 LTP and learning and memory

The neuronal processes that occur during LTP have many characteristics that are expected to occur during the learning process (reviewed by (Bliss and Collingridge, 1993). A definitive connection between LTP and learning and memory was made in 1986 when Richard Morris and colleagues discovered that administering the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (**APV**) to mice blocked both the induction of LTP and Morris water maze performance (Morris et al., 1986). Since this discovery, many reports have provided more evidence for a link between LTP and learning and memory (Barnes and McNaughton, 1985, Korol et al., 1993, Lipp and Wolfer, 1998, Chapman et al., 1999), the most notable of which are discussed in a 1993 review article by Bliss and Collingridge (Bliss and Collingridge, 1993).

1.4.5 Synaptic plasticity and PNEE

Synaptic plasticity is important during the early development of the brain (synaptogenesis, neurite outgrowth, etc) and therefore alterations in plasticity may contribute to the persistent effects of neurodevelopmental disorders such as PNEE (Galvan, 2010). Animal models have provided considerable insight into the effects of PNEE on hippocampal function. While the majority of studies examining synaptic plasticity are conducted in the CA1, studies in the DG have also been conducted, with animals being examined both at early time points (neonatal or adolescent) and in adulthood.

In the CA1, deficits in NMDA mediated synaptic plasticity have been observed with prenatal (Swartzwelder et al., 1988, Richardson et al., 2002, Izumi et al., 2005) and postnatal (i.e. 3rd trimester equivalent) (Puglia and Valenzuela, 2010b, a) exposure. In

contrast, other studies have reported no deficits in CA1 LTP following pre and perinatal ethanol exposure (Bellinger et al., 1999, Krahl et al., 1999, Byrnes et al., 2004). These differences are most likely due to variations in the period of ethanol exposure, dose of ethanol, method of ethanol delivery, species used, age at testing, recording technique (*in vitro* vs. *in vivo*) or stimulation used to induce LTP (which would differentially affect NMDA receptor activation). It is possible that NMDA receptors are altered due to PNEE, and consistent with this, reductions in NMDA currents and glutamate binding have been observed (Morrisett et al., 1989, Savage et al., 1991).

PNEE also causes long lasting deficits in synaptic plasticity in the DG – in particular, LTP is decreased in adolescent and adult offspring (Sutherland et al., 1997, Christie et al., 2005, Varaschin et al., 2010, Titterness and Christie, 2012, Brady et al., 2013). Interestingly, the majority of these studies have been conducted in males, and the only study that also examines LTP in females (Titterness and Christie, 2012) observed an increase in LTP in the DG of adolescent females. The sex differences in LTP in the DG of PNEE animals are examined in more detail in Chapter 3.

The mechanism behind the decreases in synaptic plasticity following PNEE are unclear, but it is possible that an increase in oxidative stress caused by PNEE could be responsible, due to the fact that oxidative stress negatively impacts the ability of a system to elicit LTP (Pellmar et al., 1991, Auerbach and Segal, 1997) possibly through hypofunction of the NMDA receptor (Steullet et al., 2006) (see section 5.1). This hypothesis is explored in Chapter 5 of this thesis.

1.5 Therapeutic interventions to treat hippocampal deficits associated with FASD

1.5.1 Omega-3 fatty acids

Omega-3 fatty acids are members of the PUFA family, one of the three families of fatty acids found in the human body (Marszalek and Lodish, 2005). The PUFAs, which contain multiple double bonds, are divided into three groups – omega-3 fatty acids, omega-6 fatty acids and omega-9 fatty acids, depending on the placement of the first double bond. While omega-3, omega-6 and omega-9 fatty acids are essential fatty acids required by the body for multiple functions, omega-3 fatty acids have more significant benefits for the brain due to their anti-inflammatory (Wall et al., 2010), anti-apoptotic (Kim et al., 2000, Akbar et al., 2005) and antioxidant properties (Sarsimaz et al., 2003); reviewed by (Gomez-Pinilla, 2008). The two major omega-3 fatty acids are eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**). DHA accumulation in the body depends on the amount and types of omega-3 fatty acids in the diet and on dietary intake of omega-6 fatty acids (such as arachidonic acid, **AA**), which interact and compete with omega-3 fatty acids in the fatty acid metabolic pathway (Arbuckle et al., 1994, Simopoulos, 2009b). Omega-3 fatty acids are found in high concentrations in neuronal membranes where they are acetylated into the sn-2 position of phospholipids (Simopoulos, 2009a). DHA is the most abundant omega-3 fatty acid in the brain, comprising 40% of the brain PUFAs (Singh, 2005), and DHA-containing phospholipids can act to improve membrane fluidity, enhance synaptic transmission, and increase receptor trafficking (see review by (Gomez-Pinilla, 2008). DHA-containing phospholipids can also activate energy-generating metabolic pathways that increase levels of growth factors including brain-derived neurotrophic factor (**BDNF**) and insulin-

like growth factor-1 (**IGF-1**) (Gomez-Pinilla, 2008). These growth factors in turn can activate signalling cascades, through the TrkB and IGF receptors respectively, and enhance synaptic plasticity and transcription of genes involved in neuronal growth and development (Levine et al., 1998, Huang and Reichardt, 2001). DHA and EPA can also be cleaved from membrane phospholipids by the action of phospholipase A_2 (**PLA_2**) which produces eicosanoids and docosanoids such as the anti-inflammatory neuroprotectin D1 (**NPD1**; (Bazan, 2009).

1.5.1.1 Omega-3 fatty acids and FASD

The optimal concentration of DHA in the hippocampus during development is thought to be 12-16% of total fatty acids (Innis, 2005). The majority of DHA accumulation in the brain occurs in the perinatal period; from the third trimester through to two years after birth in humans, and from GD 7 to PND 21 in rats (Dobbing and Sands, 1979, Martinez, 1992, Green et al., 1999). There is a link between low maternal DHA and poor child neural development (Innis et al., 2001, Oken et al., 2005) and increasing maternal DHA decreases the risk of poor visual and neural development in infants and children (Innis et al., 2001, Helland et al., 2003, Oken et al., 2005, Hibbeln et al., 2007).

PNEE decreases brain concentrations of DHA (Burdge and Postle, 1995, Wen and Kim, 2004). The DHA content in the brain is lowered by ethanol consumption particularly from the phospholipid phosphotidylserine (**PS**) (Harris et al., 1984, Pawlosky and Salem, 1995). The effect of ethanol on DHA accumulation was also tested *in vivo* by Wen and Kim (2004), who examined the phospholipid profile in developing rat hippocampi after chronic PNEE and found that the total neuronal level of PS was 15-20% lower than controls at both PND 0 and PND 21 (Wen and Kim, 2004). The depletion of

DHA, and therefore PS, by ethanol may significantly affect signalling pathways in the CNS, as PS is the main negatively charged phospholipid in the cell membrane and it influences several signalling proteins such as protein kinases (Kim, 2008), which can in turn modulate synaptic plasticity and other processes linked to learning and memory. Astroglial release of DHA also decreases with long-term ethanol exposure (Garcia et al., 1997). The decreased neuronal supply of DHA could therefore explain some of the neurotoxic effects of ethanol.

Treatments that act at the level of the structural and functional deficits observed in the brains of individuals with FASD may ameliorate the cognitive manifestations of this syndrome and improve certain aspects of behavior such as learning and memory. Because omega-3 content is decreased with PNEE, and omega-3 supplementation has been repeatedly shown to have multiple beneficial effects such as antioxidant protection (Sarsilmaz et al., 2003, Songur et al., 2004), promotion of LTP (McGahon et al., 1999, Martin et al., 2002) and learning and memory (Lim and Suzuki, 2001, Wu et al., 2008); see section 6.1), in Chapter 6 we tested the effects of omega-3 fatty acid supplementation in ethanol-exposed animals.

1.6 Summary and objectives

It is well established that PNEE causes long-term deficits in learning and memory and cognition. It is also clear that the DG is a region of the hippocampus that plays a prominent role in pattern separation, spatial learning and memory. Learning and memory processes are thought to be the result of synaptic plasticity. There is substantial behavioural evidence that suggests that DG function is impaired with PNEE. However, it

is still not completely understood how PNEE leads to DG dysfunction in general and impaired synaptic plasticity in particular.

Objective 1: To characterize the deficits in DG synaptic plasticity that occur in the adult male and female brain following PNEE (Chapter 3).

Objective 2: To examine the temporal vulnerability of the developing brain to ethanol, by examining perinatal exposure to ethanol during the three trimester equivalents (Chapter 4).

Objective 3: To investigate the link between GSH, the major endogenous antioxidant, and LTP in the DG and determine whether increasing GSH in the brain can enhance LTP in the DG of adult ethanol-exposed animals (Chapter 5).

Objective 4: To examine the benefits of omega-3 fatty acid supplementation on DG function in FASD, by measuring markers of oxidative stress and examining synaptic plasticity (Chapter 6).

2. General Methods

2.1. Animals and breeding

Male (300 – 350g) and virgin female (250 – 275g) Sprague-Dawley rats were obtained from Charles River Laboratories (Quebec, Canada) and housed in a colony room at the University of Victoria Animal Care Unit. Females were housed in pairs and breeding males were housed individually in clear polycarbonate cages (46 X 24 X 20 cm) with Carefresh contact bedding (Absorption Corp., Bellingham, WA, USA). The room was maintained on a 12-hour light:dark cycle (lights on at 7 am) with constant humidity and temperature (22^{o}C). Following an acclimation period in the unit for at least one week, females and males were housed together and a vaginal smear using 0.9% sodium chloride (**NaCl**) was performed at the beginning of each light cycle to determine the presence of sperm. The swab was examined on a microscope slide with an Olympus Microscope with a 10X objective (Olympus CX21, Center Valley, PA, USA). If sperm was detected this indicated pregnancy (i.e. GD 1) and the female was immediately removed to a private container supplied with nesting material and placed on one of three prenatal diets: Ethanol, Pair-fed and *Ad libitum* control (Section 2.2). The model of FASD utilized is well established (for review see (Gil-Mohapel et al., 2010) and has been used in our laboratory (Christie et al., 2005, Titterness and Christie, 2012) and by others (Weinberg, 1985, Weinberg et al., 1996, Lan et al., 2006, Hellemans et al., 2008, Lan et al., 2009, Uban et al., 2010) for several years. All animal procedures were performed in accordance with the University of Victoria Animal Care Committee and the Canadian Council for Animal Care Policies.

2.1.1 Prenatal diets

On GD 1 pregnant dams were randomly assigned to one of three prenatal diets:

(1) Ethanol – *Ad libitum* access to a liquid diet containing 35.5% ethanol derived calories (6.61% v/v). Ethanol dams were gradually introduced to the liquid diet over a three-day period (GDs 1 – 3). On GD 1, one third of the ethanol diet was combined with two thirds of the pair-fed diet (see below), on GD 2 two thirds of the ethanol diet were combined with one third of the pair-fed diet and on GD 3 (and all subsequent days of the pregnancy) 100% of the ethanol diet was supplied to the dam.

(2) Pair-fed – a liquid diet with maltose-dextrin isocalorically substituted for the ethanol derived calories. This liquid diet was not provided *ad libitum*. To control for stress and malnutrition (discussed in section 7.2.1), the pair-fed animals received the same amount of food in g/kg/day as their matched ethanol dams.

(3) *Ad libitum* control – a regular chow diet (Lab Diets 5001, LabDiets, Richmond, IN, USA) that animals had *ad libitum* access throughout pregnancy.

All diets were given to the animals two hours prior to the beginning of the dark phase each day of the pregnancy. This was done to ensure there were no shifts in the circadian rhythm (Weinberg and Gallo, 1982). When liquid diet bottles were replaced, the bottle from the previous day was weighed to determine the amount of liquid consumed each day. Females were weighed on GDs 1, 7, 14 and 21.

Liquid diets were obtained from Dyets (Bethlehem, PA, USA) where they are sold as Weinberg/Keiver high protein liquid diet-control (no. 710109) for the pair-fed diet and Weinberg/Keiver high protein liquid diet-experimental (no. 710324) for the ethanol diet. These liquid diets have been nutritionally fortified to ensure that adequate nutrition is provided to the pregnant rats throughout pregnancy (Weinberg, 1985).

2.1.2 Litters and weaning

Dams and pups were not disturbed for the first 24 – 36 hours post-partum to facilitate bonding. Litters were culled to ten pups (4 females and 6 males, or 6 females and 4 males where possible) on PND 2. Pups from all litters were weighed once a week between PNDs 2 – 22[1]. Pups were weighed and weaned at 23 days of age and housed in pairs (based on sex) in standard caging. Pups were left undisturbed (except for cage changes and water/food refills conducted by animal care staff) until they reached young adulthood. All animals were used for experimentation between PNDs 55 – 70. Please see Figure 2-1 for an overview of the breeding process.

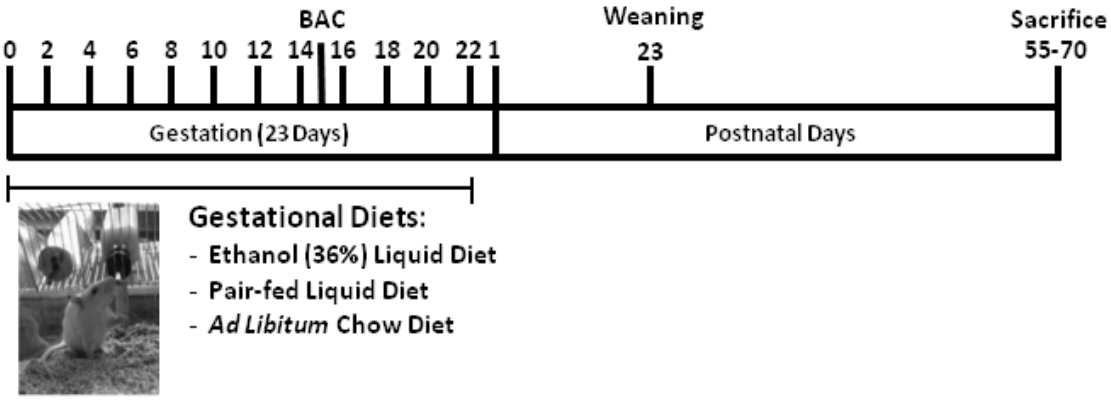

Figure 2-1 Overview of the breeding procedures.
On gestational day (GD) 1 animals were assigned to one of three prenatal diets (Ethanol, Pair-fed or *ad libitum* control). On GD 15 a blood sample was taken to assess blood alcohol concentration (BAC). Pups were born on GD 23 (assigned postnatal day (PND) 1) and were weaned from their mother at PND 23. Animals were used for experimentation when they reached young adulthood (between PNDs 55 – 70). For all experiments, a maximum of two females and two males per litter were included in any given experimental group.

2.2. Blood samples to determine blood alcohol concentration

For all ethanol-fed dams a single tail blood sample was obtained on GD 15, approximately two hours after beginning of the dark phase. Blood was collected in a

[1] In Chapter 4 animals were weighed on PNDs 2, 6, 10, 14, 18 and 22.

microcentrifuge tube (approximately 1 mL) and allowed to clot overnight at 4^{o}C. Samples were centrifuged the following day at 3,000 *g* and the serum (supernatant) was then stored at -20^{o}C until analysed. Analysis of blood alcohol concentration (**BAC**) was determined using the Analox Alcohol Analyzer (Model AMI; Analox Instruments, Lunenberg, MA) and expressed as mg/dl of serum.

2.3 *In vivo* electrophysiology

Male and female offspring were used for *in vivo* electrophysiology between the age of PNDs 55 – 70. Female subjects were examined each day for at least five days before experimentation using the lavage technique where a vaginal smear using 0.9% NaCl was performed at the beginning of each light cycle to determine the estrous cycle. Females were not used for experimentation if they were in proestrous, where estrogen levels are highest, as high levels of estrogen may modulate LTP (Spencer et al., 2008, Ooishi et al., 2012).

Animals were anaesthetized with urethane (1.5 mg/kg, intraperitoneal (**i.p.**)) and placed on a Kopf stereotaxic apparatus. Body temperature was maintained at 37 ± 0.5°C throughout the experiment with a grounded homeothermic temperature control unit (Harvard Instruments, MA, USA). Extracellular field potentials were recorded by inserting a 125µm stainless-steel recording electrode into the hilus of the DG through a hole above the dorsal hippocampus (3.5 mm anterior, 2.0 mm lateral to bregma; (Paxinos and Watson, 2007) and a 125µm monopolar stimulating electrode through a hole above the ipsilateral medial perforant path (7.4 mm anterior, 4.2 mm lateral to bregma; (Paxinos and Watson, 2007) (Figure 2-2). A ground electrode was placed posterior to lambda, and a reference electrode was placed anterior to Bregma. Stimulating and recording

electrodes were lowered to elicit a maximal response and the stimulation required to induce a 1 mV population spike was determined.

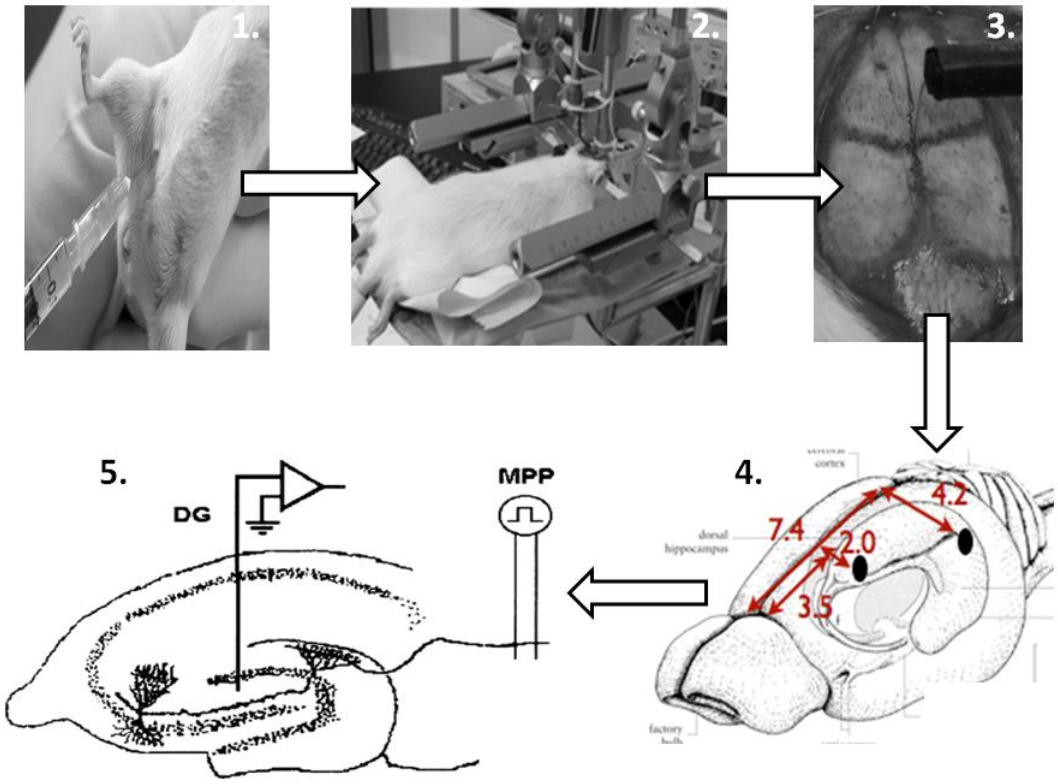

Figure 2-2 *In vivo* electrophysiology experimental protocol.
1. The animal was anaesthetised with 1.5mg/kg urethane (i.p.). 2. Once anaesthetised, the animal was placed into a stereotaxic apparatus. 3. The skull was exposed and a stimulating electrode was moved to the intercept between Bregma and the midline of the skull. 4. From this position co-ordinates were used to drill holes for the stimulating and recording electrode. 5. Schematic representation of the hippocampus, showing the stimulating electrode inserted into the medial perforant path (MPP) and the recording electrode placed in the hilus region of the dentate gyrus (DG).

Basal recordings were first obtained by administering a pulse (0.12 ms in duration) at 0.067 Hz. Once a stable baseline was observed for at least 15 minutes, LTP was induced by applying TBS consisting of 10 bursts of 5 pulses at 400Hz with an inter-burst interval of 200 ms. This protocol was repeated 4 times at 30 second intervals. The pulse duration was changed to 0.25 ms during TBS. Following TBS, baseline stimulation resumed for

60 minutes (see Figure 2-3). This protocol has been used previously in our laboratory when examining LTP in ethanol-exposed animals (Titterness and Christie, 2012).

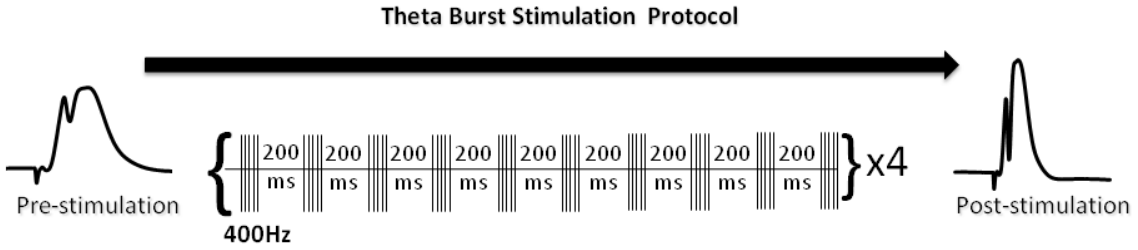

Figure 2-3 *In vivo* electrophysiology recording protocol.
Electrophysiological experiments were conducted to examine LTP in the DG. Basal recordings were first obtained by administering a pulse (0.12 ms in duration) at 0.067 Hz (Pre-stimulation). Once a stable baseline was observed for at least 15 minutes, LTP was induced by applying theta burst stimulation (TBS) consisting of 10 bursts of 5 pulses at 400 Hz with an inter-burst interval of 200 ms, which was repeated 4 times at 30 second intervals. The pulse duration was changed to 0.25 ms during TBS. Following TBS, baseline stimulation resumed for 60 minutes (Post Stimulation).

Signals from the DG region were collected using custom-made software (Lee Campbell; Getting Instruments). Signals were amplified (Getting Instruments), filtered (1Hz – 3 kHz) and digitized at 5kHz. For analysis, the slope of the rising phase of the field EPSP was used to determine alterations in the level of synaptic efficacy. All EPSP slope data are presented as the mean percent change from the pre-conditioning baseline ± standard error of the mean (**S.E.M.**).

2.4 Preparation of samples for biochemical analysis

All animals were sacrificed by rapid decapitation and the DG sub-region of the hippocampus was crudely microdissected following the procedure described by Hagihara et al. (2009). For animals that had undergone electrophysiological experiments, only the contralateral hemisphere to the stimulation was used for biochemical analyses. For all other animals, both hemispheres were used. Briefly, each brain was cut along the longitudinal fissure of the cerebrum and the midbrain, hindbrain and cerebellum were

removed. With the cerebral hemisphere medial side facing up, the diencephalon was removed, which exposed the medial side of the hippocampus and allowed for the visualization of the DG, as the boundary between the DG and CA subsections is identifiable with the naked eye. A surgical spatula was used to separate the DG from the CA region by sliding the spatula along the septo-temporal axis of the hippocampus (Hagihara et al., 2009).

2.5 General statistical analysis

Statistical analyses were performed using the Statistica 7.1 analytical software (StatSoft Inc., Tulsa, OK, USA). All data are presented as means ± S.E.M. Developmental data (pup weights) were analyzed using repeated measures analysis of variance (**ANOVA**). All other data were analyzed with either a one-way, two-way or three-way ANOVA as appropriate. Post-hoc analyses were conducted using the Tukey post-hoc test. A p value of < 0.05 was considered to be statistically significant. Details on the specific statistical analyzes used for each particular experiment are outlined in the Methods and Results sections of the succeeding chapters.

3. Examining the Differences in Synaptic Plasticity in Males and Females Following PNEE

This Chapter is based in part on the following manuscript:

Helle Sickmann, **Anna R. Patten,** Kristin Morch, Scott Sawchuk, Connie Zhang, Ellie Parton, Larissa Szlavik and Brian R. Christie (2012). Gender-differences in hippocampal long-term potentiation in adult rats following prenatal ethanol exposure. *Hippocampus. (under review).*

3.1 Background

Ethanol exposure can have a profound impact on hippocampal structure and function in humans and animals (Gil-Mohapel et al., 2011; Reyes et al., 1989; Richardson et al., 2002; Ryan et al., 2008; Titterness and Christie, 2012; Uecker and Nadel, 1998; reviewed in Gil Mohapel et al 2010) (see section 1.3.1.4 and section 1.3.3 for a more detailed discussion). Previous studies have shown that LTP in the CA1 (Swartzwelder et al., 1988, Richardson et al., 2002) and the DG (Sutherland et al., 1997, Christie et al., 2005, Varaschin et al., 2010, Titterness and Christie, 2012, Brady et al., 2013) is attenuated in juvenile and adult males following chronic PNEE. Conversely, the only study that has examined LTP in females following PNEE observed an enhancement in LTP during adolescence (Titterness and Christie, 2012). Adolescence is a period of significant hormonal change, and it may be that these results do not reflect the long-term impact of PNEE in the female adult brain. However, to date there are no studies examining LTP in the DG of adult females that were exposed to ethanol during the period of brain development.

3.1.1 The effects of ovarian-produced steroids on LTP

Differences between male and female animals may result from the differential effects of sex hormones on the brain. In particular, there is evidence to suggest that estrogen may play a protective role in the female brain and it has been shown to enhance LTP and/or learning and memory in the CA1 of the hippocampus (Foy et al., 1999, Ooishi et al., 2012). The hippocampus in particular displays structural differences in response to different gonadal hormones (Sutcliffe, 2011) and contains both α and β estrogen receptors (**ERs**) (Cahill, 2006). In humans, imaging studies indicate that the hippocampus is larger in women than in men when total brain size is adjusted (Goldstein et al., 2001). Interestingly, in animal studies it is the male hippocampus that has higher volume, with significantly more pyramidal cells in the CA1 and a higher density of granule cells in the DG (Madeira and Lieberman, 1995).

In rats and mice, levels of estrogen fluctuate on approximately a 4-day cycle, with the period defined as pro-estrous corresponding to the peak of estrogen levels. Research has examined the reasons underlying the increase in CA1 LTP that occurs during pro-estrous and it has been suggested that estrogen can augment NMDA and AMPA receptor mediated currents (Wong and Moss, 1992, Foy et al., 1999). Indeed, during pro-estrous, the number of dendritic spines and synaptic density in the CA1 is 30% higher than during estrous, when estrogen levels are lowest (Gould et al., 1990, Woolley and McEwen, 1992). This mechanism is probably due to underlying differences in glutamatergic receptor activation and sensitivity during the different stages of the estrous cycle (Woolley et al., 1997, Daniel and Dohanich, 2001, Liu et al., 2008). Furthermore, a reduction in GABA inhibition in the hippocampus during pro-estrous may also contribute to the effects of estrogen on LTP (Murphy et al., 1998). Estrogen may also modulate the

production and release of acetylcholine, which can influence cognitive ability by enhancing LTP (Daniel and Dohanich, 2001, Sutcliffe, 2011). In this thesis female rats that were in pro-estrous were not included in experimental analyses.

3.1.2 Objectives of this Chapter

The aim of this chapter was to determine the effects of PNEE on LTP in the DG of both male and female adult rats (designated cohort 1). We started by examining the effect of PNEE on LTP in the DG of male and female offspring. Since significant differences between the sexes were observed, we subsequently determined whether circulating ovarian steroids could be responsible for these differences (designated cohort 2).

Because estrogen is known to have a positive effect on LTP, removing estrogen (and other ovarian-produced sex steroids) from the circulation through ovariectomy (**OVX**) was undertaken in *ad libitum* control and ethanol-exposed females prior to sexual maturation. When the ovaries are removed, the levels of estrogen and progesterone, as well as the levels of testosterone and androstenedione are drastically reduced (Laughlin et al., 2000, Alagwu and Nneli, 2005). In this study OVX was carried out in juvenile animals (prior to sexual maturation), and when animals reached experimental age (PNDs 55 – 70) *in vivo* electrophysiology was performed to examine whether OVX had an effect on LTP in the DG following PNEE.

3.2 Animals and methods

A*d libitum*, pair-fed and ethanol-exposed dams were produced for cohort 1 (analysis of sex differences in LTP following PNEE) as described in detail in Chapter 2 (section 2.1). Between PNDs 55 – 70 two males and two females from each litter were used for *in*

vivo electrophysiology experiments. *In vivo* electrophysiology to examine LTP in the DG sub-region of the hippocampus was conducted as described in Chapter 2 (section 2.3).

3.2.1 Ovariectomy

A second cohort of ethanol-exposed and *ad libitum* control animals was generated as described in Chapter 2 (section 2.1). Between PNDs 26 – 30, four females from each litter were submitted to the OVX procedure. Two had the complete OVX procedure performed, while the other two were sham controls and underwent similar surgical procedures without removal of the ovaries. Animals were anaesthetized with isoflurane, shaved and cleaned. A 2.5 cm incision was made on the animals' flank, between the hip bone and the rib cage. The ovary was located and clamped at the level of the fallopian tube. A suture was placed around the fallopian tube and the ovary was removed. The clamp was removed and if no blood was observed the fallopian tube was repositioned and the muscle and skin were sutured closed. Animals were maintained on the analgesic buprenorphine (0.01 mg/kg, subcutaneous) for three days post-surgery. Following OVX, animals were housed singly and monitored every 12 hours for three consecutive days and once daily for a remaining week. Between PNDs 55 – 70, OVX, sham and control animals from each group were used for *in vivo* electrophysiology experiments. LTP in the DG was examined as described in Chapter 2 (section 2.3).

3.2.2 Statistical analysis

All data are presented as means ± S.E.M. A one-way ANOVA was used to determine the effect of prenatal treatment on weight gain across pregnancy and litter size. A repeated measures ANOVA for prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed) was used to analyze pup weights. A two-way ANOVA for sex (male,

female) and prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed) was used to analyze weights at experimental age (PNDs 55 - 70) for cohort 1 and a significant effect of sex was observed [$F(1, 58) = 347.9$, $p = 0.001$], with males being significantly heavier than females at this age ($p < 0.001$) As such, male and female weights at the experimental age were analysed separately using a one-way ANOVA. For cohort 2 (OVX females) a two-way ANOVA for prenatal condition (*ad libitum* control or ethanol-exposed) and treatment (OVX, sham-operated or control) was used to analyze weights at experimental age (PNDs 55 - 70).

For LTP data from cohort 1, a two-way ANOVA for prenatal condition (*ad libitum*, pair-fed, ethanol) and sex (male, female) also showed a significant main effect of sex [$F(1, 46) = 1.87$, $p = 0.05$] with males showing more LTP than females ($p < 0.05$). Thus, female and male LTP data were subsequently analyzed separately. For the LTP data generated from cohort 2 a two-way ANOVA for prenatal condition (*ad libitum*, ethanol-exposed) and treatment (OVX, sham-operated or control) was used. Post-hoc analyses were conducted using Tukey's test. A p value of < 0.05 was considered to be statistically significant.

3.3 Results

3.3.1 PNEE leads to a reduction in LTP in the DG of adult male but not female offspring

3.3.1.1 Developmental data

Pregnant dams were weighed on GDs 1, 7, 14 and 21. The percentage weight gain over the course of pregnancy did not differ between prenatal conditions [$F(2, 15) = 2.24$, $p = 0.14$, Table 3-1]. Litter size was also comparable among prenatal conditions [$F(2, 15) = 0.40$, $p = 0.68$, Table 3-1].

Table 3-1 Gestational data for *ad libitum*, pair-fed and ethanol-exposed dams from cohort 1.
Ad libitum, Pair-fed and Ethanol exposed dams gained weight comparably across pregnancy. Litter sizes were also comparable between prenatal conditions. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. n = 6 dams for all groups.

	Ad libitum	**Pair-fed**	**Ethanol-exposed**
Weight gain over pregnancy (%)	56.8 ± 3.5	39.6 ± 7.1	48.4 ± 6.0
Number of pups per litter	13.5 ± 0.4	14.8 ± 1.8	13.3 ± 1.3

Offspring weight was determined during the lactation period on PNDs 2, 8, 15 and 22 to evaluate whether prenatal condition altered offspring weight gain (Tables 3-2). A repeated measures ANOVA revealed that no significant sex differences in weight occurred at these time points [$F(4, 27) = 1.0$, $P = 0.42$] so data from both sexes were combined. A significant main effect of prenatal condition was observed [$F(8, 60) = 6.14$, $p = 0.00001$]. Post-hoc analyses revealed that at PND 2 pair-fed ($p < 0.01$) and ethanol-exposed ($p < 0.01$) offspring weighed significantly less than *ad libitum* control animals. At PND 8 the same effect was observed with pair-fed ($p < 0.05$) and ethanol-exposed ($p < 0.01$) offspring still weighing less than *ad libitum* controls. At PND 15 no significant differences in weight were observed between groups, but at PND 22 when animals were weaned, pair-fed ($p < 0.01$) offspring weighed significantly less than *ad libitum* controls and ethanol-exposed offspring weighed significantly less than both *ad libitum* control ($p < 0.001$) and pair-fed ($p < 0.05$) offspring (Table 3-2).

Table 3-2 Weight comparisons of *ad libitum*, pair-fed and ethanol-exposed offspring during development (cohort 1).

All offspring gained weight over the lactation period. A repeated measures ANOVA was used to analyze offspring weights taken on PNDs 2, 8, 15 and 22. There were no sex differences between male and female offspring between PNDs 2 - 22. *Ad libitum* offspring weighed significantly more than pair-fed and ethanol-exposed offspring at most time points (see text for details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, as compared to *ad libitum* control. $^{\#}$ = $p < 0.05$, as compared to pair-fed control.

Offspring Weight (g)	*Ad libitum*	Pair-fed	Ethanol-exposed
PND2	7.8 ± 0.2	6.9 ± 0.4**	6.9 ± 0.4**
PND8	17.7 ± 0.8	15.0 ± 1.2*	14.0 ± 1.0**
PND15	32.5 ± 1.3	28.7 ± 2.6	28.8 ± 1.4
PND22	60.1 ± 1.8	52.2 ± 2.6**	46.1 ± 1.8*** #

When animals reached experimental age males and females were analyzed separately. In both males [$F(2, 32) = 0.72$, $p = 0.49$, Table 3-4], and females [$F(2, 26) = 0.01$, $p = 0.99$, Table 3-3], no differences in weight were observed between prenatal conditions.

Table 3-3 Weights at experimental age for animals used in LTP experiments (cohort 1)

When animals reached experimental age (PNDs 55-70) a significant main effect of sex on body weight was observed, so male and female data was analyzed separately. For both males and females there was no significant effect of prenatal condition. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. *** = $p < 0.001$ compared to male offspring.

Offspring Weight (g)	*Ad libitum*	Pair-fed	Ethanol-exposed
Males	371.0 ± 8.4	366.4 ± 8.8	380.2 ± 6.9
Females	246.7 ± 7.6***	248.4 ± 5.7***	247.0 ± 8.6***

3.3.1.2 Intoxication levels

Peak BAC levels were measured two hours after the dark cycle commenced on GD 15 of pregnancy. The mean BAC level was 146.32 ± 4.75 mg/dl, which is in accordance with the levels obtained in previous studies that used this liquid diet model of PNEE (Patten et al., 2012; Titterness and Christie 2012; Christie et al., 2005).

3.3.1.3 PNEE causes a long-term reduction in LTP in the male DG but the female DG is unaffected

LTP in the DG of the hippocampus after MPP activation was affected differently in adult males and females that were exposed to ethanol during gestation. A two-way ANOVA revealed a significant main effect of sex [$F(1, 46) = 1.87$, $p = 0.05$], and therefore male and female data were subsequently analyzed independently. In males, a one-way ANOVA revealed a significant effect of prenatal condition [$F(2, 24) = 10.6$, $p = 0.0005$], with the capacity for LTP being significantly reduced in ethanol-exposed males ($25.9 \pm 4.3\%$) when compared to both *ad libitum* control ($47.4 \pm 1.0\%$; $p < 0.001$) and pair-fed ($43.4 \pm 1.0\%$; $p < 0.01$) males (Figure 3-1). Exposure to the pair-fed diet during gestation did not impact the degree of LTP in adult males when compared to their *ad libitum* counterparts ($p = 0.71$), indicating that caloric restriction during gestation does not significantly impair LTP capacity *in vivo*.

Interestingly, in adult females the percentage of LTP ranged between 31-36% irrespective of prenatal condition [$F(2, 24) = 1.24$, $p = 0.31$; Figure 3-1], indicating that PNEE affects synaptic plasticity in a sex-dependent manner.

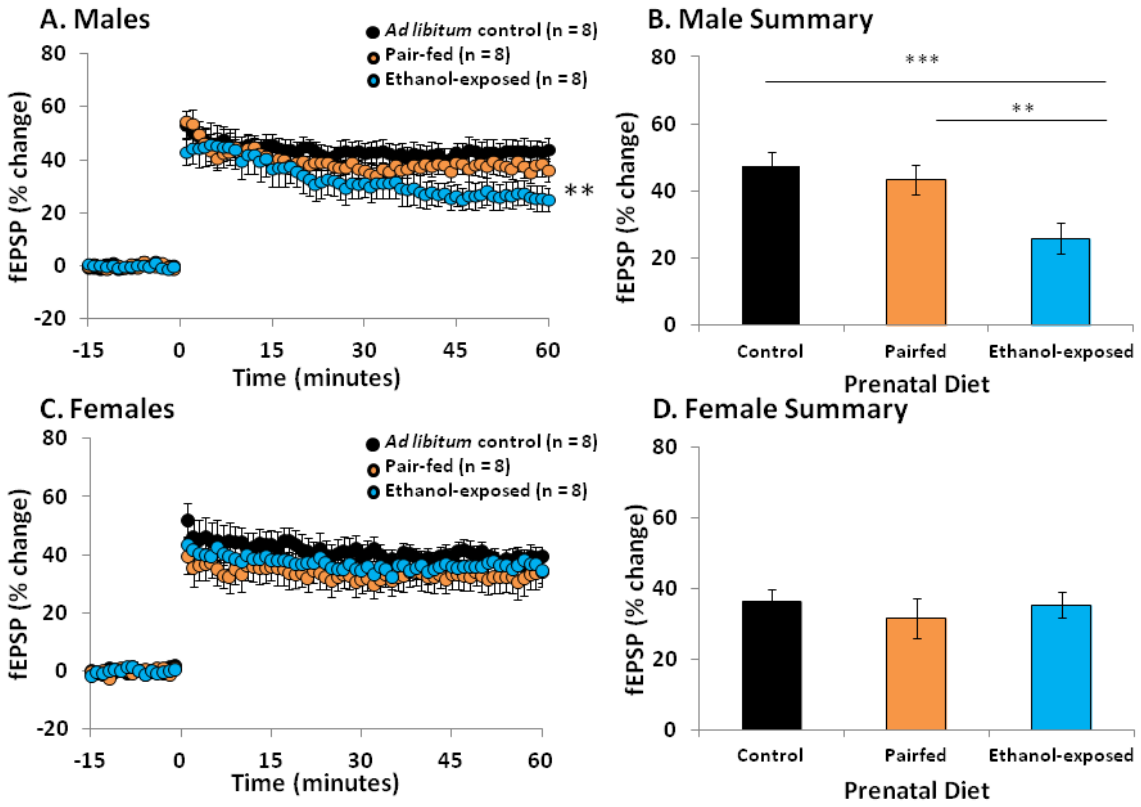

Figure 3-1 Sex-specific effects of PNEE on hippocampal DG LTP in the adult rodent brain. A. PNEE significantly decreases the strength of LTP in PNEE males when compared to their respective *ad libitum* and pair-fed controls. B. Summary of LTP results for male animals showing that PNEE significantly reduces LTP in the male offspring. C. The degree of LTP was similar in females irrespective of prenatal diet. D. Summary of LTP results for female animals showing that PNEE did not reduce LTP in the female offspring. Tetanus protocol was 4 trains (10x 5 pulses at 400Hz, inter-burst interval = 200ms, inter-train interval = 30s, pulse-width 0.25ms).

3.3.2 Ovarian sex steroids do not contribute to LTP in females following PNEE

3.3.2.1 Developmental data

Pregnant dams were weighed on GDs 1, 7, 14 and 21. The percentage weight gain over the course of pregnancy did not differ between prenatal conditions [$F(1, 8) = 1.11$, $p = 0.32$, Table 3-4]. Litter size was also comparable among prenatal conditions [$F(1, 8) = 2.52$, $p = 0.15$, Table 3-4].

Table 3-4 Gestational data for *ad libitum* and ethanol-exposed dams from cohort 2.
Ad libitum and ethanol-exposed dams both gained weight similarly across pregnancy and had comparable litter sizes. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. n = 4 dams per group.

	Ad libitum	**Ethanol-exposed**
Weight gain over pregnancy (%)	38.0 ± 3.8	33.5 ± 1.7
Number of pups per litter	14.2 ± 1.4	16.8 ± 0.9

Only female offspring were used in these experiments. A repeated measures ANOVA for weights between PNDs 2 – 22 did not reveal a significant effect of prenatal condition [$F(4, 5) = 2.14$, $p = 0.21$, Table 3-5].

Table 3-5 Weight comparisons of *ad libitum* and ethanol-exposed offspring during development (cohort 2).
Only female offspring were used for these experiments. In contrast to Table 3-3, in this cohort of animals a repeated measures ANOVA did not reveal significant differences between *ad libitum* control and ethanol-exposed offspring during the postnatal period analyzed. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$.

Offspring Weight (g)	***Ad libitum***	**Ethanol-exposed**
PND2	8.1 ± 0.5	7.4 ± 0.3
PND8	18.5 ± 1.9	14.1 ± 1.0
PND15	37.7 ± 2.2	32.2 ± 2.8
PND22	59.3 ± 2.7	54.9 ± 1.7

When animals reached experimental age, a two-way ANOVA for prenatal condition and treatment (control, sham or OVX) was used to evaluate their effects on offspring weight. Prenatal condition did not significantly impact body weight [$F(1, 48) = 0.14$, $p = 0.71$] and neither did OVX surgery [$F(2, 48) = 0.09$, $p = 0.91$] (Table 3-6).

Table 3-6 The effect of OVX on weight gain.
Prenatal condition did not significantly affect body weight at experimental age. Animals that underwent ovariectomy (OVX) or sham surgeries did not significantly differ in weight when compared to their *ad libitum* control counterparts when they reached aduthood. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$.

Weight (g)	**Control**	**Sham-operated**	**OVX**
Ad libitum	262.9 ± 4.4	267.3 ± 3.1	269.1 ± 4.9
Ethanol-exposed	266.6 ± 4.2	263.4 ± 4.7	264.6 ± 7.2

3.3.2.2 LTP is unaffected following OVX

A cohort of animals was used to examine whether ovarian steroids were responsible for the lack of deficits in LTP observed in ethanol-exposed female offspring following PNEE. OVX surgeries were performed on *ad libitum* control and ethanol-exposed females prior to sexual maturation, and synaptic plasticity was examined in adulthood. A two-way ANOVA for prenatal condition (*ad libitum*, ethanol-exposed) and treatment (control, sham-operated or OVX) revealed no significant differences among prenatal conditions [$F(1, 48) = 0.534$, $p = 0.468$] or treatments [$F(2, 48) = 0.052$, $p = 0.949$, Figure 3-2].

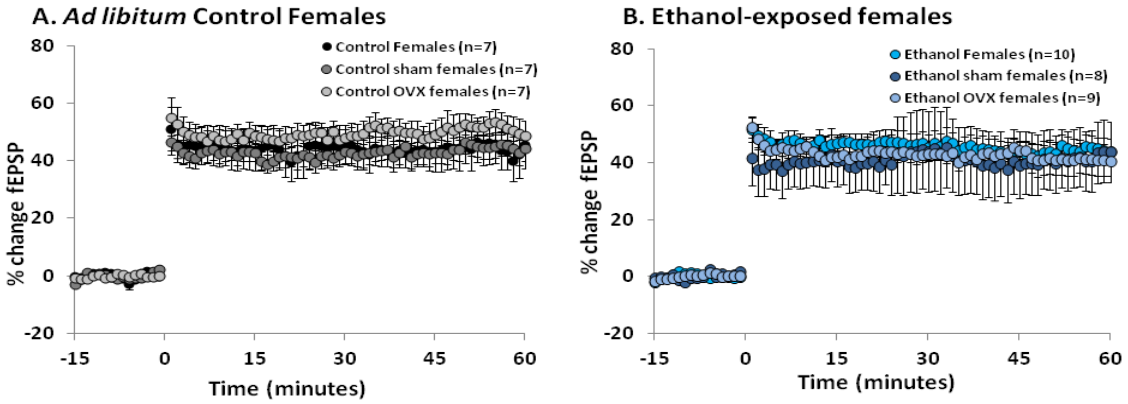

Figure 3-2 The effects of OVX on LTP in the DG of ethanol-exposed and *ad libitum* control females.
A. OVX had no effect on LTP in the DG of control animals. B. OVX had no effect on LTP in the DG of ethanol-exposed animals. Results are presented as means ± SEM and were considered statistically significant when different from $p < 0.05$.

3.4 Discussion

3.4.1 PNEE causes a long-lasting reduction in LTP in male offspring but does not alter the level of LTP in female offspring

The main finding of this study was that PNEE affected synaptic plasticity in the DG of young adult rats in a sex dependent manner. We found that adult males have reduced LTP following PNEE, which is in agreement with previous studies (Swartzwelder et al., 1988, Sutherland et al., 1997, Richardson et al., 2002, Christie et al., 2005, Titterness and

Christie, 2012, Brady et al., 2013). Conversely, LTP was unchanged in adult females following PNEE. This is the first evidence that LTP in adult females is unaffected following PNEE, indicating that exposure to ethanol *in utero* leads to sex-specific differences in functional plasticity in the hippocampus at an adult age. In a previous study from our laboratory, adolescent ethanol-exposed male rats also showed a reduction in LTP whereas female rats had augmented LTP compared to *ad libitum* females (Titterness and Christie, 2012). The same rat model of ethanol-exposure (i.e. same rat strain, diet composition, ethanol concentration, and exposure period) and similar LTP induction protocols were used in both studies. These results suggest that a developmental change in the ability to elicit LTP must occur in females when they mature from adolescence to adulthood.

It is important to note that just because LTP is not affected in young adult females, this does not imply that females are immune to learning and memory deficits produced by PNEE. For example, some studies have reported that ethanol-exposed females show more deficits in the Morris water maze than their male counterparts (Kelly et al., 1988, Minetti et al., 1996) (see section 3.4.1 and Chapter 7, for a more in-depth discussion), suggesting that other mechanisms besides LTP might account for these female-specific deficits in cognition.

3.4.2 Circulating ovarian-produced steroids were not responsible for the lack of deficits in female animals following PNEE

We performed OVX surgeries on a cohort of *ad libitum* control and ethanol-exposed females prior to sexual maturation to analyze the effects of gonadal hormones on the generation of LTP following PNEE. Because estrogen can have a protective effect on the brain and is known to enhance CA1 synaptic plasticity in slice preparations (Ooishi et al.,

2012), we hypothesized that estrogen might be responsible for the protective effect observed in female animals that were exposed to ethanol during the period of brain development. However, following OVX surgeries, ethanol-exposed females still retained their capacity to elicit LTP similarly to controls. We therefore must reject our hypothesis and assume that circulating female sex steroids are not the primary cause of the resistance to the deleterious effects of ethanol exposure that is observed in the female DG.

While numerous studies indicate that estrogen can enhance LTP *in vitro* and *in vivo* in the CA1, in the DG estradiol may in fact have the opposite effect. In the DG, males exhibit higher levels of LTP in response to TBS stimulation compared to females (Maren et al., 1994). This may be due to potentiation of $GABA_A$ currents by estradiol in this region (Maren et al., 1994). ERs are found in much higher density and are localized to interneurons in the DG (Weiland et al., 1997), whereas in the CA1 they can be found on both interneurons and pyramidal cells. Therefore, while estradiol can only enhance GABA currents in the DG, it can enhance both GABA and glutamate currents in the CA1. This may explain the opposing effects of estradiol in this region.

The inability of OVX to enhance LTP in the DG observed in these studies (Figure 3-2) is not completely understood. This result agrees with those of Maren *et al.,* (1994) who showed that while EPSP-population spike potentiation was enhanced following OVX, LTP was not. It may be that the DG adapts to the decrease in estrogen in the time between OVX and experimentation. This is known to occur in other measures following OVX – For example, while OVX is initially followed by a decrease in proliferation and neurogenesis, approximately one month after OVX, these levels have returned to normal.

Hippocampal neurons are capable of synthesizing estradiol *de novo* (Wehrenberg et al., 2001, Prange-Kiel et al., 2003). Therefore circulating estrogen may not affect synaptic plasticity as robustly as changes in locally synthesized estrogen (Kretz et al., 2004). Indeed, while exogenous application of estrogen is known to enhance LTP (Foy et al., 1999, Ooishi et al., 2012), other studies have shown that reducing circulating estrogen levels through OVX does not reduce or enhance LTP (Day and Good, 2005, Vierk et al., 2012) and it has been indicated that locally synthesized estrogen may play a more prominent role in the maintenance of hippocampal synapses (Kretz et al., 2004, Vierk et al., 2012). This is reflected in our results, as in control and ethanol-exposed animals OVX did not influence the ability to elicit LTP, and locally synthesized estrogen may be responsible for enhancing GABA in the absence of circulating estradiol.

During development in males estradiol levels are high and are responsible for masculinizing the brain. In females, estradiol is bound to α-fetoprotein and does not influence the brain. Ethanol potentiates GABAergic responses (Grant, 1994), so during development, if the male brain is exposed to high levels of ethanol plus estradiol, synergistic GABAergic responses may occur in the DG causing an overexpression of $GABA_A$ receptors or a decrease in excitatory receptors. This increased inhibition could be long-lasting due to the organizational effects of estradiol during this period, and may explain the sex differences in LTP observed in PNEE males only. The sexually dichotic effects we observe in these studies are examined further in Chapter 4, and reasons for these differences are discussed in more detail in Chapter 7.

3.4.3 Conclusions

In this chapter we have determined that PNEE affects the male and female DG differently. In males, LTP is reduced following PNEE, whereas in females no reductions are observed. The lack of effects in females is not due to circulating ovarian steroids and other factors are likely to account for these results.

4. Determining the period of ethanol exposure that renders the developing brain more vulnerable to deficits in LTP

This Chapter is based in part on the following manuscript:

Anna R. Patten, Joana Gil-Mohapel, Patricia S. Brocardo, Athena Noonan, Ryan Wortman and Brian R. Christie (2013). Hippocampal Synaptic Plasticity is Differentially Affected by Exposure to Ethanol during Distinct Periods of Brain Development. *Brain Sciences. (under review).*

4.1 Background

The sensitivity of the CNS to the effects of ethanol varies throughout the perinatal period, with specific cell types being more sensitive during certain stages of development (West, 1987, Brodie and Vernadakis, 1990, Davis et al., 1990, Cartwright and Smith, 1995). Studies from our laboratory have shown that the effects of ethanol on hippocampal oxidative stress are more pronounced when exposure occurred during all three-trimester equivalents (Brocardo et al., 2012) as compared to the 1st and 2nd trimester equivalents (Patten et al., 2012); Chapter 6). This indicates that the 3rd trimester equivalent (which corresponds to the 'brain growth spurt' in humans (Dobbing and Sands, 1979, West, 1987) might be particularly sensitive to the effects of ethanol. However, exposure during the first two trimester equivalents is enough to induce a long-lasting impairment in hippocampal synaptic plasticity (Chapter 3). Furthermore, a recent large-scale clinical study has shown that drinking during the 1st trimester (when many women are not yet aware of their pregnancy) has a strong association with signs of alcohol damage to the fetus (Feldman et al., 2012). On the other hand, a study revealed that during the 2nd trimester, ethanol is cleared from the mother's blood faster than from the amniotic fluid, suggesting that the fluid may act as an ethanol reservoir (Nava-Ocampo et al., 2004).

4.1.1 Objectives of this Chapter

It is of particular relevance to further characterize the differential effects that ethanol exposure may have during the distinct phases of brain development. As such, the objective of this Chapter was to determine which trimester equivalent during the period of brain development was the most susceptible to the effects of ethanol exposure on adult hippocampal synaptic plasticity.

4.2 Animals and methods

To examine the specific periods of vulnerability to ethanol exposure, seven different perinatal treatment groups were used. The ethanol liquid diet and pair-fed liquid diet administered were identical to those described in Chapter 2 (section 2.1.1). However, the period of exposure and the route of administration differed between groups as explained below. Dams were weighed on GDs 1, 7, 14 and 21 as described in Chapter 2 (section 2.1). See Figure 4-1 for an experimental outline.

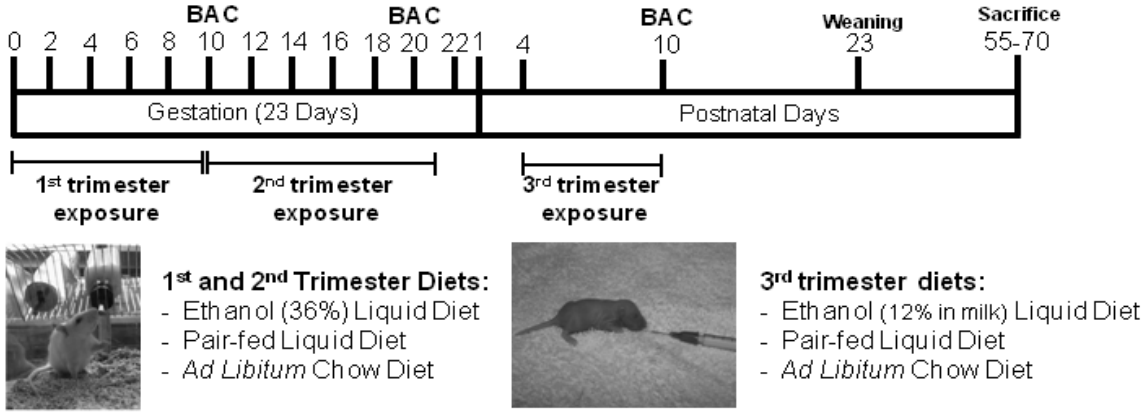

Figure 4-1 Experimental outline for the trimester equivalent experiments.
On GD 1 animals were assigned to one of seven treatment groups: *Ad libitum* controls, 1st trimester ethanol exposure, 1st trimester pair-fed, 2nd trimester ethanol exposure, 2nd trimester pair-fed, 3rd trimester ethanol exposure, or 3rd trimester pair-fed (see section 2.1.1 for a detailed explanation of the various groups). Blood samples to assess BAC were taken on GD 10 for the 1st trimester exposure condition, GD 20 for the 2nd trimester exposure condition, and PND 10 for the 3rd trimester exposure condition. When animals reached experimental age (PNDs 55 – 70) they were used for *in vivo* electrophysiological experiments to examine LTP in the DG of the hippocampus. **Abbreviations:** Blood alcohol concentration (BAC); Dentate gyrus (DG); Gestational day (GD); Long-term potentiation (LTP); Postnatal day (PND).

4.2.1 Perinatal Diets

On GD 1 pregnant dams were randomly assigned to one of seven groups:

1st or 2nd trimester equivalent ethanol exposure – *Ad libitum* access to a liquid diet containing 35.5% ethanol derived calories during either the 1st trimester equivalent (GDs 1 - 10) or 2nd trimester equivalent (GDs 11 – 21). Ethanol dams were gradually introduced to the liquid diet over a three-day period (GDs 1 - 3 or GDs 11 - 13). On GD1 or GD 11, one third of the ethanol diet was combined with two thirds of the pair-fed diet (see below), on GD2 or GD 12, two thirds of the ethanol diet was combined with one third of the pair-fed diet and on GD 3 or GD 13, 100% of the ethanol diet was supplied to the dam. Dams exposed to ethanol during the 1st trimester equivalent received regular chow (Lab Diets 5001, LabDiets, Richmond, IN, USA) from GD 11 onwards. Dams exposed to the ethanol liquid diet during the 2nd trimester consumed regular chow from GDs 1 – 10 and were then were switched to a regular chow diet on the final day of pregnancy (GD 22).

3rd trimester ethanol exposure - Pregnant females were left undisturbed throughout gestation. Between PNDs 4 – 10 (3rd trimester equivalent) pups received a dose of 4 g/kg/day of 12% (v/v) ethanol in milk solution. Ethanol was dissolved in a nutritional milk solution similar in composition to rat milk (West et al., 1984) and supplemented with a specially formulated vitamin mix (Bio-Serv; Frenchtown, NJ, USA). Solutions were administered by intragastric intubation as previously described by our laboratory (Boehme et al., 2011, Gil-Mohapel et al., 2011, Brocardo et al., 2012). The solution was administered in two separate intubations 2 h apart. An additional feeding of pure milk solution was supplied to ethanol-exposed pups in the evening to counteract the

inadequate nutrition, low birth weight and high mortality rate of ethanol-exposed pups (Boehme et al., 2011, Gil-Mohapel et al., 2011).

1st or 2nd trimester pair-fed diet – The pair-fed groups received a liquid diet with maltose-dextrin isocalorically substituted for the ethanol derived calories. This liquid diet was not provided *ad libitum*. To control for stress and malnutrition, the pair-fed animals received the same amount of food in g/kg/day as their matched ethanol dams. As above, dams in the 1st trimester equivalent exposure group were given the pair-fed diet between GDs 1 - 10. On GD 11, animals were placed back on a regular chow diet for the remainder of the pregnancy. Dams in the 2nd trimester equivalent exposure group received regular rat chow from GDs 1 – 10 and were switched to the pair-fed diet between GDs 11 – 21. On GD 22 the dams were switched back to the chow diet.

3rd trimester pair-fed diet – As above, pregnant females were left undisturbed throughout gestation. Between PNDs 4 – 10 (3rd trimester equivalent) pups received a dose of an iso-caloric and iso-volumic maltose-dextrin milk solution. Maltose-dextrin was dissolved in a nutritional milk solution as described above. The solution was administered in two separate intubations 2 h apart. The pair-fed pups were sham-intubated during the third feeding, as extra feeding could cause an excess weight gain in these animals (see review by (Gil-Mohapel et al., 2010).

***Ad libitum* control** – animals had *ad libitum* access to a regular chow diet (Lab Diets 5001, LabDiets, Richmond, IN, USA) throughout pregnancy. Pups were left undisturbed during the third-trimester equivalent.

Animals were weaned at PND 23 and *in vivo* electrophysiology was performed on the DG when they reached experimental age (PNDs 55 - 70) as described in section 2.3.

4.2.2 Blood samples to assess BAC

To assess BACs in the ethanol-exposed animals, a single tail blood sample was obtained on GD 10 (for 1st trimester equivalent exposure) or GD 20 (for 2nd trimester equivalent exposure), approximately two hours after the beginning of the dark phase. For animals that were exposed to ethanol during the 3rd trimester equivalent, a tail blood sample was obtained from the pups on PND10, approximately two hours after the last dose of ethanol diet of the day.

4.2.3 Statistical analysis

All data are presented as means ± S.E.M. A one-way ANOVA was used to determine the effect of perinatal condition on weight gain across pregnancy and litter size. A repeated measures ANOVA for perinatal condition (Ethanol-1, Ethanol-2, Ethanol-3, Pair-fed-1, Pair-fed-2, Pair-fed-3 or *Ad libitum* control) was used to analyze pup weights taken on PNDs 2, 6, 10, 14, 18 and 22. A two-way ANOVA for sex (male or female) and perinatal condition (Ethanol-1, Ethanol-2, Ethanol-3, Pair-fed-1, Pair-fed-2, Pair-fed-3 or *Ad libitum* control) was used to analyze weights at experimental age (PNDs 55 - 70). A significant main effect of sex was obtained at this age [$F(1, 101) = 1481.6$, $p < 0.0001$], with males weighing significantly more than females ($p < 0.001$). Therefore, male and female data were subsequently analysed separately using a one-way ANOVA.

A one-way ANOVA was used to determine the effect of perinatal condition (Ethanol-1, Ethanol-2, Ethanol-3, Pair-fed-1, Pair-fed-2, Pair-fed-3 or *Ad libitum* control) on LTP of either males or females. Post-hoc analyses were conducted using Tukey's test. A p value < 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Developmental data

Weight data was taken from the dams on GDs 1, 7, 14 and 21. The percentage weight gain over the course of pregnancy did not differ between perinatal conditions [$F(6, 21) = 1.7$, $p = 0.17$]. However, there was a significant effect of perinatal condition on litter size [$F(6, 21) = 2.8$, $p = 0.04$], with 1st trimester pair-fed animals having increased litter size compared to 1st trimester ethanol exposed animals ($p < 0.05$). These data are summarized in Table 4-1.

Table 4-1 Gestational data for the trimester equivalent study.
There were no significant differences in weight gain over pregnancy between perinatal conditions. 1st trimester pair-fed animals had significantly more pups than 1st trimester ethanol exposed animals, but all other groups had comparable litter sizes. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. * = $p < 0.05$, as compared to 1st trimester ethanol exposed dams.

	% weight gain over pregnancy	Number of pups per litter
Ad libitum	61.6 ± 11.3	13.7 ± 2.3
Ethanol 1st	74.4 ± 3.8	12.8 ± 1.3
Pair-fed 1st	66.3 ± 9.3	18 ± 1.7*
Ethanol 2nd	51.1 ± 3.2	15.3 ± 0.5
Pair-fed 2nd	63.1 ± 4.3	13.0 ± 0.6
Ethanol 3rd	65.4 ± 4.3	14.0 ± 0.8
Pair-fed 3rd	69.7 ± 5.2	15.6 ± 0.2

Offspring weight was determined during the lactation period on PNDs 2, 6, 10, 14, 18 and 22 to determine whether perinatal condition altered offspring weight gain. A repeated measures ANOVA revealed that there was no significant effect of sex [$F(6, 37) = 2.0$, $p = 0.99$], and therefore, data from both males and females were combined and a repeated measures ANOVA for perinatal condition (*ad libitum*, pair-fed1, pair-fed2, pair-fed3, ethanol1, ethanol2, ethanol3) revealed a significant effect of perinatal condition [F(36,

196) = 3.0, $p < 0.0001$]. Further post-hoc analysis revealed that at PND 2, 1st trimester pair-fed animals weighed significantly less than *ad libitum* ($p < 0.05$) and 1st trimester ethanol-exposed animals ($p < 0.001$). There were no significant differences in weight between PNDs 6 - 14 for all conditions. At PND 18, 3rd trimester pair-fed animals weighed significantly more than 1st trimester pair-fed animals ($p < 0.05$) and 2nd trimester pair-fed animals ($p < 0.01$). This difference remained at PND 22, with 3rd trimester pair-fed animals still weighing significantly more than 1st trimester pair-fed animals ($p < 0.001$) and 2nd trimester pair-fed animals ($p < 0.01$). These data are summarized in Table 4-2.

Table 4-2 Developmental data for animals used in the trimester equivalent study.
A repeated measures ANOVA revealed that there was no significant effect of sex and therefore data from both males and females were combined. A significant main effect of perinatal condition was observed (see text for statistical details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. * = $p < 0.05$ compared to *ad libitum* controls and 1st trimester ethanol exposed animals (PND 2). # = $p < 0.05$ compared to 3rd trimester pair-fed animals (PND 18). $ = $p < 0.01$ compared to 3rd trimester pair-fed animals (PND 22).

	Ad libitum	**Ethanol-exposed**			**Pair-fed**		
	Weight (g)	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**
PND2	8.0 ± 0.3	8.3 ± 0.3	7.5 ± 0.2	8.1 ± 0.2	6.9 ± 0.4*	7.8 ± 0.2	7.7 ± 0.2
PND5	15.0 ± 0.5	14.6 ± 0.4	14.5 ± 0.4	14.9 ± 0.4	13.6 ± 0.4	14.4 ± 0.4	14.6 ± 0.4
PND10	23.6 ± 0.8	23.3 ± 0.6	23.2 ± 0.7	24.0 ± 0.7	22.2 ± 0.7	23.0 ± 0.7	24.8 ± 0.6
PND14	33.3 ± 1.1	32.8 ± 0.9	33.4 ± 0.9	34.2 ± 1.0	32.1 ± 0.9	32.0 ± 0.9	35.8 ± 0.9
PND18	43.7 ± 1.4	41.8 ± 1.2	43.2 ± 1.2	45.1 ± 1.5	41.2 ± 1.2#	41.0 ± 1.2#	47.2 ± 1.3
PND22	60.4 ± 1.9	60.4 ± 1.7	59.8 ± 1.7	63.3 ± 1.9	54.7 ± 1.7$	59.1 ± 1.7$	67.3 ± 1.7

Weight at experimental age (PNDs 55 – 70) was analyzed separately in males and females. Nevertheless, there was no significant main effect of perinatal condition on body weight at experimental age both in males [F(6, 50) = 2.05, $p = 0.077$] and females [F(6, 51) = 1.07, $p = 0.39$]. These data are summarized in Table 4-3.

Table 4-3 Weight data for experimental animals used in the trimester equivalent study.
In adulthood, males weigh significantly more than females and therefore their weights were analyzed separately. There were no significant effects of perinatal condition on body weight in either males or females (see text for details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$.

	Weight (g)	Male	Female
	Ad libitum	413.1 ± 6.2	271.5 ± 14.3
Ethanol-exposed	**1st**	417.5 ± 5.2	281.1 ± 11.4
	2nd	400.0 ± 7.0	267.0 ± 6.1
	3rd	408.4 ± 4.7	257.1 ± 5.4
Pair-fed	**1st**	413.0 ± 1.6	263.1 ± 3.0
	2nd	413.2 ± 3.4	269.7 ± 4.0
	3rd	421.6 ± 4.1	272.1 ± 5.7

4.3.2 Intoxication levels

Peak BAC levels were measured from blood taken two hours after the dark cycle commenced on GD 10 for 1st trimester equivalent exposure and GD 20 for 2nd trimester equivalent exposure. Blood taken 2 hours following the last feeding of the ethanol diet on PND 10 was used to measure peak BAC levels for 3rd trimester equivalent exposure. The BACs for 1st, 2nd and 3rd trimester equivalent exposed animals were 91.6 mg/dl, 94.3 mg/dl, and 255.1 mg/dl, respectively. These results are in agreement with the literature, with the gavage model (3rd trimester equivalent) producing considerably higher BACs than the prenatal liquid diet exposure model (Boehme et al., 2011, Gil-Mohapel et al., 2011, Brocardo et al., 2012); for review see Gil-Mohapel et al 2010).

4.3.3 LTP is affected differently by both sex and period of exposure

In Chapter 3 we established that ethanol-exposed males have a reduction in LTP in the DG during early adulthood, whereas female animals do not show such deficits. This study aimed to determine whether LTP is affected differently if exposure to ethanol occurs only during a specific trimester equivalent. In males, a one-way ANOVA for

perinatal diet (control, pair-fed1, pair-fed2, pair-fed3, ethanol1, ethanol2, ethanol3) showed no significant differences between perinatal diet groups [$F(6, 53) = 0.601$, $p = 0.728$; Figure 4-2].

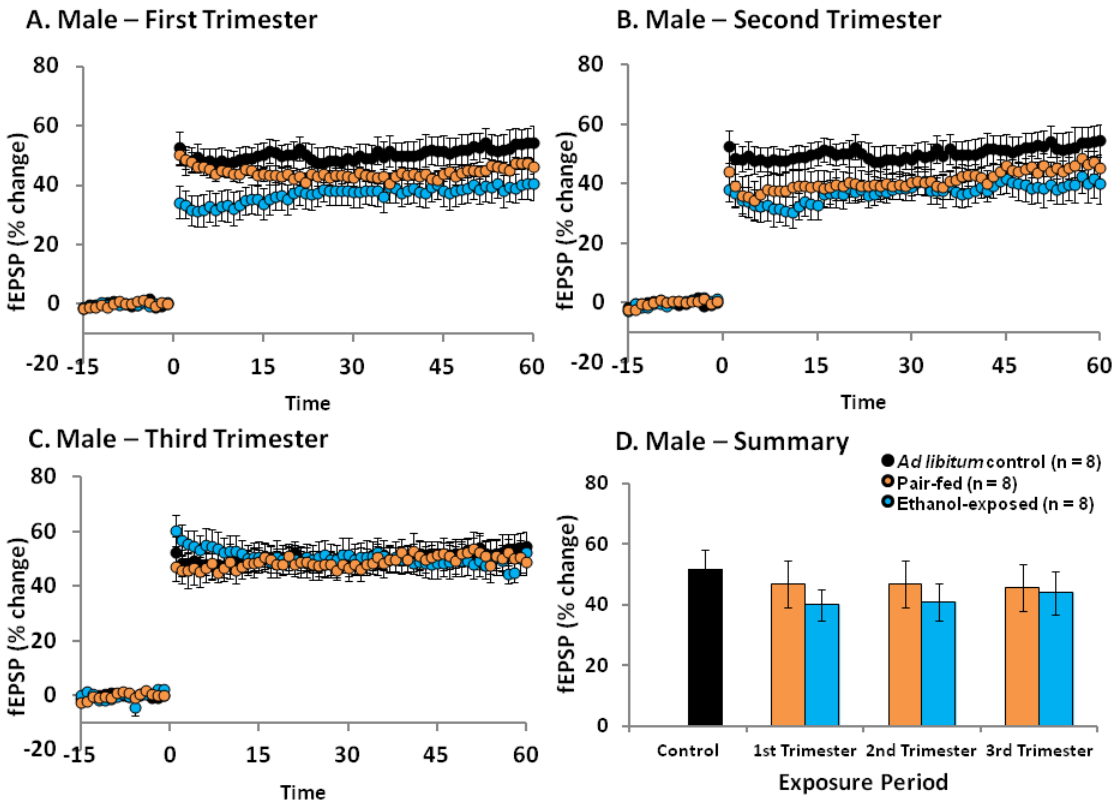

Figure 4-2 The effects of PNEE during specific trimester equivalents on LTP in the DG of adult male rats.
A) 1st trimester equivalent exposure. PNEE during the 1st trimester equivalent results in a trend towards a reduction in LTP, but this result was not statistically significant. B) 2nd trimester equivalent exposure. PNEE during the 2nd trimester equivalent also results in a trend towards a reduction in LTP levels compared to controls, but again, this result was not statistically significant. C) 3rd trimester equivalent exposure. There are no significant differences between control, ethanol-exposed and pair-fed animals. D) Summary of LTP results. Results are presented as means ± SEM and were considered statistically significant when $p < 0.05$.

In females, a one-way ANOVA for perinatal diet (control, pair-fed1, pair-fed2, pair-fed3, ethanol1, ethanol2 or ethanol3) showed no significant differences between groups [$F(6, 45) = 1.45$, $p = 0.22$] (Figure 4-3). Interestingly, if the results for each trimester

equivalent are individually analyzed, a significant increase in LTP is revealed in the 3rd trimester exposure group [$F(2,23) = 5.84$, $p < 0.01$]. Post-hoc analyses further shows that ethanol exposed females have increased LTP when compared to control ($p < 0.05$) and pair-fed ($p < 0.05$) animals.

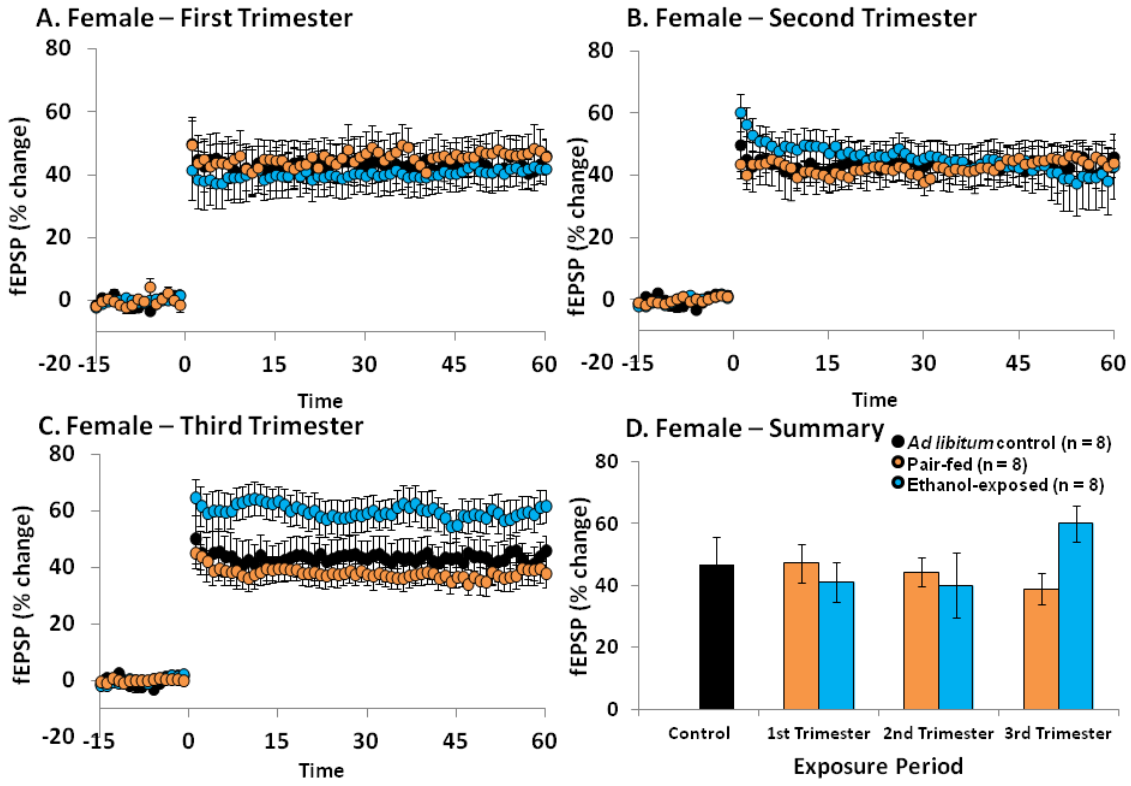

Figure 4-3 The effects of PNEE during specific trimester equivalents on LTP in the DG of adult female rats.

A) 1st trimester equivalent exposure. PNEE did not reduce LTP below control levels. B) 2nd trimester equivalent exposure. PNEE did not reduce LTP below control levels. C) 3rd trimester equivalent exposure. PNEE during the 3rd trimester equivalent results in a trend towards an increase in LTP, however this result was not statistically significant (one-way ANOVA). When each trimester was analyzed separately, a significant main effect of perinatal treatment was revealed during the 3rd trimester equivalent, with post-hoc analysis showing that 3rd trimester equivalent PNEE increased LTP in females when compared to their respective *ad libitum* ($p < 0.01$) and pair-fed ($p < 0.01$) controls. D) Summary of LTP results. Results are presented as means ± SEM and were considered statistically significant when $p < 0.05$.

4.4 Discussion

4.4.1 The DG displays temporal windows of vulnerability to ethanol during development

In this study we examined which periods during brain development are more susceptible to ethanol exposure with regards to the ability to elicit LTP. As discussed in Chapter 3, males show deficits in LTP following PNEE during gestation (1st and 2nd trimester equivalents). Not surprisingly, when alcohol exposure occurred during either the 1st, 2nd or 3rd trimester equivalent, LTP was not affected as severely. In fact, in all three exposure groups, there was no significant difference in the ability to elicit LTP when compared to control animals. However, we observed a trend towards a decrease in LTP both in the 1st and 2nd trimester equivalent exposure groups (Figure 4-2A and 4-2B). This is in contrast to the 3rd trimester equivalent exposure group, when no differences in LTP levels were detected (Figure 4-2C). These results indicate that ethanol exposure during the 1st or 2nd trimester equivalents may be more detrimental for LTP than exposure during the 3rd trimester equivalent. This is in agreement with a 1990 study conducted by Tan *et al.* who showed that when alcohol exposure (through a liquid diet with either 35% or 17.5% ethanol) occurred between GDs 8 - 22 (i.e., late 1st trimester – 2nd trimester exposure) no changes in LTP were observed in CA1 slices from PND 90 animals (Tan et al., 1990). Nevertheless, we did observe a reduction in DG LTP in a subset of males that were exposed to ethanol during either the 1st or the 2nd trimester equivalent (data not shown), suggesting that the male DG might indeed be more vulnerable to the long-term effects of ethanol during these prenatal time windows. This is in contrast to the 3rd trimester exposure group, where LTP levels are identical among all three perinatal conditions (Figure 4-2). These results are in line with previous studies that have shown

that ethanol exposure during the 3rd trimester equivalent does not affect synaptic plasticity in the CA1 (Bellinger et al., 1999, Byrnes et al., 2004) and that ethanol exposure only during the 1st, 2nd or 3rd trimester equivalent does not result in deficits in spatial navigation (Cronise et al., 2001, Byrnes et al., 2004). However, the reasons for the variable susceptibility to ethanol among males that were exposed to ethanol during either the 1st or the 2nd trimester equivalent alone in our studies are not clear. Of note, in rats the 3rd trimester equivalent occurs postnatally (during PNDs 1-10) (Dobbing and Sands, 1979, West, 1987). Thus, the mode of ethanol administration employed for the 1st and 2nd trimester equivalent groups (i.e., voluntary consumption of a liquid diet containing ethanol by the pregnant dam) was different that that used for the 3rd trimester equivalent group (i.e., intragastric intubation of the offspring with a fixed amount of ethanol). Both models have their own advantages and disadvantages (reviewed by (Gil-Mohapel et al., 2010)). Thus, while the liquid diet model usually produces BACs that more closely reflect those obtained with moderate drinking (i.e., more representative of the vast majority of alcohol consumption during pregnancy in the human population (Ethen et al., 2009) it is also associated with an increased variability when compared with the gavage model. For example, the time of exposure, the daily period of exposure, or the time during the day when the highest BAC is reached may differ dramatically among pregnant dams that have free access to an ethanol liquid diet and these factors may differentially affect their offspring. Thus, it is not surprising that higher group variability was observed in the 1st and 2nd trimester equivalent groups, making differences between groups difficult to discern. Future studies utilizing the gavage model for all periods of exposure may

provide a better understanding of how LTP is affected during each trimester equivalent alone.

Precursor cells of the DG are formed during the 1st and 2nd trimester (Andersen, 2006a), and if these are damaged, then the granule cell layer may be deformed possibly leading to the slight decrease in LTP observed following ethanol exposure during these time periods (Figure 4-2). In the 3rd trimester, granule cells of the DG have differentiated and migration and synapse formation are occurring (Steward and Falk, 1991, Andersen, 2006a). If ethanol exposure occurs during this period, cell proliferation and differentiation have already occurred and therefore will not be affected. Furthermore, ethanol may not be able to interfere with granule cell migration and synapse formation, thus explaining why no differences in DG LTP were observed in males that were exposed to ethanol exclusively during the 3rd trimester equivalent.

PNEE females show increased LTP at adolescence (Titterness and Christie, 2012) and no differences in LTP in adulthood (Patten, 2013, Sickmann, 2013). It is therefore not surprising that PNEE during either the 1st or the 2nd trimester equivalent alone did not impact LTP in the adult female DG (Figure 4-3). However, when PNEE occurred during the 3rd trimester equivalent alone, a significant increase in DG LTP was detected when compared to *ad libitum* control and pair-fed animals (Figure 4-3). This increase is similar to that observed in the DG of adolescent females that were exposed to ethanol throughout gestation (i.e., 1st and 2nd trimester equivalents combined (Titterness and Christie, 2012). Reasons for this increase are currently unknown but may be related to a dysregulation of estrogen levels with PNEE. Indeed, PNEE females exhibit an increased hypothalamic-pituitary-adrenal (HPA) sensitivity to estrogen, and estrogen levels are higher during

proestrous in PNEE females compared to controls (Lan et al., 2009). Why this enhancement is LTP is only observed after ethanol exposure during the 3rd trimester is currently unknown, but may be related to the fact that estrogen does not begin to be produced in the ovaries until PND5 (i.e., during the 3rd trimester equivalent) (Weniger et al., 1993). Perhaps the most striking result is that by examining ethanol exposure during distinct periods of brain development we have uncovered a defined time window during which exposure to ethanol results in enhanced LTP in the adult female DG.

Of note, while increased LTP may appear beneficial for learning and memory processes, this is not always the case. In fact, impairments in spatial performance have been accompanied by an increase in LTP in the CA1 (Vaillend et al., 2004). Moreover, deficits in learning and memory are common in PNEE females, particularly following 3rd trimester exposure (Kelly et al., 1988, Goodlett and Peterson, 1995, Popovic et al., 2006). However, in agreement with our results, previous studies have also shown that when ethanol exposure occurs between PNDs 7 – 9, males are more vulnerable than females with regards to learning deficits as assessed by the Morris water maze test (Goodlett and Peterson, 1995). Interestingly, if ethanol exposure occurs between PNDs 4 – 9 then females do exhibit behavioural deficits similar to those of males (Kelly et al., 1988, Goodlett and Peterson, 1995).

Interestingly, higher BACs did not correlate with greater deficits in LTP. In fact, the highest BACs were actually associated with the least detrimental effects in males and resulted in increased LTP in females. This is surprising given that higher BACs are generally associated with increased apoptosis (Ikonomidou et al., 2000), although this effect is not specific to the DG.

4.4.2 Conclusions

The results from this study indicate that exposure to ethanol during restricted periods of brain development is not as detrimental for DG LTP later in adulthood, as a more prolonged ethanol exposure that occurs across multiple trimester equivalents of brain development. As one might expect, when ethanol-exposure only occurs during one of the trimester equivalents, less robust effects on LTP are observed. However, some interesting periods of vulnerability were revealed. In males, ethanol exposure during either the 1st or 2nd trimester equivalents caused a non-significant decrease in LTP, whereas ethanol exposure during the third trimester equivalent alone (when BAC levels were the highest) did not result in significant changes of LTP levels. In females, ethanol exposure during either the first or second trimester equivalents did not impact LTP in adulthood, but following exposure during the third trimester equivalent alone, LTP was increased in the DG. These results further exemplify the disparate effects between the ability to elicit LTP in the male and female brain following PNEE and indicate that no trimester appears to be safe to consume alcohol.

5. The role of GSH in LTP following PNEE

This Chapter is based in part on the following manuscript:

Anna R. Patten, Joana Gil-Mohapel, Patricia S. Brocardo, Claire Sakiyama, Ryan Wortman, Athena Noonan and Brian R. Christie (2013). Impairments in Hippocampal Synaptic Plasticity Following Prenatal Ethanol Exposure are Dependent on Glutathione Levels. *Hippocampus. (submitted).*

5.1 Background

Oxidative stress can decrease LTP (Pellmar et al., 1991, Auerbach and Segal, 1997, Serrano and Klann, 2004). On the other hand, oxidative stress can decrease GSH levels, and GSH depletion has been shown to result in a reduction in LTP (Almaguer-Melian et al., 2000, Steullet et al., 2006), whereas an increase in the levels of this antioxidant results in enhanced CA1 LTP in slices from aged animals (Bodhinathan et al., 2010, Robillard et al., 2011). The relationship between GSH and LTP is probably due to the ability of GSH to affect the redox state of the NMDA receptor (Aizenman et al., 1989, Tang and Aizenman, 1993, Ogita et al., 1995, Janaky et al., 1999). GSH can interact with the NMDA receptor via its gamma-glutamyl moiety (Janaky et al., 1999). It seems that GSH can act as an agonist and an antagonist at the receptor, thus mediating several neuronal responses including LTP. Indeed, while GSH can displace excitatory agonists from the NMDA receptor, it can also modulate its redox state through its cysteinyl moiety leading to an increase in its activity (Janaky et al., 1999). The redox state of the NMDA receptor is an important determinant of its function (Aizenman et al., 1989, Aizenman et al., 1990, Choi and Lipton, 2000, Choi et al., 2001). Bodhinathan *et al.*, (2010) determined that age-related decreases in NMDA receptor function are due to an increase in receptor oxidation and that applying a reductant to hippocampal slices from

aged animals while recording from CA1 neurons caused a significant increase in activity, an effect that was mimicked by intracellular GSH application. The same effect was not observed in tissue from young adult animals, because at this age the receptor was probably still in a highly reduced state. In contrast, when an oxidant was added to hippocampal slices of young adult animals, NMDA receptor activity significantly declined, an effect that was not observed in aged tissue (Bodhinathan et al., 2010). These results further illustrate the importance of redox state in NMDA receptor function, and hence LTP induction. How ethanol exposure during the period of brain development affects the relationship between GSH and LTP will be the focus of this chapter.

As discussed in Chapter 1 (section 1.1.4) the mechanism(s) by which PNEE causes deleterious changes in the developing brain have not been fully elucidated, but *in vivo* and *in vitro* animal studies suggest that oxidative stress and decreased antioxidant protection are likely to be involved (Reyes et al., 1993, Henderson et al., 1995, Ramachandran et al., 2001, Heaton et al., 2002, Ramachandran et al., 2003, Siler-Marsiglio et al., 2005, Dembele et al., 2006, Lee et al., 2009, Brocardo et al., 2012), for review see (Brocardo et al., 2011). Progressive oxidative damage may be linked to deficits in cognitive function and synaptic plasticity such as those that are prevalent in FASD (Streissguth et al., 1991, Kerns et al., 1997, Sutherland et al., 1997, Christie et al., 2005); Chapter 3).

In healthy cells, antioxidants and free radical scavengers neutralize ROS/RNS and prevent cellular damage. If this process is impaired by ethanol, cells can be overcome by ROS/RNS, resulting in cell damage and eventually death (Goodlett et al., 2005). Of particular importance for FASD is the fact that levels of antioxidants are considerably

lower in the developing fetus than they are in the mature brain (Henderson et al., 1999), making developing neurons more susceptible to the deleterious effects of oxidative stress (Henderson et al., 1999). The enzymes responsible for GSH metabolism and recycling may also be disrupted by ethanol exposure (Reyes et al., 1989a), which would influence the basal levels of GSH in neurons. In addition, early ethanol exposure during development can also produce a long-lasting impairment in the capacity of neurons to handle ROS/RNS (Dembele et al., 2006, Brocardo et al., 2012).

5.1.2 Objectives of this Chapter

In this chapter the mechanism responsible for the long-term deficits in LTP in male animals is explored. Since PNEE is known to increase oxidative stress and reduce antioxidant capacity (Dembele et al., 2006, Brocardo et al., 2012, Patten et al., 2012); reviewed by Brocardo et al 2011), the role of GSH is examined (animals in this study are designated cohort 1). Furthermore, to study the interaction between GSH and LTP, we performed loss of function/gain of function experiments, where we either depleted GSH levels in control animals, or enhanced GSH levels in PNEE animals. While acute GSH depletion has been previously shown to cause a reduction in LTP in male animals (Almaguer-Melian et al., 2000, Steullet et al., 2006), it has never been explored in females. To deplete GSH to approximately 40% of control levels, diethyl maleate (**DEM**) was administered to control animals (animals in this study are designated cohort 2). DEM treatment results in the formation of GSH-DEM adducts, thus depleting the endogenous levels of this antioxidant with maximum depletion occurring approximately two hours after injection (Plummer et al., 1981, Weber et al., 1990, Mirkovic et al., 1997). To increase GSH levels in the brain we supplemented animals (animals in this study are

designated cohort 3) with N-acetyl cysteine (**NAC**), the most bioavailable precursor of GSH (Kerksick and Willoughby, 2005, Atkuri et al., 2007). Recent studies have shown that increasing GSH levels through oral supplementation with NAC, is enough to completely restore the deficits in hippocampal synaptic plasticity observed in aged mice (Robillard et al., 2011). Of note, GSH is hydrolysed by dipeptidases in the stomach and subsequently excreted, while intravenously administered GSH only has a half life of seven minutes, being rapidly excreted (Aebi et al., 1991). Furthermore, it is generally accepted that GSH cannot readily cross the BBB in its reduced form (Zeevalk et al., 2007). Therefore, the most common agent used to enhance the bioavailability of GSH in the brain is NAC, which acts as a precursor to GSH synthesis by providing cysteine and activating the GSH cycle (Dringen and Hamprecht, 1999).

5.2 Animals and methods

Ad libitum control, pair-fed and ethanol-exposed animals were generated as described in section 2.1. Two males and two females from each litter were used for GSH determination as described below (cohort 1). The remaining offspring in each litter were used for GSH supplementation studies described below (cohort 3). Cohort 2 are the animals used in GSH depletion studies that were obtained as adults (see below).

5.2.1 Tissue preparation for GSH-t analysis

On PND 60 animals in cohort 1 were anaesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA), quickly decapitated, and the brains were dissected from the skull and placed in ice-cold tris-buffered saline (0.1M, pH = 7.4). The DG sub-region of the hippocampi were crudely microdissected following the procedure described in Chapter 2 (section 2.4).

To determine GSH-t levels, samples were homogenized in cooled 0.5M perchloric acid. The homogenates were centrifuged at 15,000 *g* for two minutes and the supernatant was separated and neutralised in potassium phosphate buffer (PBS, 0.1M, pH = 7.4). The samples were immediately processed as described below.

5.2.2 Determination of GSH-t levels

GSH-t, comprising both of its reduced (GSH, which normally constitutes more than 95% of total glutathione) and oxidized (GSSG) forms, was measured by the reaction of the 5,5'-dithiobis(2-nitrobenzoic acid) (**DTNB**) GR recycling assay (Tietze, 1969). The sulfhydryl group of GSH reacts with DTNB and produces a yellow coloured product, 5-thio-2-nitrobenzoic acid (**TNB**). GS-TNB, the mixed disulfide formed between GSH and TNB, is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH measured at 412 nm. GSH-t was expressed in µmol/ g wet tissue (Figure 5-1).

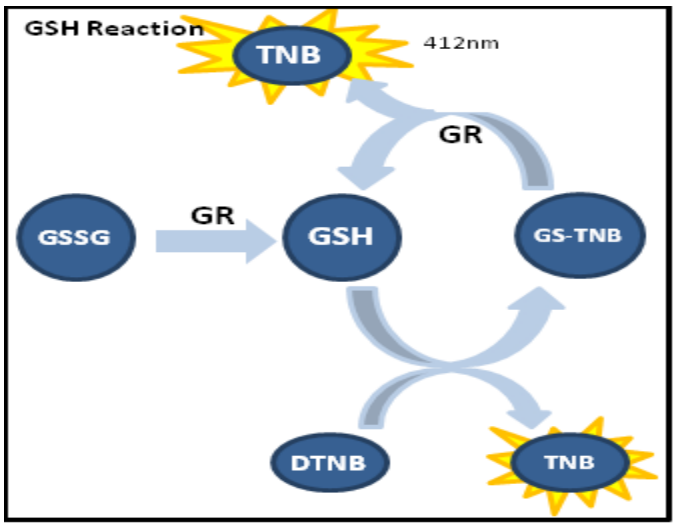

Figure 5-1 GSH-t assay reaction.
The sulfhydryl group of GSH reacts with DTNB and produces a yellow coloured product, 5-thio-2-nitrobenzoic acid (TNB). GS-TNB, the mixed disulfide formed between GSH and TNB, is

reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH measured at 412 nm.

5.2.3 Glutathione depletion

Juvenile (PND 45) male and female Sprague-Dawley rats were obtained from Charles River Laboratories (Quebec, Canada) and left undisturbed until they reached experimental age (PNDs 55 - 70). Eight females and eight males (cohort 2) received an i.p. injection of DEM (1000 mg/kg, i.e. 6 mM/Kg in corn oil) on the day of experimentation. This dose was chosen based on previous experiments as it reduces brain GSH-t levels to 40-60% of control levels (Almaguer-Melian et al., 2000). Two hours after receiving DEM, animals were used to evaluate hippocampal synaptic efficacy by *in vivo* electrophysiological techniques, as described in section 2.3. Once *in vivo* electrophysiological recordings were completed, animals were quickly decapitated and the DG hippocampal sub-region was dissected as described in section 2.4. In order to ensure that DEM induced GSH depletion in the hippocampus, samples were subsequently used to determine GSH-t (Tietze, 1969, Anderson, 1985), as described in section 5.2.2. DEM-treated animals were compared to *ad libitum* controls and ethanol-exposed offspring from Chapter 3.

5.2.4 NAC supplementation

The offspring in cohort 3 was left undisturbed until weaning (PND 23) at which point dietary supplementation with NAC began (Figure 5-2). Two males and two females from each litter received a daily dose of NAC (750 mg/Kg, administered orally via the drinking water). This dose was chosen based on previous reports (Jayalakshmi et al., 2007, Robillard et al., 2011). The water bottles were weighed every three days to track consumption and the concentration of NAC added to the water was based on the daily

water consumption curve calculated during a pilot study (data not shown). Animals received NAC supplementation until they reached experimental age (PNDs 55 – 70). At this point, animals were used to analyze LTP and *in vivo* electrophysiological recordings were obtained as described in section 2.3. Two males and two females from each litter who were not supplemented with NAC were used as controls in these experiments. Following electrophysiological experiments, the animals were rapidly decapitated and their brains were removed. The hemisphere where the electrophysiology electrodes were inserted was disposed of. The contralateral hemisphere was further dissected (as in section 2.4) and the hippocampal DG processed for analysis of GSH-t levels as described in section 5.2.2.

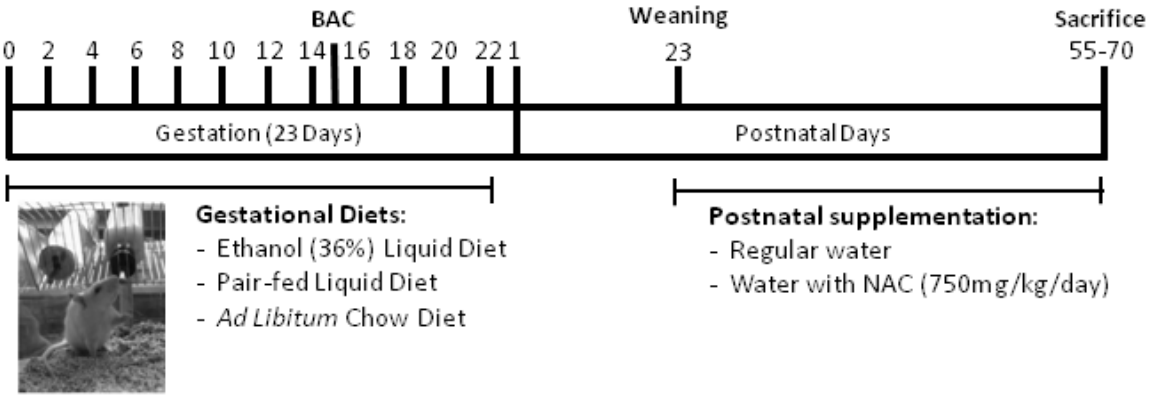

Figure 5-2 Experimental outline for the NAC supplementation experiments.
On GD 1 animals were assigned to one of three prenatal diets (Ethanol, Pair-fed or *ad libitum* control). On GD 15 a blood sample was taken to assess BAC. Pups were born on GD 23 (PND 1) and were weaned from their mother at PND 23. Upon weaning, two males and two females from each litter were given NAC supplementation. NAC was administered in the drinking water at a dose of 750 mg/kg/day. All other animals in the litter had *ad libitum* access to regular drinking water. Animals were used for *in vivo* electrophysiology when they reached young adulthood (PNDs 55 – 70). **Abbreviations:** Blood alcohol concentration (BAC); Gestational day (GD); N-acetylcysteine (NAC); postnatal day (PND).

5.2.5 Statistical analysis

A one-way ANOVA was used to determine the effect of prenatal condition on weight gain across pregnancy and litter size (cohort 1 and 3). A repeated measures ANOVA for

prenatal condition (*ad libitum*, pair-fed and ethanol-exposed) and sex was used to analyze pup weights taken between PNDs 2 – 22 for cohort 1 and 3. No significant sex differences in weight occurred at these time points [$F(4, 27) = 1.4$, $p = 0.26$] so the sexes were combined. Animals in cohort 1 were used at PND 60. At this age a sex difference between male and female body weights exists [$F(1, 58) = 679.0$, $p = 0.001$], with males being significantly heavier than their female counterparts ($p < 0.001$). Therefore, weight data from PND 60 males and females were analyzed separately. For animals in cohort 3, a three-way ANOVA for prenatal condition (*ad libitum*, pair-fed and ethanol-exposed), sex (male and female), and postnatal supplementation (NAC and regular water) was used to analyze differences in the weight of the offspring when these reached experimental age. A main effect of sex was observed [$F(1, 126) = 1851.6$, $p < 0.0001$], with males weighing significantly more than their female counterparts ($p < 0.001$). Thus, data from males and females were analyzed separately using a two-way ANOVA for prenatal condition and postnatal supplementation.

A two-way ANOVA for prenatal condition (*ad libitum*, pair-fed or ethanol-exposed) and sex was initially used to analyze GSH-t levels in the DG on animals from cohort 1. As no significant effect of sex was detected [$F(1, 24) = 0.002$, $p = 0.99$] data from males and females were combined and a one-way ANOVA for prenatal condition was performed. Similarly, for animals in cohort 2 (i.e. DEM-induced GSH depletion experiment), a two-way ANOVA for condition (*ad libitum*, DEM-treated or ethanol exposed) and sex revealed that there was no significant main effect of sex [$F(1, 43) = 1.15$, $p = 0.29$], so male and female data were combined and a one-way ANOVA for condition was performed. Finally, for animals in cohort 3 (i.e. NAC supplementation

experiment), a three-way ANOVA for prenatal condition, sex and postnatal supplementation was conducted. There was no significant main effect of sex [$F(1, 47) = 0.25$, $p = 0.62$], and therefore data from both sexes were combined and a two-way ANOVA was conducted.

Electrophysiological results from male and female subjects from cohort 2 were analyzed with a one-way ANOVA to assess the effect of prenatal condition (*ad libitum*, DEM-treated or ethanol-exposed). For cohort 3, LTP results were initially analyzed with a three-way ANOVA for prenatal condition, sex, and postnatal supplementation. A significant main effect of sex [$F(1, 104) = 6.74$, $p = 0.011$] was obtained, thus data from males and females was subsequently analysed separately using two-way ANOVAs for prenatal condition and postnatal supplementation. Post-hoc analyses were conducted using Tukey's test. A p value < 0.05 was considered to be statistically significant.

5.3 Results

5.3.1 Developmental data for animals used in GSH analysis and NAC studies

Pregnant dams were weighed on GDs 1, 7, 14 and 21. The percentage weight gain over the course of pregnancy did not differ between prenatal conditions [$F(2, 15) = 1.4$, $p = 0.28$, Table 5-1]. Litter size was also comparable among prenatal conditions [$F(2, 15) = 2.1$, $p = 0.16$, Table 5-1].

Table 5-1 Gestational data for animals used for GSH analysis and NAC studies.
The percentage weight gain over the course of pregnancy did not differ between prenatal conditions. Litter size was also comparable among prenatal conditions (see text for statistical details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$.

	Ad libitum	**Pair-fed**	**Ethanol**
Weight gain over pregnancy (%)	50.8 ± 4.7	46.9 ± 4.5	40.2 ± 4.5
Number of pups per litter	15.0 ± 0.6	17.5 ± 0.7	15.7 ± 1.3

Offspring weight was determined during the lactation period on PNDs 2, 8, 15 and 22 to evaluate whether prenatal diet altered offspring weight gain (Table 5-2). A repeated measures ANOVA revealed a significant main effect of prenatal condition [$F(8, 60) = 5.02$, $p = 0.0001$]. Post-hoc analyses revealed that at PND 2 and PND 8, pair-fed ($p < 0.001$) and ethanol-exposed ($p < 0.001$ [PND 2]; $p < 0.01$ [PND 8]) offspring weighed significantly less than *ad libitum* controls. At PND 15 pair-fed offspring ($p < 0.01$) still weighed significantly less than *ad libitum* control offspring, but by PND 22 there were no significant differences in weight among the three prenatal conditions.

Table 5-2 Offspring weights for litters used for GSH analysis and NAC analysis.
All offspring gained weight over the lactation period. A repeated measures ANOVA was used to analyze offspring weights taken on PNDs 2, 8, 15 and 22. There were no sex differences between male and female offspring between PNDs 2 - 22. Significant differences in weight are highlighted, and are presented in the text. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. ** = $p < 0.01$ and *** = $p < 0.001$, as compared to *ad libitum* controls.

Offspring Weight	*Ad libitum*	Pair-fed	Ethanol
PND2	8.4 ± 0.3	6.5 ± 0.2***	6.9 ± 0.2***
PND8	17.3 ± 0.5	13.3 ± 0.5***	14.6 ± 0.5**
PND15	31.1 ± 0.6	26.5 ± 1.1**	28.8 ± 0.9
PND22	54.1 ± 0.8	54.4 ± 0.7	52.7 ± 0.7

Cohort 1: When animals reached experimental age (PND 60), no differences in weight were observed among prenatal conditions for both males [one-way ANOVA, $F(2, 32) = 0.73$, $p = 0.49$, Table 5-3], and females [one-way ANOVA, $F(2, 26) = 1.34$, $p = 0.28$, Table 5-3].

Table 5-3 Weights at experimental age for animals used for GSH analysis (Cohort 1).
When animals reached experimental age males weighed significantly more than females and were analyzed separately. In both males and females no differences in weight were observed among the three prenatal conditions. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. *** = $p < 0.001$ compared to male animals at the same age.

Offspring Weight (g)	*Ad libitum*	Pair-fed	Ethanol-exposed
Males	362.7 ± 4.7	354.3 ± 8.7	364.4 ± 5.2
Females	239.2 ± 4.8***	224.4 ± 7.4***	232.1 ± 4.0***

Cohort 3: Animals received NAC supplementation between weaning (PND 22) and adulthood (PNDs 55 - 70). Between PNDs 55 - 70 animals were used for experimentation. For males there was no significant effect of prenatal condition [$F(2, 63) = 0.02$, $p = 0.98$] or postnatal NAC supplementation [$F(1, 63) = 0.0003$, $p = 0.99$] on body weight. Similarly, in females no significant effect of prenatal condition [$F(2, 63) = 1.82$, $p = 0.17$] or postnatal supplementation [$F(1, 63) = 1.20$, $p = 0.28$] were observed. These results are summarized in Table 5-4.

Table 5-4 Weights of NAC supplemented animals at experimental age (Cohort 3).
When animals reached experimental age (PNDs 55-70) a significant main effect of sex on body weight was observed, so male and female data was analyzed separately. For both males and females there was no significant effect of prenatal condition or postnatal supplementation (NAC; see text for statistical details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$.

Weight (g)	*Ad libitum*		Pair-fed		Ethanol-exposed	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
NAC supplementation	413.6 ± 7.2	254.5 ± 6.1	413.9 ± 8.0	241.4 ± 5.2	415.1 ± 7.8	252.0 ± 6.2
Regular water	416.0 ± 7.8	247.3 ± 5.6	414.8 ± 6.5	239.4 ± 4.9	411.5 ± 7.9	245.6 ± 6.2

5.3.2 Intoxication levels

Peak BAC levels were measured two hours after the dark cycle commenced on GD 15 of pregnancy. The mean BAC level was 101.5 ± 5.8 mg/dl, which is in accordance with

the levels obtained in previous studies from our laboratory that used the same liquid diet model of PNEE (Patten et al., 2012; Titterness and Christie 2012; Christie et al., 2005).

5.3.3 PNEE causes a significant reduction in GSH in the DG of male and female offspring

GSH-t was examined in the DG of *ad libitum*, pair-fed and ethanol-exposed offspring at PND 60. There was a significant effect of prenatal condition [$F(2, 27) = 28.0$, $p < 0.001$] and post-hoc analyses revealed that GSH-t was significantly reduced in ethanol-exposed offspring compared to both *ad libitum* controls ($p < 0.001$) and pair-fed animals ($p < 0.001$; Figure 5-3).

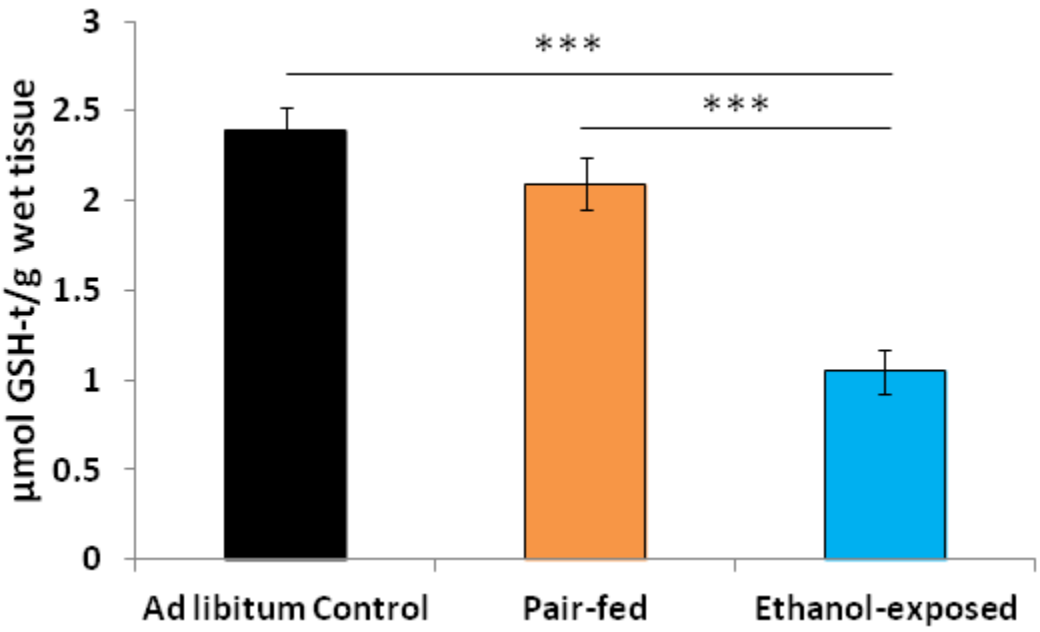

Figure 5-3 The effect of PNEE on GSH-t levels in the DG of adult animals.
PNEE significantly reduces GSH-t as compared to both *ad libitum* and pair-fed controls. Results are expressed as means ± SEM. $p < 0.05$ was considered statistically significant. *** = $p < 0.001$ compared to ethanol-exposed animals.

5.3.4 GSH depletion affects LTP in the DG differently in male and female animals

Between PNDs 55 – 70, animals treated with DEM (1000 mg/kg) were submitted to *in vivo* electrophysiology. As males and females respond differently to the induction of LTP

(Chapter 3), data from male and female animals were analyzed separately. In males, a one-way ANOVA to assess the effect of treatment on LTP revealed a significant main effect of treatment [$F(2, 26) = 8.25$, $p = 0.002$]. Post-hoc analysis revealed that ethanol-exposed males ($p < 0.01$) and DEM-treated males ($p < 0.05$) had significantly reduced levels of LTP when compared to *ad libitum* control males (Figure 5-4). In females, there was no significant effect of treatment [$F(2, 22) = 0.92$, $p = 0.41$], indicating that GSH depletion in females does not impact the levels of LTP in the DG, mirroring the effects of PNEE in the female offspring (Figure 5-4).

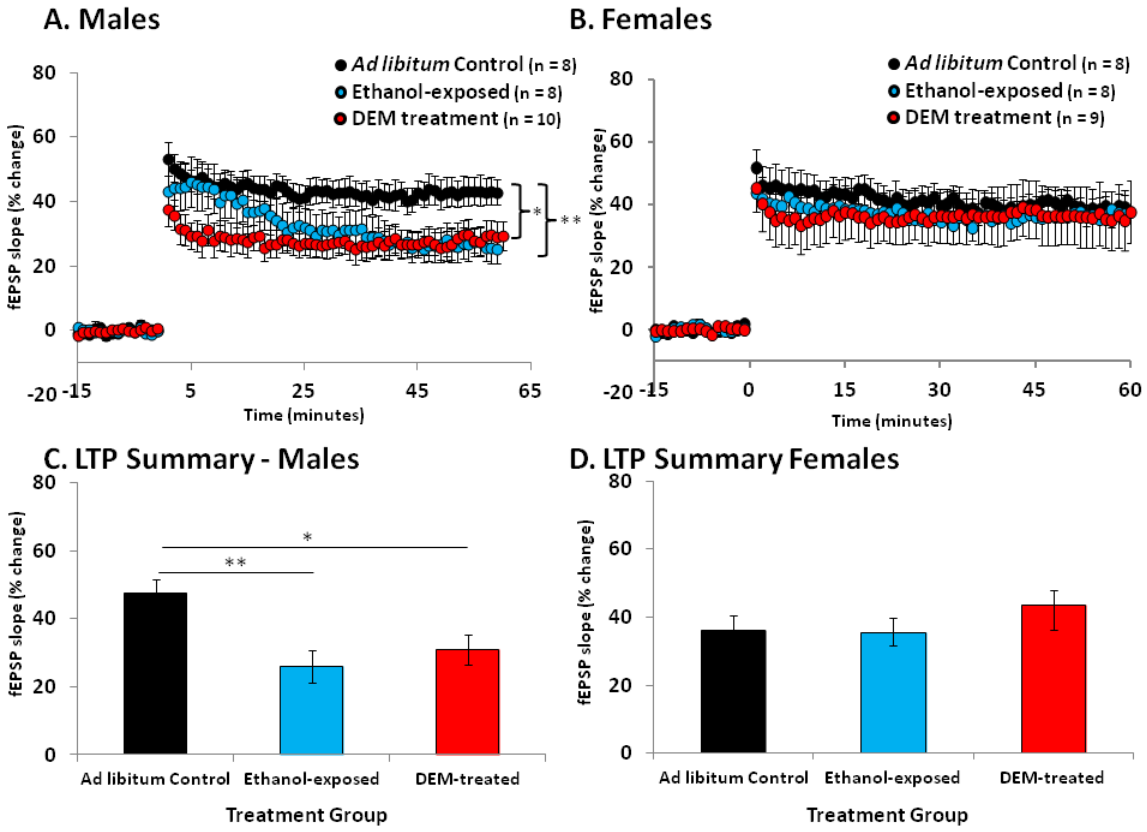

Figure 5-4 The effects of GSH depletion on LTP in the DG of male and female rats.
A) In males, PNEE causes a significant decrease in LTP as compared to control animals ($p < 0.01$). Additionally, there is a significant decrease in LTP in DEM-treated males ($p < 0.05$). B) Neither PNEE nor DEM treatment reduced LTP in females. C) LTP summary graph for males. Both PNEE and DEM-treatment reduced LTP in male animals. D) LTP summary graph for

females. Neither PNEE nor DEM-treatment affected LTP in female animals. All data are presented as means ± S.E.M. * = $p < 0.05$, ** = $p < 0.01$, as compared to *ad libitum* control.

5.3.5 GSH depletion reduces GSH to equivalent levels in the male and female DG

Following electrophysiological experiments, control, ethanol-exposed and DEM-treated animals were decapitated and their hippocampi dissected for determination of GSH-t. No effect of sex was observed using a two-way ANOVA [$F(1, 43) = 1.15$, $p = 0.29$], so male and female data were combined. A one-way ANOVA revealed that there was a significant main effect of treatment [$F(2, 43) = 28.28$, $p < 0.0001$]. Post-hoc analysis revealed that ethanol-exposed animals and DEM-treated animals had significantly reduced levels of GSH as compared to *ad libitum* controls ($p < 0.001$; Figure 5-5).

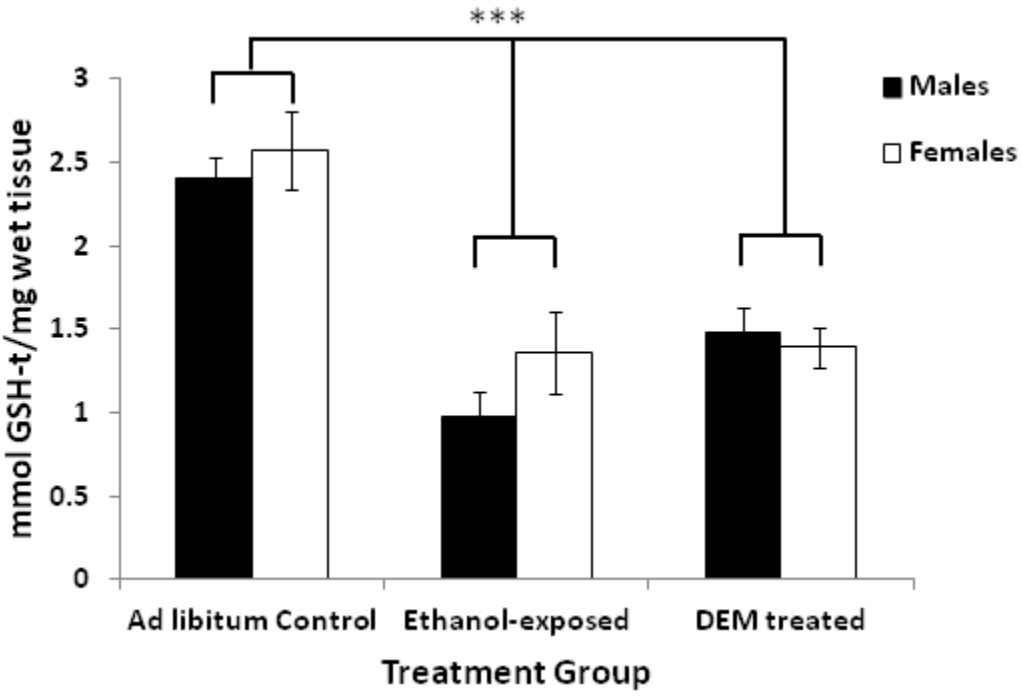

Figure 5-5 GSH-t Levels following PNEE or DEM treatment in males and females.
GSH-t was reduced in males and females following both PNEE and DEM treatment when compared to *ad libitum* control animals ($p < 0.001$). All data are presented as means ± S.E.M. *** = $p < 0.001$ compared to *ad libitum* control.

5.3.6 Postnatal NAC supplementation can rescue the deficits in LTP in male animals following PNEE

Between PNDs 55 – 70 *in vivo* electrophysiology was performed in *ad libitum* control, pair-fed and ethanol-exposed animals, half of which had been supplemented with NAC from weaning. In males, there was no significant effect of prenatal condition [$F(2, 49) = 0.86$, $p = 0.43$] or postnatal NAC supplementation [$F(1, 49) = 2.29$, $p = 0.14$] on the capacity to elicit LTP, but a significant interaction between prenatal condition and postnatal NAC supplementation [$F(2, 49) = 3.70$, $p = 0.032$] was found. Post-hoc analysis revealed a significant difference between ethanol-exposed animals in the control condition and ethanol-exposed animals supplemented with NAC ($p < 0.05$; Figure 5-6). NAC supplementation in ethanol-exposed males caused a significant increase in LTP.

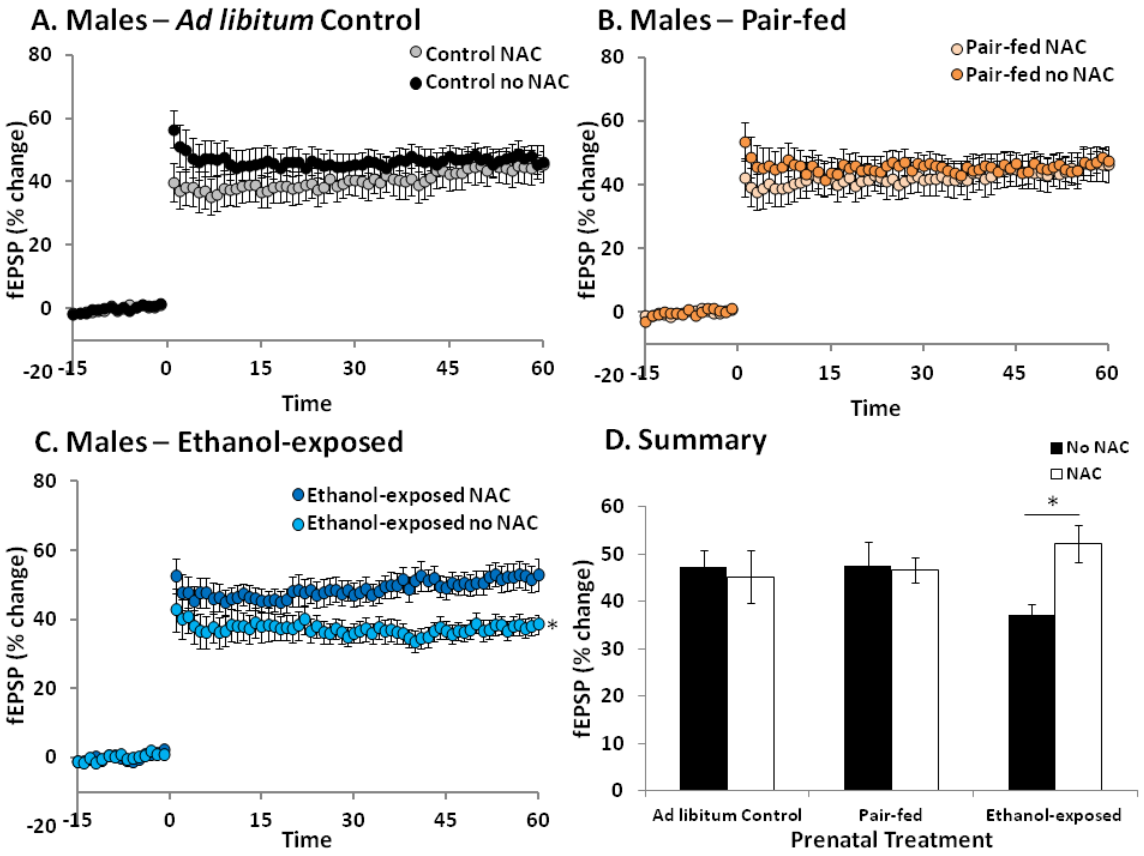

Figure 5-6 The effects of PNEE and subsequent NAC supplementation on LTP in the DG of adult male rats.

NAC supplementation did not increase LTP in *ad libitum* (A) and pair-fed (B) animals. C) NAC supplementation significantly increased LTP in PNEE animals ($p < 0.05$). D) Summary of LTP results. Results are presented as means ± SEM. A significant interaction between prenatal condition and postnatal NAC supplementation [$F(2, 51) = 3.23$, $p = 0.047$] was observed and post-hoc analysis revealed a significant difference between PNEE animals in the control condition and PNEE animals supplemented with NAC ($p < 0.05$).

In females, there was no significant effects of either prenatal condition [$F(2, 53) = 1.03$, $p = 0.36$] or postnatal NAC supplementation [$F(1, 53) = 0.019$, $p = 0.89$; Figure 5-7].

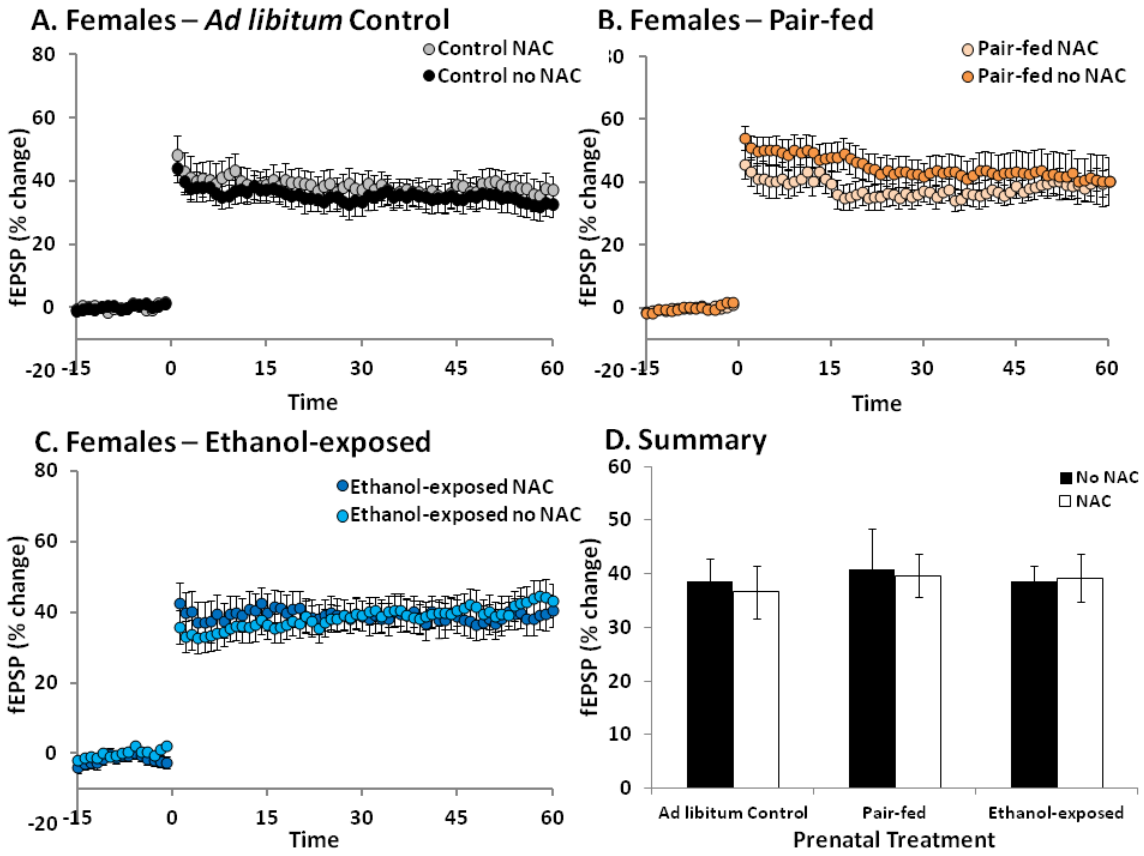

Figure 5-7 The effects of PNEE and subsequent NAC supplementation on LTP in the DG of adult female rats.

NAC supplementation did not increase LTP in *ad libitum* (A), pair-fed (B), or PNEE females (C). D) Summary of LTP results. Results are presented as means ± SEM. There was no significant effects of prenatal condition [$F(2, 53) = 1.03$, $p = 0.36$] or postnatal NAC supplementation [$F(1, 53) = 0.019$, $p = 0.89$] on the capacity to elicit LTP in the female DG.

5.3.7 Postnatal NAC supplementation increases GSH in the DG following PNEE in male and female offspring

Following electrophysiological experiments, animals were rapidly decapitated and their brains were removed in order to examine the levels of GSH-t in the hippocampal DG contralateral to the stimulation. Only *ad libitum* control and PNEE animals were used for these experiments. No significant main effect of sex was observed [$F(1, 47) = 0.25$, $p = 0.62$], and data from both sexes were combined. A two-way ANOVA revealed a significant main effect of postnatal NAC supplementation [$F(1, 51) = 16.7$, $p = 0.0002$]

and a significant interaction between prenatal condition and postnatal NAC supplementation [$F(1, 51) = 4.98$, $p = 0.03$]. Post-hoc analyses revealed that non-supplemented PNEE animals had significantly reduced GSH compared to supplemented *ad libitum* animals ($p < 0.01$). However, NAC supplementation was able to significantly increase GSH in PNEE animals ($p < 0.001$) to levels similar to those observed in *ad libitum* controls (Table 5-5).

Table 5-5 The effect of NAC supplementation on GSH levels in the DG of adult animals.
Ethanol-exposed animals have a significant reduction in GSH levels, which can be rescued by NAC supplementation. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. ** = $p < 0.01$ compared to *ad libitum* NAC-supplemented animals. $$$ = $p < 0.001$ compared to PNEE non-supplemented animals.

	Ad libitum		**Ethanol-exposed**	
	non-NAC	**NAC**	**non-NAC**	**NAC**
GSH-t (µmol/mg protein)	2.10 ± 0.14	2.4 ± 0.12	1.54 ± 0.14**	2.57 ± 0.18$$$

5.4 Discussion

5.4.1 PNEE causes a significant reduction in GSH-t in the DG of both males and females

We (Brocardo et al., 2012) and others (Dembele et al., 2006) have previously shown that ethanol exposure during the perinatal period leads to a long lasting depletion of brain GSH levels. In the present study we also found that exposure to ethanol during gestation results in a reduction of brain GSH levels in adulthood, illustrating the long-lasting and damaging effects of PNEE. In previous studies, a gavage model of PNEE was used to examine the effect of ethanol exposure during the period of brain development on GSH-t levels in the adult brain (Dembele et al., 2006, Brocardo et al., 2012). As discussed in Chapter 4, this mode of ethanol administration typically produces higher BACs and a more severe phenotype of FASD. In the current study, we used a liquid diet model of

FASD, which is considered to be more moderate, as it does not result in extremely high BAC levels (for review see Gil-Mohapel et al., 2010). Nevertheless, we found that even with lower BACs, a decrease in the levels of GSH in the DG can still be observed. It is important to note that both male and female offspring had comparable deficits in GSH-t levels, as opposed to the sex-specific impairments in synaptic plasticity described in Chapter 3.

DGCs do not develop until the third trimester equivalent (PNDs 1-10) in the rat (Rice and Barone, 2000). As such, the development of these cells occurred after the animals have been exposed to ethanol (which occurred exclusively during gestation), indicating that ethanol may have damaged the population of granule cell precursors, leading to lower GSH levels in the mature granule cells. Because developing neurons contain much higher levels of GSH than their mature counterparts, and the DG is one of the few regions of the adult brain where neurogenesis continues to occur throughout adulthood, the hippocampal DG is likely to be more vulnerable to GSH depletion (Sun et al., 2006).

5.4.2 GSH depletion in control animals shows a sexually dichotic effect on LTP

Similar to what was observed with PNEE (Chapter 3), GSH depletion also had a sexually dichotic effect on LTP. As can be seen in Figure 5-4, DEM treatment, which results in the formation of DEM-GSH adducts and reduces GSH acutely, reduces LTP in male animals but not in their female counterparts. This indicates that LTP in males is reliant on GSH concentrations, whereas in females GSH levels are not critical for maintaining LTP. This result is relevant, especially considering our PNEE results – both males and females have reduced GSH-t following PNEE, but only ethanol-exposed males show deficits in LTP, an effect that is mirrored in control animals treated with DEM.

Why LTP in females is not reduced with GSH depletion is currently unknown. Previous studies have indicated that females are more resistant to β-*N*-oxalyl amino-L-alanine (**L-BOAA**) toxicity than males (Diwakar et al., 2007). L-BOAA causes loss of GSH and inhibits Complex I of the mitochondrial electron transport chain in males, whereas female mice appear to be resistant to the effects of this drug. A similar trend is observed if mice are treated with DEM or L-propargyl glycine (Diwakar et al., 2007), indicating that, unlike their male counterparts, GSH is not critical for maintaining complex I activity in females. Interestingly, ovariectomized females, became susceptible to L-BOAA toxicity, and further analysis revealed that glutaredoxin (and not GSH) is important for neuroprotection in females (Diwakar et al., 2007). Glutaredoxin is a thiol disulfide oxidoreductase that is involved in maintaining the redox status of proteins and the recycling of GSH (Gravina and Mieyal, 1993). Females have higher levels of glutaredoxin than males, and while glutaredoxin levels are reduced in the spinal cord and some areas of the CNS following OVX, hippocampal levels of this compound remained unaffected (Diwakar et al., 2007). In Chapter 3 we showed that OVX did not result in deficits in female animals following PNEE. It is therefore possible that the resistance female animals show to PNEE or DEM-treatment could be due to preservation of mitochondrial GSH combined with higher levels of glutaredoxin (that are not reduced in the hippocampus following OVX; (Diwakar et al., 2007).

Our laboratory has previously shown that there are no differences in the activities of the antioxidant enzymes GR, GPx, GST, SOD or CAT with PNEE and that males and females have equivalent activity levels of these enzymes in adulthood (Brocardo et al., 2012, Patten et al., 2012) (see section 6.3.4). However, other studies have indicated that a

sexual dimorphism exists in the enzymes responsible for NADPH synthesis (Dukhande et al., 2009). NADPH is an important co-factor for most antioxidant enzyme reactions and GSH synthesis. NADPH can be produced by the enzymes NADP-linked isocitrate dehydrogenase (**NADP-ICDH**) and G6PDH. NADP-ICDH and G6PDH have higher basal activity in the adult female brain (Dukhande et al., 2009). Thus, it is reasonable to speculate that a reduction in NADPH levels in males may cause a delay in GSH regeneration (despite normal basal levels of this antioxidant). This in turn, may account for the deficits in LTP that are specifically observed in male animals exposed to either PNEE or DEM.

5.4.3 NAC supplementation does not increase LTP in control, pair-fed or ethanol-exposed females

In *ad libitum* control, pair-fed or ethanol-exposed females NAC supplementation did not increase LTP. The lack of effect of NAC on LTP in control animals may indicate that this supplement is only effective when a deficit in GSH and/or LTP exists, as is the case with ethanol-exposed males (see section 5.4.4). Indeed, if NAC is administered acutely, prior to *in vivo* or *in vitro* LTP induction in control animals, no increases in LTP are observed (Viggiano et al., 2008, Haxaire et al., 2012). Similar results are seen with other supplements such as omega-3 fatty acids (McGahon et al., 1999, Lonergan et al., 2002). These supplements can rescue the deficits in LTP in ethanol-exposed males, but do not influence LTP in control, pair-fed or ethanol-exposed females (see section 6.3.6). It is still unclear why NAC and other similar supplements can benefit LTP only when this form of synaptic plasticity is reduced, but it may be that adequate levels of the supplement (in the case of NAC supplementation, adequate levels of GSH) are needed to maintain LTP, and if levels of GSH are reduced, then LTP is also reduced. This is

exemplified by the changes observed in the aging brain – with age, decreases in LTP and increases in oxidative stress are apparent (McGahon et al., 1999, Martin et al., 2002, Haxaire et al., 2012), whereas supplementation with NAC (Haxaire et al., 2012), or other nutrients that are known to increase GSH and decrease oxidative stress (McGahon et al., 1999, Martin et al., 2002), can restore LTP.

It is also important to note that NAC supplementation did not increase GSH in control animals. This is in line with previous studies that have shown that NAC can restore GSH and reduce oxidative stress in models of disease or aging, but does not influence GSH in control animals where GSH levels are adequate (Nehru and Kanwar, 2007, Ljubisavljevic et al., 2011, Penugonda and Ercal, 2011). This is probably because NAC increases cysteine concentrations in the brain, which can activate GCS, one of the enzymes responsible for GSH production, thus increasing GSH levels (Griffith, 1999). However, if GSH levels are adequate, a feedback loop will inhibit GCS until GSH levels drop (Richman and Meister, 1975, Griffith, 1999, Griffith and Mulcahy, 1999). Of note, GSH was increased in ethanol-exposed females with NAC supplementation, but as shown in Figure 5-4, GSH does not seem to be critical for the maintenance of LTP in the female brain.

5.4.4 NAC supplementation can increase GSH and LTP in ethanol-exposed males

In this study, we observed that NAC supplementation from PND 23 (weaning) until adulthood (PNDs 55 – 70) was enough to reverse the deficits in LTP observed in ethanol-exposed male animals (Figure 5-6). This result is in accordance with previous literature showing that NAC supplementation can restore LTP in aged animals (Robillard et al.,

2011, Haxaire et al., 2012) and partially rescue the learning and memory impairments that occur with aging (Martinez et al., 2000).

The increase in LTP associated with NAC supplementation was accompanied by an increase in GSH in the DG (Table 5-5). This is in line with previous studies showing that NAC supplementation increases GSH in models of disease or injury where levels of this endogenous antioxidant are reduced (Nehru and Kanwar, 2007, Ljubisavljevic et al., 2011, Penugonda and Ercal, 2011). Since NAC supplementation increased GSH concentrations in the DG of ethanol-exposed male and female animals, it is tempting to assume that the increase in LTP observed in ethanol-exposed males is due to a direct increase in GSH content, which in turn can influence the redox state of the NMDA receptor and enhance synaptic plasticity (Janaky et al., 1999). Nevertheless, since NAC can also act as a free-radical scavenger (Ziment, 1988, Aruoma et al., 1989), we cannot rule out the possibility that NAC itself (and not GSH) mediates the increase in LTP observed in these animals.

5.4.5 How does GSH influence LTP in the DG?

While there is some evidence that physiological levels of ROS/RNS are necessary for the induction of LTP (Lander, 1997, Klann and Thiels, 1999), if the concentration of ROS/RNS are increased or antioxidants are decreased, LTP can be reduced (Barnes, 1979, Auerbach and Segal, 1997). This may explain why a decrease in LTP is observed following PNEE, as ethanol is a known source of ROS/RNS and can reduce the intracellular GSH pool (Brocardo et al., 2012, Patten et al., 2012) reviewed by (Brocardo et al., 2011), Figure 5-3).

The function of the NMDA receptor relies on the thiol redox state of the cell, which is influenced by GSH concentration. As stated above (section 5.1), GSH can modulate the redox state of the NMDA receptor, causing an increase in its activity (Tang and Aizenman, 1993, Janaky et al., 1999, Bodhinathan et al., 2010) and suggesting that an increase in GSH may be beneficial in restoring LTP. In agreement with this model, Yang *et al.,* (2010) discovered that the deficits in hippocampal CA1 LTP that are induced by aging, could be reversed by the reductants dithiothreitol and βmercaptoethanol (Yang et al., 2010). Interestingly, Vitamin C, a classical antioxidant, was unable to reverse the deficits, which suggests that maintaining the thiol redox status of the NMDA receptor may be more critical for restoring LTP in the damaged or aging brain than the scavenging action of other oxidants (Yang et al., 2010).

5.4.6 Conclusions

The studies conducted in this Chapter demonstrate the existence of a relationship between intracellular GSH levels and LTP in the DG of the hippocampus. With PNEE, long-term deficits in GSH occur and these lead to a decrease in LTP in male animals. Supplementing ethanol-exposed animals with NAC to boost GSH concentrations is able to restore LTP in male animals. Interestingly, reducing GSH acutely in control animals leads to a sexual dichotomy similar to that observed with PNEE: in males, DEM-induced GSH depletion leads to a reduction in LTP, whereas in females, DEM-induced GSH depletion did not affect this form of synaptic plasticity. While the nature of these sexually dichotic effects is still not completely understood, the relationship between GSH and LTP suggests that the female brain may be more resistant to a reduction in the levels of this endogenous antioxidant.

6. The effects of omega-3 fatty acids on GSH levels and synaptic plasticity following PNEE

This Chapter is based in part on the following manuscripts:

Anna R. Patten, Patricia Brocardo, Brian Christie (2012). Prenatal ethanol exposure causes long-term deficits in antioxidant capacity that can be partially restored by omega-3 supplementation. *Journal of Nutritional Biochemistry (electronic publication ahead of print).*

Anna R. Patten, Helle Sickmann, Roger Dyer, Sheila Innis and Brian R. Christie (2013). Omega-3 Fatty Acids Reverse the Long-Term Deficits in Hippocampal Synaptic Plasticity Caused by Prenatal Ethanol Exposure. *Neuroscience Letters. (under review).*

6.1 Background

6.1.1 Omega-3 fatty acids and oxidative stress

Omega-3 fatty acid supplementation can improve antioxidant status and reduce oxidative stress in healthy brains and disease models where oxidative stress is increased (Sarsilmaz et al., 2003, Sarsimaz et al., 2003, Wang et al., 2003, Songur et al., 2004, Zararsiz et al., 2006), producing significant decreases in lipid peroxidation in brain areas such as the hypothalamus (Songur et al., 2004) and hippocampus (Sarsimaz et al., 2003). Furthermore, this capacity is retained even in pathological conditions (i.e. formaldehyde exposure or glutamate excitotoxicity) (Wang et al., 2003, Zararsiz et al., 2006). In the prefrontal cortex, formaldehyde exposure decreases GPx and SOD activities and subsequently increases lipid peroxidation, but concomitant omega-3 fatty acid supplementation during the period of formaldehyde exposure was shown to prevent these alterations (Zararsiz et al., 2006). In hippocampal cultures, supplementation with omega-3 fatty acids was protective against glutamate induced excitotoxicity, increasing cell survival and activity of the antioxidant enzymes GPx and GR (Wang et al., 2003).

Perhaps the most significant benefit of omega-3 supplementation is the increase in GSH content in the brain. As discussed in Chapter 1, GSH is the most abundant and important non-enzymatic antioxidant in the brain and is responsible not only for the detoxification of ROS/RNS, but also for controlling the redox state of many proteins and receptors important in cell signaling (Janaky et al., 1999, Lash, 2006, Forman et al., 2009). Omega-3 supplementation increases GSH in healthy or diseased or damaged brains (Hossain et al., 1999, McGahon et al., 1999, Arnal et al., 2010, Patten et al., 2012).

6.1.2 Omega-3 fatty acids, learning and memory and synaptic plasticity

As well as increasing antioxidant capacity and decreasing oxidative stress, omega-3 fatty acid incorporation into the hippocampus may also be important for learning and memory. Epidemiological studies have shown that high intakes of DHA and EPA (from fish and other sea foods) are associated with a decreased risk of poor child performance on standardized IQ tests (Oken et al., 2005, Hibbeln et al., 2006, Hibbeln et al., 2007). These results are mirrored in a number of animal studies using different tests for learning and memory.

Bourre et al (1989) showed that rats fed omega-3 deficient diets had significant impairments in the shuttle box test, indicating that appropriate levels of omega-3 fatty acids in the brain may be necessary for normal learning. In line with this, Catalan et al (2002) showed that while DHA deficiency produced no deficits in simple two odour discrimination tasks, there were significant deficits in the acquisition of a 20 problem olfactory learning set, suggesting that DHA deficiency does not result in changes in sensory capacity but instead higher order learning is affected (Catalan et al., 2002). On the other hand, supplementation with omega-3 fatty acids can improve learning and

memory in control and diseased animals. Maze learning improvements are observed after one months of a diet rich in DHA ethyl esters (2g/100g diet) (Lim and Suzuki, 2001), and even 12 days of a DHA-enriched diet can improve Morris water maze performance in rats (Wu et al., 2008). There are also several studies indicating that omega-3 fatty acid supplementation can reverse learning and memory deficits associated with traumatic brain injury (Wu et al., 2004) and in models of Alzheimer's disease (Hashimoto et al., 2005).

The benefits of omega-3 fatty acid supplementation on LTP have been shown in both the DG and CA1 sub-regions of the hippocampus, but only in the aged brain or following some sort of insult (McGahon et al., 1999, Lonergan et al., 2002, Martin et al., 2002, Kawashima et al., 2009, Cao et al., 2010), similarly to what we observed with NAC supplementation (see Chapter 5). In the healthy brain, omega-3 fatty acid supplementation does not appear to affect LTP, but may prevent age related declines in LTP from occurring (McGahon et al., 1999, Martin et al., 2002, Cao et al., 2010). It is thought that omega-3 supplementation can improve this form of synaptic plasticity through reducing oxidative stress, inhibiting apoptosis and enhancing membrane fluidity (McGahon et al., 1999, Lonergan et al., 2002, Martin et al., 2002, Cao et al., 2010).

6.1.4 Objectives of this Chapter

In Chapters 3 and 5 we demonstrated that PNEE causes a long-lasting deficit in GSH in both male and female offspring, and this deficit in GSH may lead to the reduction in LTP that is observed in ethanol-exposed male offspring. In Chapter 5, we showed that enhancing intracellular levels of GSH using NAC supplementation was able to rescue the deficit in GSH levels and subsequently the deficits in LTP observed in ethanol-exposed

male animals. In this chapter, we examine whether we can restore GSH in male and female ethanol-exposed animals and rescue the deficits in LTP in male animals through postnatal omega-3 fatty acid supplementation. First, we examined the ability of omega-3 fatty acids to counteract the effects of PNEE on GSH-t levels and other cellular antioxidant enzyme systems and markers of oxidative damage in the DG. Next, we examined the benefits of omega-3 fatty acids on LTP following PNEE.

6.2 Animals and methods

Ad libitum control, pair-fed and ethanol exposed dams were bred as described in Chapter 2 (section 2.1). Two males and two females from each litter were used for biochemical experiments (see below). The remaining animals in each litter were used to examine the effect of omega-3 fatty acid supplementation on synaptic plasticity (no more than two males and two females from each litter were included in any given experiment); between PNDs 55 – 70 *in vivo* electrophysiology was performed to assess LTP in the DG according to the methods described in section 2.3.

6.2.1 Postnatal supplementation with omega-3 fatty acids

Half the dams in each prenatal condition were switched to regular rat chow (Lab Diets 5001; Table 6-1) on GD 21, while the other half was placed on an omega-3 enriched powder diet (kindly supplied by Dr. Sheila Innis, University of British Columbia, Canada; Table 6-1). The omega-3 diet was supplied in a glass jar that was placed at the bottom of the cage and was replaced every day to prevent oxidation of the omega-3 fatty acids in the diet. The dams stayed on the respective diets throughout the suckling period, and upon weaning, the pups were continued on the same diet as their mother. Animals

were given *ad libitum* access to the diet from PND 23 until experimental age (PNDs 55 - 70; see Figure 6-1 for an experimental outline).

Table 6-1 Omega-3 and omega-6 composition of the postnatal diets used in this study.

	Regular Chow Diet	Omega-3 enriched diet
Fat	**5%**	**10%**
Omega-3 fatty acids	0.29%	34.2% (24.6% DHA)
Omega-6 fatty acids	1.22%	4.1%

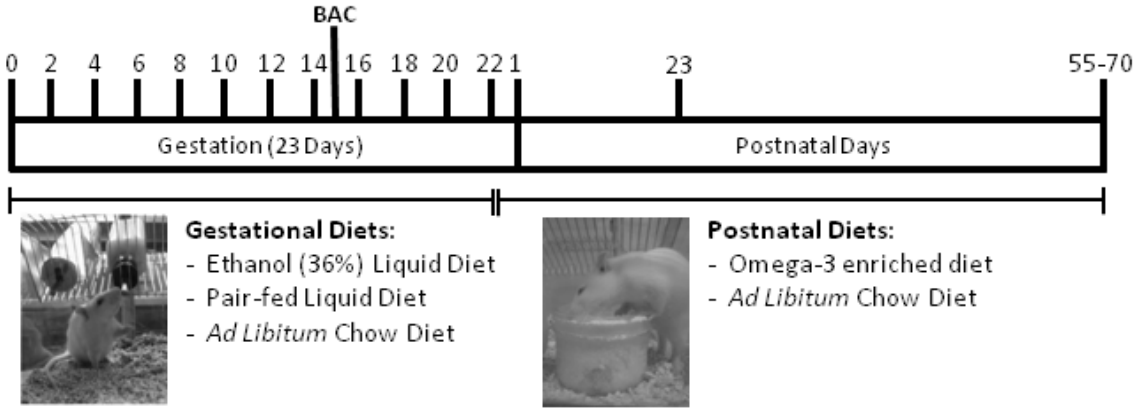

Figure 6-1 Experimental timeline for omega-3 supplementation experiments.
On gestational day (GD) 1 animals were assigned to one of three prenatal diets (Ethanol, Pair-fed or *ad libitum* control). On GD 15 a blood sample was taken to assess blood alcohol concentration (BAC). When pups were born (postnatal day (PND) 1) the dams were placed on either a regular chow diet or an omega-3 enriched diet. When pups were weaned at PND 23 they were continued on the same postnatal diet as their mothers until they reached experimental age (PND55 - 70).

6.2.3 Tissue preparation for biochemical analysis

Hippocampal tissue was prepared exactly as described in section 2.4. Samples that were used to determine the activity of endogenous antioxidant enzymes were sonicated four times for five seconds in 20 mM 4-(2-hydroxyethyl)-1-piprazineethanesulfonic acid buffer (pH = 7.4). Lysates were then centrifuged at 15,000 *g* for 30 minutes at 4°C and the supernatants were stored at -80°C until processed.

Fresh tissue was used to determine GSH-t, and was prepared according to the procedures described in section 5.2.1.

The total protein content of all the samples was determined by the bicinchoninic acid (**BCA**) method using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and following the manufacturer's instructions.

6.2.4 Analysis of antioxidants and markers of oxidative damage

The levels of the endogenous antioxidant GSH, as well as the activities of SOD and CAT, and the lipid peroxidation and protein carbonyl markers were measured using a Microplate reader (Molecular Devices, Sunnyvale, CA, USA). The activity of the endogenous enzymatic antioxidants that utilize GSH as a co-factor, GPx, GR and GST, were determined by spectrophotometry (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK). A representation of the mechanisms underlying the various enzyme assays and the mechanisms of oxidative stress evaluated in this study are shown in Figures 6-2 and 6-3.

6.2.4.1. GSH-t levels

GSH-t was measured using the method described in Chapter 5, section 5.2.2.

6.2.4.2 Glutathione reductase activity

GR activity in DG homogenates was determined by the method described by (Carlberg and Mannervik, 1985). This assay is based on the GR-mediated reduction of GSSG by NADPH. The concomitant oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm that is directly proportional to the GR activity in the sample (Figure 6-2). The assay was performed in the presence of saturating concentrations of the enzyme substrates (GSSG and NADPH). GR activity was expressed in nmol/mg protein.

6.2.4.3 Glutathione peroxidase activity

GPx activity was measured indirectly by a coupled reaction with GR, using the method described by (Wendel, 1981). In this assay, GPx catalyzes the reduction of an organic peroxide, leading to the oxidation of GSH to form GSSG. GSSG is recycled back to GSH by GR with the concomitant conversion of NADPH into NADP+, which can then be detected spectrophotometrically at 340nm (Figure 6-2). The reaction was performed at 25 °C and pH = 8.0 in the presence of saturating concentrations of exogenous GSH, GR and NADPH and was initiated by adding the organic peroxide *tert*-butyl hydroperoxide (**t-BOOH**). The reaction with t-BOOH measures the amount of GPx activity present in the sample. The rate of decrease in absorbance at 340 nm is proportional to the GPx activity in the sample, which was expressed in nmol/mg protein.

6.2.4.4 Glutathione-S-transferase activity

GST activity in the DG was measured as described by (Habig and Jakoby, 1981) using 1-chloro-2,4-dinitrobenzene (**CDNB**) as the substrate. CDNB is not specific for any particular GST isoenzyme, being able to react with a broad range of GST isoenzymes. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and GSH, which results in an increase in the absorbance at 340 nm (Figure 6-2). GST activity was expressed in nmol/mg protein.

6.2.4.5 Super oxide dismutase activity

SOD activity in the DG was determined using a SOD activity assay kit (BioVision Life Science) following the manufacturer's instructions and was measured at 450 nm (Figure 6-2). This assay uses the tetrazolium salt 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (**WST-1**), a chemical that produces a water-soluble dye, formazan, when O_2-· is reduced. SOD competes with the scavenger of O_2-·, xanthine

oxidase and because xanthine oxidase activity is linearly related to the reduction of O_2-·, a process inhibited by SOD, colormetric analysis of WST-1 formazan at 450nm can determine the inhibition activity of SOD. SOD activity was expressed in U/mg protein where 1U equals the amount of SOD activity needed to induce 50% dismutation of O_2-·.

6.2.4.6 Catalase activity

Brain CAT activity was measured using a CAT activity assay kit (BioVision Life Science, San Francisco, CA, USA) according to the manufacturer's directions. In this assay, CAT first reacts with H_2O_2 to produce water and oxygen and any unconverted H_2O_2 reacts with the OxiRed™ probe (BioVision Life Science) to produce a product, which can be measured at 570 nm (Figure 6-2). CAT activity was expressed in U/mg protein where 1U equals the amount of CAT that decomposes 1 μmole of H_2O_2 per minute.

6.2.4.7 Lipid peroxidation levels

As an index of lipid peroxidation, the formation of thiobarbituric acid reactive substances (**TBARS**) (Ohkawa et al., 1979) was measured in homogenates from the DG, using the commercially available TBARS assay kit (BioAssay Systems, Hayward, CA, USA) and following the manufacturer's instructions. TBARS (including MDA) react with thiobarbatric acid (TBA) to form a pink coloured MDA-TBA adduct that can be detected using a spectrophotometer at 535nm (Figure 6-3). The results were expressed in μM MDA/mg protein.

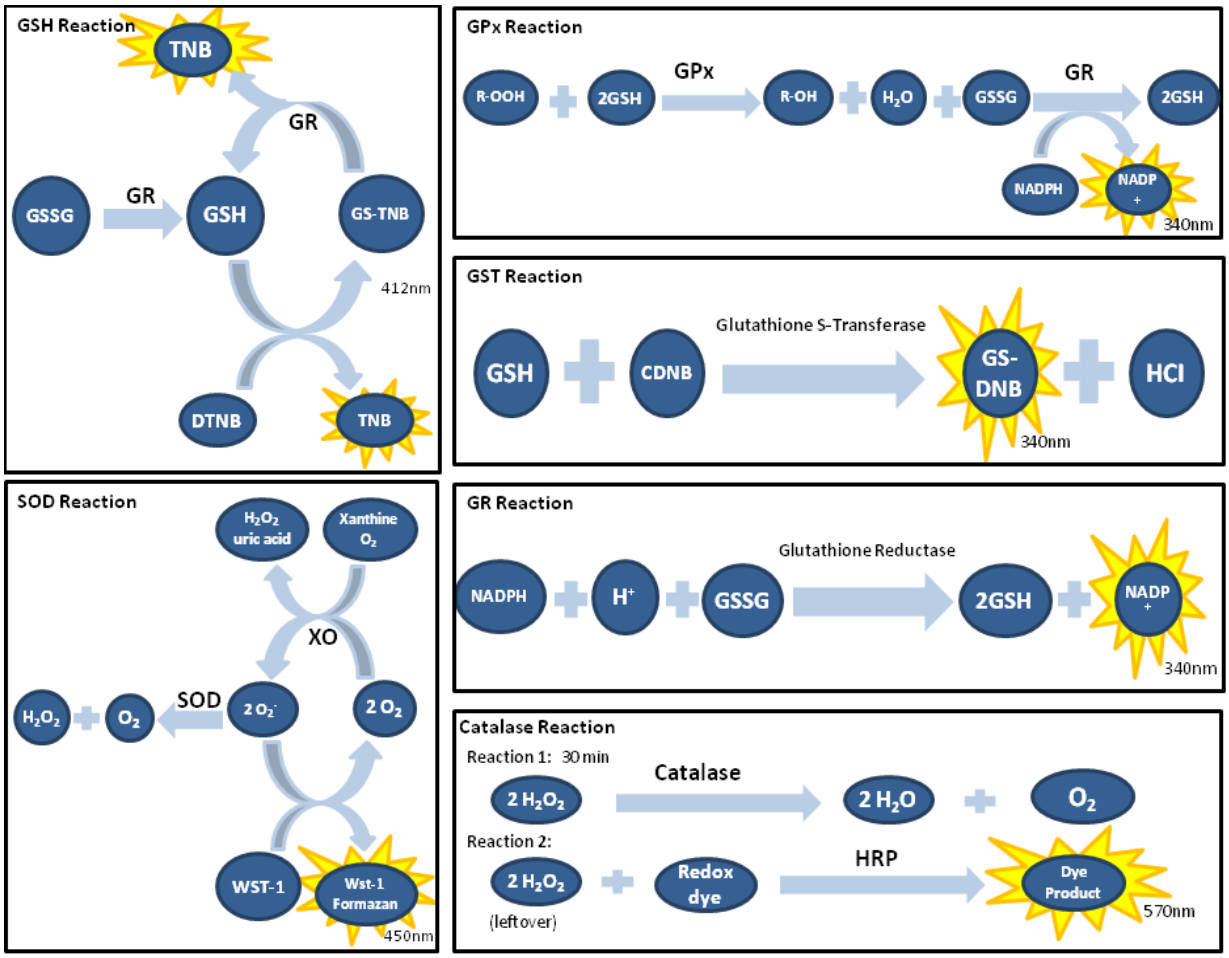

Figure 6-2 Spectrophotometric assay reactions for the detection of the major antioxidants in the brain

GSH Reaction: The sulfhydryl group of GSH reacts with DTNB and produces a yellow coloured product, 5-thio-2-nitrobenzoic acid (TNB). GS-TNB, the mixed disulfide formed between GSH and TNB, is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH measured at 412 nm.

GPx Reaction: GPx activity is measured indirectly. GSSG is produced due to reduction of a peroxide substrate, t-BOOH, by GPx. The GSSG is then recycled back to GSH by GR and NADPH. During this recycling NADPH is oxidised to NADP+. This oxidation can be measured by a decrease in absorbance at 340nm. GSH, GR and NADPH are all provided in excess, so the rate limiting step of the reaction is dependent on GPx activity.

GST Reaction: This assay utilises the xenobiotic 1-Chloro-2,4-dinitrobenzene (CDNB). GST detects CDNB and binds GSH to CDNB creating GS-DNB, which is electrophilically inactive. GS-DNB can be detected at 340nm.

GR Reaction: GR utilises the co-factor NADPH to reduce GSSG back to GSH. GR activity is determined by detecting the oxidation of NADPH to NADP+ and measuring the decrease in absorbance at 340nm.

SOD Reaction: Measuring SOD activity uses the tetrazolium salt WST-1, a chemical that produces a water-soluble dye, formazan, when O_2-· is reduced. SOD competes with xanthine oxidase and since xanthine oxidase activity is linearly related to the reduction of O_2-·, a process

inhibited by SOD, colormetric analysis of WST-1 Formazan at 450nm can determine the inhibitory activity of SOD.

CAT Reaction: The colormetric assay for catalase activity uses a redox dye to directly measure CAT degradation of H_2O_2 molecules through a chemical process involving two sequential reactions. Firstly, catalase is allowed to react with a known amount of H_2O_2 to form water and oxygen during a 30 minute incubation. Secondly, any unconverted H_2O_2 reacts with a redox dye, using an horse radish peroxidise (HRP) catalyst, to produce a product that can be detected at 570nm.

Abbreviations: 1-Chloro-2,4-dinitrobenzene (CDNB); 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB);Glutathione (GSH); Glutathione Peroxidase (GPx); Glutathione Reductase (GR); Glutathione-S-Transferase (GST); Glutathione Disulfide (GSSG); Horseradish Peroxidase (HRP); Hydrogen chloride (HCl); Hydrogen ion (H^+); Hydrogen peroxide (H_2O_2); oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$); organic alcohol (ROH); organic peroxide (ROOH); molecular oxygen (O_2); reduced nicotinamide adenine dinucleotide phosphate (NADPH); water (H_2O); 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1); xanthine oxidase (XO).

6.2.4.8 Protein carbonyl levels

The levels of protein carbonyl groups were determined based on the reaction with 2,4-dinitrophenylhydrazine (**DNPH**) and using a protein carbonyl assay kit (Cayman Chemical Company Ann Arbor, Michigan, USA) that utilizes the DNPH reaction to measure protein carbonyl content (Figure 6-3). The amount of protein-hydrozone produced was determined spectrophotometrically at an absorbance between 360-380 nm. The results were expressed in nmol/mg protein.

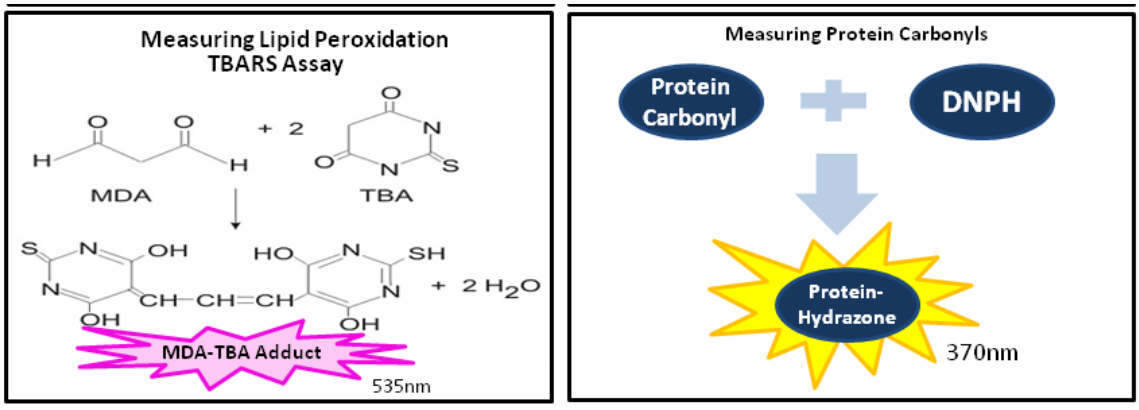

Figure 6-3 Assays to measure cellular oxidative damage.

A) Lipid Peroxidation Assay: Oxidized phospholipids and phospholipid breakdown products (such as MDA) react with thiobarbatric acid (TBA) to form a pink coloured MDA-TBA adduct that can be detected using a spectrophotometer at 535nm. B) Protein Carbonyl Assay: Protein carbonyls can be measured using a colourmetric assay. The protein carbonyl reacts with DNPH, and the amount of protein-hydrazone produced can be detected at 375nm. **Abbreviations:** 2,4-

dinitrophenylhydrazine (DNPH); Malondialdehyde (MDA); Thiobarbatric acid (TBA), Thiobarbatric acid reactive substances (TBARS).

6.2.5 Statistical analysis

All data are presented as means ± S.E.M. A one-way ANOVA was used to determine the effect of prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed) on weight gain across pregnancy and litter size. A repeated measures ANOVA for prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed), postnatal diet (omega-3, regular diet) and sex was used to analyze the body weight of the offspring taken on PNDs 2, 8, 15 and 22. No significant sex differences in weight occurred at these time points [$F(4, 113) = 0.95$, $p = 0.44$] and data from both sexes were subsequently combined. When animals reached experimental age, a three-way ANOVA for sex, prenatal condition and postnatal diet (regular diet or omega-3) was used to evaluate differences on the weight of the offspring. A significant sex difference was detected at this age [$F(1, 246) = 1193.6$, $p = 0.0001$], with males weighing significantly more than females ($p < 0.0001$), and therefore data from males and females were subsequently analyzed separately using two-way ANOVAs.

For GSH-t levels, the activity of the various antioxidant enzymes, and the levels of markers of oxidative damage, no significant effects of sex were observed. Therefore, data from males and females were combined and two-way ANOVAs for prenatal condition and postnatal diet were subsequently performed (see below for individual ANOVA results).

A three-way ANOVA for prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed), postnatal diet (omega-3 or regular diet), and sex was initially performed to analyze the electrophysiological data. As seen in Chapter 3 and in agreement with

previous studies (Titterness and Christie, 2012), a significant main effect of sex was found [F(1, 123) = 5.74, p = 0.018]. As such, male and female data were subsequently analyzed separately using a two-way ANOVA with prenatal condition and postnatal diet as between-subjects factors. Post-hoc analyses were conducted using Tukey's test. A p value < 0.05 was considered to be statistically significant.

6.3 Results

6.3.1 Developmental data

Weight data was taken from the dams on GDs 1, 7, 14 and 21. The percentage weight gain over the course of pregnancy did not differ between prenatal conditions [F (2, 30) = 0.60, p = 0.56; Table 6-2]. Litter size was also comparable between conditions [F (2, 30) = 3.15, p = 0.06; Table 6-2].

Table 6-2 Gestational data for animals used in omega-3 supplementation studies.
Dams were weighed on GDs 1, 7, 14 and 21. Percentage weight gain across pregnancy was analyzed and no significant differences between prenatal conditions were observed. The number of pups per litter was also comparable across prenatal conditions. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. n = 11 for each group.

	Ad libitum	**Pair-fed**	**Ethanol-exposed**
Weight gain over pregnancy (%)	36.7 ± 3.8	35.5 ± 3.7	32.9 ± 4.0
Number of pups per litter	13.8 ± 1.1	15.3 ± 1.3	16.8 ± 1.2

Offspring weight was determined during the lactation period on PNDs 2, 8, 15 and 22 to determine whether prenatal condition altered offspring weight gain (Table 6-3). A repeated measures ANOVA revealed significant main effects of prenatal condition [F(8, 238) = 7.85, $p < 0.0001$] and postnatal diet [F(4, 119) = 9.92, p = 0.0001]. Post-hoc analyses revealed that at PND 2, pair-fed ($p < 0.01$) and ethanol-exposed ($p < 0.001$) offspring on the regular diet weighed significantly less than *ad libitum* control offspring on the regular diet. At PND 8 a significant difference in weight was observed between

regular diet fed *ad libitum* control offspring and ethanol-exposed offspring ($p < 0.05$). At PND 15 a significant difference in weight was observed between *ad libitum* control offspring on the regular diet, and both *ad libitum* control offspring on the omega-3 diet ($p < 0.001$) and pair-fed offspring on omega-3 diet ($p < 0.001$). At PND 22 all groups weighed significantly less than *ad libitum* control offspring on the regular diet (*ad libitum* controls on omega-3 diet ($p < 0.05$); pair-fed on regular diet ($p < 0.05$); pair-fed on omega-3 diet ($p < 0.05$); ethanol-exposed on regular diet ($p < 0.001$); ethanol-exposed on omega-3 diet ($p < 0.01$); Table 6-3).

Table 6-3 Offspring weight data for animals used in omega-3 supplementation studies.
All offspring gained weight over the lactation period. A repeated measures ANOVA was used to analyze offspring weights taken on PNDs 2, 8, 15 and 22. There were no sex differences between male and female offspring. Significant differences in weight are highlighted, and are further outlined in the text. Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ as compared to *Ad libitum* controls on regular chow diet.

Offspring Weight (g)	*Ad libitum*		Pair-fed		Ethanol-Exposed	
	Regular Diet	Omega-3 Diet	Regular Diet	Omega-3 Diet	Regular Diet	Omega-3 Diet
PND2	8.3 ± 0.5	7.8 ± 0.3	7.1 ± 0.7**	7.6 ± 0.3	6.8 ± 0.3***	7.0 ± 0.4
PND8	18.5 ± 1.7	16.7 ± 1.2	16.8 ± 1.7	16.3 ± 1.0	15.2 ± 1.1*	16.9 ± 0.8
PND15	34 ± 1.9	27.6 ± 1.8***	32.1 ± 3.1	27.0 ± 2.0***	30.1 ± 1.4	30.9 ± 1.6
PND22	56.6 ± 2.6	50.5 ± 2.3*	50.6 ± 3.3*	51.9 ± 2.1*	48.6 ± 2.2***	50.0 ± 2.6**

When animals reached experimental age, the weights of males and females were analyzed separately, as at this age a main effect of sex exists, with males being significantly heavier than females ($p < 0.001$). In males there was no significant effect of prenatal condition [$F(2, 120) = 2.05$, $p = 0.13$] but there was a significant main effect of postnatal diet [$F(1, 120) = 196.7$, $p = 0.0001$; Table 6-4]. Post-hoc analyses revealed that animals on the omega-3 enriched diet weighed significantly less than those on the regular chow diet ($p < 0.001$). In females there was no significant effect of prenatal condition

[F(2, 126) = 1.72, p = 0.18, Table 6-4], but like males, a significant effect of postnatal diet was observed [F(1, 126) = 88.7, p = 0.0001], with animals on the omega-3 enriched diet weighing significantly less than those on the regular chow diet ($p < 0.001$).

Table 6-4 Weights at experimental age for animals used in omega-3 supplementation studies.
When animals reached experimental age (PNDs 55 - 70) a significant main effect of sex on body weight was observed, and male and female data were analyzed separately. For both males and females there was no significant effect of prenatal condition, but there was a significant effect of postnatal supplementation (with omega-3; see text for statistical details). Results are expressed as means ± S.E.M. Results are considered statistically significant if $p < 0.05$. ******* = $p < 0.001$ compared to offspring from same prenatal condition but who received a postnatal diet of regular chow.

Offspring weight (g)	*Ad libitum*		Pair-fed		Ethanol-Exposed	
	Regular Diet	Omega-3 Diet	Regular Diet	Omega-3 Diet	Regular Diet	Omega-3 Diet
Males	384.6 ± 9.6	303.0 ± 7.7***	374.7 ± 9.3	286.1 ± 8.1***	373.0 ± 10.7	292.1 ± 9.4***
Females	244.6 ± 5.2	205.0 ± 4.1***	231.6 ± 6.1	200 ± 6.8***	230.6 ± 5.6	208.0 ± 6.0***

6.3.2 Intoxication levels

Peak BAC levels were measured two hours after the dark cycle commenced on GD 15 of pregnancy. The mean BAC level was 135.40 ± 7.54 mg/dl.

6.3.3 GSH-t levels are reduced following PNEE but can be partially restored with omega-3 fatty acid supplementation

A three-way ANOVA for sex, prenatal condition (*ad libitum*, pair-fed or ethanol-exposed) and postnatal diet (regular chow or omega-3 enriched) showed no significant sex differences in GSH-t [F(1, 72) = 1.22, p = 0.27], and therefore male and female data were combined and two-way ANOVAs for prenatal condition and postnatal diet were used to assess the effects of PNEE and omega-3 fatty acid supplementation on GSH-t levels in the DG. Significant main effects of prenatal condition [F(2, 78) = 69.6, p = 0.001], and postnatal diet [F(1, 78) = 26.8, p = 0.001] as well as a significant interaction between prenatal condition and postnatal diet [F(2, 78) = 5.77, p = 0.005] were observed.

Post-hoc analyses revealed that ethanol-exposed animals on the regular diet had a significant reduction in GSH-t levels compared to both *ad libitum* controls ($p < 0.001$) and pair-fed animals ($p < 0.001$) on the regular diet (Figure 6-4). Omega-3 supplementation significantly increased GSH in both ethanol exposed ($p < 0.05$) and *ad libitum* control ($p < 0.01$) offspring (Figure 6-4).

These results suggest that PNEE has a detrimental and long-term effect on GSH-t levels in the DG (as demonstrated in Chapter 5) that can be partially restored with omega-3 fatty acid supplementation.

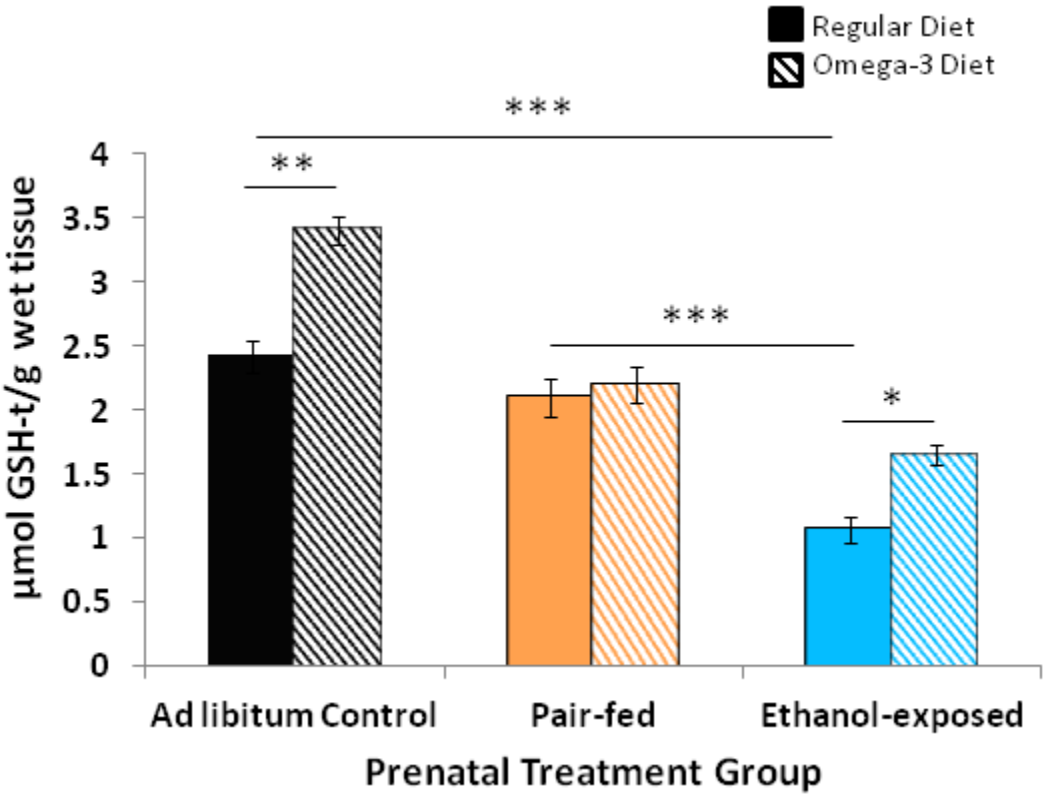

Figure 6-4 The effect of PNEE and subsequent omega-3 fatty acid supplementation on GSH levels in the DG of adult rats.

PNEE decreased GSH levels in the DG. Omega-3 fatty acid supplementation increased GSH-t levels in both control and ethanol-exposed animals. N = 14 for all groups (7 males and 7 females). Abbreviations: Glutathione (GSH-t), Prenatal Ethanol Exposure (PNEE).

6.3.4 Antioxidant enzyme activity is not affected by PNEE or omega-3 fatty acid supplementation

The activities of the various antioxidant enzymes tested were initially assessed by a three-way ANOVA for prenatal condition (*ad libitum* control, pair-fed or ethanol-exposed), postnatal diet (regular chow or omega-3 enriched) and sex. There were no significant effects of sex for any of the enzymes examined (GPx: [$F(1, 72) = 0.13$, $p = 0.71$]; GR: [$F(1, 72) = 0.01$, $p = 0.91$]; GST: [$F(1, 72) = 2.7$, $p = 0.11$]; SOD: [$F(1, 72) = 1.18$, $p = 0.28$]; CAT: [$F(1, 72) = 0.10$, $p = 0.75$]), so male and female data was combined.

For the activity levels of each of the antioxidant enzymes, a two-way ANOVA for prenatal condition and postnatal diet revealed no significant main effects of prenatal condition (GPx: [$F(2, 78) = 0.50$, $p = 0.61$]; GR: [$F(2, 78) = 0.78$, $p = 0.46$]; GST: [$F(2, 78) = 0.08$, $p = 0.93$]; SOD: [$F(1, 78) = 0.03$, $p = 0.97$]; CAT: [$F(2, 78) = 0.41$, $p = 0.66$]) or postnatal diet (GPx: [$F(1, 78) = 0.77$, $p = 0.38$]; GR: [$F(1, 78) = 0.10$, $p = 0.75$]; GST: [$F(1, 78) = 0.04$, $p = 0.83$]; SOD: [$F(1, 78) = 0.39$, $p = 0.53$]; CAT: [$F(1, 78) = 0.08$, $p = 0.78$]; Table 6-5).

These results show that PNEE does not affect antioxidant enzyme activity in the adult DG and that omega-3 supplementation does not increase the activity of any of these antioxidant enzymes in *ad libitum* control, pair-fed or ethanol-exposed offspring.

Table 6-5 The effects of PNEE and postnatal omega-3 fatty acid supplementation on the activity of antioxidant enzymes and subsequent oxidative damage in the DG of adult rats.
PNEE and an omega-3 enriched diet did not affect the activity of any of the antioxidant enzymes or protein carbonyl formation (see text for details). PNEE caused a significant increase in lipid peroxidation products, and these were reduced by supplementing animals with an omega-3 enriched diet during the postnatal period. *** = $p < 0.001$ compared to *ad libitum* and pair-fed offspring. ### = $p < 0.001$ as compared with ethanol-exposed offspring on regular chow diet. SOD: 1U = SOD activity needed to induce 50% dismutation of O_2-·. CAT: 1U = catalase activity needed to decompose 1µmole of H_2O_2 per minute. N = 14 per group (7 males/7 females).

	***Ad libitum* Control**		**Pair-fed**		**Ethanol-exposed**	
	Regular Diet	**Omega-3 Diet**	**Regular Diet**	**Omega-3 Diet**	**Regular Diet**	**Omega-3 Diet**
Antioxidant Enzymes						
GPx (nmol/mg protein)	15.9 ± 1.4	18.0 ± 1.5	17.9 ± 1.4	19.0 ± 1.3	17.8 ± 1.6	17.9 ± 1.4
GR (nmol/mg protein)	7.7 ± 1.0	8.8 ± 1.2	7.8 ± 0.7	6.6 ± 0.6	7.9 ± 0.8	7.4 ± 0.5
GST (nmol/mg protein)	16.5 ± 2.0	12.9 ± 1.4	12.9 ± 1.1	17.0 ± 1.7	14.0 ± 1.9	14.4 ± 1.8
SOD (U/mg protein)	0.50 ± 0.03	0.48 ± 0.03	0.49 ± 0.03	0.50 ± 0.03	0.46 ± 0.03	0.52 ± 0.02
CAT (U/mg protein)	0.39 ± 0.05	0.36 ± 0.03	0.39 ± 0.03	0.40 ± 0.03	0.41 ± 0.06	0.40 ± 0.02
Oxidative Damage						
Lipid Peroxidation (µmol MDA/mg protein)	0.51 ± 0.03	0.46 ± 0.01	0.52 ± 0.03	0.52 ± 0.03	0.77 ± 0.08***	0.43 ± 0.04###
Protein Carbonyls (nmol/mg protein)	0.23 ± 0.05	0.22 ± 0.04	0.24 ± 0.06	0.20 ± 0.07	0.24 ± 0.04	0.16 ± 0.03

6.3.5 Lipid peroxidation but not protein oxidation is increased following PNEE and can be rescued with omega-3 fatty acid supplementation.

A three-way ANOVA was initially used to analyze the effects of PNEE on lipid peroxidation, but as no significant main effect of sex was detected [$F(1, 84) = 0.29$, $p = 0.59$], a two-way ANOVA for prenatal condition and postnatal diet was subsequently used. This revealed a significant main effect of prenatal condition [$F(2, 90) = 7.16$, $p = 0.001$], a significant main effect of postnatal diet [$F(1, 90) = 17.4$, $p = 0.00007$] and a significant interaction between prenatal condition and postnatal diet [$F(2, 90) = 12.3$, $p = 0.00002$]. Post-hoc analyses revealed that PNEE increased lipid peroxidation in the DG when compared to both *ad libitum* controls ($p < 0.001$) and pair-fed animals ($p < 0.001$;

Table 6-5). However, omega-3 supplementation was able to reverse the ethanol-induced increase in lipid peroxidation with a significant difference between ethanol-exposed offspring on a regular diet and ethanol-exposed offspring on an omega-3 enriched diet ($p < 0.001$; Table 6-5).

A three-way ANOVA revealed no significant main effect of sex on the levels of protein carbonyls in the DG [$F(1, 82) = 1.56$, $p = 0.22$] so a two-way ANOVA for prenatal condition and postnatal diet was then used for analysis. No significant main effect of prenatal condition [$F(2, 88) = 0.22$, $p = 0.80$] or postnatal diet [$F(1, 88) = 1.13$, $p = 0.29$] were detected, indicating that protein carbonyl formation is not increased with PNEE, and that omega-3 fatty acid supplementation does not affect protein carbonyl formation in control or ethanol-exposed offspring (Table 6-5).

6.3.6 Omega-3 fatty acid supplementation can completely restore LTP in PNEE males

In Chapter 3, we showed that ethanol-exposed males have significantly reduced LTP compared to both *ad libitum* control and pair-fed male offspring. In this cohort of animals, a two-way ANOVA also showed a significant main effect of prenatal condition in males [$F (2, 50) = 6.12$, $p = 0.004$], further revealing a significant and specific deficit in LTP in ethanol-exposed males when compared to their *ad libitum* ($p < 0.001$) and pair-fed ($p < 0.05$) male counterparts. In this experiment, the two-way ANOVA also revealed a significant main effect of postnatal diet [$F (1, 50) = 10.21$, $p = 0.002$] as well as a significant interaction between prenatal condition and postnatal diet [$F (2, 50) = 7.56$, $p = 0.001$]. Post-hoc analyses revealed that in ethanol-exposed males, omega-3 supplementation from birth until adulthood led to a significant increase in LTP to control levels ($p < 0.001$) (Figure 6-5).

In females, a two-way ANOVA revealed no significant main effects of either prenatal condition [$F(2, 48) = 0.28$, $p = 0.76$] or postnatal diet [$F(1, 48) = 0.05$, $p = 0.83$], indicating that LTP in the DG is not significantly affected by either PNEE or postnatal omega-3 supplementation in adult female animals (Figure 6-6).

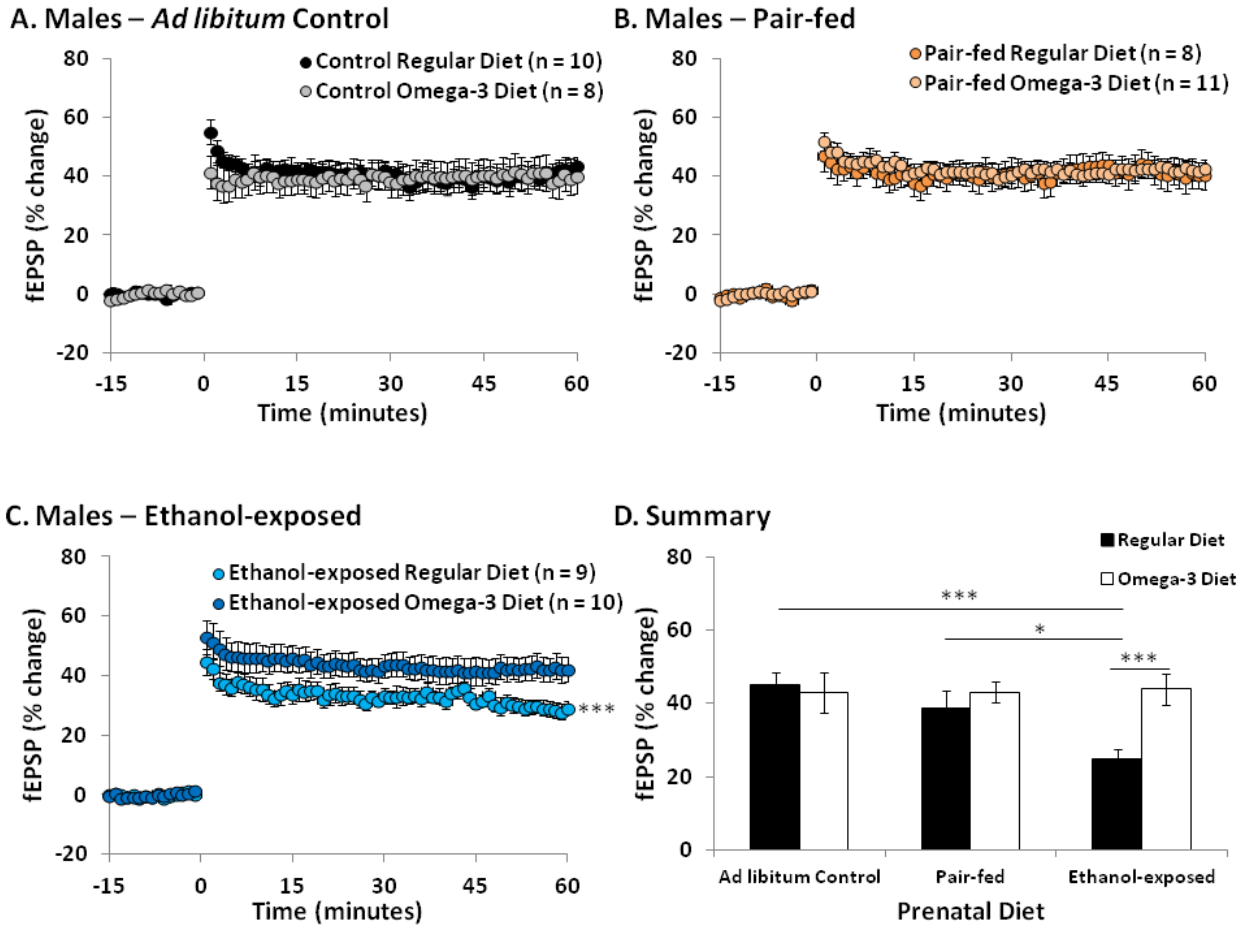

Figure 6-5 The effect of PNEE and subsequent omega-3 fatty acid supplementation on LTP in the DG of adult male rats.

Omega-3 fatty acid supplementation does not affect the level of LTP in the DG of *ad libitum* control (A) or pair-fed animals (B). C) Omega-3 fatty acid supplementation increases LTP in the DG of ethanol-exposed males. D) Summary graph showing that PNEE decreases LTP in male animals, but postnatal supplementation with omega-3 fatty acids can completely restore LTP to control levels. Results are presented as means ± SEM and were considered statistically significant when $p < 0.05$. * = $p < 0.05$, *** = $p < 0.001$.

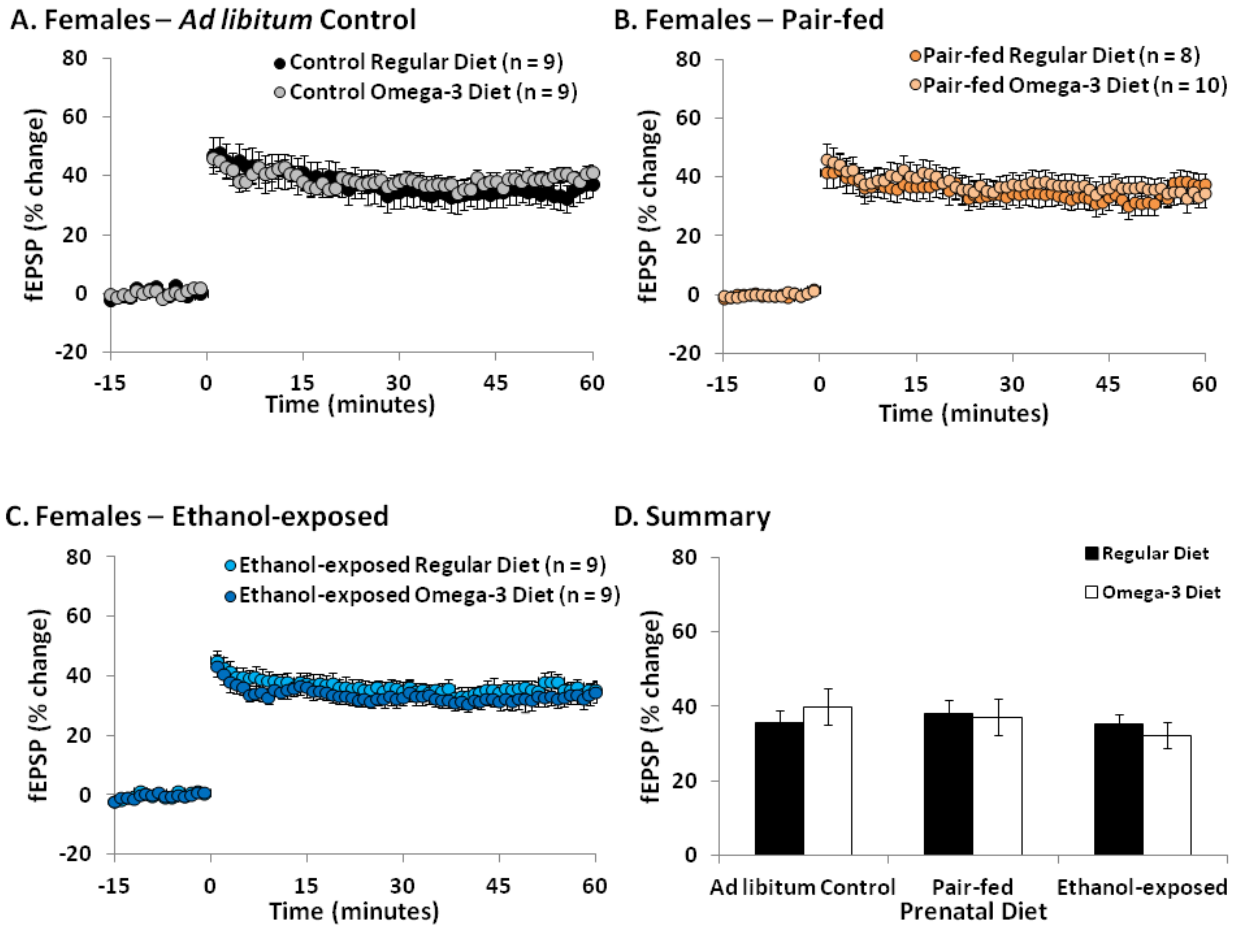

Figure 6-6 The effect of PNEE and subsequent omega-3 fatty acid supplementation on LTP in the DG of adult female rats.
Omega-3 fatty acid supplementation does not influence LTP in the DG of *ad libitum* control (A), pair-fed (B) or ethanol-exposed (C) females. D) Summary of LTP results showing that PNEE does not decrease LTP in the DG of female animals and that omega-3 fatty acid supplementation does not influence LTP in the adult female DG. Results are presented as means ± SEM and were considered statistically significant different when $p < 0.05$.

6.4 Discussion

The results of this study demonstrate that omega-3 fatty acid supplementation was able to overcome long-lasting deficits in GSH levels in the DG and rescue deficits in LTP in ethanol-exposed males. This suggests that omega-3 supplementation may be a viable treatment option for preventing oxidative stress and declines in synaptic plasticity in the brains of individuals diagnosed with FASD.

6.4.1 Omega-3 supplementation can restore GSH levels following PNEE

GSH, the major non-enzymatic antioxidant in the brain (Meister, 1988b), was significantly decreased in the DG of male and female adult rats that were exposed to ethanol during embryonic development (Figure 6-4). These deficits in GSH may account, at least in part, for the increase in oxidative stress that was observed following PNEE, as indicated by the elevated levels of lipid peroxidation (Table 6-5). Interestingly, ethanol-exposed animals that received postnatal omega-3 supplementation did not show deficits in GSH (Figure 6-4), nor an increase in lipid peroxidation (Table 6-5) showing that omega-3 supplementation can restore GSH levels and prevent oxidative stress in the adult brain. These results are in agreement with previous studies that have shown that omega-3 supplementation can increase GSH levels in both normal brains (Hossain et al., 1999) and those hampered by different disease states (Pan et al., 2009, Arnal et al., 2010).

6.4.2 Effects of PNEE and omega-3 supplementation on the activity of antioxidant enzymes

PNEE did not alter the activity of any of the antioxidant enzymes measured in this study, and thus is not surprising that omega-3 supplementation also did not affect the activity of these antioxidant enzymes. These results are in contrast with other studies that have observed deficits in the activities of certain antioxidant enzymes following PNEE (Dembele et al., 2006, Brocardo et al., 2012). However, these studies used a more robust model of ethanol exposure (i.e., intragastric intubation of a higher dose of ethanol during the entire period of brain development – the three trimester equivalents), that was likely severe enough to also induce changes in antioxidant enzyme activity. The fact that none of the antioxidant enzymes in the brain were affected by omega-3 supplementation could mean that these enzymes are already active at maximal capacity, or it could indicate that

omega-3 supplementation has a very specific effect on GSH-t levels in the brain. Indeed, GSH is the major antioxidant in the brain and is a co-factor for many antioxidant enzymatic reactions, and therefore a deficit in GSH may be more relevant than changes in the activity of any single enzyme. Alternatively, these results may also indicate that GSH plays a more specific role in the defence against oxidative stress than the enzymatic antioxidants. In fact, no major abnormalities or increased sensitivity to hypoxia have been observed in transgenic mice deficient in GPx (Ho et al., 1997). Furthermore, over-expressing GPx in the hippocampus following traumatic brain injury did not rescue deficits in neurogenesis (Potts et al., 2009).

6.4.3 Effects of PNEE and omega-3 supplementation on oxidative damage

In this study we observed an increase in lipid peroxidation in the DG following PNEE. These results are in agreement with previous studies that have examined lipid peroxidation in the adult brain following PNEE (Dembele et al., 2006, Brocardo et al., 2012). Importantly, the increase in lipid peroxidation was prevented by supplementation with an omega-3 enriched diet (Table 6-5), which is in agreement with previous studies (Hossain et al., 1999, Sarsilmaz et al., 2003, Songur et al., 2004). For example, omega-3 supplementation was shown to prevent formaldehyde-induced increase in lipid peroxidation in the prefrontal cortex (Zararsiz et al., 2006).

Omega-3 fatty acid supplementation increases GSH-t and GSH interacts with products of lipid peroxidation (in combination with GST) inhibiting their propagation, so this decrease in lipid peroxidation may be directly linked to increases in GSH-t (Xie et al., 1998).

Protein carbonyl formation, a measure of oxidative damage to cellular proteins, was less affected by PNEE and omega-3 supplementation. It is still unclear why this may be the case, but lipid peroxidation is usually one of the initial steps in oxidative cascades, and the products of lipid peroxidation (such as MDA) are known to oxidise proteins, leading to the formation of carbonyl groups (Marnett, 1999). In previous studies from our laboratory an increase in protein oxidation with PNEE was also detected (Brocardo et al., 2012), however as discussed above, differences in the models of PNEE used, the periods of exposure, and the BACs achieved in the two studies may underlie these discrepancies, and a more severe insult might be needed in order to detect oxidative damage not only to lipids but also to proteins.

6.4.4 Omega-3 supplementation rescues the deficits in DG LTP in ethanol-exposed males

In this study we found that omega-3 supplementation did not increase LTP in animals showing normal levels of this form of synaptic plasticity (i.e., *ad libitum* and pair-fed males and females as well as ethanol-exposed females) (Figure 6-5A-B, Figure 6-6). Previous research has shown that the benefits of omega-3 fatty acids depend on the timing and length of supplementation as well as the brain region, age of the animal, and whether disease or injury has occurred (McGahon et al., 1999, Lonergan et al., 2002, Martin et al., 2002, Cao et al., 2010), which is in line with these results.

In ethanol-exposed male rats, omega-3 could completely reverse the deficits in LTP following PNEE (Figure 6-5C). There is a large body of evidence suggesting that omega-3 fatty acid supplementation can enhance LTP in the aging or damaged brain (McGahon et al., 1999, Lonergan et al., 2002, Martin et al., 2002, Cao et al., 2010). Omega-3 fatty acid supplementation may enhance LTP in ethanol-exposed males by increasing the

availability of PUFAs that were lost as a result of developmental exposure to ethanol and/or by reducing the levels of oxidative stress in these animals (Figure 6-4; Table 6-5).

Interestingly, omega-3 fatty acid supplementation has already been examined for its protective effect in a liquid diet model of FASD. In a 1990 study by Wainwright and colleagues, pregnant mice were fed a diet containing 25% ethanol derived calories in combination with 20% oil (omega-6 or omega-6 + omega-3 enriched). The diets were given between GDs 7 - 17. Pups that received ethanol but no omega-3 supplementation showed decreased brain and body weights, sensory and motor developmental abnormalities, and deficits in T-maze learning (Wainwright et al., 1990). While omega-3 supplementation concomitant with ethanol exposure was able to prevent the decreases in body weight and enhance sensory development, the motor and behavioural deficits persisted in ethanol + omega-3 exposed animals, showing that concomitant omega-3 supplementation was only able to prevent some of the ethanol induced deficits in these animals.

While in most studies omega-3 fatty acids are administered concomitantly with the insult (Wainwright et al., 1990, Lonergan et al., 2002, Cao et al., 2010), in our study administration of an omega-3 enriched diet occurred postnatally, after ethanol exposure had occurred. This showcases the remarkable ability of omega-3 fatty acids to rescue rather than just prevent increases in oxidative stress and deficits in synaptic plasticity, as administration following PNEE was robust enough to increase GSH-t levels, reduce lipid peroxidation, and enhance LTP in ethanol-exposed males. While administering omega-3 fatty acids prenatally concomitantly with ethanol exposure would still be relevant, preventive strategies may be less likely to be adhered to by alcohol-drinking mothers.

Thus, the development of postnatal therapies is likely to have an increased clinical relevance for the treatment of children afflicted with FASD.

6.4.6 Conclusions

In this chapter, we have demonstrated a beneficial effect of omega-3 supplementation for treating both the biochemical and functional deficits in the ethanol-exposed DG (Figure 6-7). We have demonstrated the protective role of omega-3 fatty acids in preventing oxidative stress in the DG of rats that were exposed to ethanol during gestation by showing that omega-3 fatty acid supplementation from birth until adulthood can partially restore GSH levels and reduce PNEE-induced lipid peroxidation (Figure 6-4 and Table 6-5). Furthermore, we also showed that omega-3 fatty acid supplementation can rescue the deficits in synaptic plasticity observed in the DG of adult male rats that were exposed to ethanol during gestation (Figure 6-5). This study is the first to demonstrate the benefits of omega-3 fatty acid supplementation in the treatment of FASD. Furthermore, it is one of the few studies that used postnatal supplementation as a therapeutic intervention. Indeed, previous studies have tested the effects of other antioxidants such as silymarin (La Grange et al., 1999, Reid et al., 1999) and vitamin E (Marino et al., 2004); for review see Brocardo et al 2011) when administered concomitantly with ethanol, but have failed to investigate whether such antioxidants would still be beneficial when given after the initial insult.

These results indicate that omega-3 fatty acids may be a viable treatment option for the neurological deficits associated with FASD.

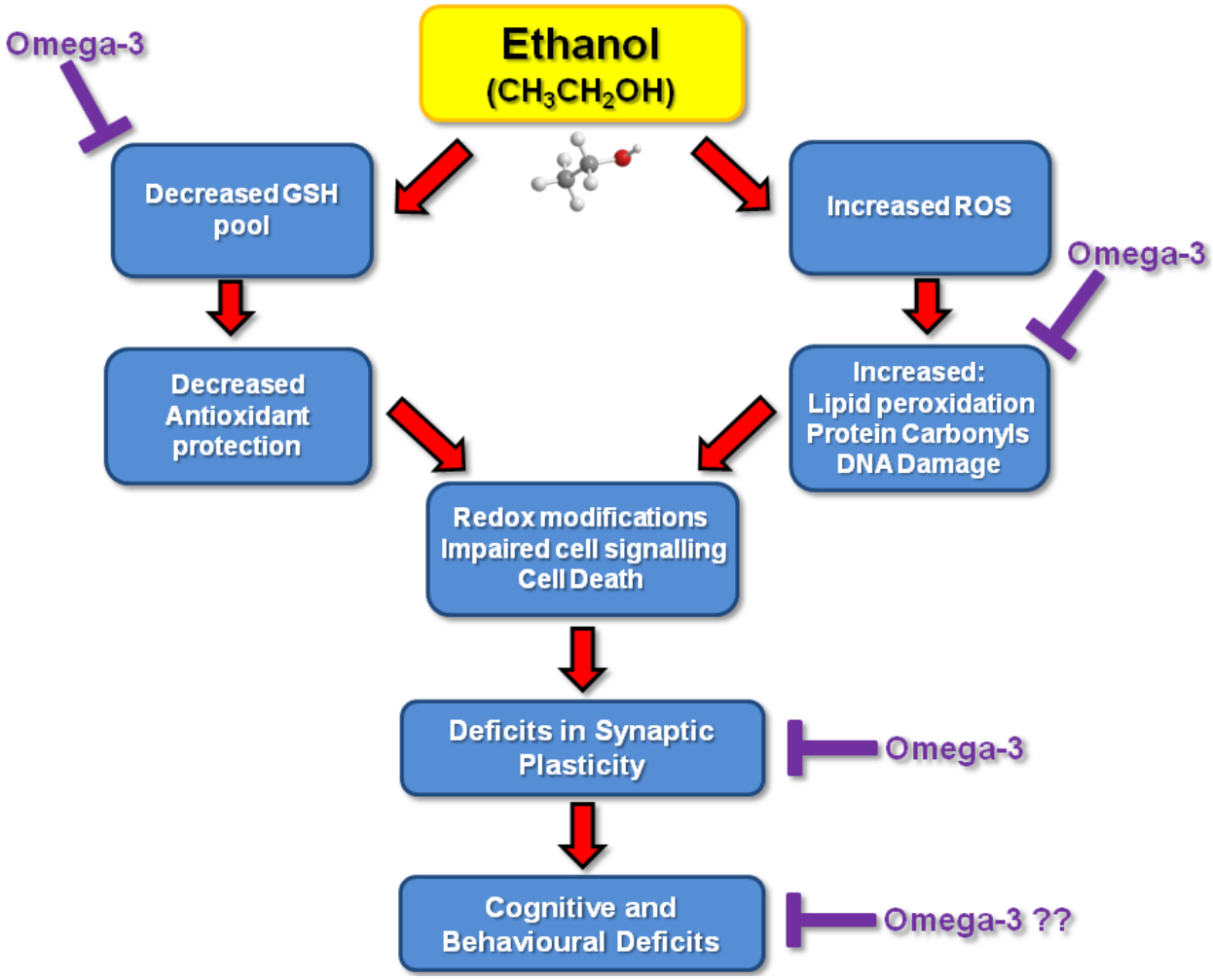

Figure 6-7 FASD and omega-3 fatty acid supplementation

Ethanol can cause oxidative damage through direct and indirect pathways. Ethanol can spontaneously produce ROS such as HO· and hydroxyethyl radicals, which cause lipid peroxidation. Indirectly, ethanol can increase oxidative stress by decreasing the GSH pool. GSH is one of the major co-factors used by antioxidant enzymes to protect the cell against oxidative damage. Decreased antioxidant protection and increased oxidative damage can lead to redox modifications, cell signalling impairments and cell death. These mechanisms may be the underlying cause of deficits in synaptic plasticity observed in the DG of ethanol-exposed males and may result in brain damage and cognitive dysfunction in children with FASD. Omega-3 fatty acid supplementation can reduce oxidative stress by decreasing lipid peroxidation and increasing GSH-t levels. Omega-3 fatty acid supplementation can also completely reverse the deficits in synaptic plasticity observed in ethanol exposed males. Ultimately this may rescue some of the cognitive and behavioural deficits associated with FASD. Abbreviations: Glutathione (GSH); Reactive oxygen species (ROS).

7. General Discussion

7.1 Summary of findings

The purpose of this thesis was to further understand the male and female differences in synaptic plasticity following PNEE, the relationship between synaptic plasticity and oxidative stress following PNEE, and to investigate postnatal therapies that could rescue any deficits observed.

7.1.1 PNEE affects the male and female hippocampus differently

In Chapter 3 we have shown that PNEE causes differential effects on LTP in the DG of adult males and females. Males have a significant deficit in LTP, whereas females do not show these deficits. We also determined that circulating sex hormones produced by the ovary do not underlie the protective mechanisms that are responsible for the lack of LTP deficits in females. This is probably because locally synthesized estrogen is more likely to be responsible for influencing LTP than circulating levels of this hormone (Hojo et al., 2008, Ooishi et al., 2012). Estrogens can act either genomically by modifying transcription through classical nuclear ERs, or non-genomically by activating extra-nuclear ERs and signaling cascades involving ERK/PKA/PKC (Hojo et al., 2008, Ooishi et al., 2012) or other non-ER mediated processes (Moosmann and Behl, 1999, Howard et al., 2001). Estrogens and androgens can be synthesized in the brain by the action of cytochrome P450s, hydroxysteroid dehydrogenases and 5-alpha-reductase (Hojo et al., 2008). Future experiments could be employed to examine the role of brain synthesized estradiol in PNEE by using locally or systemically injected inhibitors of these enzymes to block local synthesis of estrogen in males and females (control and PNEE).

In males, testosterone is metabolised to form estradiol in the brain by the enzyme aromatase (Morris et al., 2004, Wilson and Davies, 2007). Aromatase expression and activity is stimulated by testosterone acting on androgen receptors (Hutchison, 1997). Using a gavage model of ethanol exposure throughout gestation and from PNDs 2 - 10, Lugo *et al.,* (2006) found that male offspring show reduced aggression accompanied with lower testosterone levels (Lugo et al., 2006). Other studies have also observed decreased testosterone levels in ethanol-exposed male offspring (Udani et al., 1985, McGivern et al., 1988, Ward et al., 1994). Estradiol can play a modulatory role and enhance LTP when applied to slices (Foy et al., 1999, Ooishi et al., 2012), Thus, if ethanol-exposed males have reduced testosterone, we can speculate that they will also have a reduction of estradiol in the hippocampus, and this reduction may then underlie the deficit in LTP observed in these animals. In females, locally synthesized estrogen comes from cholesterol/testosterone, and to the best of our knowledge there is no evidence to suggest that the levels of these hormones are reduced in the female brain following PNEE.

In Chapter 4, we found that restricting the temporal window of ethanol exposure during brain development also led to different effects in adult male and female offspring. In males, the deficits in LTP were not as severe as when ethanol exposure occurred during both the 1st and 2nd trimester equivalent of development (Chapter 3), but a trend towards decreases in LTP were noted when exposure occurred during the 1st or 2nd trimester equivalent. In females, similar to the effect observed when ethanol-exposure occurred during the 1st and 2nd trimester equivalents (i.e. throughout gestation) there were no differences in LTP when exposure occurred only during one of these two trimester equivalents. Interestingly, when ethanol-exposure occurred only during the 3rd trimester

equivalent (i.e. from PNDs 4 - 10), LTP was unaffected in males, and was enhanced in females. These effects were unexpected; as usually, higher BACs, like those produced by the 3rd trimester equivalent exposure, produce more extreme deficits in the brain (Ikonomidou et al., 2000). This study further exemplified the variance in the vulnerability of the male and female brain to ethanol exposure during the developmental period.

In Chapter 5, we determined that a possible reason for the sex differences reported in Chapter 3 may in fact be a difference in the ability to function with low levels of the endogenous antioxidant GSH. We found that GSH-t levels were depleted in both the male and female brain following PNEE, but only males have deficits in LTP. When GSH was depleted acutely in control animals, this sexually dichotic effect was mirrored, and only males showed reductions in LTP. This leads us to believe that it is this decrease in the levels of GSH-t following PNEE that causes the synaptic deficits in males. On the other hand, the female brain is able to compensate for the low levels of GSH [possibly due to compensatory mechanisms that prevent mitochondrial damage in conditions with low GSH (Diwakar et al., 2007)], and can continue to function as efficiently as controls and therefore LTP is not diminished. It is important to note that just because decreased GSH in female animals does not reduce LTP in the DG that it does not have other serious long-term side effects. As discussed in Chapter 5, GSH is not only an antioxidant and redox modulator, but it can also control many transcription factors and influence proliferation and apoptosis (Janaky et al., 1999, Lash, 2006, Forman et al., 2009, Markovic et al., 2010). Therefore, lower levels of GSH in the female brain can potentially lead to neuronal dysfunction.

In accordance with our results showing the sex specific effects of PNEE on LTP, sex specific effects of PNEE have also been observed in various behavioural tests. For example, in the T-maze task, while both sexes show deficits in spatial reference memory, only males show deficits in spatial working memory (Zimmerberg et al., 1991). Additionally, PNEE males are more impaired than PNEE females in spatial memory, tested during probe trials of the Morris Water Maze, even though both showed similar deficits in acquisition (Blanchard et al., 1987). Similarly, using a postnatal gavage model of ethanol exposure (PNDs 4 - 10), Goodlett and Peterson (1995) observed that males and females had comparable deficits in Morris water maze acquisition when they reached adolescence. However, if ethanol exposure was limited to PNDs 7 - 9, deficits were only observed in male animals (Goodlett and Peterson, 1995).

It is important to note that LTP deficits in this thesis were observed in the DG and were not examined in other regions of the hippocampus. Other studies have shown that PNEE either has no effect or can impair LTP in the CA1 of both male and female animals (Swartzwelder et al., 1988, Tan et al., 1990, Bellinger et al., 1999, Krahl et al., 1999, Richardson et al., 2002, Byrnes et al., 2004). Overall, these results tell us that while females may be resistant to reductions in DG specific LTP following PNEE that the same may not be true for the rest of the hippocampus, and therefore mechanisms of learning and memory that are dependent on other sub-regions of the hippocampus may not be intact. In fact, a single dose of ethanol on GD 8 (5.8 mg/kg i.p) had a more severe effect on females with respect to their performance on probe trials of the Morris water maze (Minetti et al., 1996). Other studies using the postnatal gavage model of alcohol exposure in pups between the ages of PNDs 4 - 10 have also found that females showed greater

deficits than males in Morris water maze acquisition (Kelly et al., 1988). There is a paucity of data on the effects of PNEE on DG-specific behaviours, and future research is warranted to examine whether the differences in DG LTP observed in this thesis manifest in differences in DG specific behaviours.

7.1.2 Mechanisms underlying the long-lasting effects of PNEE on synaptic plasticity

As discussed in Chapter 1, ethanol can increase the generation of ROS/RNS and reduce antioxidant protection (Montoliu et al., 1995, Haorah et al., 2008). In the developing brain, this increase in oxidative stress can lead to cell damage as the free radicals produced accumulate and self perpetuate over time. These ROS/RNS can attack carbohydrates, proteins, lipids and nucleic acids (reviewed by (Brocardo et al., 2011). Increased oxidative stress and/or lack of antioxidants such as GSH are known to reduce LTP (Pellmar et al., 1991, Auerbach and Segal, 1997, Almaguer-Melian et al., 2000, Serrano and Klann, 2004, Steullet et al., 2006). In Chapter 5, we determined that GSH was reduced in both male and female offspring following PNEE, and in Chapter 6, we showed that lipid peroxidation (a marker of oxidative damage) is also increased in both males and females. It is likely that this increased oxidative stress may be one of the major contributors to the LTP deficits observed in male animals. As mentioned above, females may be more resistant to the deleterious effects of oxidative damage on synaptic plasticity (Diwakar et al., 2007), which may explain why deficits in LTP are not observed in female ethanol-exposed offspring.

7.1.3 Postnatal supplementation as a treatment for FASD

Perhaps the most exciting result of this thesis is that postnatal supplementation with omega-3 fatty acids and NAC can be used to rescue the deficits in GSH and LTP that are

observed in the DG of adult animals that were exposed to ethanol during development. These results demonstrate that the damage caused by ethanol can be reversed, at least to some extent, providing hope for future therapies for children with FASD. At present, the only available options for these children are behavioural and educational support, with specific drugs prescribed for concomitant issues such as attention deficit hyperactivity disorder (Kalberg and Buckley, 2007, Bertrand, 2009). While other therapeutics, including other antioxidants, have been examined for their ability to counteract the effects of alcohol during pregnancy (Marino et al., 2004, Grisel and Chen, 2005); reviewed by Brocardo et al 2011), in this thesis we focused on therapies that could be administered postnatally, after alcohol exposure had occurred, in order to offer treatment to individuals born with these disorders. Strikingly, we found that postnatal supplementation with either NAC or omega-3 fatty acids is robust enough to rescue the deficits in GSH and LTP that occur in the DG following PNEE. In particular, NAC administration commenced at weaning, indicating that even if supplementation occurs later in life (i.e. in childhood or adolescence rather than at birth), the benefits are still considerable.

7.2 Limitations and pitfalls

7.2.1 Use of a “pair-fed” group

Because ethanol-exposed rats tend to consume less food than their *ad libitum* control counterparts (Weinberg, 1985), a pair-fed group was included to account for any impact of such calorie restriction. There is however a lot of contention over the use of a pair-fed group in FASD studies. In our model, the use of a pair-fed group is nevertheless indispensable, due to the fact that animals receiving the ethanol-containing liquid diet

consume less food (and therefore less calories) than those on a non-ethanol (i.e. *ad libitum*) containing diet. Thus, to ensure that the results we obtained are due to ethanol consumption and not to lower calorie intake, we utilized the pair-fed group. However, caloric restriction alone can be perceived as a stressful stimuli, and therefore stress can be a confounding factor in these studies (Titterness and Christie, 2012). While ethanol-exposed animals eat less food voluntarily, pair-fed animals are forced to eat less and spend many hours of the day hungry. We replace the bottles of food daily at approximately the same time of day, and at this time all pair-fed animals' bottles are empty and the animals appear desperate for food.

Another potential problem with this model is that ethanol has inflammatory effects in the stomach (reviewed by (Wang et al., 2010). This means that any food that is ingested may not be metabolised as efficiently, and nutrients from the food that is consumed may not be absorbed (Jian et al., 1986, Thomson et al., 1991). Unfortunately, this side-effect of ethanol consumption cannot be replicated in pair-fed animals, and therefore we cannot be entirely certain that the results we observe are not due to a lack of absorption of nutrients. However, in mothers consuming ethanol during pregnancy, this mal-absorption would also occur, therefore the effects we see are reflective of what occurs in alcohol consuming mothers.

In contrast to other studies of PNEE (Gianoulakis, 1990, Uban et al., 2010, Titterness and Christie, 2012), in this thesis we did not encounter any major pair-fed effects; The weight of the offspring, as well as the levels of antioxidants such as GSH and the levels of LTP were not affected in pair-fed animals. The only instance where the pair-fed group could have possibly confounded our results was in Chapter 4, where in male animals, 1st

or 2nd trimester exposure to the ethanol or pair-fed diet caused similar non-significant decreases in LTP. Nevertheless, considering the overall lack of pair-fed effects reported in this thesis, we can be confident that the results we observe in ethanol-exposed animals are purely due to ethanol and not to malnutrition and lower calorie consumption. It may be the case that it takes time for the brain to compensate for the effects of calorie restriction during development, as many studies that observe deficits in pair-fed animals observe them only in younger animals (Gianoulakis, 1990, Titterness and Christie, 2012). In fact, Gianoulakis (1990) examined spatial learning and memory in animals at PNDs 40, 60 and 90, and while ethanol-exposed animals showed deficits at all time points, pair-fed animals only had deficits when examined at PND40.

7.2.2 Ethanol effects are not limited to the hippocampus

Ethanol causes long-lasting deficits throughout the brain (West, 1987, West and Goodlett, 1990, Mattson et al., 1994, Goodlett and Eilers, 1997, Guerri and Renau-Piqueras, 1997). It is therefore possible that the deficits in synaptic plasticity that we observed in the DG are caused by, or occur in parallel with, deficits in other areas of the brain. Indeed, the DG receives input from several different regions aside from the EC, including the pre- and parasubiculum, medial septum, hypothalamus and the ventral tegmental area (reviewed by (Amaral, 2007), all of which may have been altered by ethanol exposure during development. In support of this idea, oxidative stress is increased throughout the brain of PNEE animals (Dembele et al., 2006, Brocardo et al., 2012, Patten et al., 2012) reviewed by (Brocardo et al., 2011) and therefore increases in oxidative stress in other brain regions might then underlie the hippocampal deficits reported here.

7.2.3 Semi-synthetic omega-3 enriched diet

The omega-3 fatty acid supplemented diet used in these studies was not identical in composition to the regular chow diet that control rats received. This may have caused some discrepancies in our results. As shown in Table 6-1, the omega-3 diet used in these studies had 10% fat, as opposed to the regular chow diet, which only contained 5% fat. Furthermore, the majority of the fat in the omega-3 enriched diet came from omega-3 fatty acids and other PUFAs, whereas in the regular chow diet, omega-3 fatty acid concentration was very low, and there were higher amounts of saturated and monounsaturated fatty acids. While we are confident that the majority of the effects we observed were due to the high concentrations of omega-3 fatty acids in the diet, we cannot rule out that other factors present in the diet may have contributed to these effects, either alone, or in synergy with the omega-3 fatty acids. Another possible way to examine omega-3 fatty acid supplementation would be to use identical lab chow diets for both groups, but provide additional omega-3 fatty acids orally or systemically. While this would control for differences in dietary composition, it would also increase the calorie intake in the omega-3 supplemented animals and would potentially decrease the protein to energy proportions in each group. Thus, we believe that our approach was best to examine the benefits of omega-3 fatty acid enriched diets.

7.3 Future directions

7.3.2 Testing DG-specific behaviours in PNEE animals

While many behavioural studies have shown that ethanol-exposed animals have deficits in hippocampal function (Blanchard et al., 1987, Kelly et al., 1988, Goodlett and Peterson, 1995, Thomas et al., 1996, Johnson and Goodlett, 2002, Richardson et al., 2002, Christie et al., 2005), there is a paucity of data where DG-specific learning and

memory has been examined in PNEE models. These experiments are crucial in order to verify whether the deficits in DG LTP we observed in ethanol-exposed males are accompanied by specific behavioural deficits. Furthermore, it is important to examine whether DG-specific learning and memory is affected in DEM-treated control animals. Similarly to PNEE, acute DEM treatment reduces GSH-t levels in both males and females, and causes deficits in LTP in males. Thus, it would be interesting to see whether PNEE and DEM (i.e., GSH depletion) also have similar effects regarding DG-specific behaviours.

In line with this, it will be important to examine whether the therapeutic supplements we examined in this thesis, NAC and omega-3 fatty acids, also improve behaviour in PNEE animals. While a strong correlation exists between LTP and learning and memory (reviewed by (Bliss and Collingridge, 1993), this is not always the case and therefore these experiments are necessary to ensure that the rescue in LTP we observe in ethanol-exposed males following these two regimes of postnatal supplementation manifests in benefits for hippocampal learning and memory as well.

7.3.3 Determining dosage and time-window for maximum effects of supplementation

In this thesis we used two different supplements to rescue LTP in the DG of ethanol-exposed males. NAC supplementation from weaning until adulthood, and omega-3 fatty acids from birth to adulthood were both reliable at rescuing LTP. It is now important to determine the optimal dosage and time window of supplementation before these therapies can be further developed for use in a human population. As discussed above (Section 7.2.3), the omega-3 enriched diet employed in these studies was semi-synthetic and the majority of fat came from omega-3 fatty acids (Table 6-1). It will now be important to

determine the ideal concentration of omega-3 fatty acids in the diet necessary to obtain maximal beneficial effects. Furthermore, the development of combination therapies where omega-3 is administered in combination with other saturated and unsaturated fatty acids or even other antioxidant compounds, should also be explored. It is also important to determine whether supplementation needs to be immediate (i.e. starting directly from birth) or can occur later in life. Our NAC results would suggest that supplementation beginning later in life (i.e. during childhood) can still have beneficial effects by increasing LTP and GSH-t levels, but this may not be the case with omega-3 fatty acids. Omega-3 fatty acids are critical for brain development (reviewed by (Innis, 2007, 2008), so it may be that omega-3 supplementation is more effective when initiated at birth (when the rat brain is still developing). Starting supplementation with these fatty acids later in life may not produce such a robust rescue effect, as incorporation of omega-3 fatty acids into neuronal membranes early in life may be needed in order to increase GSH and improve synaptic plasticity later on in life.

7.3.4 Combination therapy approaches

Today there is increasing awareness that brain structure and function can be positively boosted by physical exercise. In humans it has been shown that exercise can improve learning (Winter et al., 2007) and lead to decreased risks of cognitive impairment, dementia (Laurin et al., 2001, Ahlskog et al., 2011) reviewed by (Cotman and Berchtold, 2002), and stroke (Guo et al., 2008). Furthermore, with normal aging, the cortex and hippocampus begin to undergo atrophy, which correlates with a decrease in memory (Golomb et al., 1996), and this may be attenuated with exercise (Hillman et al., 2008, Marlatt et al., 2012). Indeed, studies in animal models have revealed that voluntary

exercise is beneficial for dendritic structure (Eadie et al., 2005, Redila and Christie, 2006), cell proliferation and neurogenesis (van Praag et al., 1999a, van Praag et al., 1999b, Cotman and Berchtold, 2002, Redila et al., 2006), synaptic plasticity (van Praag et al., 1999a, Farmer et al., 2004, Kronenberg et al., 2006, Liu et al., 2011, Titterness et al., 2011) and spatial learning (Fordyce and Wehner, 1993, van Praag et al., 1999a), in both rats and mice.

Previous studies from our laboratory have shown that voluntary exercise can also alleviate the deficits in LTP observed in males following PNEE (Christie et al., 2005), and upregulate both hippocampal neurogenesis and BDNF levels (Boehme et al., 2011) in both females and males that were exposed to ethanol during the entire period of brain development (i.e. the three trimester equivalents). Other studies have indicated that exercise may rescue spatial learning and memory deficits in ethanol-exposed animals (Thomas et al., 2008). The combination of exercise and omega-3 fatty acids has been shown to have a synergistic effect in healthy rodents (Wu et al., 2008) and therefore it is possible that the same enhanced effects may be observed in ethanol-exposed animals. Future studies should determine whether the combination of supplementation to reduce oxidative stress, and exercise to increase blood flow and growth factor levels can have a synergistic effect in ethanol-exposed animals.

Aside from exercise, there are other pharmacological or dietary strategies that have shown benefits in treating the learning and memory deficits associated with FASD. For example, both choline (Thomas et al., 2010, Monk et al., 2012, Otero et al., 2012, Thomas and Tran, 2012) and vitamin E (Marino et al., 2004) were shown to have some beneficial effects when administered concomitantly or following ethanol exposure

(reviewed by (Brocardo et al., 2011). Thus, perhaps the best strategy for treating the learning and memory deficits associated with FASD would be to use a multi-supplement approach, combining omega-3 fatty acids, choline and other antioxidants such as vitamin E. In fact "medical food cocktails" are currently being tested both in mice and humans for their potential to treat neurodegenerative diseases such as Alzheimer's disease, and significant improvements in cognition accompanied by reductions in oxidative stress have been observed in these studies (Suchy et al., 2009, Parachikova et al., 2010).

7.4 Overall conclusions

PNEE causes a long lasting decrease in LTP in the DG of adult male animals but this deficit is not observed in female animals. This reduction may be caused by a decrease in the levels of the endogenous antioxidant GSH. However, PNEE causes a reduction in GSH-t levels in both male and female animals. Interestingly, depleting GSH in control animals also reveals a sexually dichotic response, since GSH depletion reduces LTP in males but not in females. These results indicate that the female brain is more resistant to changes in GSH-t and there are possible compensatory mechanisms that occur in the female brain in response to GSH depletion that are not present in males. Increasing the level of GSH in the DG of PNEE animals using NAC can rescue the deficits in LTP that occur in male animals. Since LTP deficits could be rescued by enhancing GSH-t levels, omega-3 fatty acid supplementation was tested in PNEE animals due to the antioxidant properties of these compounds. Postnatal omega-3 supplementation was robust enough to increase endogenous GSH levels in the hippocampus and completely rescue LTP in the DG of male animals, indicating that this dietary supplement may be beneficial in individuals with FASD. This thesis provides further evidence of male and female specific

effects that result from PNEE and gives insight into possible mechanisms that may underlie these differences. Based on these results, specific therapies were used to replenish the brain of nutrients lost due to PNEE (omega-3 fatty acids and GSH through NAC supplementation). Strikingly, these strategies were able to completely reverse the PNEE-induced deficits in LTP. Based on the results presented here, we suggest that pharmacological therapies aimed at alleviating the learning and memory deficits in children and adults with FASD should target GSH using supplements such as omega-3 fatty acids.

Bibliography

Abel EL (In utero alcohol exposure and developmental delay of response inhibition. Alcohol Clin Exp Res 6:369-376.1982).

Aebi S, Assereto R, Lauterburg BH (High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine. Eur J Clin Invest 21:103-110.1991).

Ahlskog JE, Geda YE, Graff-Radford NR, Petersen RC (Physical exercise as a preventive or disease-modifying treatment of dementia and brain aging. Mayo Clin Proc 86:876-884.2011).

Aizenman E, Hartnett KA, Reynolds IJ (Oxygen free radicals regulate NMDA receptor function via a redox modulatory site. Neuron 5:841-846.1990).

Aizenman E, Lipton SA, Loring RH (Selective modulation of NMDA responses by reduction and oxidation. Neuron 2:1257-1263.1989).

Akbar M, Calderon F, Wen Z, Kim HY (Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. Proc Natl Acad Sci U S A 102:10858-10863.2005).

Alagwu EA, Nneli RO (Effect of ovariectomy on the level of plasma sex hormones in albino rats. Niger J Physiol Sci 20:90-94.2005).

Alfonso-Loeches S, Guerri C (Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain. Crit Rev Clin Lab Sci 48:19-47.2011).

Almaguer-Melian W, Cruz-Aguado R, Bergado JA (Synaptic plasticity is impaired in rats with a low glutathione content. Synapse 38:369-374.2000).

Altman J, Bayer SA (Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J Comp Neurol 301:365-381.1990).

Altman J, Das GD (Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.1965).

Amaral D, Lavenex, P. (2006) Hippocampal Neuroanatomy. In: The Hippocampus Book(Andersen P, M. R., Amaral D, Bliss T, O'Keefe J., ed): Oxford University Press.

Amaral D, Lavenex, P. (2007) Hippocampal Neuroanatomy. In: The Hippocampus Book(Andersen P, M. R., Amaral D, Bliss T, O'Keefe J., ed): Oxford University Press.

Andersen P (2006a) The Hippocampus Book. New York: Oxford University Press.

Andersen P, Holmqvist B, Voorhoeve PE (Entorhinal activation of dentate granule cells. Acta Physiol Scand 66:448-460.1966).

Andersen P, Morris, R., Amarel, D., Bliss, T, O'Keefe, J. (2006b) The Hippocampal Formation. In: The Hippocampus Book(Andersen, P., Morris, R., Amarel, D., Bliss, T, O'Keefe, J., ed): Oxford university Press.

Andersen P, Sundberg SH, Sveen O, Wigstrom H (Specific long-lasting potentiation of synaptic transmission in hippocampal slices. Nature 266:736-737.1977).

Anderson ME (Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548-555.1985).

Anderson ME (Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111-112:1-14.1998).
Anderson P, Bliss TV, Skrede KK (Lamellar organization of hippocampal pathways. Exp Brain Res 13:222-238.1971).
Aoyama K, Watabe M, Nakaki T (Regulation of neuronal glutathione synthesis. J Pharmacol Sci 108:227-238.2008).
Arbuckle LD, MacKinnon MJ, Innis SM (Formula 18:2(n-6) and 18:3(n-3) content and ratio influence long-chain polyunsaturated fatty acids in the developing piglet liver and central nervous system. J Nutr 124:289-298.1994).
Arnal E, Miranda M, Barcia J, Bosch-Morell F, Romero FJ (Lutein and docosahexaenoic acid prevent cortex lipid peroxidation in streptozotocin-induced diabetic rat cerebral cortex. Neuroscience 166:271-278.2010).
Aruoma OI, Halliwell B, Hoey BM, Butler J (The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 6:593-597.1989).
Association AP (2000) Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition: DSM-IV-TR®: American Psychiatric Association.
Astley SJ, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, Davies J, Dorn S, Gendler B, Jirikowic T, Kraegel P, Maravilla K, Richards T (Functional magnetic resonance imaging outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. J Neurodev Disord 1:61-80.2009a).
Astley SJ, Richards T, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, Davies J, Dorn S, Gendler B, Jirikowic T, Kraegel P, Maravilla K (Magnetic resonance spectroscopy outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. Magn Reson Imaging 27:760-778.2009b).
Atkuri KR, Mantovani JJ, Herzenberg LA (N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency. Curr Opin Pharmacol 7:355-359.2007).
Auerbach JM, Segal M (Peroxide modulation of slow onset potentiation in rat hippocampus. J Neurosci 17:8695-8701.1997).
Autti-Ramo I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L (MRI findings in children with school problems who had been exposed prenatally to alcohol. Dev Med Child Neurol 44:98-106.2002).
Bains JS, Shaw CA (Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res Brain Res Rev 25:335-358.1997).
Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J (Regional dissociations within the hippocampus--memory and anxiety. Neurosci Biobehav Rev 28:273-283.2004).
Barnes CA (Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol 93:74-104.1979).
Barnes CA, McNaughton BL (An age comparison of the rates of acquisition and forgetting of spatial information in relation to long-term enhancement of hippocampal synapses. Behav Neurosci 99:1040-1048.1985).

Barnes DE, Walker DW (Prenatal ethanol exposure permanently reduces the number of pyramidal neurons in rat hippocampus. Brain Res 227:333-340.1981).
Bawin SM, Sheppard AR, Mahoney MD, Adey WR (Influences of sinusoidal electric fields on excitability in the rat hippocampal slice. Brain Res 323:227-237.1984).
Bayer SA (Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography. J Comp Neurol 190:87-114.1980).
Bayer SA, Altman J (The effects of X-irradiation on the postnatally-forming granule cell populations in the olfactory bulb, hippocampus, and cerebellum of the rat. Exp Neurol 48:167-174.1975).
Bazan NG (Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease. J Lipid Res 50 Suppl:S400-405.2009).
Beck MJ, McLellan C, Lightle RL, Philbert MA, Harris C (Spatial glutathione and cysteine distribution and chemical modulation in the early organogenesis-stage rat conceptus in utero. Toxicol Sci 62:92-102.2001).
Becker HC, Randall CL, Riley EP (Effect of prenatal ethanol exposure on response to abrupt reward reduction. Neurotoxicol Teratol 10:121-125.1988).
Beckman JS (Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. Ann N Y Acad Sci 738:69-75.1994).
Bellinger FP, Bedi KS, Wilson P, Wilce PA (Ethanol exposure during the third trimester equivalent results in long-lasting decreased synaptic efficacy but not plasticity in the CA1 region of the rat hippocampus. Synapse 31:51-58.1999).
Bergamini CM, Gambetti S, Dondi A, Cervellati C (Oxygen, reactive oxygen species and tissue damage. Curr Pharm Des 10:1611-1626.2004).
Berlett BS, Stadtman ER (Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 272:20313-20316.1997).
Berman RF, Hannigan JH (Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. Hippocampus 10:94-110.2000).
Bertrand J (Interventions for children with fetal alcohol spectrum disorders (FASDs): overview of findings for five innovative research projects. Res Dev Disabil 30:986-1006.2009).
Bhave SV, Hoffman PL (Ethanol promotes apoptosis in cerebellar granule cells by inhibiting the trophic effect of NMDA. J Neurochem 68:578-586.1997).
Blanchard BA, Riley EP, Hannigan JH (Deficits on a spatial navigation task following prenatal exposure to ethanol. Neurotoxicol Teratol 9:253-258.1987).
Bliss TV, Collingridge GL (A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.1993).
Bliss TV, Lomo T (Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.1973).
Blumenfeld H (2010) Neuroanatomy Through Clinical Cases: Sinauer Associates.
Bodhinathan K, Kumar A, Foster TC (Intracellular redox state alters NMDA receptor response during aging through Ca2+/calmodulin-dependent protein kinase II. J Neurosci 30:1914-1924.2010).

Boehme F, Gil-Mohapel J, Cox A, Patten A, Giles E, Brocardo PS, Christie BR (Voluntary exercise induces adult hippocampal neurogenesis and BDNF expression in a rodent model of fetal alcohol spectrum disorders. Eur J Neurosci 33:1799-1811.2011).

Bon CL, Garthwaite J (On the role of nitric oxide in hippocampal long-term potentiation. J Neurosci 23:1941-1948.2003).

Brady ML, Diaz MR, Iuso A, Everett JC, Valenzuela CF, Caldwell KK (Moderate Prenatal Alcohol Exposure Reduces Plasticity and Alters NMDA Receptor Subunit Composition in the Dentate Gyrus. J Neurosci 33:1062-1067.2013).

Bramham CR (Control of synaptic consolidation in the dentate gyrus: mechanisms, functions, and therapeutic implications. Prog Brain Res 163:453-471.2007).

Bramham CR, Milgram NW, Srebro B (Activation of AP5-sensitive NMDA Receptors is Not Required to Induce LTP of Synaptic Transmission in the Lateral Perforant Path. Eur J Neurosci 3:1300-1308.1991).

Brocardo PS, Boehme F, Patten A, Cox A, Gil-Mohapel J, Christie BR (Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: Protective effects of voluntary physical exercise. Neuropharmacology 62:1607-1618.2012).

Brocardo PS, Gil-Mohapel J, Christie BR (The Role of Oxidative Stress in Fetal Alcohol Spectrum Disorders. Brain Res Rev.2011).

Brodal A (The hippocampus and the sense of smell; a review. Brain 70:179-222.1947).

Brodie C, Vernadakis A (Critical periods to ethanol exposure during early neuroembryogenesis in the chick embryo: cholinergic neurons. Brain Res Dev Brain Res 56:223-228.1990).

Brown KL, Calizo LH, Goodlett CR, Stanton ME (Neonatal alcohol exposure impairs acquisition of eyeblink conditioned responses during discrimination learning and reversal in weanling rats. Dev Psychobiol 49:243-257.2007).

Brucato FH, Levin ED, Mott DD, Lewis DV, Wilson WA, Swartzwelder HS (Hippocampal long-term potentiation and spatial learning in the rat: effects of GABAB receptor blockade. Neuroscience 74:331-339.1996).

Burd L, Martsolf JT (Fetal alcohol syndrome: diagnosis and syndromal variability. Physiol Behav 46:39-43.1989).

Burdge GC, Postle AD (Effect of maternal ethanol consumption during pregnancy on the phospholipid molecular species composition of fetal guinea-pig brain, liver and plasma. Biochim Biophys Acta 1256:346-352.1995).

Buzsaki G (Theta oscillations in the hippocampus. Neuron 33:325-340.2002).

Byrnes ML, Richardson DP, Brien JF, Reynolds JN, Dringenberg HC (Spatial acquisition in the Morris water maze and hippocampal long-term potentiation in the adult guinea pig following brain growth spurt--prenatal ethanol exposure. Neurotoxicol Teratol 26:543-551.2004).

Cahill L (Why sex matters for neuroscience. Nat Rev Neurosci 7:477-484.2006).

Cao XJ, Cao JJ, Chen TT, Chen WH, Ruan DY (Protective effects of omega-3 fish oil on lead-induced impairment of long-term potentiation in rat dentate gyrus in vivo. Sheng Li Xue Bao 62:225-230.2010).

Carlberg I, Mannervik B (Glutathione reductase. Methods Enzymol 113:484-490.1985).

Cartwright MM, Smith SM (Stage-dependent effects of ethanol on cranial neural crest cell development: partial basis for the phenotypic variations observed in fetal alcohol syndrome. Alcohol Clin Exp Res 19:1454-1462.1995).

Catalan J, Moriguchi T, Slotnick B, Murthy M, Greiner RS, Salem N, Jr. (Cognitive deficits in docosahexaenoic acid-deficient rats. Behav Neurosci 116:1022-1031.2002).

Cavara NA, Hollmann M (Shuffling the deck anew: how NR3 tweaks NMDA receptor function. Mol Neurobiol 38:16-26.2008).

Ceccanti M, Alessandra Spagnolo P, Tarani L, Luisa Attilia M, Chessa L, Mancinelli R, Stegagno M, Francesco Sasso G, Romeo M, Jones KL, Robinson LK, Del Campo M, Phillip Gossage J, May PA, Eugene Hoyme H (Clinical delineation of fetal alcohol spectrum disorders (FASD) in Italian children: comparison and contrast with other racial/ethnic groups and implications for diagnosis and prevention. Neurosci Biobehav Rev 31:270-277.2007).

Chance B, Sies H, Boveris A (Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527-605.1979).

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, Hsiao KK (Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci 2:271-276.1999).

Choi Y, Chen HV, Lipton SA (Three pairs of cysteine residues mediate both redox and zn2+ modulation of the nmda receptor. J Neurosci 21:392-400.2001).

Choi YB, Lipton SA (Redox modulation of the NMDA receptor. Cell Mol Life Sci 57:1535-1541.2000).

Christie BR, Kerr DS, Abraham WC (Flip side of synaptic plasticity: long-term depression mechanisms in the hippocampus. Hippocampus 4:127-135.1994).

Christie BR, Swann SE, Fox CJ, Froc D, Lieblich SE, Redila V, Webber A (Voluntary exercise rescues deficits in spatial memory and long-term potentiation in prenatal ethanol-exposed male rats. Eur J Neurosci 21:1719-1726.2005).

Clamp PA, Lindsley TA (Early events in the development of neuronal polarity in vitro are altered by ethanol. Alcohol Clin Exp Res 22:1277-1284.1998).

Clarren SK, Alvord EC, Jr., Sumi SM, Streissguth AP, Smith DW (Brain malformations related to prenatal exposure to ethanol. J Pediatr 92:64-67.1978).

Coan EJ, Collingridge GL (Magnesium ions block an N-methyl-D-aspartate receptor-mediated component of synaptic transmission in rat hippocampus. Neurosci Lett 53:21-26.1985).

Colino A, Malenka RC (Mechanisms underlying induction of long-term potentiation in rat medial and lateral perforant paths in vitro. J Neurophysiol 69:1150-1159.1993).

Collingridge GL, Kehl SJ, McLennan H (The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. J Physiol 334:19-31.1983).

Compston A (The hippocampus and the sense of smell. A review, by Alf Brodal. Brain 1947: 70; 179-222. Brain 133:2509-2513.2010).

Corkin S, Amaral DG, Gonzalez RG, Johnson KA, Hyman BT (H. M.'s medial temporal lobe lesion: findings from magnetic resonance imaging. J Neurosci 17:3964-3979.1997).

Cotman CW, Berchtold NC (Exercise: a behavioral intervention to enhance brain health and plasticity. Trends Neurosci 25:295-301.2002).
Cronise K, Marino MD, Tran TD, Kelly SJ (Critical periods for the effects of alcohol exposure on learning in rats. Behav Neurosci 115:138-145.2001).
Cull-Candy SG, Leszkiewicz DN (Role of distinct NMDA receptor subtypes at central synapses. Sci STKE 2004:re16.2004).
Danglot L, Triller A, Marty S (The development of hippocampal interneurons in rodents. Hippocampus 16:1032-1060.2006).
Daniel JM, Dohanich GP (Acetylcholine mediates the estrogen-induced increase in NMDA receptor binding in CA1 of the hippocampus and the associated improvement in working memory. J Neurosci 21:6949-6956.2001).
Davies DL, Smith DE (A Golgi study of mouse hippocampal CA1 pyramidal neurons following perinatal ethanol exposure. Neurosci Lett 26:49-54.1981).
Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S (The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J Neurosci 20:4563-4572.2000).
Davis WL, Crawford LA, Cooper OJ, Farmer GR, Thomas DL, Freeman BL (Ethanol induces the generation of reactive free radicals by neural crest cells in vitro. J Craniofac Genet Dev Biol 10:277-293.1990).
Day M, Good M (Ovariectomy-induced disruption of long-term synaptic depression in the hippocampal CA1 region in vivo is attenuated with chronic estrogen replacement. Neurobiol Learn Mem 83:13-21.2005).
Dean RT, Fu S, Stocker R, Davies MJ (Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 324 (Pt 1):1-18.1997).
Deltour L, Ang HL, Duester G (Ethanol inhibition of retinoic acid synthesis as a potential mechanism for fetal alcohol syndrome. FASEB J 10:1050-1057.1996).
Dembele K, Yao XH, Chen L, Nyomba BL (Intrauterine ethanol exposure results in hypothalamic oxidative stress and neuroendocrine alterations in adult rat offspring. Am J Physiol Regul Integr Comp Physiol 291:R796-802.2006).
Deng W, Aimone JB, Gage FH (New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339-350.2010).
Desmond NL, Levy WB (Granule cell dendritic spine density in the rat hippocampus varies with spine shape and location. Neurosci Lett 54:219-224.1985).
Diaz Vivancos P, Wolff T, Markovic J, Pallardo FV, Foyer CH (A nuclear glutathione cycle within the cell cycle. Biochem J 431:169-178.2010).
Diwakar L, Kenchappa RS, Annepu J, Ravindranath V (Downregulation of glutaredoxin but not glutathione loss leads to mitochondrial dysfunction in female mice CNS: implications in excitotoxicity. Neurochem Int 51:37-46.2007).
Dobbing J, Sands J (Quantitative growth and development of human brain. Arch Dis Child 48:757-767.1973).
Dobbing J, Sands J (Comparative aspects of the brain growth spurt. Early Hum Dev 3:79-83.1979).
Dragunow M (A role for immediate-early transcription factors in learning and memory. Behav Genet 26:293-299.1996).

Dringen R (Metabolism and functions of glutathione in brain. Prog Neurobiol 62:649-671.2000).
Dringen R, Hamprecht B (N-acetylcysteine, but not methionine or 2-oxothiazolidine-4-carboxylate, serves as cysteine donor for the synthesis of glutathione in cultured neurons derived from embryonal rat brain. Neurosci Lett 259:79-82.1999).
Dukhande VV, Isaac AO, Chatterji T, Lai JC (Reduced glutathione regenerating enzymes undergo developmental decline and sexual dimorphism in the rat cerebral cortex. Brain Res 1286:19-24.2009).
Eadie BD, Redila VA, Christie BR (Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density. J Comp Neurol 486:39-47.2005).
Eichenbaum H, Wiener SI, Shapiro ML, Cohen NJ (The organization of spatial coding in the hippocampus: a study of neural ensemble activity. J Neurosci 9:2764-2775.1989).
Ekstrom AD, Kahana MJ, Caplan JB, Fields TA, Isham EA, Newman EL, Fried I (Cellular networks underlying human spatial navigation. Nature 425:184-188.2003).
Erecinska M, Cherian S, Silver IA (Energy metabolism in mammalian brain during development. Prog Neurobiol 73:397-445.2004).
Esterbauer H, Eckl P, Ortner A (Possible mutagens derived from lipids and lipid precursors. Mutat Res 238:223-233.1990).
Esterbauer H, Schaur RJ, Zollner H (Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11:81-128.1991).
Ethen MK, Ramadhani TA, Scheuerle AE, Canfield MA, Wyszynski DF, Druschel CM, Romitti PA (Alcohol consumption by women before and during pregnancy. Matern Child Health J 13:274-285.2009).
Fanselow MS, Dong HW (Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65:7-19.2010).
Farmer J, Zhao X, van Praag H, Wodtke K, Gage FH, Christie BR (Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. Neuroscience 124:71-79.2004).
Fattoretti P, Bertoni-Freddari C, Casoli T, Di Stefano G, Giorgetti G, Solazzi M (Ethanol-induced decrease of the expression of glucose transport protein (Glut3) in the central nervous system as a predisposing condition to apoptosis: the effect of age. Ann N Y Acad Sci 1010:500-503.2003).
Feldman HS, Jones KL, Lindsay S, Slymen D, Klonoff-Cohen H, Kao K, Rao S, Chambers C (Prenatal alcohol exposure patterns and alcohol-related birth defects and growth deficiencies: a prospective study. Alcohol Clin Exp Res 36:670-676.2012).
Ferrer I, Galofre F, Lopez-Tejero D, Llobera M (Morphological recovery of hippocampal pyramidal neurons in the adult rat exposed in utero to ethanol. Toxicology 48:191-197.1988).
Finkel T, Holbrook NJ (Oxidants, oxidative stress and the biology of ageing. Nature 408:239-247.2000).

Fordyce DE, Wehner JM (Physical activity enhances spatial learning performance with an associated alteration in hippocampal protein kinase C activity in C57BL/6 and DBA/2 mice. Brain Res 619:111-119.1993).
Forman HJ, Zhang H, Rinna A (Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med 30:1-12.2009).
Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW (17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol 81:925-929.1999).
Frey U, Frey S, Schollmeier F, Krug M (Influence of actinomycin D, a RNA synthesis inhibitor, on long-term potentiation in rat hippocampal neurons in vivo and in vitro. J Physiol 490 (Pt 3):703-711.1996).
Fridovich I (Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem 272:18515-18517.1997).
Frotscher M, Zhao S, Forster E (Development of cell and fiber layers in the dentate gyrus. Prog Brain Res 163:133-142.2007).
Fyhn M, Molden S, Witter MP, Moser EI, Moser MB (Spatial representation in the entorhinal cortex. Science 305:1258-1264.2004).
Gabriel KI, Johnston S, Weinberg J (Prenatal ethanol exposure and spatial navigation: effects of postnatal handling and aging. Dev Psychobiol 40:345-357.2002).
Galimberti I, Gogolla N, Alberi S, Santos AF, Muller D, Caroni P (Long-term rearrangements of hippocampal mossy fiber terminal connectivity in the adult regulated by experience. Neuron 50:749-763.2006).
Galvan A (Neural plasticity of development and learning. Hum Brain Mapp 31:879-890.2010).
Garcia MC, Kim KY, Hough C, Kim HY (Effects of chronic ethanol on the mobilization of arachidonate and docosahexaenoate stimulated by the type 2A serotonin receptor agonist (+/-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride in C6 glioma cells. Alcohol Clin Exp Res 21:1465-1470.1997).
Ge Y, Belcher SM, Light KE (Alterations of cerebellar mRNA specific for BDNF, p75NTR, and TrkB receptor isoforms occur within hours of ethanol administration to 4-day-old rat pups. Brain Res Dev Brain Res 151:99-109.2004).
Gianoulakis C (Rats exposed prenatally to alcohol exhibit impairment in spatial navigation test. Behav Brain Res 36:217-228.1990).
Gil-Mohapel J, Boehme F, Kainer L, Christie BR (Hippocampal cell loss and neurogenesis after fetal alcohol exposure: insights from different rodent models. Brain Res Rev 64:283-303.2010).
Gil-Mohapel J, Boehme F, Patten A, Cox A, Kainer L, Giles E, Brocardo PS, Christie BR (Altered adult hippocampal neuronal maturation in a rat model of fetal alcohol syndrome. Brain Res 1384:29-41.2011).
Goldstein JM, Seidman LJ, Horton NJ, Makris N, Kennedy DN, Caviness VS, Jr., Faraone SV, Tsuang MT (Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic resonance imaging. Cereb Cortex 11:490-497.2001).
Golomb J, Kluger A, de Leon MJ, Ferris SH, Mittelman M, Cohen J, George AE (Hippocampal formation size predicts declining memory performance in normal aging. Neurology 47:810-813.1996).

Gomez-Pinilla F (Brain foods: the effects of nutrients on brain function. Nat Rev Neurosci 9:568-578.2008).
Goodlett CR, Eilers AT (Alcohol-induced Purkinje cell loss with a single binge exposure in neonatal rats: a stereological study of temporal windows of vulnerability. Alcohol Clin Exp Res 21:738-744.1997).
Goodlett CR, Horn KH, Zhou FC (Alcohol teratogenesis: mechanisms of damage and strategies for intervention. Exp Biol Med (Maywood) 230:394-406.2005).
Goodlett CR, Peterson SD (Sex differences in vulnerability to developmental spatial learning deficits induced by limited binge alcohol exposure in neonatal rats. Neurobiol Learn Mem 64:265-275.1995).
Gould E, Woolley CS, Frankfurt M, McEwen BS (Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J Neurosci 10:1286-1291.1990).
Grant KA (Emerging neurochemical concepts in the actions of ethanol at ligand-gated ion channels. Behav Pharmacol 5:383-404.1994).
Gravina SA, Mieyal JJ (Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. Biochemistry 32:3368-3376.1993).
Green P, Glozman S, Kamensky B, Yavin E (Developmental changes in rat brain membrane lipids and fatty acids. The preferential prenatal accumulation of docosahexaenoic acid. J Lipid Res 40:960-966.1999).
Greenstein YJ, Pavlides C, Winson J (Long-term potentiation in the dentate gyrus is preferentially induced at theta rhythm periodicity. Brain Res 438:331-334.1988).
Gressens P, Lammens M, Picard JJ, Evrard P (Ethanol-induced disturbances of gliogenesis and neuronogenesis in the developing murine brain: an in vitro and in vivo immunohistochemical and ultrastructural study. Alcohol Alcohol 27:219-226.1992).
Griffith OW (Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med 27:922-935.1999).
Griffith OW, Mulcahy RT (The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. Adv Enzymol Relat Areas Mol Biol 73:209-267, xii.1999).
Grisel JJ, Chen WJ (Antioxidant pretreatment does not ameliorate alcohol-induced Purkinje cell loss in the developing rat cerebellum. Alcohol Clin Exp Res 29:1223-1229.2005).
Guerri C, Bazinet A, Riley EP (Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour. Alcohol Alcohol 44:108-114.2009).
Guerri C, Montoliu C, Renau-Piqueras J (Involvement of free radical mechanism in the toxic effects of alcohol: implications for fetal alcohol syndrome. Adv Exp Med Biol 366:291-305.1994).
Guerri C, Renau-Piqueras J (Alcohol, astroglia, and brain development. Mol Neurobiol 15:65-81.1997).
Guo M, Lin V, Davis W, Huang T, Carranza A, Sprague S, Reyes R, Jimenez D, Ding Y (Preischemic induction of TNF-alpha by physical exercise reduces blood-brain barrier dysfunction in stroke. J Cereb Blood Flow Metab 28:1422-1430.2008).
Habig WH, Jakoby WB (Glutathione S-transferases (rat and human). Methods Enzymol 77:218-231.1981).

Hagihara H, Toyama K, Yamasaki N, Miyakawa T (Dissection of hippocampal dentate gyrus from adult mouse. J Vis Exp.2009).
Halliwell B (Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 91:14S-22S.1991).
Halliwell B (Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141:312-322.2006).
Halliwell B (Biochemistry of oxidative stress. Biochem Soc Trans 35:1147-1150.2007).
Halliwell B, Gutteridge JM (The definition and measurement of antioxidants in biological systems. Free Radic Biol Med 18:125-126.1995).
Hamby-Mason R, Chen JJ, Schenker S, Perez A, Henderson GI (Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. Alcohol Clin Exp Res 21:1063-1072.1997).
Hamilton DA, Kodituwakku P, Sutherland RJ, Savage DD (Children with Fetal Alcohol Syndrome are impaired at place learning but not cued-navigation in a virtual Morris water task. Behav Brain Res 143:85-94.2003).
Hamilton GF, Murawski NJ, St Cyr SA, Jablonski SA, Schiffino FL, Stanton ME, Klintsova AY (Neonatal alcohol exposure disrupts hippocampal neurogenesis and contextual fear conditioning in adult rats. Brain Res 1412:88-101.2011).
Haorah J, Ramirez SH, Floreani N, Gorantla S, Morsey B, Persidsky Y (Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic Biol Med 45:1542-1550.2008).
Harris RA, Baxter DM, Mitchell MA, Hitzemann RJ (Physical properties and lipid composition of brain membranes from ethanol tolerant-dependent mice. Mol Pharmacol 25:401-409.1984).
Hashimoto M, Tanabe Y, Fujii Y, Kikuta T, Shibata H, Shido O (Chronic administration of docosahexaenoic acid ameliorates the impairment of spatial cognition learning ability in amyloid beta-infused rats. J Nutr 135:549-555.2005).
Haxaire C, Turpin FR, Potier B, Kervern M, Sinet PM, Barbanel G, Mothet JP, Dutar P, Billard JM (Reversal of age-related oxidative stress prevents hippocampal synaptic plasticity deficits by protecting D-serine-dependent NMDA receptor activation. Aging Cell 11:336-344.2012).
He FQ (Prenatal Ethanol Exposure Increases Depressive-Like Behavior and Central Estrogen Receptor α and Oxytocin Expressions in Adult Female Mandarin Voles. Zoological studies 51:1.2012).
Heaton MB, Paiva M, Madorsky I, Mayer J, Moore DB (Effects of ethanol on neurotrophic factors, apoptosis-related proteins, endogenous antioxidants, and reactive oxygen species in neonatal striatum: relationship to periods of vulnerability. Brain Res Dev Brain Res 140:237-252.2003).
Heaton MB, Paiva M, Mayer J, Miller R (Ethanol-mediated generation of reactive oxygen species in developing rat cerebellum. Neurosci Lett 334:83-86.2002).
Hebb DO (1949) The Organization of Behavior: A Neuropsychological Theory. New York: Wiley.
Helfer JL, Goodlett CR, Greenough WT, Klintsova AY (The effects of exercise on adolescent hippocampal neurogenesis in a rat model of binge alcohol exposure during the brain growth spurt. Brain Res 1294:1-11.2009).

Helland IB, Smith L, Saarem K, Saugstad OD, Drevon CA (Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. Pediatrics 111:e39-44.2003).

Hellemans KG, Verma P, Yoon E, Yu W, Weinberg J (Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. Ann N Y Acad Sci 1144:154-175.2008).

Henderson GI, Chen JJ, Schenker S (Ethanol, oxidative stress, reactive aldehydes, and the fetus. Front Biosci 4:D541-550.1999).

Henderson GI, Devi BG, Perez A, Schenker S (In utero ethanol exposure elicits oxidative stress in the rat fetus. Alcohol Clin Exp Res 19:714-720.1995).

Hibbeln JR, Davis JM, Steer C, Emmett P, Rogers I, Williams C, Golding J (Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. Lancet 369:578-585.2007).

Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE (Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. Am J Clin Nutr 83:1483S-1493S.2006).

Hillman CH, Erickson KI, Kramer AF (Be smart, exercise your heart: exercise effects on brain and cognition. Nat Rev Neurosci 9:58-65.2008).

Ho YS, Magnenat JL, Bronson RT, Cao J, Gargano M, Sugawara M, Funk CD (Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. J Biol Chem 272:16644-16651.1997).

Hojo Y, Murakami G, Mukai H, Higo S, Hatanaka Y, Ogiue-Ikeda M, Ishii H, Kimoto T, Kawato S (Estrogen synthesis in the brain--role in synaptic plasticity and memory. Mol Cell Endocrinol 290:31-43.2008).

Hopper RA, Garthwaite J (Tonic and phasic nitric oxide signals in hippocampal long-term potentiation. J Neurosci 26:11513-11521.2006).

Hossain MS, Hashimoto M, Gamoh S, Masumura S (Antioxidative effects of docosahexaenoic acid in the cerebrum versus cerebellum and brainstem of aged hypercholesterolemic rats. J Neurochem 72:1133-1138.1999).

Howard SA, Brooke SM, Sapolsky RM (Mechanisms of estrogenic protection against gp120-induced neurotoxicity. Exp Neurol 168:385-391.2001).

Huang EJ, Reichardt LF (Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677-736.2001).

Hunsaker MR, Mooy GG, Swift JS, Kesner RP (Dissociations of the medial and lateral perforant path projections into dorsal DG, CA3, and CA1 for spatial and nonspatial (visual object) information processing. Behav Neurosci 121:742-750.2007).

Hutchison JB (Gender-specific steroid metabolism in neural differentiation. Cell Mol Neurobiol 17:603-626.1997).

Hutson JR, Magri R, Gareri JN, Koren G (The incidence of prenatal alcohol exposure in Montevideo Uruguay as determined by meconium analysis. Ther Drug Monit 32:311-317.2010).

Idanpaan-Heikkila J, Jouppila P, Akerblom HK, Isoaho R, Kauppila E, Koivisto M (Elimination and metabolic effects of ethanol in mother, fetus, and newborn infant. Am J Obstet Gynecol 112:387-393.1972).

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW (Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056-1060.2000).

Incerti M, Vink J, Roberson R, Wood L, Abebe D, Spong CY (Reversal of alcohol-induced learning deficits in the young adult in a model of fetal alcohol syndrome. Obstet Gynecol 115:350-356.2010).

Innis SM (Essential fatty acid transfer and fetal development. Placenta 26 Suppl A:S70-75.2005).

Innis SM (Dietary (n-3) fatty acids and brain development. J Nutr 137:855-859.2007).

Innis SM (Dietary omega 3 fatty acids and the developing brain. Brain Res 1237:35-43.2008).

Innis SM, Gilley J, Werker J (Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants? J Pediatr 139:532-538.2001).

Iqbal U, Dringenberg HC, Brien JF, Reynolds JN (Chronic prenatal ethanol exposure alters hippocampal GABA(A) receptors and impairs spatial learning in the guinea pig. Behav Brain Res 150:117-125.2004).

Izumi Y, Kitabayashi R, Funatsu M, Izumi M, Yuede C, Hartman RE, Wozniak DF, Zorumski CF (A single day of ethanol exposure during development has persistent effects on bi-directional plasticity, N-methyl-D-aspartate receptor function and ethanol sensitivity. Neuroscience 136:269-279.2005).

Janaky R, Ogita K, Pasqualotto BA, Bains JS, Oja SS, Yoneda Y, Shaw CA (Glutathione and signal transduction in the mammalian CNS. J Neurochem 73:889-902.1999).

Janaky R, Varga V, Hermann A, Saransaari P, Oja SS (Mechanisms of L-cysteine neurotoxicity. Neurochem Res 25:1397-1405.2000).

Jang JH, Surh YJ (Potentiation of cellular antioxidant capacity by Bcl-2: implications for its antiapoptotic function. Biochem Pharmacol 66:1371-1379.2003).

Jayalakshmi K, Singh SB, Kalpana B, Sairam M, Muthuraju S, Ilavazhagan G (N-acetyl cysteine supplementation prevents impairment of spatial working memory functions in rats following exposure to hypobaric hypoxia. Physiol Behav 92:643-650.2007).

Jian R, Cortot A, Ducrot F, Jobin G, Chayvialle JA, Modigliani R (Effect of ethanol ingestion on postprandial gastric emptying and secretion, biliopancreatic secretions, and duodenal absorption in man. Dig Dis Sci 31:604-614.1986).

Johnson TB, Goodlett CR (Selective and enduring deficits in spatial learning after limited neonatal binge alcohol exposure in male rats. Alcohol Clin Exp Res 26:83-93.2002).

Jones KL (The fetal alcohol syndrome. Addict Dis 2:79-88.1975).

Jones KL, Smith DW (Recognition of the fetal alcohol syndrome in early infancy. Lancet 302:999-1001.1973).

Jung MW, McNaughton BL (Spatial selectivity of unit activity in the hippocampal granular layer. Hippocampus 3:165-182.1993).

Kalberg WO, Buckley D (FASD: what types of intervention and rehabilitation are useful? Neurosci Biobehav Rev 31:278-285.2007).

Kawashima A, Harada T, Kami H, Yano T, Imada K, Mizuguchi K (Effects of eicosapentaenoic acid on synaptic plasticity, fatty acid profile and phosphoinositide 3-kinase signaling in rat hippocampus and differentiated PC12 cells. J Nutr Biochem.2009).

Kelly SJ, Goodlett CR, Hulsether SA, West JR (Impaired spatial navigation in adult female but not adult male rats exposed to alcohol during the brain growth spurt. Behav Brain Res 27:247-257.1988).

Kerksick C, Willoughby D (The antioxidant role of glutathione and N-acetyl-cysteine supplements and exercise-induced oxidative stress. J Int Soc Sports Nutr 2:38-44.2005).

Kerns KA, Don A, Mateer CA, Streissguth AP (Cognitive deficits in nonretarded adults with fetal alcohol syndrome. J Learn Disabil 30:685-693.1997).

Kesner RP (Behavioral functions of the CA3 subregion of the hippocampus. Learn Mem 14:771-781.2007).

Kesner RP (An analysis of the dentate gyrus function. Behav Brain Res.2013).

Kesner RP, Lee I, Gilbert P (A behavioral assessment of hippocampal function based on a subregional analysis. Rev Neurosci 15:333-351.2004).

Kim CK, Kalynchuk LE, Kornecook TJ, Mumby DG, Dadgar NA, Pinel JP, Weinberg J (Object-recognition and spatial learning and memory in rats prenatally exposed to ethanol. Behav Neurosci 111:985-995.1997).

Kim H-Y (Biochemical and biological functions of docosahexaenoic acid in the nervous system: modulation by ethanol. Chemistry and Physics of Lipids 153:34.2008).

Kim HY, Akbar M, Lau A, Edsall L (Inhibition of neuronal apoptosis by docosahexaenoic acid (22:6n-3). Role of phosphatidylserine in antiapoptotic effect. J Biol Chem 275:35215-35223.2000).

Klann E, Thiels E (Modulation of protein kinases and protein phosphatases by reactive oxygen species: implications for hippocampal synaptic plasticity. Prog Neuropsychopharmacol Biol Psychiatry 23:359-376.1999).

Klausberger T, Magill PJ, Marton LF, Roberts JD, Cobden PM, Buzsaki G, Somogyi P (Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. Nature 421:844-848.2003).

Konorski J (1948) Conditioned reflexes and neuron organization: University Press.

Korol DL, Abel TW, Church LT, Barnes CA, McNaughton BL (Hippocampal synaptic enhancement and spatial learning in the Morris swim task. Hippocampus 3:127-132.1993).

Krahl SE, Berman RF, Hannigan JH (Electrophysiology of hippocampal CA1 neurons after prenatal ethanol exposure. Alcohol 17:125-131.1999).

Krebs C, Weinberg J, Akesson E (2011) Lir: Neuroscience (Us Ed): Lippincott Williams & Wilkins.

Kretz O, Fester L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, Prange-Kiel J, Naumann T, Jarry H, Frotscher M, Rune GM (Hippocampal synapses depend on hippocampal estrogen synthesis. J Neurosci 24:5913-5921.2004).

Kronenberg G, Bick-Sander A, Bunk E, Wolf C, Ehninger D, Kempermann G (Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus. Neurobiol Aging 27:1505-1513.2006).

Kuge T, Asayama T, Kakuta S, Murakami K, Ishikawa Y, Kuroda M, Imai T, Seki K, Omoto M, Kishi K (Effect of ethanol on the development and maturation of synapses in the rat hippocampus: a quantitative electron-microscopic study. Environ Res 62:99-105.1993).

La Grange L, Wang M, Watkins R, Ortiz D, Sanchez ME, Konst J, Lee C, Reyes E (Protective effects of the flavonoid mixture, silymarin, on fetal rat brain and liver. J Ethnopharmacol 65:53-61.1999).

Lan N, Yamashita F, Halpert AG, Ellis L, Yu WK, Viau V, Weinberg J (Prenatal ethanol exposure alters the effects of gonadectomy on hypothalamic-pituitary-adrenal activity in male rats. J Neuroendocrinol 18:672-684.2006).

Lan N, Yamashita F, Halpert AG, Sliwowska JH, Viau V, Weinberg J (Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal function across the estrous cycle. Alcohol Clin Exp Res 33:1075-1088.2009).

Lander HM (An essential role for free radicals and derived species in signal transduction. FASEB J 11:118-124.1997).

Larson J, Wong D, Lynch G (Patterned stimulation at the theta frequency is optimal for the induction of hippocampal long-term potentiation. Brain Res 368:347-350.1986).

Lash LH (Mitochondrial glutathione transport: physiological, pathological and toxicological implications. Chem Biol Interact 163:54-67.2006).

Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Muhlen D (Hysterectomy, oophorectomy, and endogenous sex hormone levels in older women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:645-651.2000).

Laurin D, Verreault R, Lindsay J, MacPherson K, Rockwood K (Physical activity and risk of cognitive impairment and dementia in elderly persons. Arch Neurol 58:498-504.2001).

Lee JH, Tajuddin NF, Druse MJ (Effects of ethanol and ipsapirone on the expression of genes encoding anti-apoptotic proteins and an antioxidant enzyme in ethanol-treated neurons. Brain Res 1249:54-60.2009).

Lee MH, Rabe A (Infantile handling eliminates reversal learning deficit in rats prenatally exposed to alcohol. Alcohol 18:49-53.1999).

Lenton KJ, Therriault H, Wagner JR (Analysis of glutathione and glutathione disulfide in whole cells and mitochondria by postcolumn derivatization high-performance liquid chromatography with ortho-phthalaldehyde. Anal Biochem 274:125-130.1999).

Leutgeb JK, Leutgeb S, Moser MB, Moser EI (Pattern separation in the dentate gyrus and CA3 of the hippocampus. Science 315:961-966.2007).

Levine ES, Crozier RA, Black IB, Plummer MR (Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci U S A 95:10235-10239.1998).

Levy WB, Steward O (Synapses as associative memory elements in the hippocampal formation. Brain Res 175:233-245.1979).

Lewen A, Matz P, Chan PH (Free radical pathways in CNS injury. J Neurotrauma 17:871-890.2000).

Liesi P (Ethanol-exposed central neurons fail to migrate and undergo apoptosis. J Neurosci Res 48:439-448.1997).

Lim S, Suzuki H (Changes in maze behavior of mice occur after sufficient accumulation of docosahexaenoic acid in brain. J Nutr 131:319-324.2001).

Lindsley TA, Clarke S (Ethanol withdrawal influences survival and morphology of developing rat hippocampal neurons in vitro. Alcohol Clin Exp Res 28:85-92.2004).

Lindsley TA, Kerlin AM, Rising LJ (Time-lapse analysis of ethanol's effects on axon growth in vitro. Brain Res Dev Brain Res 147:191-199.2003).

Lipp HP, Wolfer DP (Genetically modified mice and cognition. Curr Opin Neurobiol 8:272-280.1998).

Lisman JE, McIntyre CC (Synaptic plasticity: a molecular memory switch. Curr Biol 11:R788-791.2001).

Liu F, Day M, Muniz LC, Bitran D, Arias R, Revilla-Sanchez R, Grauer S, Zhang G, Kelley C, Pulito V, Sung A, Mervis RF, Navarra R, Hirst WD, Reinhart PH, Marquis KL, Moss SJ, Pangalos MN, Brandon NJ (Activation of estrogen receptor-beta regulates hippocampal synaptic plasticity and improves memory. Nat Neurosci 11:334-343.2008).

Liu HL, Zhao G, Cai K, Zhao HH, Shi LD (Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. Behav Brain Res 218:308-314.2011).

Livy DJ, Miller EK, Maier SE, West JR (Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicol Teratol 25:447-458.2003).

Ljubisavljevic S, Stojanovic I, Pavlovic D, Sokolovic D, Stevanovic I (Aminoguanidine and N-acetyl-cysteine supress oxidative and nitrosative stress in EAE rat brains. Redox Rep 16:166-172.2011).

Lochry EA, Riley EP (Department of Psychology, State University of New York at Albany. Neurobehav Toxicol 2:107-115.1980).

Lonergan PE, Martin DS, Horrobin DF, Lynch MA (Neuroprotective effect of eicosapentaenoic acid in hippocampus of rats exposed to gamma-irradiation. J Biol Chem 277:20804-20811.2002).

Lonze BE, Ginty DD (Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.2002).

Lugo JN, Jr., Marino MD, Gass JT, Wilson MA, Kelly SJ (Ethanol exposure during development reduces resident aggression and testosterone in rats. Physiol Behav 87:330-337.2006).

Luo J, Miller MW (Ethanol inhibits basic fibroblast growth factor-mediated proliferation of C6 astrocytoma cells. J Neurochem 67:1448-1456.1996).

Luo J, Miller MW (Growth factor-mediated neural proliferation: target of ethanol toxicity. Brain Res Brain Res Rev 27:157-167.1998).

Lupton C, Burd L, Harwood R (Cost of fetal alcohol spectrum disorders. Am J Med Genet C Semin Med Genet 127C:42-50.2004).

Lynch G, Larson J, Kelso S, Barrionuevo G, Schottler F (Intracellular injections of EGTA block induction of hippocampal long-term potentiation. Nature 305:719-721.1983).

Madeira MD, Lieberman AR (Sexual dimorphism in the mammalian limbic system. Prog Neurobiol 45:275-333.1995).

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554-557.1989).

Malenka RC, Nicoll RA (Long-term potentiation--a decade of progress? Science 285:1870-1874.1999).

Malisza KL, Allman AA, Shiloff D, Jakobson L, Longstaffe S, Chudley AE (Evaluation of spatial working memory function in children and adults with fetal alcohol spectrum disorders: a functional magnetic resonance imaging study. Pediatr Res 58:1150-1157.2005).

Maren S, De Oca B, Fanselow MS (Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. Brain Res 661:25-34.1994).

Marino MD, Aksenov MY, Kelly SJ (Vitamin E protects against alcohol-induced cell loss and oxidative stress in the neonatal rat hippocampus. Int J Dev Neurosci 22:363-377.2004).

Markovic J, Garcia-Gimenez JL, Gimeno A, Vina J, Pallardo FV (Role of glutathione in cell nucleus. Free Radic Res 44:721-733.2010).

Marlatt MW, Potter MC, Lucassen PJ, van Praag H (Running throughout middle-age improves memory function, hippocampal neurogenesis and BDNF levels in female C57Bl/6J mice. Dev Neurobiol.2012).

Marnett LJ (Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 424:83-95.1999).

Marszalek JR, Lodish HF (Docosahexaenoic acid, fatty acid-interacting proteins, and neuronal function: breastmilk and fish are good for you. Annu Rev Cell Dev Biol 21:633-657.2005).

Martin DS, Spencer P, Horrobin DF, Lynch MA (Long-term potentiation in aged rats is restored when the age-related decrease in polyunsaturated fatty acid concentration is reversed. Prostaglandins Leukot Essent Fatty Acids 67:121-130.2002).

Martin SJ, Grimwood PD, Morris RG (Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23:649-711.2000).

Martinez M (Tissue levels of polyunsaturated fatty acids during early human development. J Pediatr 120:S129-138.1992).

Martinez M, Hernandez AI, Martinez N (N-Acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. Brain Res 855:100-106.2000).

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H (Structural basis of long-term potentiation in single dendritic spines. Nature 429:761-766.2004).

Matthews DB, Simson PE (Prenatal exposure to ethanol disrupts spatial memory: effect of the training-testing delay period. Physiol Behav 64:63-67.1998).

Mattson MP (Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. Trends Neurosci 21:53-57.1998).

Mattson SN, Goodman AM, Caine C, Delis DC, Riley EP (Executive functioning in children with heavy prenatal alcohol exposure. Alcohol Clin Exp Res 23:1808-1815.1999).

Mattson SN, Riley EP (Implicit and explicit memory functioning in children with heavy prenatal alcohol exposure. J Int Neuropsychol Soc 5:462-471.1999).

Mattson SN, Riley EP, Gramling L, Delis DC, Jones KL (Neuropsychological comparison of alcohol-exposed children with or without physical features of fetal alcohol syndrome. Neuropsychology 12:146-153.1998).

Mattson SN, Riley EP, Jernigan TL, Garcia A, Kaneko WM, Ehlers CL, Jones KL (A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. Neurotoxicol Teratol 16:283-289.1994).

May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE (Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Dev Disabil Res Rev 15:176-192.2009).

McGahon BM, Martin DS, Horrobin DF, Lynch MA (Age-related changes in synaptic function: analysis of the effect of dietary supplementation with omega-3 fatty acids. Neuroscience 94:305-314.1999).

McGivern RF, Raum WJ, Salido E, Redei E (Lack of prenatal testosterone surge in fetal rats exposed to alcohol: alterations in testicular morphology and physiology. Alcohol Clin Exp Res 12:243-247.1988).

McNaughton BL (Evidence for two physiologically distinct perforant pathways to the fascia dentata. Brain Res 199:1-19.1980).

McNaughton BL, Douglas RM, Goddard GV (Synaptic enhancement in fascia dentata: cooperativity among coactive afferents. Brain Res 157:277-293.1978).

Meister A (Glutathione metabolism and its selective modification. J Biol Chem 263:17205-17208.1988a).

Meister A (On the discovery of glutathione. Trends Biochem Sci 13:185-188.1988b).

Miller MW (Effect of early exposure to ethanol on the protein and DNA contents of specific brain regions in the rat. Brain Res 734:286-294.1996a).

Miller MW (Limited ethanol exposure selectively alters the proliferation of precursor cells in the cerebral cortex. Alcohol Clin Exp Res 20:139-143.1996b).

Miller MW, Dow-Edwards DL (Structural and metabolic alterations in rat cerebral cortex induced by prenatal exposure to ethanol. Brain Res 474:316-326.1988).

Miller MW, Luo J (Effects of ethanol and basic fibroblast growth factor on the transforming growth factor beta1 regulated proliferation of cortical astrocytes and C6 astrocytoma cells. Alcohol Clin Exp Res 26:671-676.2002).

Miller MW, Robertson S (Prenatal exposure to ethanol alters the postnatal development and transformation of radial glia to astrocytes in the cortex. J Comp Neurol 337:253-266.1993).

Minetti A, Arolfo MP, Virgolini MB, Brioni JD, Fulginiti S (Spatial learning in rats exposed to acute ethanol intoxication on gestational day 8. Pharmacol Biochem Behav 53:361-367.1996).

Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, Meyn RE (Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. Oncogene 15:1461-1470.1997).

Molina JC, Moyano HF, Spear LP, Spear NE (Acute alcohol exposure during gestational day 8 in the rat: effects upon physical and behavioral parameters. Alcohol 1:459-464.1984).

Monk BR, Leslie FM, Thomas JD (The effects of perinatal choline supplementation on hippocampal cholinergic development in rats exposed to alcohol during the brain growth spurt. Hippocampus 22:1750-1757.2012).
Montoliu C, Sancho-Tello M, Azorin I, Burgal M, Valles S, Renau-Piqueras J, Guerri C (Ethanol increases cytochrome P4502E1 and induces oxidative stress in astrocytes. J Neurochem 65:2561-2570.1995).
Montuschi P, Barnes PJ, Roberts LJ, 2nd (Isoprostanes: markers and mediators of oxidative stress. FASEB J 18:1791-1800.2004).
Moosmann B, Behl C (The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. Proc Natl Acad Sci U S A 96:8867-8872.1999).
Morris JA, Jordan CL, Breedlove SM (Sexual differentiation of the vertebrate nervous system. Nat Neurosci 7:1034-1039.2004).
Morris RG, Anderson E, Lynch GS, Baudry M (Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319:774-776.1986).
Morris RG, Garrud P, Rawlins JN, O'Keefe J (Place navigation impaired in rats with hippocampal lesions. Nature 297:681-683.1982).
Morrisett RA, Martin D, Wilson WA, Savage DD, Swartzwelder HS (Prenatal exposure to ethanol decreases the sensitivity of the adult rat hippocampus to N-methyl-D-aspartate. Alcohol 6:415-420.1989).
Muller RU, Kubie JL (The effects of changes in the environment on the spatial firing of hippocampal complex-spike cells. J Neurosci 7:1951-1968.1987).
Murawski NJ, Stanton ME (Variants of contextual fear conditioning are differentially impaired in the juvenile rat by binge ethanol exposure on postnatal days 4-9. Behav Brain Res 212:133-142.2010).
Murphy DD, Cole NB, Greenberger V, Segal M (Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. J Neurosci 18:2550-2559.1998).
Naber PA, Lopes da Silva FH, Witter MP (Reciprocal connections between the entorhinal cortex and hippocampal fields CA1 and the subiculum are in register with the projections from CA1 to the subiculum. Hippocampus 11:99-104.2001).
Nagahara AH, Handa RJ (Fetal alcohol exposure produces delay-dependent memory deficits in juvenile and adult rats. Alcohol Clin Exp Res 21:710-715.1997).
Nakazawa K, Quirk MC, Chitwood RA, Watanabe M, Yeckel MF, Sun LD, Kato A, Carr CA, Johnston D, Wilson MA, Tonegawa S (Requirement for hippocampal CA3 NMDA receptors in associative memory recall. Science 297:211-218.2002).
Nava-Ocampo AA, Velazquez-Armenta Y, Brien JF, Koren G (Elimination kinetics of ethanol in pregnant women. Reprod Toxicol 18:613-617.2004).
Nehru B, Kanwar SS (Modulation by N-acetylcysteine of lead-induced alterations in rat brain: reduced glutathione levels and morphology. Toxicol Mech Methods 17:289-293.2007).
Nguyen PV, Kandel ER (A macromolecular synthesis-dependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. J Neurosci 16:3189-3198.1996).

O'Hare ED, Lu LH, Houston SM, Bookheimer SY, Mattson SN, O'Connor MJ, Sowell ER (Altered frontal-parietal functioning during verbal working memory in children and adolescents with heavy prenatal alcohol exposure. Hum Brain Mapp 30:3200-3208.2009).

O'Keefe J, Conway DH (Hippocampal place units in the freely moving rat: why they fire where they fire. Exp Brain Res 31:573-590.1978).

O'Keefe J, Dostrovsky J (The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res 34:171-175.1971).

O'Leary-Moore SK, McMechan AP, Mathison SN, Berman RF, Hannigan JH (Reversal learning after prenatal or early postnatal alcohol exposure in juvenile and adult rats. Alcohol 38:99-110.2006).

O'Leary CM, Bower C, Zubrick SR, Geelhoed E, Kurinczuk JJ, Nassar N (A new method of prenatal alcohol classification accounting for dose, pattern and timing of exposure: improving our ability to examine fetal effects from low to moderate alcohol. J Epidemiol Community Health 64:956-962.2010a).

O'Leary CM, Nassar N, Kurinczuk JJ, de Klerk N, Geelhoed E, Elliott EJ, Bower C (Prenatal alcohol exposure and risk of birth defects. Pediatrics 126:e843-850.2010b).

O'Reilly RC, Norman KA (Hippocampal and neocortical contributions to memory: advances in the complementary learning systems framework. Trends Cogn Sci 6:505-510.2002).

Ogita K, Enomoto R, Nakahara F, Ishitsubo N, Yoneda Y (A possible role of glutathione as an endogenous agonist at the N-methyl-D-aspartate recognition domain in rat brain. J Neurochem 64:1088-1096.1995).

Ohkawa H, Ohishi N, Yagi K (Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358.1979).

Oken E, Wright RO, Kleinman KP, Bellinger D, Amarasiriwardena CJ, Hu H, Rich-Edwards JW, Gillman MW (Maternal fish consumption, hair mercury, and infant cognition in a U.S. Cohort. Environ Health Perspect 113:1376-1380.2005).

Olson HC, Feldman JJ, Streissguth AP, Sampson PD, Bookstein FL (Neuropsychological deficits in adolescents with fetal alcohol syndrome: clinical findings. Alcohol Clin Exp Res 22:1998-2012.1998).

Ooishi Y, Kawato S, Hojo Y, Hatanaka Y, Higo S, Murakami G, Komatsuzaki Y, Ogiue-Ikeda M, Kimoto T, Mukai H (Modulation of synaptic plasticity in the hippocampus by hippocampus-derived estrogen and androgen. J Steroid Biochem Mol Biol 131:37-51.2012).

Otero NK, Thomas JD, Saski CA, Xia X, Kelly SJ (Choline supplementation and DNA methylation in the hippocampus and prefrontal cortex of rats exposed to alcohol during development. Alcohol Clin Exp Res 36:1701-1709.2012).

Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (Mitochondria, oxidative stress and cell death. Apoptosis 12:913-922.2007).

Pacher P, Beckman JS, Liaudet L (Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315-424.2007).

Pallardo FV, Markovic J, Garcia JL, Vina J (Role of nuclear glutathione as a key regulator of cell proliferation. Mol Aspects Med 30:77-85.2009).

Pan HC, Kao TK, Ou YC, Yang DY, Yen YJ, Wang CC, Chuang YH, Liao SL, Raung SL, Wu CW, Chiang AN, Chen CJ (Protective effect of docosahexaenoic acid against brain injury in ischemic rats. J Nutr Biochem 20:715-725.2009).

Paoletti P, Neyton J (NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol 7:39-47.2007).

Papez JW (A proposed mechanism of emotion. 1937. J Neuropsychiatry Clin Neurosci 7:103-112.1995).

Parachikova A, Green KN, Hendrix C, LaFerla FM (Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. PLoS One 5:e14015.2010).

Patten A, Sickmann, HM., Dyer, R., Innis, SM., Christie, BR. (Omega-3 Fatty Acids Reverse the Long-Term Deficits in Hippocampal Synaptic Plasticity Caused by Prenatal Ethanol Exposure. Neuroscience Letters (Submitted).2013).

Patten AR, Brocardo PS, Christie BR (Omega-3 supplementation can restore glutathione levels and prevent oxidative damage caused by prenatal ethanol exposure. J Nutr Biochem.2012).

Pauli J, Wilce P, Bedi KS (Spatial learning ability of rats following acute exposure to alcohol during early postnatal life. Physiol Behav 58:1013-1020.1995).

Pavlides C, Greenstein YJ, Grudman M, Winson J (Long-term potentiation in the dentate gyrus is induced preferentially on the positive phase of theta-rhythm. Brain Res 439:383-387.1988).

Pawlosky RJ, Salem N, Jr. (Ethanol exposure causes a decrease in docosahexaenoic acid and an increase in docosapentaenoic acid in feline brains and retinas. Am J Clin Nutr 61:1284-1289.1995).

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. San Diego: Academic Press.

Pellmar TC, Hollinden GE, Sarvey JM (Free radicals accelerate the decay of long-term potentiation in field CA1 of guinea-pig hippocampus. Neuroscience 44:353-359.1991).

Penugonda S, Ercal N (Comparative evaluation of N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA) on glutamate and lead-induced toxicity in CD-1 mice. Toxicol Lett 201:1-7.2011).

Perez HD, Villanueva JE, Salas JM (Behavioral and Hippocampal Morphological Changes Induced by Ethanol Administered to Pregnant Rats. Annals of the New York Academy of Sciences 625:300-304.1991).

Perin-Dureau F, Rachline J, Neyton J, Paoletti P (Mapping the binding site of the neuroprotectant ifenprodil on NMDA receptors. J Neurosci 22:5955-5965.2002).

Peuchen S, Bolanos JP, Heales SJ, Almeida A, Duchen MR, Clark JB (Interrelationships between astrocyte function, oxidative stress and antioxidant status within the central nervous system. Prog Neurobiol 52:261-281.1997).

Phillips DE (Effects of limited postnatal ethanol exposure on the development of myelin and nerve fibers in rat optic nerve. Exp Neurol 103:90-100.1989).

Pick CG, Cooperman M, Trombka D, Rogel-Fuchs Y, Yanai J (Hippocampal cholinergic alterations and related behavioral deficits after early exposure to ethanol. Int J Dev Neurosci 11:379-385.1993).

Plummer JL, Smith BR, Sies H, Bend JR (Chemical depletion of glutathione in vivo. Methods Enzymol 77:50-59.1981).

Pokorny J, Yamamoto T (Postnatal ontogenesis of hippocampal CA1 area in rats. II. Development of ultrastructure in stratum lacunosum and moleculare. Brain Res Bull 7:121-130.1981).

Popovic M, Caballero-Bleda M, Guerri C (Adult rat's offspring of alcoholic mothers are impaired on spatial learning and object recognition in the Can test. Behav Brain Res 174:101-111.2006).

Potts MB, Rola R, Claus CP, Ferriero DM, Fike JR, Noble-Haeusslein LJ (Glutathione peroxidase overexpression does not rescue impaired neurogenesis in the injured immature brain. J Neurosci Res 87:1848-1857.2009).

Prange-Kiel J, Wehrenberg U, Jarry H, Rune GM (Para/autocrine regulation of estrogen receptors in hippocampal neurons. Hippocampus 13:226-234.2003).

Prevention CfDCa (Alcohol Use Among Pregnant and Nonpregnant Women of Childbearing Age - United States, 1991-2005. MMWR Morb Mortal Wkly Rep 58:529-532.2009).

Puglia MP, Valenzuela CF (Ethanol acutely inhibits ionotropic glutamate receptor-mediated responses and long-term potentiation in the developing CA1 hippocampus. Alcohol Clin Exp Res 34:594-606.2010a).

Puglia MP, Valenzuela CF (Repeated third trimester-equivalent ethanol exposure inhibits long-term potentiation in the hippocampal CA1 region of neonatal rats. Alcohol 44:283-290.2010b).

Ramachandran V, Perez A, Chen J, Senthil D, Schenker S, Henderson GI (In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal. Alcohol Clin Exp Res 25:862-871.2001).

Ramachandran V, Watts LT, Maffi SK, Chen J, Schenker S, Henderson G (Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons. J Neurosci Res 74:577-588.2003).

Ratan RR, Murphy TH, Baraban JM (Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem 62:376-379.1994).

Redila VA, Christie BR (Exercise-induced changes in dendritic structure and complexity in the adult hippocampal dentate gyrus. Neuroscience 137:1299-1307.2006).

Redila VA, Olson AK, Swann SE, Mohades G, Webber AJ, Weinberg J, Christie BR (Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. Hippocampus 16:305-311.2006).

Reid C, Edwards J, Wang M, Manybeads Y, Mike L, Martinez N, La Grange L, Reyes E (Prevention by a silymarin/phospholipid compound of ethanol-induced social learning deficits in rats. Planta Med 65:421-424.1999).

Reyes E, Ott S, Robinson B (Effects of in utero administration of alcohol on glutathione levels in brain and liver. Alcohol Clin Exp Res 17:877-881.1993).

Reyes E, Wolfe J, Marquez M (Effects of prenatal alcohol on gamma-glutamyl transpeptidase in various brain regions. Physiol Behav 46:49-53.1989a).

Reyes E, Wolfe J, Savage DD (The effects of prenatal alcohol exposure on radial arm maze performance in adult rats. Physiol Behav 46:45-48.1989b).

Rice D, Barone S, Jr. (Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108 Suppl 3:511-533.2000).

Richardson DP, Byrnes ML, Brien JF, Reynolds JN, Dringenberg HC (Impaired acquisition in the water maze and hippocampal long-term potentiation after chronic prenatal ethanol exposure in the guinea-pig. Eur J Neurosci 16:1593-1598.2002).

Richman PG, Meister A (Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem 250:1422-1426.1975).

Riikonen R, Salonen I, Partanen K, Verho S (Brain perfusion SPECT and MRI in foetal alcohol syndrome. Dev Med Child Neurol 41:652-659.1999).

Riikonen RS, Nokelainen P, Valkonen K, Kolehmainen AI, Kumpulainen KI, Kononen M, Vanninen RL, Kuikka JT (Deep serotonergic and dopaminergic structures in fetal alcoholic syndrome: a study with nor-beta-CIT-single-photon emission computed tomography and magnetic resonance imaging volumetry. Biol Psychiatry 57:1565-1572.2005).

Riley EP (The long-term behavioral effects of prenatal alcohol exposure in rats. Alcohol Clin Exp Res 14:670-673.1990).

Riley EP, Lochry EA, Shapiro NR, Baldwin J (Response perseveration in rats exposed to alcohol prenatally. Pharmacol Biochem Behav 10:255-259.1979).

Riley EP, Vorhees CV (1986) Handbook of behavioral teratology: Plenum Press.

Robillard JM, Gordon GR, Choi HB, Christie BR, MacVicar BA (Glutathione restores the mechanism of synaptic plasticity in aged mice to that of the adult. PLoS One 6:e20676.2011).

Rose GM, Dunwiddie TV (Induction of hippocampal long-term potentiation using physiologically patterned stimulation. Neurosci Lett 69:244-248.1986).

Rubert G, Minana R, Pascual M, Guerri C (Ethanol exposure during embryogenesis decreases the radial glial progenitorpool and affects the generation of neurons and astrocytes. J Neurosci Res 84:483-496.2006).

Sarsilmaz M, Songur A, Ozyurt H, Kus I, Ozen OA, Ozyurt B, Sogut S, Akyol O (Potential role of dietary omega-3 essential fatty acids on some oxidant/antioxidant parameters in rats' corpus striatum. Prostaglandins Leukot Essent Fatty Acids 69:253-259.2003).

Sarsimaz M, Songur A, Kus I, Ozyurt B, Gulec M, Sogut S, Ilhan A, Akyol O (The regulatory role of dietary ω-3 essential fatty acids on oxidant/antioxidant balance in rat hippocampus. Neuroscience Research Communications 33:114-123.2003).

Savage DD, Montano CY, Otero MA, Paxton LL (Prenatal ethanol exposure decreases hippocampal NMDA-sensitive [3H]-glutamate binding site density in 45-day-old rats. Alcohol 8:193-201.1991).

Scoville WB, Milner B (Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry 20:11-21.1957).

Sen CK, Packer L (Antioxidant and redox regulation of gene transcription. FASEB J 10:709-720.1996).

Seress L (Comparative anatomy of the hippocampal dentate gyrus in adult and developing rodents, non-human primates and humans. Prog Brain Res 163:23-41.2007).

Serrano F, Klann E (Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Res Rev 3:431-443.2004).

Sharma G, Stevens CF (Interactions between two divalent ion binding sites in N-methyl-D-aspartate receptor channels. Proc Natl Acad Sci U S A 93:14170-14175.1996).

Shibley IA, Jr., Pennington SN (Metabolic and mitotic changes associated with the fetal alcohol syndrome. Alcohol Alcohol 32:423-434.1997).

Sickmann H, Patten, AR, Morch, K., Sawchuk, S., Zhang, C., Parton, R., Svlavik, L., Christie, BR. (Gender-differences in hippocampal long-term potentiation in adult rats following prenatal ethanol exposure. Hippocampus (Submitted).2013).

Siegenthaler JA, Miller MW (Ethanol disrupts cell cycle regulation in developing rat cortex interaction with transforming growth factor beta1. J Neurochem 95:902-912.2005).

Sies H (Oxidative stress: from basic research to clinical application. Am J Med 91:31S-38S.1991).

Siler-Marsiglio KI, Pan Q, Paiva M, Madorsky I, Khurana NC, Heaton MB (Mitochondrially targeted vitamin E and vitamin E mitigate ethanol-mediated effects on cerebellar granule cell antioxidant defense systems. Brain Res 1052:202-211.2005).

Simopoulos AP (Omega-3 fatty acids, the brain and retina. Preface. World Rev Nutr Diet 99:VII-XII.2009a).

Simopoulos AP (Omega-6/omega-3 essential fatty acids: biological effects. World Rev Nutr Diet 99:1-16.2009b).

Singh M (Essential fatty acids, DHA and human brain. Indian J Pediatr 72:239-242.2005).

Smith DE, Davies DL (Effect of perinatal administration of ethanol on the CA1 pyramidal cell of the hippocampus and Purkinje cell of the cerebellum: an ultrastructural survey. J Neurocytol 19:708-717.1990).

Snyder AK, Jiang F, Singh SP (Effects of ethanol on glucose utilization by cultured mammalian embryos. Alcohol Clin Exp Res 16:466-470.1992).

Snyder AK, Singh SP (Effects of ethanol on glucose turnover in pregnant rats. Metabolism 38:149-152.1989).

Sokol RJ, Delaney-Black V, Nordstrom B (Fetal alcohol spectrum disorder. JAMA 290:2996-2999.2003).

Sokoloff L (Energetics of functional activation in neural tissues. Neurochem Res 24:321-329.1999).

Songur A, Sarsilmaz M, Sogut S, Ozyurt B, Ozyurt H, Zararsiz I, Turkoglu AO (Hypothalamic superoxide dismutase, xanthine oxidase, nitric oxide, and malondialdehyde in rats fed with fish omega-3 fatty acids. Prog Neuropsychopharmacol Biol Psychiatry 28:693-698.2004).

Sowell ER, Lu LH, O'Hare ED, McCourt ST, Mattson SN, O'Connor MJ, Bookheimer SY (Functional magnetic resonance imaging of verbal learning in children with heavy prenatal alcohol exposure. Neuroreport 18:635-639.2007).

Spadoni AD, Bazinet AD, Fryer SL, Tapert SF, Mattson SN, Riley EP (BOLD response during spatial working memory in youth with heavy prenatal alcohol exposure. Alcohol Clin Exp Res 33:2067-2076.2009).

Spencer JL, Waters EM, Milner TA, McEwen BS (Estrous cycle regulates activation of hippocampal Akt, LIM kinase, and neurotrophin receptors in C57BL/6 mice. Neuroscience 155:1106-1119.2008).

Spencer JP, Jenner P, Daniel SE, Lees AJ, Marsden DC, Halliwell B (Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: possible mechanisms of formation involving reactive oxygen species. J Neurochem 71:2112-2122.1998).

Stade B, Ali A, Bennett D, Campbell D, Johnston M, Lens C, Tran S, Koren G (The burden of prenatal exposure to alcohol: revised measurement of cost. Can J Clin Pharmacol 16:e91-102.2009).

Stadtman ER, Levine RL (Protein oxidation. Ann N Y Acad Sci 899:191-208.2000).

Stadtman ER, Oliver CN (Metal-catalyzed oxidation of proteins. Physiological consequences. J Biol Chem 266:2005-2008.1991).

Staubli U, Scafidi J (Time-dependent reversal of long-term potentiation in area CA1 of the freely moving rat induced by theta pulse stimulation. J Neurosci 19:8712-8719.1999).

Steullet P, Neijt HC, Cuenod M, Do KQ (Synaptic plasticity impairment and hypofunction of NMDA receptors induced by glutathione deficit: relevance to schizophrenia. Neuroscience 137:807-819.2006).

Steward O, Falk PM (Selective localization of polyribosomes beneath developing synapses: a quantitative analysis of the relationships between polyribosomes and developing synapses in the hippocampus and dentate gyrus. J Comp Neurol 314:545-557.1991).

Stone WS, Altman HJ, Hall J, Arankowsky-Sandoval G, Parekh P, Gold PE (Prenatal exposure to alcohol in adult rats: relationships between sleep and memory deficits, and effects of glucose administration on memory. Brain Res 742:98-106.1996).

Streissguth AP, Aase JM, Clarren SK, Randels SP, LaDue RA, Smith DF (Fetal alcohol syndrome in adolescents and adults. JAMA 265:1961-1967.1991).

Streissguth AP, Barr HM, Olson HC, Sampson PD, Bookstein FL, Burgess DM (Drinking during pregnancy decreases word attack and arithmetic scores on standardized tests: adolescent data from a population-based prospective study. Alcohol Clin Exp Res 18:248-254.1994).

Streissguth AP, Barr HM, Sampson PD (Moderate prenatal alcohol exposure: effects on child IQ and learning problems at age 7 1/2 years. Alcohol Clin Exp Res 14:662-669.1990).

Streissguth AP, Bookstein FL, Sampson PD, Barr HM (Neurobehavioral effects of prenatal alcohol: Part III. PLS analyses of neuropsychologic tests. Neurotoxicol Teratol 11:493-507.1989).

Streissguth AP, LaDue RA (Fetal alcohol. Teratogenic causes of developmental disabilities. Monogr Am Assoc Ment Defic 1-32.1987).

Suchy J, Chan A, Shea TB (Dietary supplementation with a combination of alpha-lipoic acid, acetyl-L-carnitine, glycerophosphocoline, docosahexaenoic acid, and

phosphatidylserine reduces oxidative damage to murine brain and improves cognitive performance. Nutr Res 29:70-74.2009).

Sulik KK (Genesis of alcohol-induced craniofacial dysmorphism. Exp Biol Med (Maywood) 230:366-375.2005).

Sulik KK, Johnston MC, Webb MA (Fetal alcohol syndrome: embryogenesis in a mouse model. Science 214:936-938.1981).

Sun X, Shih AY, Johannssen HC, Erb H, Li P, Murphy TH (Two-photon imaging of glutathione levels in intact brain indicates enhanced redox buffering in developing neurons and cells at the cerebrospinal fluid and blood-brain interface. J Biol Chem 281:17420-17431.2006).

Sun Y, Oberley LW (Redox regulation of transcriptional activators. Free Radic Biol Med 21:335-348.1996).

Sutcliffe JS (Female rats are smarter than males: influence of test, oestrogen receptor subtypes and glutamate. Curr Top Behav Neurosci 8:37-56.2011).

Sutherland RJ, McDonald RJ, Savage DD (Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. Hippocampus 7:232-238.1997).

Suzuki K (Neuropathology of developmental abnormalities. Brain Dev 29:129-141.2007).

Swartzwelder HS, Farr KL, Wilson WA, Savage DD (Prenatal exposure to ethanol decreases physiological plasticity in the hippocampus of the adult rat. Alcohol 5:121-124.1988).

Swayze VW, 2nd, Johnson VP, Hanson JW, Piven J, Sato Y, Giedd JN, Mosnik D, Andreasen NC (Magnetic resonance imaging of brain anomalies in fetal alcohol syndrome. Pediatrics 99:232-240.1997).

Sweatt JD (The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem 76:1-10.2001).

Sweatt JD (2003) Mechanisms of Memory: Academic Press.

Swindle EJ, Metcalfe DD (The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. Immunol Rev 217:186-205.2007).

Tan S, Wood M, Maher P (Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem 71:95-105.1998).

Tan SE, Berman RF, Abel EL, Zajac CS (Prenatal alcohol exposure alters hippocampal slice electrophysiology. Alcohol 7:507-511.1990).

Tanaka H, Nasu F, Inomata K (Fetal alcohol effects: decreased synaptic formations in the field CA3 of fetal hippocampus. Int J Dev Neurosci 9:509-517.1991).

Tang LH, Aizenman E (The modulation of N-methyl-D-aspartate receptors by redox and alkylating reagents in rat cortical neurones in vitro. J Physiol 465:303-323.1993).

Taube JS, Muller RU, Ranck JB, Jr. (Head-direction cells recorded from the postsubiculum in freely moving rats. II. Effects of environmental manipulations. J Neurosci 10:436-447.1990).

Thomas JD, Idrus NM, Monk BR, Dominguez HD (Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. Birth Defects Res A Clin Mol Teratol 88:827-837.2010).

Thomas JD, Sather TM, Whinery LA (Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt. Behav Neurosci 122:1264-1273.2008).

Thomas JD, Tran TD (Choline supplementation mitigates trace, but not delay, eyeblink conditioning deficits in rats exposed to alcohol during development. Hippocampus 22:619-630.2012).

Thomas JD, Wasserman EA, West JR, Goodlett CR (Behavioral deficits induced by bingelike exposure to alcohol in neonatal rats: importance of developmental timing and number of episodes. Dev Psychobiol 29:433-452.1996).

Thomson AB, Keelan M, Clandinin MT (Feeding rats a diet enriched with saturated fatty acids prevents the inhibitory effects of acute and chronic ethanol exposure on the in vitro uptake of hexoses and lipids. Biochim Biophys Acta 1084:122-128.1991).

Tietze F (Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27:502-522.1969).

Titterness AK, Christie BR (Prenatal ethanol exposure enhances NMDAR-dependent long-term potentiation in the adolescent female dentate gyrus. Hippocampus 22:69-81.2012).

Titterness AK, Wiebe E, Kwasnica A, Keyes G, Christie BR (Voluntary exercise does not enhance long-term potentiation in the adolescent female dentate gyrus. Neuroscience 183:25-31.2011).

Tomlinson D, Wilce P, Bedi KS (Spatial learning ability of rats following differing levels of exposure to alcohol during early postnatal life. Physiol Behav 63:205-211.1998).

Tran TD, Kelly SJ (Critical periods for ethanol-induced cell loss in the hippocampal formation. Neurotoxicol Teratol 25:519-528.2003).

Treves A, Tashiro A, Witter MP, Moser EI (What is the mammalian dentate gyrus good for? Neuroscience 154:1155-1172.2008).

Uban KA, Sliwowska JH, Lieblich S, Ellis LA, Yu WK, Weinberg J, Galea LA (Prenatal alcohol exposure reduces the proportion of newly produced neurons and glia in the dentate gyrus of the hippocampus in female rats. Horm Behav 58:835-843.2010).

Udani M, Parker S, Gavaler J, Van Thiel DH (Effects of in utero exposure to alcohol upon male rats. Alcohol Clin Exp Res 9:355-359.1985).

Uecker A, Nadel L (Spatial locations gone awry: object and spatial memory deficits in children with fetal alcohol syndrome. Neuropsychologia 34:209-223.1996).

Uecker A, Nadel L (Spatial but not object memory impairments in children with fetal alcohol syndrome. Am J Ment Retard 103:12-18.1998).

Vaillend C, Billard JM, Laroche S (Impaired long-term spatial and recognition memory and enhanced CA1 hippocampal LTP in the dystrophin-deficient Dmd(mdx) mouse. Neurobiol Dis 17:10-20.2004).

van Praag H, Christie BR, Sejnowski TJ, Gage FH (Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96:13427-13431.1999a).

van Praag H, Kempermann G, Gage FH (Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266-270.1999b).

Vanderwolf CH (Hippocampal electrical activity and voluntary movement in the rat. Electroencephalogr Clin Neurophysiol 26:407-418.1969).

Varaschin RK, Akers KG, Rosenberg MJ, Hamilton DA, Savage DD (Effects of the cognition-enhancing agent ABT-239 on fetal ethanol-induced deficits in dentate gyrus synaptic plasticity. J Pharmacol Exp Ther 334:191-198.2010).

Vargha-Khadem F, Gadian DG, Watkins KE, Connelly A, Van Paesschen W, Mishkin M (Differential effects of early hippocampal pathology on episodic and semantic memory. Science 277:376-380.1997).

Vierk R, Glassmeier G, Zhou L, Brandt N, Fester L, Dudzinski D, Wilkars W, Bender RA, Lewerenz M, Gloger S, Graser L, Schwarz J, Rune GM (Aromatase inhibition abolishes LTP generation in female but not in male mice. J Neurosci 32:8116-8126.2012).

Viggiano A, Viggiano E, Monda M, Ascione S, Amaro S, De Luca B (Intracerebroventricular injection of oxidant and antioxidant molecules affects long-term potentiation in urethane anaesthetized rats. Physiol Res 57:269-273.2008).

Wainwright PE, Huang YS, Simmons V, Mills DE, Ward RP, Ward GR, Winfield D, McCutcheon D (Effects of prenatal ethanol and long-chain n-3 fatty acid supplementation on development in mice. 2. Fatty acid composition of brain membrane phospholipids. Alcohol Clin Exp Res 14:413-420.1990).

Wall R, Ross RP, Fitzgerald GF, Stanton C (Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 68:280-289.2010).

Wang HJ, Zakhari S, Jung MK (Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. World J Gastroenterol 16:1304-1313.2010).

Wang X, Zhao X, Mao ZY, Wang XM, Liu ZL (Neuroprotective effect of docosahexaenoic acid on glutamate-induced cytotoxicity in rat hippocampal cultures. Neuroreport 14:2457-2461.2003).

Ward IL, Ward OB, Winn RJ, Bielawski D (Male and female sexual behavior potential of male rats prenatally exposed to the influence of alcohol, stress, or both factors. Behav Neurosci 108:1188-1195.1994).

Weber CA, Duncan CA, Lyons MJ, Jenkinson SG (Depletion of tissue glutathione with diethyl maleate enhances hyperbaric oxygen toxicity. Am J Physiol 258:L308-312.1990).

Wehrenberg U, Prange-Kiel J, Rune GM (Steroidogenic factor-1 expression in marmoset and rat hippocampus: co-localization with StAR and aromatase. J Neurochem 76:1879-1886.2001).

Weiland NG, Orikasa C, Hayashi S, McEwen BS (Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats. J Comp Neurol 388:603-612.1997).

Weinberg J (Effects of ethanol and maternal nutritional status on fetal development. Alcohol Clin Exp Res 9:49-55.1985).

Weinberg J, Gallo PV (Prenatal ethanol exposure: pituitary-adrenal activity in pregnant dams and offspring. Neurobehav Toxicol Teratol 4:515-520.1982).

Weinberg J, Taylor AN, Gianoulakis C (Fetal ethanol exposure: hypothalamic-pituitary-adrenal and beta-endorphin responses to repeated stress. Alcohol Clin Exp Res 20:122-131.1996).

Wen Z, Kim HY (Alterations in hippocampal phospholipid profile by prenatal exposure to ethanol. J Neurochem 89:1368-1377.2004).

Wendel A (Glutathione peroxidase. Methods Enzymol 77:325-333.1981).

Weniger JP, Zeis A, Chouraqui J (Estrogen production by fetal and infantile rat ovaries. Reprod Nutr Dev 33:129-136.1993).

West JR (Fetal alcohol-induced brain damage and the problem of determining temporal vulnerability: a review. Alcohol Drug Res 7:423-441.1987).

West JR, Dewey SL, Pierce DR, Black AC, Jr. (Prenatal and early postnatal exposure to ethanol permanently alters the rat hippocampus. Ciba Found Symp 105:8-25.1984).

West JR, Goodlett CR (Teratogenic effects of alcohol on brain development. Ann Med 22:319-325.1990).

West JR, Hamre KM (Effects of alcohol exposure during different periods of development: changes in hippocampal mossy fibers. Brain Res 349:280-284.1985).

Westergren S, Rydenhag B, Bassen M, Archer T, Conradi NG (Effects of prenatal alcohol exposure on activity and learning in Sprague-Dawley rats. Pharmacol Biochem Behav 55:515-520.1996).

Wigal T, Amsel A (Behavioral and neuroanatomical effects of prenatal, postnatal, or combined exposure to ethanol in weanling rats. Behav Neurosci 104:116-126.1990).

Willoughby KA, Sheard ED, Nash K, Rovet J (Effects of prenatal alcohol exposure on hippocampal volume, verbal learning, and verbal and spatial recall in late childhood. J Int Neuropsychol Soc 14:1022-1033.2008).

Wilson CA, Davies DC (The control of sexual differentiation of the reproductive system and brain. Reproduction 133:331-359.2007).

Winson J (Patterns of hippocampal theta rhythm in the freely moving rat. Electroencephalogr Clin Neurophysiol 36:291-301.1974).

Winson J (Loss of hippocampal theta rhythm results in spatial memory deficit in the rat. Science 201:160-163.1978).

Winter B, Breitenstein C, Mooren FC, Voelker K, Fobker M, Lechtermann A, Krueger K, Fromme A, Korsukewitz C, Floel A, Knecht S (High impact running improves learning. Neurobiol Learn Mem 87:597-609.2007).

Wong M, Moss RL (Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. J Neurosci 12:3217-3225.1992).

Woolley CS, McEwen BS (Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12:2549-2554.1992).

Woolley CS, Weiland NG, McEwen BS, Schwartzkroin PA (Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. J Neurosci 17:1848-1859.1997).

Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ (Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. Neurobiol Dis 17:403-414.2004).

Wu A, Ying Z, Gomez-Pinilla F (Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. J Neurotrauma 21:1457-1467.2004).

Wu A, Ying Z, Gomez-Pinilla F (Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neuroscience 155:751-759.2008).

Wu J, Rowan MJ, Anwyl R (Long-term potentiation is mediated by multiple kinase cascades involving CaMKII or either PKA or p42/44 MAPK in the adult rat dentate gyrus in vitro. J Neurophysiol 95:3519-3527.2006).

Xie C, Lovell MA, Markesbery WR (Glutathione transferase protects neuronal cultures against four hydroxynonenal toxicity. Free Radic Biol Med 25:979-988.1998).

Yang SN, Tang YG, Zucker RS (Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. J Neurophysiol 81:781-787.1999).

Yang YJ, Wu PF, Long LH, Yu DF, Wu WN, Hu ZL, Fu H, Xie N, Jin Y, Ni L, Wang JZ, Wang F, Chen JG (Reversal of aging-associated hippocampal synaptic plasticity deficits by reductants via regulation of thiol redox and NMDA receptor function. Aging Cell 9:709-721.2010).

Yanni PA, Lindsley TA (Ethanol inhibits development of dendrites and synapses in rat hippocampal pyramidal neuron cultures. Brain Res Dev Brain Res 120:233-243.2000).

Yuan L, Kaplowitz N (Glutathione in liver diseases and hepatotoxicity. Mol Aspects Med 30:29-41.2009).

Zadak Z, Hyspler R, Ticha A, Hronek M, Fikrova P, Rathouska J, Hrnciarikova D, Stetina R (Antioxidants and vitamins in clinical conditions. Physiol Res 58 Suppl 1:S13-17.2009).

Zararsiz I, Kus I, Akpolat N, Songur A, Ogeturk M, Sarsilmaz M (Protective effects of omega-3 essential fatty acids against formaldehyde-induced neuronal damage in prefrontal cortex of rats. Cell Biochem Funct 24:237-244.2006).

Zeevalk GD, Manzino L, Sonsalla PK, Bernard LP (Characterization of intracellular elevation of glutathione (GSH) with glutathione monoethyl ester and GSH in brain and neuronal cultures: relevance to Parkinson's disease. Exp Neurol 203:512-520.2007).

Zhang FX, Rubin R, Rooney TA (Ethanol induces apoptosis in cerebellar granule neurons by inhibiting insulin-like growth factor 1 signaling. J Neurochem 71:196-204.1998).

Ziment I (Acetylcysteine: a drug that is much more than a mucokinetic. Biomed Pharmacother 42:513-519.1988).

Zimmerberg B, Mattson S, Riley EP (Impaired alternation test performance in adult rats following prenatal alcohol exposure. Pharmacol Biochem Behav 32:293-299.1989).

Zimmerberg B, Sukel HL, Stekler JD (Spatial learning of adult rats with fetal alcohol exposure: deficits are sex-dependent. Behav Brain Res 42:49-56.1991).