Not All is Lost; Prenatal Ethanol Exposure Impairs Bidirectional Synaptic Plasticity in the Juvenile Dentate Gyrus

by

Christine Jessie Fontaine
B.Sc. Honours, Memorial University of Newfoundland, 2013

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Supervisory Committee

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Abstract

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Fetal alcohol spectrum disorders (FASDs) are among the leading preventable disorders in North America and are caused by prenatal ethanol exposure (PNEE). Ethanol is a teratogen, and prenatal exposure leads to structural and functional impairments that depend on the amount, timing and duration of exposure. PNEE is commonly associated with learning and memory impairments, which are paralleled by deficits in synaptic plasticity. A number of studies have shown deficits in long-term potentiation (LTP) of synaptic plasticity in the hippocampus, however, to date few studies have determined how PNEE impacts long-term depression (LTD). Here, we examine the effect of PNEE on the dynamic range of synaptic plasticity, by studying both LTP and LTD in the juvenile Dentate Gyrus (DG) of male and female offspring. We find that PNEE impairs N-methyl-D-aspartate receptor (NMDAR)-dependent LTP in both sexes. This appears to be the result of a change in the threshold for induction, as increasing the amount of stimuli administered can restore the LTP to control levels. We found that LTD was significantly reduced in male, but not female, offspring following PNEE. As with LTP, these deficits could be rescued by increasing the stimulation used to elicit synaptic depression. Unlike LTP, which was NMDAR dependent, LTD induction required the activation of both metabotropic glutamate 5 receptors ($mGluR_5$) and cannabinoid type 1 (CB1) receptors. These data are the first to describe the impact of PNEE on the dynamic range of synaptic plasticity in the DG of juvenile male and female offspring. The findings in this dissertation further describe the potential mechanistic underpinnings of learning and memory deficits, and help identify new therapeutic targets to examine for enhancing hippocampal function in young people afflicted with FASD.

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2-AG	2-arachidonoyl-glycerol	**IP3**	inositol triphosphate
ACPD	1-amino-1,3-dicarboxycyclopentane		
aCSF	artificial cerebrospinal fluid	**LEC**	lateral entorhinal cortex
ADH	alcohol dehydrogenase	**LFS**	low frequency stimulation
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide	**LPP**	lateral perforant path
AMPAR	α-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid	**LTCC**	L-type calcium channel
		LTD	long-term depression
ARBD	alcohol-related behavioural disorder	**LTP**	long-term potentiation
ARND	alcohol-related neurological disorder	**MAPK**	map-activated protein kinase
BEC	blood ethanol concentration	**MEC**	medial entorhinal cortex
BIC	bicuculline methiodide	**mGluR**	metabotropic glutamate receptor
CA	cornu ammonis	**MPEP**	2-Methyl-6-(phenylethynyl)pyridine
CaMKII	calcium-calmodulin dependent protein kinase II	**MPP**	medial perforant path
cAMP	cyclic adenosine monophosphate	**MWM**	morris water maze
CB1	cannabinoid type 1	**NAD+**	nicotinamide adenine dinucleotide
CNS	central nervous system	**NIMO**	nimodipine
CO2	carbon dioxide	**NMDAR**	N-methyl-D-aspartate receptor
CREB	cAMP response element binding protein	**NT**	neurotransmitter
CS	conditioning stimulus	**P**	postnatal day
CYP2E1	cytochrome P450	**pFAS**	partial FAS
DAG	diacylglycerol	**PI3K**	phosphoinositide 3-kinase
DG	dentate gyrus	**PIP2**	phosphatidylinositol 4,5-biphosphate
DHPG	(S)-3,5-dihydroxyphenylglycine	**PKA**	protein kinase A
DL-APV	DL-2-Amino-5-phosphonopentanoic acid	**PKC**	protein kinase C
EC	entorhinal cortex	**PLC**	phospholipase C
eCB	endocannabinoid	**PNEE**	prenatal ethanol exposure
EDC	ethanol-derived calories	**PNS**	peripheral nervous system
ERα	estrogen receptor alpha	**PP1**	protein phosphatase 1
ERβ	estrogen receptor beta	**PP2B**	protein phosphatase 2 B

ERK	extracellular signal-regulated kinase
FAEE	fatty acid ethyl esters
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorders
fEPSP	field excitatory postsynaptic potential
fMRI	functional magnetic resonance imaging
GABAR	gamma-aminobutyric acid
GD	gestational day
HFS	high frequency stimulation
I-1	inhibitor 1
I/O	input-output
PPP	paired pulse plasticity
PTP	post tetanic potentiation
ROS	reactive oxygen species
SEM	standard error of the mean
SGZ	subgranular zone
STD	short term depression
TrK	tyrosine kinase
TrKB	tyrosine receptor kinase beta
VGCC	voltage-gated calcium channels

Acknowledgments

First and foremost, I have to thank Dr. Brian Christie for taking me on as a student and fostering my growth, both personal and professional. You provided me with the opportunity to work independently and expand my managerial and mentorship skill-sets for which I am so grateful. During my time in your lab I have learned technical, theoretical and communication skills that will leave a lasting impact on me for years to come.

Thank you to my supervisory committee members Dr. Joanne Weinberg and Dr. Raad Nashmi for your support, enthusiasm for my work and guidance as a neuroscientist-in-training. I am inspired by the faculty in the Neuroscience Graduate Program for their dedication and tireless passion for their research areas. Dr. Pedro Grandes, it has been a joy getting to know you and working with you over the last 2 years of my PhD – thank you for giving me the opportunity to help with your studies and for your positive attitude and interest in my own work. Dr. Anna (Patten) MacDonald, you have been an amazing mentor (personally and professionally) and have set an amazing example for me to follow. I am so happy that I was able to learn from you and the fact that we could speed-write a great review paper together is a testament to your abilities as a leader and an indication that we should keep working as a team!

Thank you to other members of the Christie Lab – in particular to Cristina Pinar, my co-author and co-conspirator, I am so glad that we were able to work together and that I could learn from you. I am looking forward to the opportunity to keep teaming up to accomplish any goal we set together. I wish that I could dedicate a whole page to you and to the rest of the lab because you and the rest of the lab have truly had a significant impact on my progression and growth. Thank you for your patience as I tried to learn some conversational Spanish. Thank you to past and present members, Penny's favourite uncle - Ryan Wortman, to Melissa Clarkson for your unending jokes and for giving Penny her favorite pal to play with, to Katie Neale for your friendship and support, to Juan Trivino-Paredes for your calming presence and scientific input, and to Dr. Luis Bettio for your humor and positive attitude. In the short time that I have known you, Erin Grafe, I can already tell that you are the best student to be following up this body of work. I am happy to know that the FASD projects are being left in great hands. Graduate school is never easy for anyone but together your influence on me has given me the ability to push forward and be here today. I am immensely appreciative of all of the undergraduate students that have helped throughout this dissertation, whether it came to making diet, helping with litters, making solutions and helping run slices, I could not have done this without your assistance. To have been a mentor to each and every one of you has been a gift of which I am so grateful. I thank my incredible students Waisley Yang, Angela Pang, Konrad Suesser, James Choi and Courtney Zoschke. I hope that our experience together has helped you accomplish your goals and I am so proud of each of you. Thank you for also putting up with my, at times embarrassing, enthusiasm at your defenses and poster presentations.

My female academic mentors have always been a source of strength and support for me over the last 5 years. I would never have had the confidence to branch out and pursue graduate school so far away from home if it weren't for the unparalleled mentorship of Dr. Qi Yuan. You facilitated my passion for neuroscience and your advice and encouragement have picked me up at the darkest of times and you are my inspiration. I hope that I can follow your example and help the next generation of students reach their fullest potential, as you do. Dr. Christina Thorpe, you have also been a major beacon of guidance for me for the last decade and have supported me every step of the way. From a young, naïve high school student in 2008 who would never leave the lab to a graduate student and now a PhD, you have always told me that I could have it all and I would never have thought to pursue a career in science and research without your example.

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Dedication

This body of work is dedicated to the legacy of groundbreaking women scientists that have come before me and empowered the next generation to pursue their interests in science without trepidation.

This dissertation is also dedicated to the memory of Jessica McErlean, my first trainee/mentee. I am grateful for our time together as your mentor in the lab, running rats through the various mazes, and especially while we worked together with Women in Science and Engineering Newfoundland and Labrador to advance opportunities for other girls in science and engineering. I will always remember your enthusiasm and how you applied your scientific mind to your creative goals, whether it be learning ukulele, practicing your art or becoming a rock climber. Your positivity, drive, adventurous spirit, selflessness and genuine curiosity-driven mind continue to be an inspiration to me and to your peers.

Jessica McErlean's life was tragically cut short in a rock climbing accident in Flatrock, Newfoundland August 21st, 2015.

1.0 Introduction

This chapter is based in part on the following review papers:

Pinar C*, **Fontaine CJ***, Trivino-Paredes J*, Lottenberg CP, Gil-Mohapel J & Christie BR (2017) Revisiting the flip side: Long-term depression of synaptic efficacy in the hippocampus. *Neuroscience and Biobehavioral Reviews 80,* 394-413.

Fontaine CJ*, Patten AR*, Sickmann H, Helfer J & Christie BR (2016) Effects of pre-natal alcohol exposure on hippocampal synaptic plasticity: Sex, age and methodological considerations. *Neuroscience and Biobehavioral Reviews* 64, 12-34.

Patten, AR*, **Fontaine CJ*,** & Christie BR (2014) A comparison of different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Frontiers in Pediatrics* 2(93).

1.1 Fetal Alcohol Spectrum Disorders; An umbrella of dysfunction

Alcohol-related developmental disorders are among the most common preventable disorders in North America. They were first termed fetal alcohol syndrome (FAS) in the early 1970s (Jones et al., 1973; Jones and Smith, 1973), although there is a French study from 1968 (originally published in Ouest Medical (8)476-482, translated in English and republished in the early 2000s) that associated heavy alcohol consumption with facial dysmorphologies, growth retardation and psycho-motor abnormalities (Lemoine et al., 2003). During this initial period, further studies of alcoholic mothers began to associate prenatal alcohol consumption with the impairments described above and continued to contribute to the understanding of FAS (Jones and Smith, 1975; Mulvihill et al., 1976; Ulleland, 1972; Ulleland et al., 1970), despite disbelief and criticism surrounding the idea that a compound as common as alcohol could have such detrimental effects that had gone unnoticed until this time. As a result of this work, In the 1980s the United States Surgeon General released a warning regarding the consumption of alcohol during pregnancy. Over the next 20 years the understanding of the teratogenic effects of alcohol underwent a significant evolution and is now recognized to lead to a spectrum of disorders. As of 2005, following a public release from Vice Admiral Richard Carmona, the U.S. Surgeon General (https://www.cdc.gov/ncbddd/fasd/documents/surgeongenbookmark.pdf) at the time,

pregnant women are advised not to consume alcohol and this was accompanied by the statement that no amount of alcohol during pregnancy is considered safe, which is the standing public message across North America. Despite this message and decades of efforts directed at prevention, alcohol consumption during pregnancy, and the diagnosis of FASD persists.

Currently, nearly half a century after the initial work of Jones, Smith and their colleagues, alcohol consumption during pregnancy is known to lead to the umbrella term fetal alcohol spectrum disorders (FASD), which describes the structural and functional damage caused by alcohol exposure *in utero* and encompasses conditions from FAS, the most severe form, to the less severe partial FAS (pFAS), alcohol-related neurodevelopmental disorder (ARND) and alcohol-related birth defects (ARBD) categories. While the facial dysmorphologies classically associated with FAS are important for accurate diagnosis across FASD, as we will see, it is possible for offspring to present with central nervous system (CNS) defects but without the obvious physical malformations necessary for diagnosis (see (Astley, 2012; Benz et al., 2009; Coriale et al., 2013) for review). The effects of prenatal ethanol exposure (PNEE) on the CNS can be widespread affecting neuroanatomy and neurophysiology, leading to a wide range of possible impairments in motor skills, cognition, language, academic achievement, intelligence, learning and memory, attention, executive function including impulse control and hyperactivity, affect regulation and social skills including social communication and adaptive behaviour (Guerri et al., 2009; Hannigan and Riley, 1988; Mattson and Riley, 1998; Riley et al., 2011; Riley and McGee, 2005). The presence and degree of impairments in these neurobehavioural domains varies across the spectrum of FASD as a result of variability in a number of factors including how much alcohol was consumed, when it was consumed, pattern of consumption, maternal nutritional status, other drug abuse (nicotine, opioids, etc.), maternal health status, and genetic predispositions among others (Benz et al., 2009; May and Gossage, 2011). Clearly these pervasive impairments have the capacity to affect an individual's personal and professional life and well-being possibly throughout the lifespan and with the capacity to affect future generations of offspring. In humans there are many unique challenges

associated with the study of FASDs including the variability that results from the factors described above in addition to social pressures and stigmas against mothers that impact diagnosis which then has downstream effects on estimates of prevalence and our understanding of the human condition across the wide spectrum of this disorder. The following sections will briefly describe relevant CNS effects of FASD for this dissertation.

1.1.1 Prevalence

Assessing instances of FASDs can be challenging due to the range and variability of the disorder and its symptoms which can be attributed to differences in the amount, timing and pattern of alcohol consumption during pregnancy. Furthermore, the influence of societal norms and stigmas on new moms can inhibit the likelihood of reports of alcohol consumption during pregnancy, which as we will see is a critical component of diagnosis for FASDs. In addition, many of the behavioural effects associated with FASD are common to other neurodevelopmental disorders, and without maternal confirmation of alcohol consumption or the salient facial features it can lead to reduced rates of diagnosis. Despite these factors, and the recommendations against alcohol consumption during pregnancy, the rate of FASD diagnosis in North America has been estimated to be between 1-5% of births, which equates to more than 300,000 cases in Canada alone, although for the reasons described above, this number may be an underestimate (May et al., 2018; Popova et al., 2017). In fact 48% of pregnancies are estimated to be unintended in North America in women aged 15-44 and recent survey data has indicated that approximately 50% of women aged 15-44 consume alcohol with reports of ~23% binge alcohol consumption (Ahrnsbrak et al., 2016; Moos et al., 2008; Singh et al., 2010). In a 2011 survey, ~10% of women in Canada reported consuming alcohol while pregnant (Walker et al., 2011). To complicate matters, many women are unaware that they are pregnant, particularly in the case of unintended pregnancies for the early gestational period and may still be consuming alcohol during this time (McCormack et al., 2017; O'Leary et al., 2010a, 2010b).

Certain populations have emerged as having higher reported rates of FASDs than others. For example South Africa has one of the highest rates of FASDs in the world,

with a reported incidence of upwards of 10% of births being associated with FASD (Benz et al., 2009; Cook et al., 2016; Popova et al., 2017). The direct and indirect healthcare costs of the most severe form of FASD, FAS, were estimated at $6.7 million in Canada almost a decade ago (Popova et al., 2012). This cost estimate reflects only a small portion of the likely cost of the entire spectrum of FASD which can include healthcare costs associated with diagnosis, specialist time for therapies and assessments, costs associated with potential hospitalization or with incarceration and rehabilitation. As such, while FASD may be considered preventable, the prevalence and cost of this spectrum of dysfunction is of paramount importance in our society. Despite having equal likelihood to be exposed to alcohol prenatally, there is emerging evidence of sex differences in some of the effects of FASD. A recent study of the prevalence of FASD in Alberta found 1.4 times higher prevalence of FASD in males over a 10 year study (Thanh et al., 2014). Furthermore a study from Washington state found that young males were more predominant among those affected by mild or severe ARBD, although no sex differences existed in cases of the more severe FAS (Astley, 2010).

1.2. Early Brain Development

To understand how alcohol can impact development, one needs to appreciate the progression of developmental milestones *in utero*. Human and rodent development understandably occur on very different timescales, and one important distinction is prenatal development where human gestation is approximately 38-40 weeks in duration while rat gestation is three weeks long (~21-22 days) with the first postnatal week being considered the third trimester equivalent of development. Some of the major steps in the development of the CNS will be described below and are depicted visually in Figure 1.

Prior to the development of the CNS, gastrulation occurs which is the formation of three germ layers, the endoderm, that will later form the lining of the gut and internal organs, mesoderm, which forms the musculature, skeletal and circulatory systems and the ectoderm that will eventually for the skin and CNS (Rice and Barone, 2000; Rodier, 1995; Seely, 2000; Spear, 2000). The formation of three germ layers is attributed to triploblasts and is common amongst all vertebrates. Neurulation follows this process, which begins with the development of a small invagination in the ectoderm called the

primitive pit which elongates to form the primitive streak that is visible in humans around gestational day (GD) 18. Beneath these obvious physical features of the ectoderm, there is a thickening of mesodermal cells that will form the notochord, a defining characteristic of all chordates. The notochord is a critical component of embryology as it, along with the primitive streak, initially define the rostral-caudal axis along the midline of the embryo and play roles in releasing inductive signals that help differentiate between various cell populations. Despite the importance of this structure in patterning the early nervous system, its existence is transient in most vertebrates but is retained in more primitive organisms such as lamprey, hagfish and coelacanth (Shimeld and Donoghue, 2012). The portion of the ectoderm that overlies the notochord is called the neuroectoderm, and its cells become neuroectodermal precursor cells. These cells then form the neural plate, whose edges fold inward to form a tube-like structure that closes around GD 22-24 in humans and GD 10-11 in rats, and is called the neural tube, that will then give rise to the CNS and much of the peripheral nervous system (PNS). Proximity of various aspects of the closed neural tube such as the notochord, somites, and sensory ganglion help pattern the stem cells of the neural tube and define cellular populations that will further develop into various aspects of the nervous system.

The brain itself begins as having three major regions, or vesicles, the prosencephalon (to become forebrain), mesencephalon (to become midbrain) and rhombencephalon (to become hindbrain) by GD 26 in humans and GD 10-11 in rats. Followed by a five vesicle stage where the prosencephalon gives rise to the telencephalon and diencephalon and the rhombencephalon gives rise to the metencephalon and myelencephalon around GD 33 in humans and GD 11-12 in rats. Throughout and following this process neurogenesis, proliferation, migration, differentiation, synaptogenesis, apoptosis and myelination occur to finally complete the development of the CNS and PNS (See Figure 1) (Rice and Barone, 2000). While the majority of these processes occur prior to birth in humans and rodents, synaptogenesis, apoptosis and myelination persist until, in some structures, adulthood. Importantly, the first postnatal week in rats is considered the third-trimester equivalent and has commonly been

described as the 'brain growth spurt' where many structures undergo the final stages of maturation (Johnson and Goodlett, 2002b).

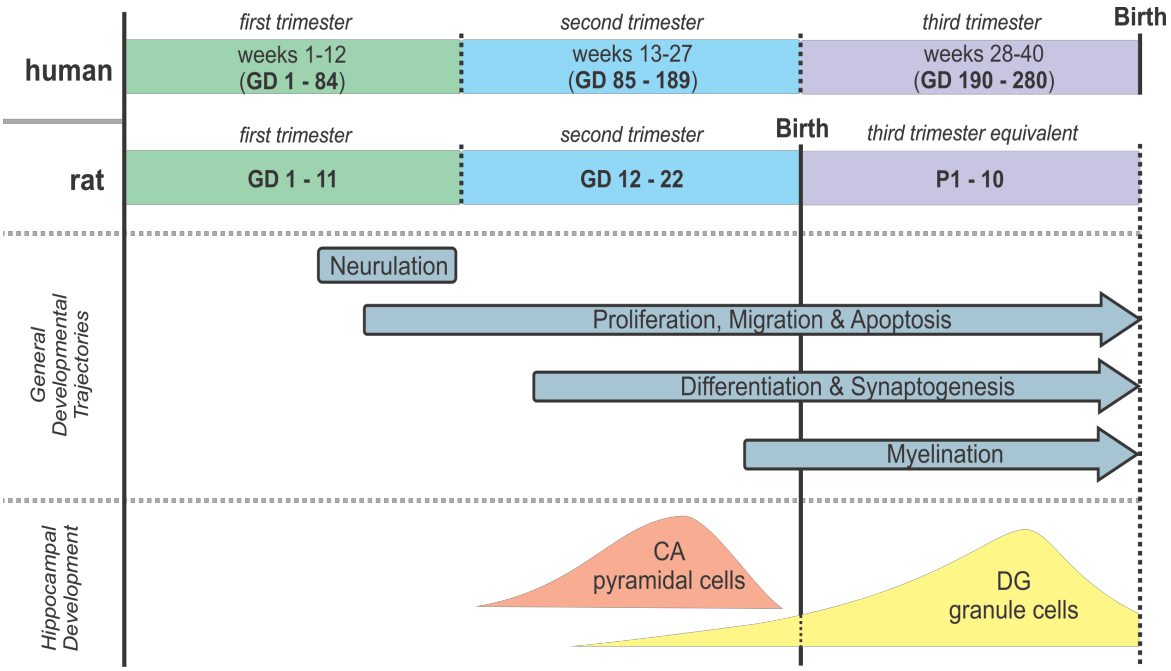

Figure 1. Prenatal Brain and Hippocampal Development

The approximate human and rat gestational lengths are depicted in the tricoloured bars above with the first trimester in green, second in blue and third or third-trimester equivalent in purple. Aside from differences in the length of gestation an important distinction between human and rat gestation is that the first 10 postnatal days are considered the third-trimester equivalent of development. The grey bars and arrows below depict major developmental events and their relative timecourses in the rat. The curves represent principal cell generation in the CA region (orange) and DG (yellow) with peak pyramidal cell formation around GD 15-16 and peak granule cell development around P7. By birth, it is estimated that only 15% of granule cells have formed in the DG. Abbreviations: CA: Cornu Ammonis; DG: Dentate Gyrus; GD: Gestational Day; P: Postnatal Day.

1.3. Ethanol

Finally, in order to appreciate how ethanol leads to the structural and functional deficits associated with FASD we must describe the pathway by which ethanol enters and is metabolized by the body of the mother and can reach and be cleared from the embryo-fetal compartment. Naturally, there is some variability in each of these elements that will lead to the variability that exists across the spectrum of FASDs.

1.3.1. Ethanol Metabolism

Ingested ethanol enters the bloodstream primarily via passive diffusion in small intestine and the stomach (Figure 2A). Once in the bloodstream, ethanol metabolism, primarily by the liver occurs (Figure 2B), either via oxidative or non-oxidative processes (Zakhari, 2006) whose actions are interrelated. Ethanol can also be metabolized by extrahepatic structures such as the stomach and the brain (Deitrich et al., 2006; Zimatkin and Deitrich, 1997). Once consumed, the blood ethanol concentration (BEC) rises steadily as ethanol enters the bloodstream and is metabolized. The degree of damage caused by ethanol is generally attributed to the level, duration and pattern of elevations of BEC therefore rapid metabolism is key to mitigate the impact of this compound on the body.

The majority of ethanol metabolism in the body occurs via the oxidative pathway (Figure 2B) which generally consists of either adding oxygen or removing hydrogen. Ethanol metabolism has been reviewed extensively in the literature but is described in brief below (Zakhari, 2006; Zimatkin and Deitrich, 1997). Alcohol dehydrogenase (ADH) is an enzyme present in the cytosol of hepatocytes and converts ethanol to acetaldehyde which involves nicotinamide adenine dinucleotide (NAD^+) as an intermediate electron carrier that is reduced to NADH. Even through this well-described mechanism, metabolism rates vary between individuals based on genetic variants in ADH that can be more or less active in addition to a number of factors including general health, nutrition, pattern of consumption among others (Chen et al., 1995; Weinberg, 1985b; Zakhari, 2006). The byproduct of this metabolism, acetaldehyde, is in and of itself highly toxic and reactive and has been thought to contribute to the teratogenic effects of ethanol and possibly to its addictive qualities (Hayashi, 1991; McBride et al., 2002; O'Shea and Kaufman, 1979; Quertemont, 2004; Webster et al., 1983). Acetaldehyde is then metabolized by aldehyde dehydrogenase (ALDH) in mitochondria to form NADH and the byproduct acetate which is oxidized to carbon dioxide (CO_2) primarily outside of the liver. Cytochrome P450 (CYP2E1) also contributes to ethanol metabolism at high concentrations in the liver and in the brain where ADH levels are low by acting in

microsomes on the endoplasmic reticulum of cells. Ethanol metabolism by CYP2E1 produces reactive oxygen species (ROS) that lead to subsequent cellular damage.

Metabolism of ethanol can also occur through non-oxidative pathways, although non-oxidative metabolism is less responsible for the breakdown of ethanol in the body, the by-products of this method of metabolism can be deposited in different tissues and cells in the body and can assist in the accurate diagnosis of FASDs. First, by interacting with the enzyme fatty acid ethyl ester (FAEE) synthase, FAEEs are produced that can be deposited in keratenized tissues such as hair and fingernails long after the ethanol exposure has ended. Additionally, FAEEs can be detected in meconium, the first bowel movement of the baby at or after birth, and can aid in accurate diagnoses of FASDs for the offspring (Bearer et al., 2005; Burd and Hofer, 2008; Caprara et al., 2006). At higher ethanol concentrations the enzyme phospholipase D (PLD) metabolizes ethanol to produce phosphatidyl ethanol, which is poorly metabolized by the body. The metabolism of ethanol by PLD detracts from its normal physiological function which involves the production of phosphatidic acid, which plays an important role in cell signalling.

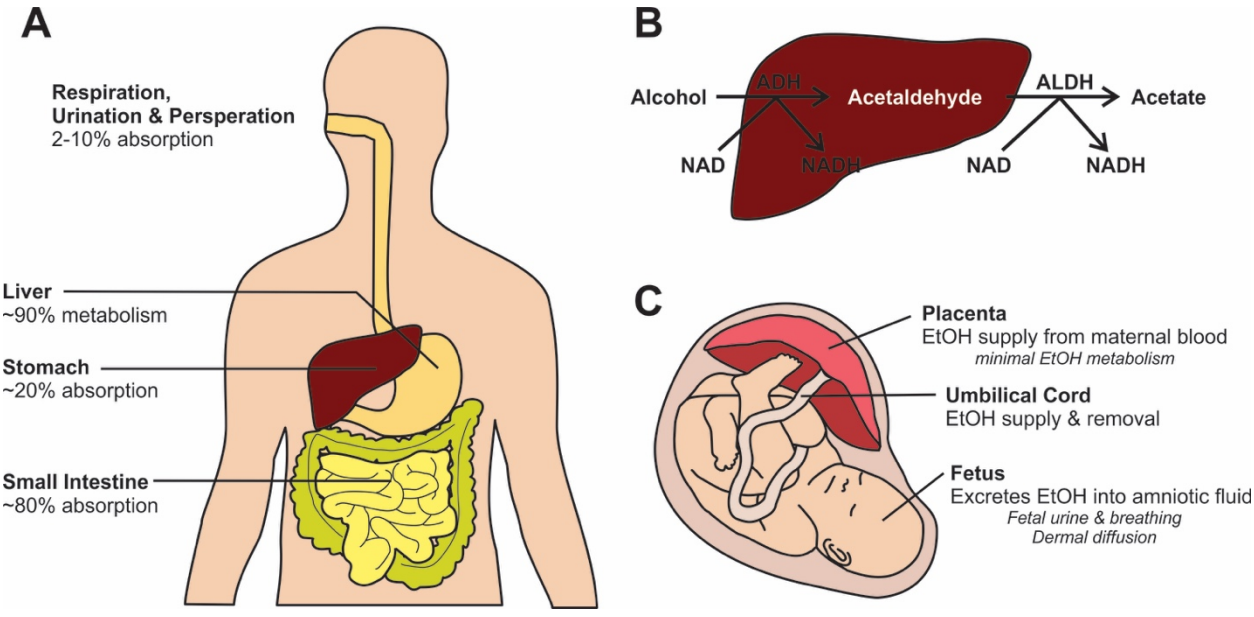

Figure 2. Ethanol Absorption, Metabolism & Passage to the Fetal Compartment.
(**A**) Ethanol is primarily absorbed from the stomach and small intestine, at which point it freely enters the bloodstream and can diffuse in and out of tissues with relative ease prior to metabolism by the liver. (**B**) The liver conducts most of the ethanol metabolism for the body. Depicted above

is one of the oxidative pathways of ethanol metabolism, where alcohol is converted to acetaldehyde and then to acetate via enzymatic activity of alcohol dehydrogenase (ADH/ALDH). (**C**) When ethanol is consumed during pregnancy, it can diffuse freely through the placenta and into fetal blood and amniotic fluid. The fetus is incapable of significant ethanol metabolism until late in gestation, thus ethanol can become trapped and can diffuse into the fetal skin and through amniotic fluid consumed during fetal breathing, then excretion back into the amniotic sac. Ethanol in the fetal blood and amniotic fluid is gradually removed via maternal blood, thus levels of this teratogen remain elevated in the fetal compartment longer than in maternal blood alone (Burd et al., 2007b; Heller and Burd, 2014). Abbreviations: ADH: Alcohol Dehydrogenase; ALDH: Aldehyde Dehydrogenase; EtOH: Ethanol; NAD: Nicotinamide Adenine Dinucleotide; NADH: Reduced Nicotinamide Adenine Dinucleotide.

1.3.2. Passage of Ethanol to the Fetus

Despite having interrelated and overlapping mechanisms by which the body can metabolize ethanol, this compound circulates in the bloodstream and can cause damage to tissues around the body. This is particularly critical for pregnant women, who via the placenta share a blood supply with the developing fetus which is unable to metabolize teratogens like ethanol until late in gestation. The placenta hosts the exchange of gases, nutrients and wastes in the labyrinth zone where the maternal sinusoid and fetal capillaries interact which then feed into and from the developing offspring through umbilical vessels (Bridgman, 1948; Jollie, 1990; Jones et al., 1981). The simplistic chemical structure of ethanol also allows it to diffuse freely through cell membranes, including those of the placenta that normally act as a barrier separating maternal and fetal circulation.

The pre-term placenta and fetal liver have poor metabolic capacities for toxins, allowing for ethanol to directly affect the embryofetus (Syme et al., 2004). Ethanol metabolism for the pre-term embryofetus is almost completely dependent upon the maternal system. Perhaps in order to account for this vulnerability, maternal ethanol metabolism is increased during pregnancy (Badger et al., 2005; Nava-Ocampo et al., 2004). Other maternal factors, such as poor nutrition and other drug use, such as nicotine can exacerbate the teratogenic effects of ethanol on the fetus (Shankar et al., 2007; Syme et al., 2004). Ethanol levels rise in the amniotic fluid at a relatively similar rate as in the maternal blood within an hour after consumption (Idänpään-Heikkilä et al., 1972). While ethanol accumulates rapidly in the amniotic fluid, the fetus is exposed to ethanol long

after ethanol has been metabolized in the maternal bloodstream because the amniotic fluid itself can act as a sink for ethanol (Burd et al., 2012). A recent analytical review estimated that it could take up to three hours for ethanol from a single alcoholic beverage to be completely cleared from amniotic fluid and metabolized by the maternal system (Burd et al., 2007b). While in the amniotic compartment or fetal blood, ethanol can freely diffuse through fetal tissues without being broken down, including diffusion through fetal skin prior to keratinization (20-24 weeks gestation in humans).

In addition to direct effects on the developing offspring, ethanol can impact nutrient transport to the fetus by reducing blood flow through the placental and umbilical cord (Falconer, 1990) or by inhibiting nutrient transport across the placenta to the fetus. Ethanol can impact levels prostaglandin E, prostacyclin and thromboxane in the placenta that each play a role in vasoconstriction and vasodilation of blood vessels in this structure (Randall et al., 1996; Randall and Saulnier, 1995; Siler-Khodr et al., 2000). Ethanol can also to cause spasms of the umbilical blood vessels in a dose-dependent fashion (Altura et al., 1982; Savoy-Moore et al., 1989). Together the combination of these effects of ethanol on blood circulation can also impact both nutrient access and waste removal from the fetus and can also contribute to slow removal of ethanol from the amniotic fluid by maternal metabolism.

1.3.3. Acute Ethanol and the Brain

With the capacity to easily diffuse across cell membranes, ethanol and has multi-faceted effects on cell structure, health and function ((Freund, 1973) see Figure 3). Of particular relevance to the present dissertation is the impact of ethanol on glutamatergic and GABAergic receptors in the cell membrane. Ethanol acts as an antagonist on the NMDAR (Abdollah and Brien, 1995; Chandrasekar, 2013; Hendricson et al., 2004; Ikonomidou, 2000; Mameli et al., 2005; Sanna et al., 1993; Savage et al., 1991) and a positive allosteric modulator to the GABARs (Davies, 2003; Lobo and Harris, 2008). Presynaptic neurotransmitter release can also be attenuated by ethanol through its actions on voltage-gated calcium channels (Mameli et al., 2005). This drug also has widespread effects on other neurotransmitter, neuroendocrine and neurotrophic signalling systems,

their related receptors and even on the cell membrane itself (see (Davis, 2008; Deitrich et al., 1989; Diamond and Gordon, 1997; Goodlett and Horn, 2001; Grant, 1994; Vengeliene et al., 2008) for review).

Inside the cell, ethanol can negatively impact cell health. Ethanol metabolism produces ROS and induces a state of increased oxidative stress inside the cell (Guerri et al., 1994) and increases neuroinflammation (Franke et al., 1997; Pascual et al., 2007). Furthermore ethanol can activate caspase signalling that can initiate DNA fragmentation, degeneration and ultimately cell death (Creeley and Olney, 2014; Olney et al., 2002a, 2002b).

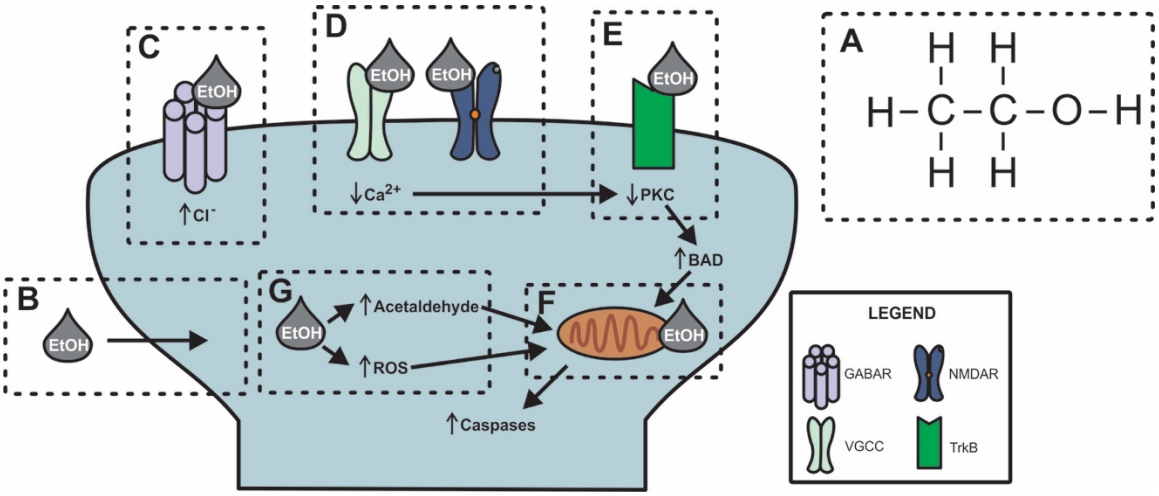

Figure 3. Acute Ethanol & The Cell

Diagram of some basic effects of acute ethanol on the cell. (**A**) The chemical structure of ethanol. Its simplicity and physical characteristics allow it to move freely through extracellular and intracellular spaces. (**B**) Ethanol is capable of diffusing across cell membranes to have direct impact on intracellular signalling cascades. (**C**) Ethanol is a positive allosteric modulator of GABARs. (**D**) Through inhibitory effects on both the voltage-gated calcium channels (VGCCs; left) and NMDARs (right), ethanol can reduce calcium entry into the cell. (**E**) Ethanol can downregulate neurotrophic receptor expression, such as the tropomyosin receptor kinase B (TrKB), which, combined with the effects of D lead to reduce protein kinase C (PKC) activity and the initiation of cell death pathways. (**G**) Ethanol metabolism causes the production of the toxic byproduct acetaldehyde as well as the production of reactive oxygen species (ROS), which with E can lead to further cellular and mitochondrial damage as depicted in (**F**). Modified from (Fontaine et al., 2016). Abbreviations: BAD: a pro-apoptotic protein; Ca^{2+}: Calcium; Cl^-: Chloride; EtOH: ethanol; GABAR: gamma-aminobutyric acid; NMDAR: N-methyl-D-

aspartate receptor; PKC: protein kinase C; ROS: reactive oxygen species; TrKB: Tyrosine receptor kinase B: VGCC: voltage-hgated calcium channel.

Acute ethanol exposure also impairs synaptic plasticity in the hippocampus, and these plasticity impairments are thought to potentially underlie some of the effects of intoxication on learning and memory (see (Zorumski et al., 2014) for review). Specifically long-term potentiation (LTP) is impaired by acute ethanol exposure in both the cornu ammonis 1 (CA1) and dentate gyrus (DG) subregions of the hippocampus in both *in vivo* and *in vitro* preparations (Fujii et al., 2008; Izumi et al., 2005; Pyapali et al., 1999; Swartzwelder et al., 1995). Acute exposure to ethanol also reversibly inhibits CA1 long-term depression (LTD) in rats around puberty (postnatal day (P) 30-32)(Izumi et al., 2005) but has not been reported to enhance LTD in younger animals (Hendricson, 2002). Another by-product of ethanol metabolism, acetaldehyde, is embryolethal (O'Shea and Kaufman, 1979), and may at least partially mediate the effects of high concentrations of acute ethanol on hippocampal LTP (Tokuda et al., 2013) as alone this metabolite inhibits LTP (Abe et al., 1999). Likely related to the hippocampal plasticity and behavioural deficits are ethanol-induced cell loss and dendritic atrophy after chronic ethanol exposure (King et al., 1988; Riley and Walker, 1978; Walker et al., 1973, 1980, 1981).

1.4. FASDs in Clinical & Pre-Clinical Populations

1.4.1. Physical Features of FASD

As can be inferred from the preceeding sections, the impact of ethanol on the fetus can be quite variable depending on the mothers drinking behaviour. Classic dysmorphology associated with more severe forms of FASD can serve as diagnostic indicators in humans, and these have been mimicked in some animal models of PNEE (del Campo and Jones, 2017). The key craniofacial dysmorphologies in FAS include a shorter horizontal palpebral fissure, a smooth philtrum and thin upper lip. The palpebral fissure is the opening between the eyelids, and its horizontal value, between the endocanthion and the exocanthion is reduced in cases of FASD, and the eye in particular has been reported to be vulnerable to prenatal alcohol (Jones, 2011). Similarly, the philtrum which is the vertical groove between the nose and the upper lip and significant

smoothness of this value according to a 5 point scale can be used to diagnose children with this disorder (Astley and Clarren, 1996). A thin upper lip has historically been associated with FASD however, as with many of these facial features they can vary with ethnicity (Hoyme et al., 2015; May et al., 2010). Other facial features common to FASD include: midface hypoplasia, a low or flat nasal bridge and microcephaly. Given the diagnostic importance of these facial features some groups have sought to use 3D images of faces to assign strict numerical characterizations of these abnormalities by age, sex and ethnicity (Suttie et al., 2013, 2017). Similar facial dysmorphology has been modeled in mice when alcohol is delivered only on GD 7 (Godin et al., 2010; O'Leary-Moore et al., 2011). The dysmorphology was less apparent at GD 8.5, indicating that the timing of alcohol consumption is an important consideration for particular deficits (Lipinski et al., 2012). The investigation of the underlying causes and timecourses for the classic facial features of FASD have also been investigated in non-rodent models such as zebrafish and chicken (Kiecker, 2016) and provide convergent evidence for ethanol-induced facial dysmorphologies.

In addition to facial dysmorphology, FASDs are also associated with heart defects, altered osteogenesis and other organ damage. Congenital heart defects are common to humans with FASD and include atrial, ventricular and septal defects (Burd et al., 2007a) and have also been shown in animal models (Fang et al., 1987; Sarmah and Marrs, 2017). Furthermore other organs, like the kidney, can be reduced in weight and nephron number, showing that alcohol is a teratogen that can impact any developing tissues (Gallo and Weinberg, 1986; Gray et al., 2010; Hofer and Burd, 2009). Bone development is also impaired by PNEE and results in reduced bone volume overall which may underlie general growth retardation, the craniofacial defects described above and later vulnerability to osteoporosis (Simpson et al., 2005; Snow and Keiver, 2007).

1.4.2. **Diagnosis**

The nature of the spectrum of dysfunction and variability in teratogen exposure across FASDs make diagnosis challenging and as a result the diagnostic criteria have undergone significant restructuring and improvement over the last decade alone with the help of basic and applied research. The CanFASD Research Network sought to develop

new diagnostic guidelines for FASDs in Canada in 2015 in order to update clinical diagnosis for individuals using the latest findings from pre-clinical animal models (Cook et al., 2016). A brief summary of the 2015 diagnostic guidelines are depicted in Table 1. Clear diagnostic criteria are critical for spectra such as these in order to provide accurate treatment and medical advice to patients (Astley, 2012; Coriale et al., 2013). A key component for diagnosis of FASDs is confirmation of alcohol consumption during pregnancy, however a recent meta-analysis conducted in Alberta estimated that 30-50% of children in foster care suffer from FASD and in many cases access to accurate information about maternal alcohol consumption can be restricted (Government of Alberta). Furthermore, societal stigma against alcohol consumption during pregnancy is a barrier to accurate maternal reports for diagnosis. In cases where the maternal consumption is unknown, offspring must exhibit the three sentinel facial features and impairment in at least three of the neurodevelopmental domains depicted in the far-right column of Table 1. As such, the accurate diagnosis of FASDs requires the input from a team of medical specialists which has been estimated as requiring 32-47 hours to reach a diagnosis per individual putting a significant burden on the healthcare system costing anywhere from $ 3.2 to $7.3 million in Canada (Popova et al., 2013). In offspring with unknown maternal consumption but without the three sentinel facial features no diagnosis of FASD can be made. Given the importance of being able to confirm alcohol consumption by the mother while pregnant, many research groups have sought to uncover biomarkers such as FAEE deposition in hair, fingernails and meconium, the first bowel movement after birth (Bearer et al., 2005; Burd and Hofer, 2008; Caprara et al., 2006; Pragst and Yegles, 2008). Many of the neurodevelopmental effects of PNEE tend to become apparent, particularly measures such as academic achievement, attention, memory and language when children are school-aged and surrounded by their age-matched peers in the classroom.

Table 1. Summary of 2015 Diagnostic Guidelines of FASD in Canada

Summary of the major diagnostic components for diagnosis of FASDs based on the findings of (Cook et al., 2016). A severe impairment is defined as a score that is ≥ 2 standard deviations below the mean for that given measure.

Confirmation of Exposure	Sentinel Facial Features	Neurodevelopmental Domains
Yes	Short palpebral fissure	Motor skills
No	Smooth philtrum	Neuroanatomy/neurophysiology
Unknown	Thin upper lip	Cognition
		Language
		Academic achievement
		Memory
		Attention
		Executive function
		Impulse control/hyperactivity
		Affect regulation
		Adaptive behaviour/social skills/ communication

The timing, amount and pattern of exposure in addition to maternal and paternal health factors as well as genetics can all impact the presentation of neuroanatomical and neuronal functional deficits as well as the subsequent neurobehavioural impairments implicated with FASDs (O'Leary et al., 2010c). As such it is necessary to use pre-clinical animal models to control some of these factors to more clearly demonstrate the relationships between PNEE and the developing brain.

1.4.3. Animal Models of PNEE

Animal models of FASD provide the experimental control that is critical for understanding the mechanistic underpinnings of ethanol-induced damage to brain structure and function, and can lead to the discovery of new therapeutic strategies. The two main classes of animal models can roughly be divided into "Injection" and "Ingestion", and each have been used to model different aspects of FASD in different

species (see (Patten et al., 2014) for review). Each model is accompanied by their own advantages and disadvantages and care must be taken to choose the appropriate model that best suits the research question and field of study.

Injection models: Ethanol injection paradigms typically target the intraperitoneal or subcutaneous space, and ethanol is administered in relation to the subjects weight to deliver a predetermined BEC. Ethanol passes easily through cell membranes and into the vasculature, allowing it to be distributed throughout tissues of the body as well as the embryo-fetal compartment. With this model, ethanol can be delivered at specific times and patterns and at specific concentrations during gestation to exert its teratogenic effects on the developing fetus. For example, in studies described above modeling the craniofacial defects associated with FASD, ethanol injections were delivered on specific GDs to better understand periods of vulnerability to this teratogen (Godin et al., 2010; O'Leary-Moore et al., 2011; Parnell et al., 2009; Webster et al., 1983). Comparatively, injections of ethanol require relatively little potential handling-induced stress as compared to some other models described below, although the injections themselves can induce stress. The downside to this model however is that it does not fully model the effects of ethanol ingestion in humans, including the impact on nutritional absorption in the maternal digestive tract. Furthermore injection into the intraperitoneal space causes a rapid spike immediately following the injection in the amniotic fluid in a guinea pig model of PNEE, unlike the natural timecourse observed when ethanol is ingested (Brien et al., 1985; Clarke et al., 1985; Hayashi, 1991).

Ingestion models: Ingestion of ethanol more closely mimics the widespread effects of PNEE in the human experience of FASD, and there are a number of models of ethanol ingestion that exist. These include intragastric intubation (or gavage), administering ethanol in the drinking water, and as is used in the present dissertation, feeding ethanol as part of a liquid diet. Intragastric intubation, or gavage, involves delivering ethanol directly to the stomach through the use of metal or plastic tubing. Using this method, ethanol can be delivered throughout gestation and even to the pups at any point after birth (Boehme et al., 2011a; Brocardo et al., 2012; Gil-Mohapel et al., 2011). Similar to

ethanol injection, this method allows the experimenter to have control over the exact volumes and concentrations of ethanol delivered to the animal and the drug is absorbed into the maternal bloodstream through the digestive system as with humans. This method is more invasive, as well as labour-intensive, and requires significant handling of the animals throughout the period of exposure. While it is advantageous to examine the impact of ethanol on the early postnatal pup, care must be taken to ensure that the animal is receiving adequate nutrition but ultimately this method can be associated with greater pup mortality during this time. Conversely voluntary drinking of ethanol-containing drinking water or of ethanol-containing liquid diet requires less animal handling, is less invasive and is less labour-intensive on the part of the researcher. Ethanol in drinking water typically involves daily *ad libitum* access to this water solution that contains a sweetener to entice animals to consume it (Allan et al., 2003; Choi et al., 2005). Ethanol-containing liquid diets deliver food in the form of a blended smoothie that can be provided *ad libitum* but in lieu of standard rat chow. These diets are specially formulated to contain all of the dietary requirements for pregnant dams (Weinberg, 1985a). In both cases fresh ethanol-water or ethanol-diet are provided daily by the experimenter and the volume or weight consumed are measured therefore the exact amount of ethanol consumed cannot be controlled and will naturally vary between dams. Furthermore, this model may not be ideal for the study of binge exposure as animals are unlikely to consume higher concentrations and amounts of ethanol in limited period of time. For studies using any method of ingestion, it is important to accurately determine the ethanol consumption for each dam whether it be calculated from the amount of diet consumed or the BEC. For the present dissertation the ethanol-containing liquid diet was chosen as the model of PNEE, originally developed by Charles Leiber and Leonore DeCarli then further modified to best suit pregnant rats by Joanne Weinberg and Kathy Keiver (Gallo and Weinberg, 1986; Keiver et al., 1996; Lieber and DeCarli, 1982; Weinberg, 1985a; Weinberg and Gallo, 1982).

While there are many benefits to each model discussed here and existing elsewhere in the literature to model different aspect of FASD in animals, the diversity in models,

concentration of ethanol, timecourses of delivery and more can make comparisons between studies more complex and lead to at times, variable results across measures.

1.4.4. **Neurobehavioural Consequences of PNEE**

Perhaps most widespread across the spectrum of FASD are the CNS impairments caused by PNEE. The critical impairments for diagnosis are described in the third column of Table 1 and have been summarized in a recent review from our laboratory (Patten et al., 2014). For the purposes of this dissertation motor skills, executive function, social behaviour, affect regulation and learning and memory will be described however other CNS effects are described in detail elsewhere (Mattson et al., 2011; Mattson and Riley, 1998; Rasmussen et al., 2008; Riley and McGee, 2005; Sokol et al., 2003). Many of the neurodevelopmental domains of damage by PNEE have been modeled in animals which will be reviewed in this section, however the impact of PNEE on learning and memory will be discussed in more detail.

The cerebellum is vulnerable to the effects of PNEE and damage to this area can underlie some of the motor impairments seen in FASD. Children with FASD display impaired directed reaching behaviour (Domellöf et al., 2011), postural balance (Roebuck et al., 1998) and saccade accuracy (Green et al., 2007; Paolozza et al., 2013, 2015). Animal models of FASD have determined that the early postnatal period appears to result in specific damage to cerebellar purkinje cells (Maier et al., 1999; Marcussen et al., 1994). This damage also translates to behavioural defects on tasks such as the rotating beam and runway and in the analysis of gait, which may be more pronounced in younger offspring (Bond and Di Giusto, 1977; Cebolla et al., 2009; Hannigan and Riley, 1988).

Poor social skills and altered social behaviour are common amongst individuals with FASD and have been demonstrated in animal models of PNEE (Kelly et al., 2000, 2009a). In humans with FASD, social dysfunction persists throughout the lifespan (Streissguth et al., 1991), which can lead to issues integrating with societal norms. Young children with FASD can display increase irritability, poor coping abilities and interpersonal skills and increased aggression toward peers (Greenbaum et al., 2009;

McGee et al., 2008, 2009; Thomas et al., 1998). Even prior to weaning rodent models of PNEE can show impairments in critical social communication through ultrasonic vocalizations with the dam (Marino et al., 2002). Play behaviour is characteristic of juvenile rodents, is sexually dimorphic and is considered to play an important role in social development of young animals (Auger and Olesen, 2009). Despite its importance, social play is reduced and aggression increased in animal models of PNEE (Hamilton et al., 2010; Krsiak et al., 1977; Meyer and Riley, 1986; Royalty, 1990). Similarly, social recognition memory is impaired by PNEE in both sexes, however exposure impaired memory retention in males whereas it impaired memory encoding in females (Kelly et al., 2009b). In addition to being observed throughout the lifespan, poor social skills of PNEE dams in particular can be passed onto the next generation by inadequate maternal behaviour (Curley et al., 2008; Hård et al., 2009). In an effort to address differences in maternal care some groups cross-foster offspring with dams that have not been exposed to ethanol.

Executive functioning is also known to be impaired by FASD, likely due to structural and functional damage to higher-order brain structures. Impairments in executive function are largely reported in the human literature which includes response inhibition, decision-making, planning, directed attention, strategy development and more (Mattson et al., 1999; Paolozza et al., 2014). Furthermore, working memory dysfunction is also associated with FASD, and are apparent across the human lifespan (in young children and in adults), which may be related to changes in activity patterns in the frontal cortex as measured by fMRI (Malisza et al., 2005). There is also evidence that working memory deficits exist in adult male but not in adult female rats following PNEE (Zimmerberg et al., 1991). Attention deficit hyperactivity disorder is a common psychiatric comorbidity in FASD that may be associated with executive functioning, where males with FASD are more likely to be diagnosed than females (Herman et al., 2008). Impairments in executive function can have life-long consequences and likely play a role in these offspring experiencing problems with the law. As such, the cost to the Canadian correctional system due to incarceration of youth with FASD alone has been estimated at $ 17.5 million (Popova et al., 2015).

1.5. Sex and the Brain

In order to best interpret how male and female offspring may be differentially affected by PNEE we must first understand the effect of biological sex and the brain. Males have by and large represented the vast majority of subjects in the study of the underpinnings of basic neuroscientific processes in general and the same has historically been true in the field of FASD. Emerging interest paired with changing policies from major funding agencies has increased our understanding of how the male and female brain differ and in particular how they are differentially affected in neurodevelopmental disorders.

1.5.1. Sexual Differentiation & Maturation

The effects of sex hormones on the CNS and the rest of the body are most simply separated into two phases having either organizational or activational effects, terms first described by Phoenix in 1959 (Phoenix et al., 1959). Embryonic sexual differentiation begins around GD 18 in rats (gestational week 13 in humans) when the developing male testes begin secreting testosterone and no sex hormones are secreted from the developing female ovaries (Weisz and Ward, 1980). The early testosterone spike in males persists for the first few days after birth however until as late as P 10 is considered the end of a critical period for sexual differentiation of the offspring. During this period experimental manipulations of sex hormones in either sex can shape future outcomes from neuroanatomy to behaviour (Arnold and Breedlove, 1985). Under control conditions, testosterone in the brain is aromatized into estradiol, which paradoxically is thought to masculinize the male brain. In fact when estradiol or testosterone are experimentally administered to developing females, it induces masculinization of the female brain (McEwen et al., 1977; Phoenix et al., 1959). Later in normal development, the early, permanent organizing effects of testosterone (or lack thereof in females) are activated by increases in testosterone or estrogens and progesterone produced by the testes and ovaries in males and females respectively during puberty, which occurs between P 30-40 in the rat (Schwarz, 2016) with females typically entering pubertal stages ahead of males in this

approximate range. In female offspring puberty can be roughly approximated by the onset of the first estrus and vaginal opening (Gaytan et al., 2017) and in males by preputial separation (Gaytan et al., 2009). The interactions between testosterone and estradiol during sexual differentiation and development are critical but are also influenced by various other factors and signals, such as exposure to prenatal stressors (Choleris et al., 2018; Konkle and McCarthy, 2011; McCarthy, 2008, 2009; McCarthy and Arnold, 2011; Schneider, 2008; Ward and Weisz, 1984; Weisz et al., 1982).

1.5.2. Sex Differences in the Brain

The male and female brain can differ in their anatomy and in subsequent behavioural outcomes (see (Lenroot and Giedd, 2010) for a review in humans). Historically across the field of neuroscience as a whole male subjects have been used disproportionately more than females although new guidelines set by federal funding agencies in North America are increasing the number of studies examining both sexes. As such, the study of sex differences across the brain and behaviour is an emerging area across neuroscience. There are several well-defined sexually dimorphic nuclei in the brain such as the medial preoptic area, spinal nucleus of the bulbocavernosus and the bed nucleus of the stria terminalis, which are in general, larger in the male than in the female possibly due to the protection against normally-occurring apoptosis in these areas (see (Schwarz, 2016) for review).

Although not considered part of the classically sexually dimorphic areas of the brain, hippocampal structure and function is also considered to differ between the sexes. In humans there have been reports of sex differences both in the overall size and in growth rates of the hippocampus (Giedd et al., 1996, 1997, 2012) which are reported to favor females when overall brain size is taken into account. Furthermore sex hormones have differential effects on hippocampal neurogenesis, cell proliferation and cell survival (Mahmoud et al., 2016) as well as dendritic morphology, dendritic spines in both sexes and throughout the lifespan (see (Choleris et al., 2018; Triviño-Paredes et al., 2016) for review). It is important to note in the discussion of the impact of sex hormones on hippocampal structure and function that estradiol and testosterone can both be

extragonadally synthesized in the hippocampus itself (Hojo et al., 2004) and the enzymes necessary for this synthesis are localized to this region of the brain, among others (Mensah-Nyagan et al., 1999). it may be possible that ovariectomy and castration may not completely remove the influence of sex hormones in the brain. In fact, many neurosteroid enzymes are found in the brain and sex hormones such as testosterone and estradiol can be synthesized from dehydroepiandrosterone (DHEA) and DHEA sulfate as opposed to cholesterol, as is common for synthesis outside of the CNS (Compagnone and Mellon, 2000; Mellon, 1994; Mellon and Griffin, 2002). These enzymes are also reported to be differentially expressed throughout the lifespan and may reflect the changing needs of the brain over the course of development, puberty, sexual maturity and aging (Baulieu, 1998; Mensah-Nyagan et al., 1999; Plassart-Schiess and Baulieu, 2001).

1.6. The Hippocampal Formation

The hippocampal formation is a paleocortical temporal lobe structure that is a part of the limbic system and plays a role in learning and memory, spatial navigation, emotional regulation among other functions. The term hippocampus originates from the Greek word for its seahorse-like shape first described by Arantius in 1587. For an excellent review of hippocampal literature see (Anderson et al., 2007).

1.6.1. Anatomy & Basic Circuitry

Generally, the hippocampus is comprised of the CA region, named in Latin after the ram-headed god Ammon's horn for its shape, the DG region, named for its toothlike indented structure, or dentate in Latin and the subiculum which is named in Latin for 'support'. The hippocampus receives inputs from across the brain primarily via the entorhinal cortex (EC) and is illustrated in Figure 4. Simply, the major pathways in the hippocampus are excitatory and unidirectional starting with the EC, where axons from cells originating in layer II form the perforant path which projects to the granule cells of the DG that then project to CA3 pyramidal neurons forming a pathway called the mossy fibres. The axons from these neurons form the schaffer collaterals that synapse on CA1 pyramidal neurons that finally project to the subiculum and EC. The subiculum itself also

projects to the EC. Generally, the subiculum is the primary source of subcortical projections whereas the EC is the primary source of neocortical projections. Together this simplified circuit (EC → DG → CA3 → CA1) is termed the trisynaptic circuit however there are direct projections from the EC the CA3, CA1 and subiculum in addition to other more complex connections.

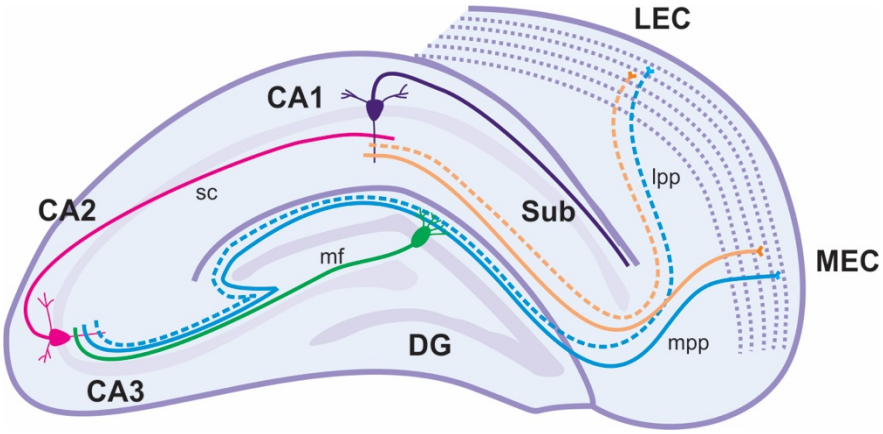

Figure 4. Simplified Hippocampal Circuitry.

Simplified schematic of hippocampal circuitry outlining the trisynaptic circuit. Briefly, the lateral and medial entorhinal cortices (LEC and MEC) form the lateral and medial perforant paths (LPP and MPP) respectively (blue) that provide input to the dentate gyrus (DG) granule cells. The granule cells axons form the mossy fibres (green) that project to the *cornu ammonis* (CA) 3 region pyramidal cells. The CA3 pyramidal cell axons form the schaffer collateral projection (pink) to the CA1 pyramidal cells, which project to the subiculum (purple). Abbreviations: CA1: cornu ammonis 1; CA2: cornu ammonis 2; CA3: cornu ammonis 3; DG: dentate gyrus; LEC: lateral entorhinal cortex; LPP: lateral perforant path; MEC: medial entorhinal cortex; MF: mossy fibres; MPP: medial perforant path; SC: schaffer collaterals; SUB: subiculum.

1.6.2. Cornu Ammonis

The CA region is subdivided into three regions, CA1, CA2 and CA3. A region termed CA4 has also been historically described by Lorente de No which was clarified as being a part of the DG. The primary cell type in the CA regions is the pyramidal cell whose cell bodies are located in the pyramidal cell layer, separating the stratum radiatum and stratum oriens. The cell bodies of these principal neurons vary based on subregion tending towards larger cell bodies in CA2 and CA3 (ranging from 20-30µm in diameter) and smaller cell bodies in CA1 (~15µm in diameter). These cells have basal dendritic arbours that extend into the stratum oriens and apical arbours that extend through the

stratum radiatum. While CA2 and CA3 play important functional roles in spatial memory and processing for the purposes of this dissertation the impact of PNEE on CA1 synaptic plasticity will be discussed in greater detail as it is the hippocampal subregion that has been best studied on this topic.

1.6.3. Dentate Gyrus

The DG is a unique area of the brain, exhibiting both structural and functional plasticity and playing a complex role in behavioural learning and memory. The DG is classically described as being U-shaped around the pyramidal cell layer of CA3, and is composed of three layers; the molecular, granule cell and polymorphic cell layers (Figure 5). The upper blade of the DG is referred to as the suprapyramidal blade while the lower blade is termed the infrapyramidal blade, connected by the genu or 'knee' of the DG.

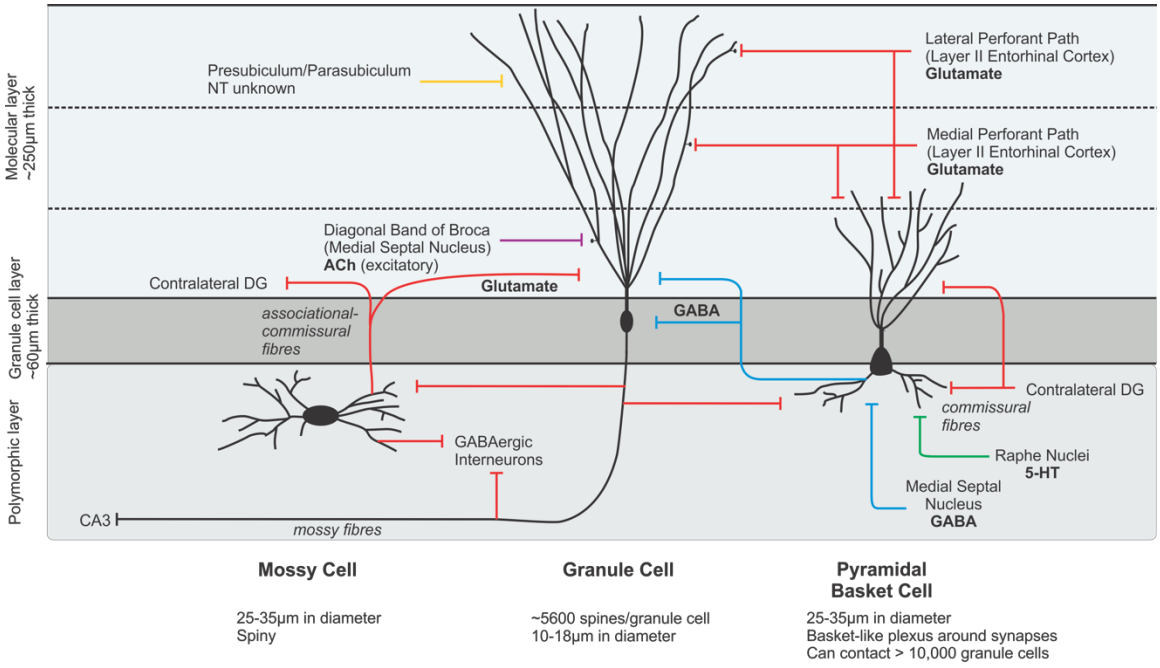

Figure 5. Inputs to DG Granule Cells.

Diagram depicting an overview of some input to dentate gyrus granule cells. Granule cells of the dentate gyrus (DG) receive input from diverse brain regions as well as from neighboring cells such as the mossy cells (left) and pyramidal basket cells (right). The primary excitatory input to these cells are from the entorhinal cortex via the lateral and medial perforant paths (LPP and MPP respectively; upper right), which also provide input to the inhibitory basket cells. Mossy cells are primarily responsible for communicating with the contralateral DG and also provide glutamatergic input to local granule cells. The pyramidal basket cells (and other inhibitory

interneurons) play a critical role in plasticity in this region given that they can provide feed forward inhibition (from LPP and MPP inputs) and feedback inhibition (from granule cells) to modulate granule cell activity. Abbreviations: Ach: acetylcholine; CA3; cornu ammonis 3; DG: dentate gyrus; GABA: gamma-aminobutyric acid; NT: neurotransmitter; 5-HT: serotonin.

The anatomy of the DG has been well-characterized and is described in good detail by a variety of book chapters and review papers (Amaral and Lavenex, 2007; Anderson et al., 2007; Frotscher and Seress, 2007). The molecular layer is approximately 250μm wide and is subdivided into thirds with the outer and middle thirds housing the perforant path inputs originating from the lateral and medial EC respectively. The inner third of the molecular layer houses intrinsic connections as well as strong feed-forward and feed-back inhibitory connections with interneurons. The molecular layer comprises for the most part dendrites of the granule cells and associated excitatory and inhibitory cells as well as the axons of the perforant path, however some inhibitory cell bodies can be found sparsely within this region such as the molecular layer perforant-path associated cells.

The granule cell layer is home to the primary cell type of the DG, the granule cells, which in general have smaller cell bodies (~10μm in diameter) than their neighboring pyramidal counterparts in the CA regions, and receive glutamatergic inputs from the perforant path in addition to various other neurotransmitter (NT) input throughout the molecular and polymorphic regions. Furthermore, dense inhibitory connections are made between granule cells and surrounding interneurons that generate both feed-forward and feed-back inhibition circuits. Granule cells in the suprapyramidal blade tend to have greater dendritic arbours and greater dendritic spine densities (~3500μm; 1.6 spines/μm) than those located on the infrapyramidal blade (~2800μm; 1.3 spines/μm). The cell layer itself is approximately 60μm thick, densely packed with granule cells to total an estimated 1.2×10^6 cells per DG in the adult rat, although this density can be affected by the generation of new neurons throughout the lifespan, or neurogenesis, as well as experimental manipulations and neurological disease that can impact cell health and neurogenic processes. Importantly, a well-known inhibitory interneuron known as the pyramidal basket cell resides in the deep granule cell layer and

performs essential inhibitory functions on the granule cells. The apical dendritic arbours of these cells extend into the molecular layer, receiving input from the perforant path. These pyramidal basket cells thus can provide feed-forward inhibition from EC inputs to their inhibitory connections on over 10 000 granule cells per basket cells. They are so-named for their basket-like plexus that surround their synapses (see (Amaral and Lavenex, 2007; Ribak and Shapiro, 2007) for a review of DG anatomy).

The polymorphic layer of the DG is often also described as the hilus and houses both inhibitory and excitatory neurons. Notably this layer is separated from the granule cell layer by the subgranular zone (SGZ) best known for being a locus of neurogenesis. Mossy cells exist in the polymorphic layer that make associational bilateral connections between granule cells of both hemispheres and are characterized as being glutamatergic. Their nomenclature originates from receiving mossy input from DG axons as well as for their 'spiny' appearance due to large, dense spines. Interneurons in this region remain poorly described but include hilar perforant-path associated cells that provide inhibitory input to DG granule cells (see (Houser, 2007) for a review of interneuron types in the DG).

The DG plays a complex role in behavioural learning and memory, which is only beginning to be unraveled. As will be discussed later in this dissertation, the DG participates in identifying and distinguishing intricate differences between objects in space but yet may also decode and avail of larger directional cues or objects to orient a subject in their surroundings (Kesner, 2007; Treves et al., 2008). Specifically, the DG is classically known for its ability for pattern separation, which allows a subject to differentiate between highly similar circumstances or objects. This specialized property of the DG is shared with other regions of the brain such as the piriform cortex, or olfactory cortex, which is responsible for pattern separation of different odourants and odour mixtures (Barnes et al., 2008; Leutgeb et al., 2007; Shakhawat et al., 2014).

1.6.4. Perforant Paths

The primary glutamatergic inputs to the DG originate from the perforant path, formed of axons from layer II pyramidal neurons in the EC that are so-named for the fact that they perforate the subiculum in order to access the DG. Perforant path input originating from the lateral EC form the lateral perforant path (LPP) whereas inputs originating from the medial EC form the medial perforant path (MPP). Axons of the LPP and MPP occupy the outer and middle thirds of the molecular layer, respectively. These inputs form excitatory synapses on the apical dendritic arbours of granule cells as well as on dendrites of inhibitory interneurons such as the pyramidal basket cells (Andersen et al., 1971; Hjorth-Simonsen and Jeune, 1972; McNaughton, 1980; Witter, 2007). While the focus of the present dissertation is centered on DG plasticity there is evidence that both MPP and LPP projections can directly synapse in both CA3 and CA1 (Do et al., 2002; Steward and Scoville, 1976).

Recent evidence has suggested that the MPP and LPP inputs differ in their pharmacological and electrophysiological properties and perhaps in their functional roles in the behaving animal. Pharmacologically the MPP may be subject to greater cholinergic innervation and perhaps modulation than the LPP (Kahle and Cotman, 1989) as well as divergent distribution and function of group II and group III metabotropic glutamate receptors (mGluR)(Macek et al., 1996).

Electrophysiologically, there has been evidence to suggest divergence in vesicle release probability between these inputs, with the MPP having a greater release probability than the LPP (McNaughton, 1980) although this depends on the inter-pulse intervals and simulation intensities used (Petersen et al., 2013). Specifically, the differences between the two pathways in their PPRs are reduced at greater inter-pulse-intervals (200ms) and are most obvious at shorter intervals such as those used in the present dissertation (50ms). Furthermore, subtle changes in the kinetics of the fEPSPs that can be evoked in the MPP and LPP can be detected where the latency of the peak amplitude in the fEPSPs evoked in the MPP were slightly reduced compared to those evoked in the LPP (Petersen et al., 2013). Additionally synaptic plasticity in both

pathways appear to be of differential origin with LTP in the MPP being dependent on NMDARs and in the LPP on μ opioid receptors (Breindl et al., 1994; Dahl et al., 1990; Derrick et al., 1992; Do et al., 2002). Electrophysiological differentiation of the MPP and LPP remains unclear at this time however it warrants further investigation in order to dissociate the roles of these two inputs to the hippocampal circuit in learning and memory behaviours. It has been thought that each pathway is important for different kinds of information and subsequent learning. In a study from the laboratory of Hunsaker, the roles for LTP in the MPP and LPP were dissociated through the administration of NMDAR and μ-opioid antagonists to block plasticity in each pathway respectively and examine subsequent behavioural abnormalities in either a spatial or non-spatial (novel object) learning paradigm (Hunsaker et al., 2008). The findings from these experiments showed that blockade of either NMDARs or μ-opioid receptors impaired spatial memory whereas novel object memory was disrupted only by blockade of the latter presumably signifying that if LTP in the LPP did indeed require activation of μ-opioid receptors these inputs are important for processing non-spatial information. As for spatial information it is possible that the DG is involved in incorporating critical information from both the MPP and LPP prior to projecting to CA3. Interestingly, when these experiments were completed in CA1 it was found that the roles of the MPP and LPP in these behaviours were completely separate and activated distinct neuronal populations in this subregion. It has also been reported that projections from specialized grid cells located in the medial EC are contained within the MPP that are responsible for encoding spatial topographic maps of environments (Hafting et al., 2005; Witter and Moser, 2006).

1.6.5 Hippocampal Development

The telencephalon is of special interest for the present dissertation because it gives rise to the hippocampal formation, in addition to forebrain cortical structures and the neocortex. The majority of hippocampal development occurs prior to birth in rodents (Frotscher and Seress, 2007; Khalaf-Nazzal and Francis, 2013), however the first postnatal week is considered to be the third trimester equivalent to humans and is often known colloquially as the ‘brain growth spurt’ (see Figure 1). The various subregions of

the hippocampus develop at different rates with CA pyramidal cells developing between GD 11 to 21, and peak generation of principal neurons occurring between GD 15 and 16. Meanwhile granule cells of the DG first appear on GD 16 and their growth curve is much slower, with only approximately 15% of granule cells being present at birth in rodents (Bayer, 1980; Bayer and Altman, 1975, 1987). Development of the DG granule cells is more or less complete as of the second to third week after birth in rats, peaking at up to 50 000 new granule cells being generated daily between P 5-7 (Schlessinger et al., 1975). Despite the late development of the granule cells, their inputs from the EC can correctly target the appropriate areas of the molecular layer as early as GD 18 with the guidance of a combination of repulsive signals from the EC, neurotrophic factors from the extracellular matrix and from 'pioneer' Cajal-Retzius cells (Frotscher and Seress, 2007; Supèr and Soriano, 1994). The dendritic spine numbers on granule cells of the DG have been reported to be at approximately a similar level as in adults by the third postnatal week (Cotman et al., 1973). The total number of granule cells at 1 month of age in Sprague-Dawley rats is relatively constant throughout adulthood, although this can vary in other strains (Boss et al., 1985).

1.7. Hippocampal Synaptic Plasticity

The term synaptic plasticity was first introduced by Jerzy Konorski then formally hypothesized by the Canadian neuroscientist Donald Hebb in the mid-1900s to describe the long-lasting changes in the brain that were thought to underlie memory storage and has since been widely used (Hebb, 1949; Konorski, 1948). Now nearly 70 years later, the term retrieves over 50 000 scientific articles using the PubMed online database and its varieties, functions and molecular underpinnings have been extensively studied both under healthy and disease conditions across a range of brain regions. Short term forms of plasticity in addition to longer forms such as LTP and LTD will be further discussed below. These forms of plasticity can be recorded in the whole animal (anesthetized or awake) termed *in vivo* or in hippocampal slices or cell culture termed *in vitro*. While the effects of PNEE on synaptic plasticity have been examined using both techniques, *in vitro* hippocampal slice electrophysiology will be used for the work contained in this dissertation.

1.7.1. **Paired Pulse Plasticity**

Paired pulse (PP) plasticity is measured by delivering two pulses of stimulation separated by short interpulse intervals, ranging from a few to several hundred milliseconds and has been used as a relative indicator of presynaptic release probability. In the present dissertation PP is used to determine whether our experimental manipulation (PNEE) impacts NT release probability from MPP inputs. Plasticity is typically measured as the ratio of the slope of the second fEPSP relative to the slope of the first and is commonly used as a relative measure of release probability. When this ratio value is greater than one (i.e. the second pulse has a greater slope than the first) this is called paired pulse facilitation while when the ratio value is less than one (i.e. the second pulse has a smaller slope than the first) this is paired pulse depression (see (Regehr, 2012; Zucker and Regehr, 2002) for review). The simplest mechanistic explanation for this type of plasticity is illustrated in Figure 6. In the first pulse of stimulation vesicles in the readily releasable pool in the presynaptic terminal bind to the lipid bilayer and exocytose their contents (neurotransmitters) into the synaptic cleft to subsequently bind to postsynaptic receptors, allowing ions to flux and leading to a measurable fEPSP. In the second pulse the same process is repeated however depending on the release probability of the presynaptic terminal this second fEPSP will be, on average larger or smaller. In terminals with a high release probability, more vesicles would be released during the first pulse which can lead to fewer vesicles docked and in the readily releasable pool to be released during the second pulse, leading to a smaller second fEPSP and therefore paired pulse depression. Meanwhile in terminals with a lower release probability, fewer vesicles may be available for release upon the first stimulation and residual calcium in the presynaptic terminal may facilitate vesicle release upon the second pulse leading to a larger second fEPSP and therefore paired pulse facilitation (Katz and Miledi, 1968). It should be noted however that many other presynaptic factors can be involved in affecting paired pulse plasticity such as the relative presence of calcium buffers and their affinities for calcium. For example, it is possible that calcium buffers could be saturated with calcium rather than a mechanism dependent on increased calcium entry into the presynaptic terminal.

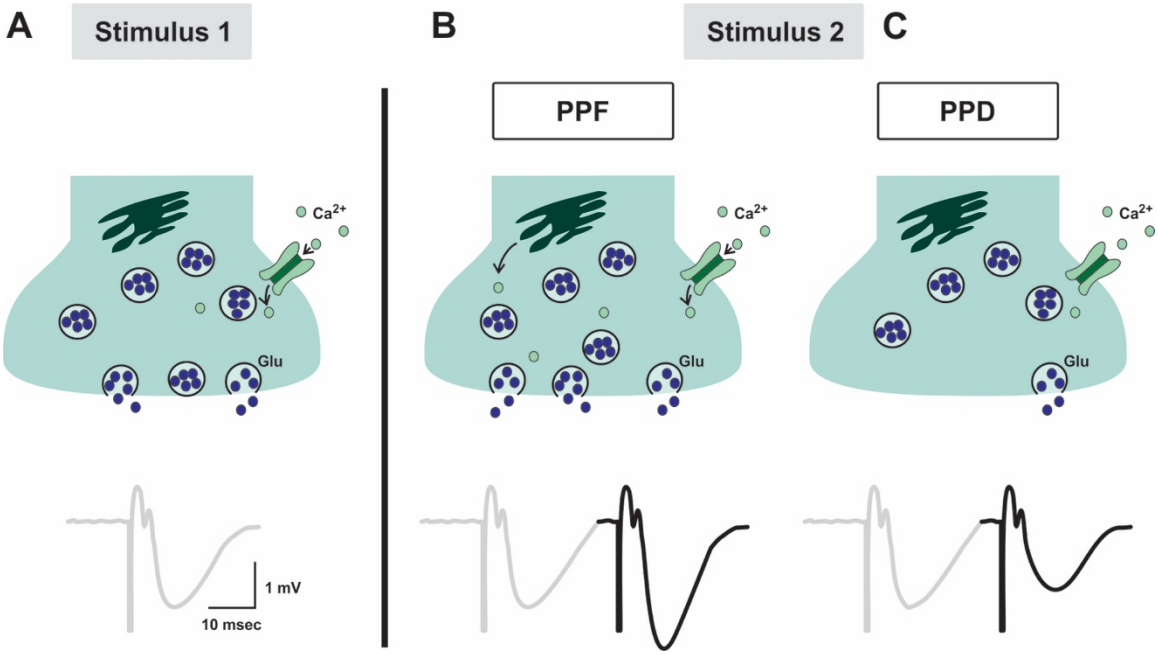

Figure 6. Paired Pulse Plasticity.

Pairs of stimuli can be delivered in close temporal proximity to give an estimate of the effect of a treatment on presynaptic NT release. (**A**) A depiction of the glutamatergic presynaptic terminal processes following delivery of the first pulse of stimulation. An example fEPSP is provided below in grey. Vesicles loaded with glutamate (Glu; dark blue circles) exist in the presynaptic terminal. Upon electrical stimulation, voltage-gated calcium channels (light teal channel, upper right) flux calcium (CA^{2+}) ions that are involved in vesicle fusion and NT release. Vesicles in the readily releasable pool fuse with the presynaptic membrane and release their contents in the synaptic cleft. (**B**) In a terminal with a low release probability the second pulse (black fEPSP below) will have a greater slope than the first possibly due to residual calcium in the terminal. (**C**) In a terminal with a high release probability, the first pulse may deplete the vesicles in the readily releasable pool, leading to fewer being ready for release during the second pulse, yielding a second fEPSP (black fEPSP below) that has a smaller slope. Used with permission from (Fontaine et al., 2016). Abbreviations: Ca^{2+}: calcium; Glu: glutamate; PPD: paired-pulse depression; PPF: paired-pulse facilitation.

1.7.2. Post Conditioning Short-Term Plasticity

Another form of short term plasticity relevant to this dissertation is post-tetanic potentiation (PTP) and its pseudo-inverse form termed short-term depression (STD). These temporary increases and decreases follow the delivery of the conditioning stimulus necessary for either LTP or LTD and decay quickly. PTP is a term used to describe the brief increase in the size and slope of the fEPSP that immediately follows high-frequency stimulation (HFS) and its duration can be extended with longer bouts of HFS (Magleby,

1987) and is accompanied by augmentation which has a shorter duration. The mechanistic underpinnings of PTP are thought to be related to increases in calcium, either by increased influx or increased residual calcium that will subsequently lead to facilitation of vesicle release (Delaney et al., 1989; Habets and Borst, 2005, 2006; Kamiya and Zucker, 1994). Similarly following low frequency stimulation (LFS) a brief period of accentuated synaptic depression is typically observed and has been described as STD. The mechanisms underlying this short term plasticity are considered to be related to a depletion of the readily releasable pool of vesicles caused by the prolonged nature of LFS (typically in the range of tens of minutes)(Schneggenburger et al., 2002). Other possible mechanisms related to calcium involve an LFS-induced reduction in presynaptic calcium influx leading to reduced transmission (Kamiya and Zucker, 1994; Regehr, 2012; Xu and Wu, 2005; Zucker and Regehr, 2002).

1.7.3. Long-Term Potentiation

Arguably one of the most popular topics in the study of neuroscience, LTP has been widely studied throughout the brain and has long been known as one of the top candidates for the mechanisms underlying behavioural learning and memory. First published in 1973, Tim Bliss and Terje Lomo showed that at the MPP-DG synapse of the hippocampus (Bliss and Lomo, 1973) brief bouts of tetanic stimulation yielded increaseds in the amplitude of their recorded field excitatory postsynaptic potentials (fEPSPs) in the rabbit *in vivo*. Later this long-lasting potentiation was given the name by which it is now known – LTP (Douglas and Goddard, 1975). As such numerous excellent reviews of the literature have been written (Abraham, W. C. & Williams, 2003; Bear and Malenka, 1994; Bliss et al., 2007; Bortolotto et al., 2011; Larkman and Jack, 1995; Malenka, 1994; Malenka and Bear, 2004; Malinow and Malenka, 2002; Stevens, 1998). Simply, LTP is a long-lasting increase in synaptic efficacy as a result of prior synaptic activation at a high frequency and exists in many forms throughout the brain and within the hippocampus itself. The form of LTP observed can depend on the conditioning stimulus or induction paradigm used which can vary in terms of their intensities, frequencies, durations and patterns. For example, HFS tuned to the theta rhythm can also be used to induce LTP.

The phenomenon of increased synaptic efficacy has been observed to last from hours and days to weeks.

The most commonly described form of glutamatergic LTP in the hippocampus requires the activity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropoionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs; Figure 7). Upon stimulation of glutamatergic inputs such as, in the case of this dissertation, the MPP, glutamate is released into the synaptic cleft where it binds to postsynaptic glutamatergic receptors including the AMPAR, NMDAR and mGluRs, among others. The AMPAR is an ionotropic receptor that fluxes sodium and potassium ions, which leads to an accumulation of increasingly positive charge (*i.e.* depolarization) in the postsynaptic terminal, which will dislodge the positively-charged magnesium ion blocking the pore of neighboring NMDARs where glutamate is already bound (in addition to co-agonists such as glycine). Once the NMDAR pore is open it fluxes important calcium ions in addition to sodium and potassium into the postsynaptic cell. It should also be noted that AMPARs that do not contain the GluA2 subunit are also capable to fluxing calcium although to a lesser extent (see (Isaac et al., 2007) for review).

Calcium entry into the postsynaptic cell is a critical component of this plasticity as it unleashes second messenger cascades that contribute to long-lasting changes in synaptic efficacy through effects on both the pre- and postsynaptic cells. This sharp rise in intracellular calcium activates calmodulin and subsequently the calcium-calmodulin dependent protein kinase II (CaMKII). CaMKII consists of regulatory and catalytic subunits, which separate upon binding with the calcium-bound calmodulin complex. The catalytic subunit then can go on to phosphorylate a variety of substrates including the AMPAR itself. Furthermore, LTP is associated with a number of other second messenger signalling cascades including activation of other kinases such as protein kinase A (PKA), protein kinase C (PKC) map-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and other elements such as cyclic adenosine monophosphate (cAMP), cAMP response element binding protein (CREB) among many others. Ultimately these signaling cascades can lead to long-term changes to the synapse and cell

itself that support long-lasting increases in synaptic efficacy. One of the best studied mechanisms of increased postsynaptic responsiveness as a result of an LTP-inducing stimulus (such as HFS) is altered AMPAR dynamics. The GluA1 and GluA2 subunits of the AMPAR are best studied in the context of synaptic plasticity due to a variety of phosphorylation sites that are targeted by protein and tyrosine kinases associated with LTP (Wang et al., 2005). In particular, the GluA1 subunit has two well-characterized phosphorylation sites at ser-831 and ser-845 which are phosphorylated by PKC or CaMKII and PKA, respectively increasing AMPAR conductance, currents, as well as channel open probability, which are associated with LTP (Barria et al., 1997; Derkach et al., 2007; Esteban et al., 2003; Lisman and Zhabotinsky, 2001; Lledo et al., 1995; Roche et al., 1996). PKC activation also results in phosphorylation of ser-880 on the GluA2 subunit (Matsuda et al., 2002; McDonald et al., 2001). In addition to the aforementioned effects of phosphorylation at distinct sites on AMPAR kinetics, this can also lead to enhanced insertion of new AMPARs into the postsynaptic membrane, which has been classically associated with LTP. The long-lasting changes in synaptic efficacy in LTP can also result in structural changes such as extension of the postsynaptic density, widening of the dendritic spine head and even the development of new spines among others (Bell et al., 2014; Watson et al., 2016). It is likely that the latter changes support longer-lasting forms of LTP beyond the scope of the present dissertation.

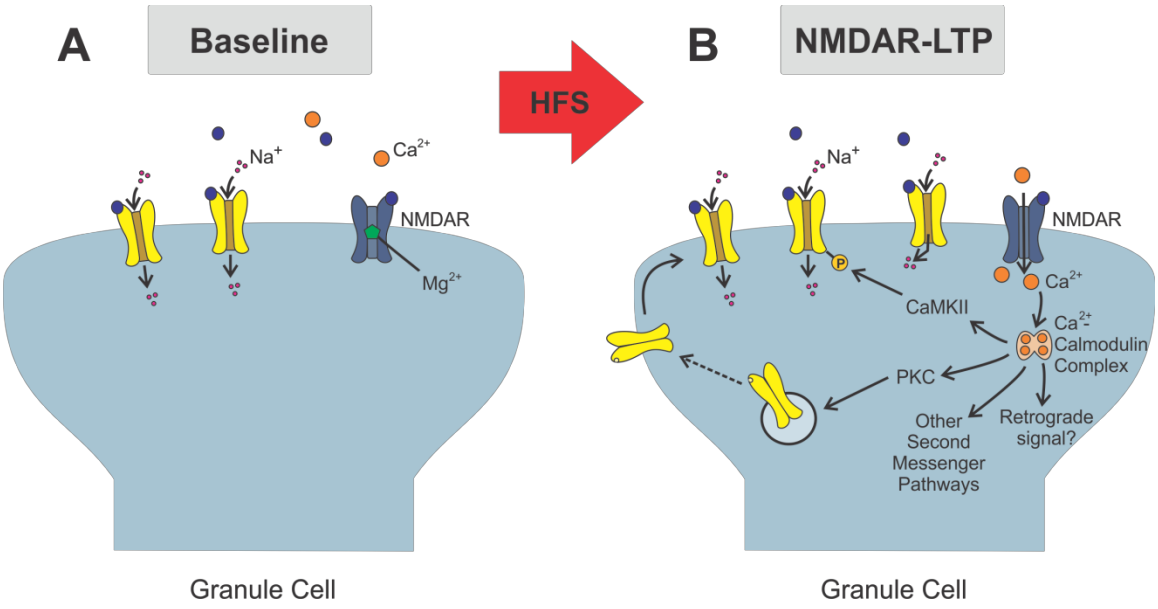

Figure 7. Simplified Mechanisms of NMDAR-LTP

(A) Under baseline conditions at glutamatergic terminals, glutamate (Glu) in the synaptic cleft binds to and opens AMPARs that flux sedum (Na^+) ions into the postsynaptic cell. Glu also binds to the NDMAR however without sufficient membrane depolarization the magnesium (Mg^{2+}) remains in the pore of the NMDAR, blocking flux of cations sodium and calcium (Ca^{2+}). (B) Following high-frequency stimulation, Glu binds to both AMPARs and NMDARs where sodium flux through the AMPAR causes sufficient depolarization of the membrane to expel the Mg^{2+} block in the NMDAR, allowing it to flux both Na^+ and Ca^{2+}. The increase in postsynaptic Ca^{2+} activates CaMKII among other second messenger signalling pathways that ultimately lead to the insertion of AMPARs into the postsynaptic membrane and potentiation of the fEPSP. Abbreviations: AMPAR: α-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid; Ca^{2+}: calcium; CaMKII: calcium-calmodulin-dependent protein kinase II; LTP: long-term potentiation; Mg^{2+}: magnesium; Na^+: sodium; NMDAR: N-methyl-D-aspartate receptor.

LTP is characterized by 3 key properties: cooperativity, associativity and input-specificity. Cooperativity refers to the fact that there is a threshold for the stimulus intensity necessary to induce LTP by recruiting sufficient afferent fibres to initiate the postsynaptic second messenger signalling cascades that we now know are implicated in LTP. In these initial studies taking place in the DG, McNaughton and colleagues examined the relationship between stimulus intensity and resulting increases in the subsequent size of fEPSPs (McNaughton et al., 1978). Of special interest to the present dissertation is a set of experiments in this study where the authors delivered repeated bursts of HFS in order to saturate the potentiation observed in the DG. Also studied by this group was the concept of associativity where weak inputs could be potentiated if a

strong stimulus was coincidentally delivered in another input (Levy and Steward, 1979; McNaughton et al., 1978). Finally, LTP is input-specific such that inputs that are not active during HFS are not involved in the subsequent potentiation observed in that pathway (Andersen et al., 1977; Lynch et al., 1977).

1.7.4. Long-Term Depression

The so-called flipside of this LTP, LTD was first shown experimentally by Lynch et al. to occur at synapses adjacent to those being potentiated, and was termed heterosynaptic, or non-associative, LTD because it occurred at unstimulated synapses (Dunwiddie and Lynch, 1978; Lynch et al., 1977). Homosynaptic, or associative LTD, was later shown to occur at stimulated synapses in a single input using prolonged LFS from a baseline condition, (*i.e. de novo* or without prior plasticity or priming) and within a single pathway by Serena Dudek and Mark Bear nearly 20 years after the initial observations of LTP (Dudek and Bear, 1992). As opposed to the widely studied, mechanistically-explained and well-known LTP, its 'flipside' LTD remains under-investigated. It is likely that both LTP and LTD play important cooperative roles in behavioural learning and memory. LTD is the long-lasting decrease in synaptic efficacy that follows LFS and also exists in many forms throughout the CNS. As with LTP within the hippocampus there are various mechanisms that support each form of LTD, although the best-characterized forms of this type of plasticity in the DG are NMDAR or mGluR-dependent (see Figure 8).

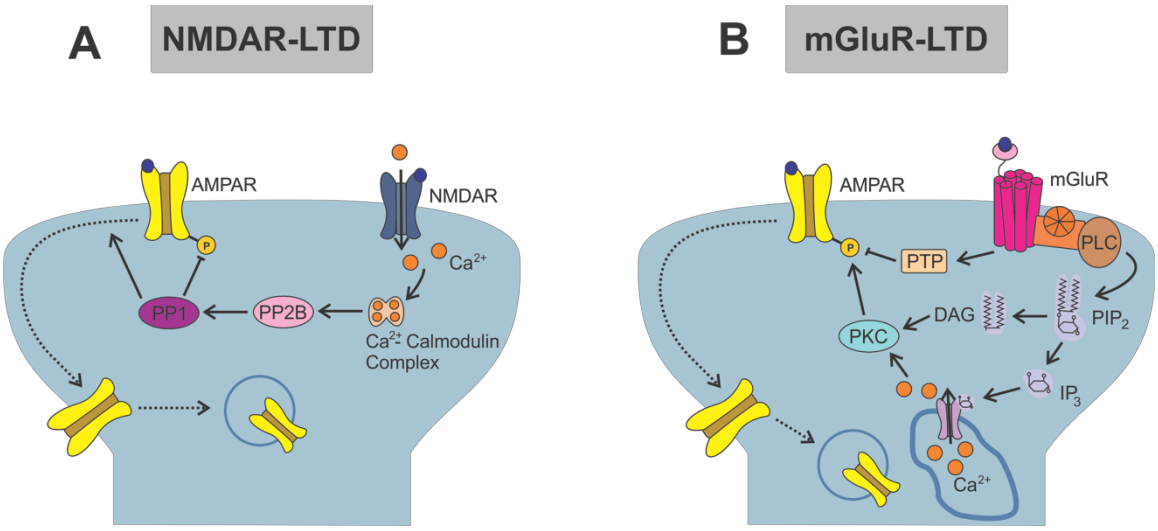

Figure 8. Simplified Mechanisms of NMDAR and mGluR LTD.

(**A**) In the classic example of NMDAR-dependent LTD at glutamatergic terminals, low frequency stimulation (LFS) leads to reduced activation of NMDARs and thus reduced calcium (Ca^{2+}) entry, which bind to less calmodulin forming fewer calcium-calmodulin complexes, which preferentially activate protein phosphatase 2B (PP2B), which activates protein phosphatase 1 (PP1), dephosphorylating the GluA1 subunit of the AMPARs at serine-845, leading to their eventual lateral diffusion and internalization. (**B**) In mGluR LTD LFS activated phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG can then activate protein kinase C (PKC), while IP_3 binds to calcium receptors on the endoplasmic reticulum to cause intracellular calcium release, also activating PKC. PKC phosphorylates the GluA2 subunit of the AMPARs at serine 880 leading to their lateral diffusion and eventual internalization. This pathway also includes activation of protein tyrosine phosphatases (PTPs) that can dephosphorylate the GluA2 subunit of the AMPAR and similarly lead to AMPAR internalization. It should be noted that these pathways are overly simplified for illustration processes and that many other second messenger signaling cascades can be activated in these pathways contributing the long-term depression (LTD). Used with permission from (Pinar et al., 2017). Abbreviations: AMPAR: α-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid; Ca^{2+}: calcium; DAG: diacylglycerol; IP_3: inositol triphosphate; mGluR: metabotropic glutamate receptor; NMDAR: N-methyl-D-aspartate receptor; PIP_2: phosphatidylinositol 4,5-biphosphate; PKC: protein kinase C; PLC: phospholipase C; PP1: protein phosphatase 1; protein phosphatase 2B; PTP; protein tyrosine phosphatase.

Hippocampal LTD can be induced through a variety of protocols based on the technique of electrophysiology, age of the animal and desired form of LTD (see (Pinar et al., 2017) for review). This form of plasticity has long been known to be age-dependent, with the greatest magnitudes of LTD being measured in young and aged animals. The most commonly-used protocols for inducing LTD are LFS that typically consist of stimulation at low frequencies (0.5-5 Hz) for long periods of time (600-900 pulses) (Dudek and Bear, 1992, 1993; Dunwiddie and Lynch, 1978). Other groups have extended

LFS to beyond 900 pulses (1200-2400) to yield either a greater magnitude of LTD or to induce LTD in older animals (Kamal et al., 1999; Nosyreva and Huber, 2005; Raymond et al., 2003). These classic protocols have been modified and applied to *in vivo* electrophysiology where *de novo* LTD has been historically challenging to induce. In some cases the LFS is delivered in pairs of pulses rather than single pulses as in the experiments by Dudek, Bear and colleagues (Thiels et al., 1994). Hippocampal LTD can also be induced pharmacologically by infusion of NMDA (for NMDAR-dependent LTD) or mGluR agonists such as (S)-3,5-dihydroxyphenylglycine (DHPG) or 1-amino-1,3-dicarboxycyclopentane (ACPD) (Mukai et al., 2007a; Murakami et al., 2015; O'Mara et al., 1995; Overstreet et al., 1997; Shiroma et al., 2005).

The best-studied forms of hippocampal LTD can be simply classified as either being NMDAR or mGluR-dependent and each activate distinct second messenger signalling cascades to support long-term changes at the level of the synapse that are thought to result in AMPAR endocytosis and removal from the synapse, resulting in a long-lasting depression in synaptic efficacy. AMPARs undergo regular insertion (exocytosis) and removal (endocytosis) from the post-synaptic density (PSD) through interactions with various scaffolding proteins (Man et al., 2000). In particular, there are tyrosine residues on the GluA2 subunits that are targets for plasticity-related phosphorylation that are related to the anchoring of AMPARs in the PSD (see Figure 9 for a summary of GluA1 and GluA2 phosphorylation sites related to LTP and LTD (Ahmadian et al., 2004)). Specifically, phosphorylation at Tyr-876 by tyrosine receptor kinases (Trks) inhibits the association of the GluA2 with cytoskeletal proteins and leads to internalization of the receptor (Hayashi and Huganir, 2004). Interestingly, blockade of phosphorylation on tyrosine residues on the GluA2 inhibits AMPAR endocytosis, LTD and spatial memory consolidation in the morris water maze (MWM) task (Ahmadian et al., 2004; Ge et al., 2010). Very recent evidence however suggests that LTD is associated not always directly with increased endocytosis of the GluA1-subunit-containing AMPAR but rather with a suppression of typical exocytosis that will still lead to fewer net AMPARs present in the post-synaptic density (Fujii et al., 2018). While there may be disagreement in the literature as to what specifically about AMPAR trafficking (receptor

subunits and mechanisms involved) contributes to hippocampal LTD, it is clear that the dynamics of the movement of these receptors in and out of the post-synaptic membrane are integral to plasticity. It should also be noted that most studies examining the specific dynamics of AMPAR endocytosis have either been examined in hippocampal cell cultures or in the CA1 region, and whether there are regional differences in AMPAR dynamics is unknown.

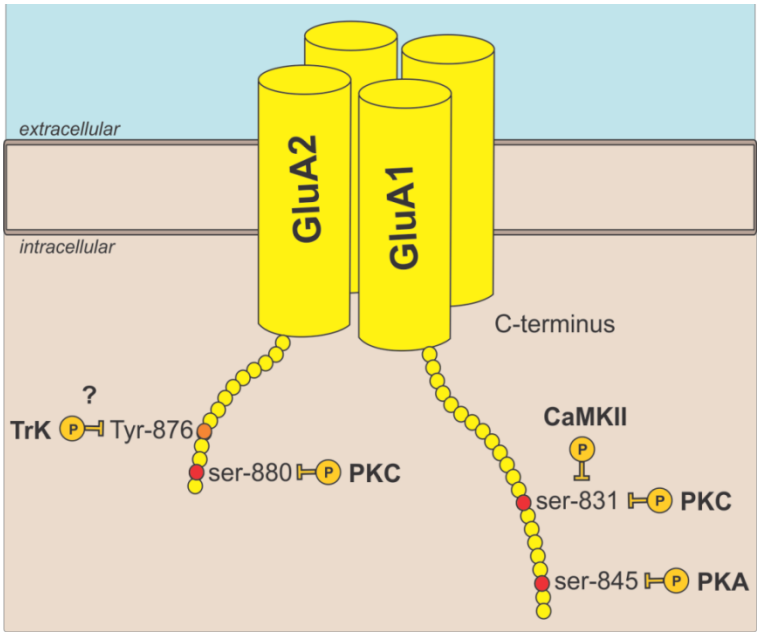

Figure 9. Plasticity-Related Phosphorylation Sites on the AMPAR

The GluA1 and GluA2 subunits of the AMPAR are the best studied in the context of synaptic plasticity. On the GluA1 subunit there are two principal serine residues that are known to be phosphorylated and dephosphorylated in synaptic plasticity. At ser-831 phosphorylation can occur via activation of CaMKII or PKC whereas phosphorylation at ser-845 is accomplished by activation of PKA. Although it is less-studied, the GluA2 subunit has one key serine residue, ser-880 that is phosphorylated following activation of PKC meanwhile tyrosine residues have been associated with anchoring the AMPAR to the appropriate cytoskeletal proteins. Specifically, the tyr-876 residue is phosphorylated by Trks which inhibits the association between the receptor and the anchoring proteins, leading to its eventual internalization.

It may be tempting to view these forms of NMDAR-dependent and mGluR-dependent LTD, as well as the many other possible mechanisms, as distinct processes; but they are likely not as the hippocampus and its subregions are capable to supporting several distinct pathways for LTD (Oliet et al., 1997; Pöschel and Stanton, 2007).

In NMDAR-LTD, calcium ions enter via the NMDAR itself or through voltage-gated calcium channels, which, as in LTP, will then trigger a signalling cascade that is thought to involve the internalization of AMPARs. In the case of LTD, the calcium-calmodulin complex preferentially activates protein phosphatase 2B (PP2B) or calcineurin, which subsequently dephosphorylates inhibitor 1 (I-1) that normally inhibits protein phosphatase 1 (PP1) in its phosphorylated state. Through dephosphorylation of I-1, PP1 is activated, which dephosphorylates the AMPARs at the synapse at the serine-845 site and leads to reduced ion flux and AMPAR internalization (Beattie et al., 2000; Carroll et al., 2001; Lee et al., 1998, 2000; Malenka and Bear, 2004; Mockett et al., 2011; Mulkey et al., 1993, 1994). Both LTP and LTD can be initiated through intracellular calcium signals, however the valence of the subsequent effect on synaptic efficacy (potentiation or depression) is related to the affinity of CaMKII and calcineurin for the calcium-calmodulin complex. PP2B, or calcineurin, has a higher affinity for the calcium-calmodulin complex than does CaMKII, meaning that it will be preferentially activated at lower calcium concentrations during the induction of NMDAR-LTD (see (Xia and Storm, 2005) for a review of the role of calmodulin in plasticity). Calcineurin itself can also reduce the open time of NMDARs thereby reducing further calcium entry into the postsynaptic cell, which may also contribute to LTD (Shi et al., 2000). Through these, and likely other, postsynaptic signalling cascades synaptic efficacy is reduced as compared to pre-conditioning recordings.

The mechanisms underlying mGluR-LTD are less clear and are also dependent on the mGluR subtype activated (see (Sanderson et al., 2016) for review). Activation of group I mGluRs ($mGluR_1$ and $mGluR_5$) is linked with activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) from the lipid bilayer of the cell into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 can then bind to its intracellular receptor to release calcium from intracellular stores, while DAG can activate a number of kinases including PKC, MAPK, ERK and phosphoinositide 3-kinase (PI3K) that can phosphorylate the serine-880 site of the GluA2 subunit of the AMPAR which can lead to lateral diffusion and internalization of the AMPAR (Gladding

et al., 2009; Lüscher and Huber, 2010). While these mechanisms can obviously be activated by the use of agonists ACPD and DHPG, mGluR-LTD can also be initiated through electrical mechanisms (Huber et al., 2000). Some forms of mGluR-LTD are also thought to be expressed presynaptically, and independently of the classic postsynaptic, calcium-related mechanisms (Fitzjohn et al., 2001). What differentiates NMDAR-LTD from mGluR-LTD in terms of electrical induction remains a question, and there are clearly overlapping activation mechanisms within the second messenger cascade and expression of these forms of LTD.

Recent work has implicated endocannabinoid (eCB) signalling in LTD in the brain (Chevaleyre et al., 2006). eCBs have long been thought to play a role as retrograde signals involved in synaptic plasticity but are now known to play various roles in plasticity and brain function (see (Araque et al., 2017; Castillo, 2012; Castillo et al., 2012; Heifets and Castillo, 2009; Ohno-Shosaku and Kano, 2014; Südhof and Malenka, 2008) for review). The role of eCBs in synaptic function is not strictly limited to retrograde neurotransmission, as their g-protein coupled cannabinoid type 1 (CB1) receptor can also be found in the postsynaptic membrane and on astrocytes (Gutiérrez-Rodríguez et al., 2017, 2018). Early work has suggested that the presynaptic activity of eCBs plays a role in the short-term component of DHPG-induced mGluR LTD (Rouach and Nicoll, 2003). Most recently, using electrical LFS (10 Hz for 10 minutes) in the DG yielded a form of eCB-LTD that was dependent on group I mGluR activation and release of calcium from intracellular stores (Chiu and Castillo, 2008).

1.7.5. Depotentiation

In order for a biological system to maintain capacity to edit existing learning or LTP logically there must be some mechanism by which this process could be reversed, which is now known as depotentiation (Barrionuevo et al., 1980; Staubli and Lynch, 1990). Depotentiation is the apparent reversal of established LTP by LFS and is readily observed in the hippocampus. In fact, the study of depotentiation preceded that of *de novo* LTD (Barrionuevo et al., 1980; Dunwiddie and Lynch, 1978; Staubli and Lynch, 1990). This may perhaps be due to the age-dependency for the induction of de novo LTD

as we now know, using typical LFS (Dudek and Bear, 1992, 1993). Bidirectional plasticity may in fact be mediated by distinct AMPAR subunit phosphorylation and dephosphorylation where previously depressed synapses can be repotentiated by HFS possibly through phosphorylation at ser-845 on the GluA1, meanwhile previously potentiated synapses can be depotentiated through dephosphorylation at ser-831 on the GluA1 (See Figure 10; (Lee et al., 2000, 2003)).

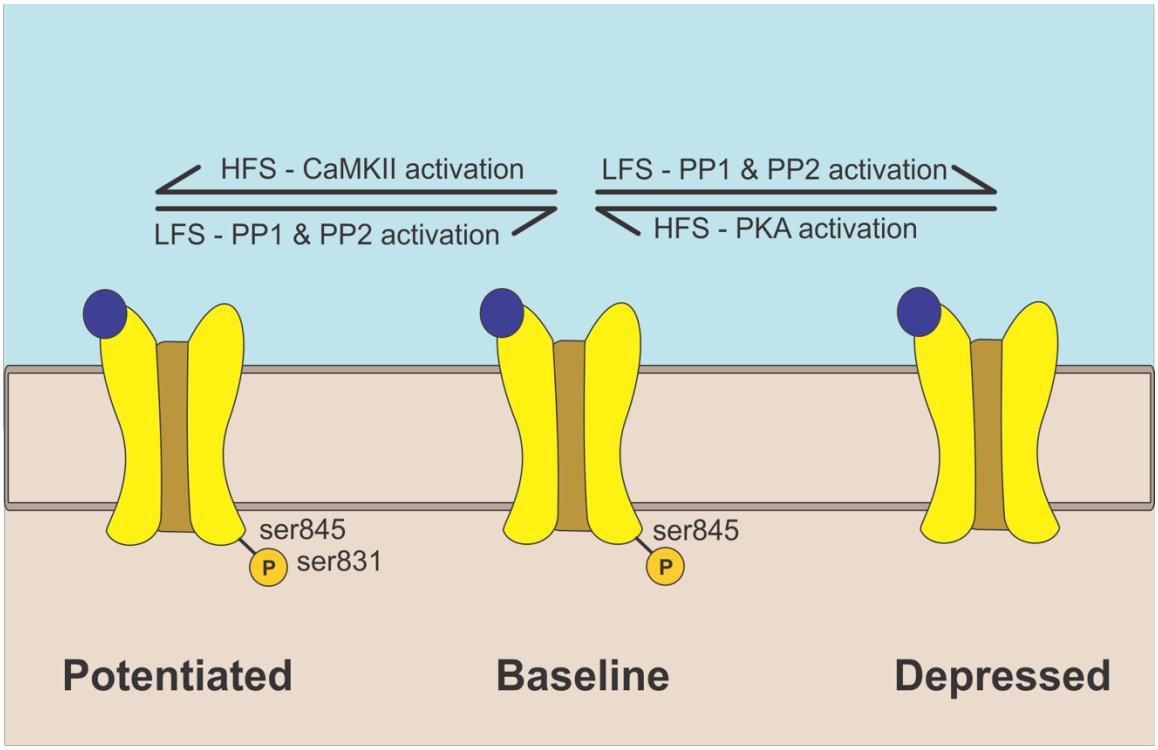

Figure 10. GluA1 Phosphorylation States in Bidirectional Synaptic Plasticity.

Different serine residues are phosphorylated or dephosphorylated depending on the previous activation state of the AMPAR. At baseline conditions (center) the GluA1 subunit of the AMPAR is phosphorylated at serine-845. Following low frequency stimulation (LFS) this site is dephosphorylated (right) via activation of protein phosphatases 1 and 2 (PP1 and PP2 respectively) among other cascades. High frequency stimulation (HFS) from baseline leads to phosphorylation at serine-831 (left). If LFS is delivered as in depotentiation it is hypothesized that this leads to dephosphorylation at serine-831but not a serine-845. It should be noted that this process is overly simplified and other subunits of the AMPAR can be phosphorylated and dephosphorylated to affect its function and ultimately possibly the lateral diffusion and internalization of the receptor as in long-term depression.

1.7.6. Involvement of Synaptic Plasticity in Learning & Memory

Synaptic plasticity in the form of both LTP and LTD have long been thought to underlie behavioural learning and memory. The synaptic plasticity and memory hypothesis is a basic foundation that has been used to study the involvement of LTP, LTD and other forms of synaptic plasticity in learning and memory (Martin et al., 2000). This hypothesis states that synaptic plasticity occurs during normal brain activity and that this plasticity is both necessary and sufficient for memory formation and storage.

Intense investigation has sought to provide direct evidence that synaptic plasticity, as it is known, is responsible for learning and memory. The functional significance of LTP in the behaving animal is far better understood than that of LTD, and has been reviewed by others (Bliss and Collingridge, 1993; Lynch, 2004). Behavioural learning in simple associative tasks has been associated with potentiation of the fEPSP in the CA1 as measured *in vivo* (Gruart et al., 2006; Whitlock et al., 2006). These potentiated fEPSPs decayed during extinction of the learned association and the potentiation, as well as the learning itself were found to be NMDAR-dependent (Gruart et al., 2006). The seemingly learning-induced potentiation of the fEPSPs occluded further electrophysiologically-stimulated LTP and was associated with increased phosphorylation at ser-831 of the AMPAR (Whitlock et al., 2006). Pharmacological blockade of NMDARs (via delivery of antagonists either intracerebroventricularly or intrahippocampally) blocks spatial memory at concentrations known to impair LTP *in vitro* (Davis et al., 1992; Morris, 1989; Morris et al., 1986). In the piriform cortex, a paleocortical structure thought to be important for olfactory encoding similar to the hippocampus, NMDAR blockade is sufficient to impair odor preference memory and an agonist of β-adrenergic receptors delivered directly to this brain area while the animal is exposed to an aversive odor will produce a preference memory for that scent without any other behavioural CS (Morrison et al., 2013). In this same study, prior behavioural training occluded further LTP induction in the trained piriform cortex without affecting LTP induction in the untrained contralateral cortex.

The case for the involvement of LTD in behavioural learning and memory processes is more unclear. Most simply, LTD has been thought to be erasure of LTP and

therefore forgetting of memory; however this differentiation between both 'sides' of bidirectional plasticity is overly simplistic as there is now data to support an integral role for LTD in memory formation (Christie et al., 1994; Kemp and Manahan-Vaughan, 2007; Malenka and Bear, 2004; Pinar et al., 2017; Staubli and Lynch, 1990). In fact, LTD has been associated with the reversal of memories of spatial locations in the MWM task, and suggest the involvement of its mechanisms in object exploration tasks (Dong et al., 2012, 2013). In freely-moving awake animals, exploration of a novel environment facilitated CA1 LTP whereas exploring novel objects within that environment facilitated CA1 LTD (Kemp and Manahan-Vaughan, 2004). This CA1 LTD could also be induced by moving the location of previously-explored objects within an environment. Depotentiation was also observed in this study when the animals were exposed to a novel object-containing environment following delivery of the HFS. In an extension of this work a similar phenomenon was found in the DG where LTP was facilitated by exploration of an empty novel environment however with respect to LTD a subregion-specific effect was found where CA1 LTD was facilitated by small novel features of objects while DG LTD was facilitated by large novel cues that provided orientational information (Kemp and Manahan-Vaughan, 2008). Together these studies support a role for hippocampal LTD in novel object investigation and the use of these objects as navigational cues to help orient the organism within an environment.

1.7.8. A Dynamic Range of Synaptic Plasticity

While it may be tempting to consider LTP, LTD and depotentiation as separate, unique entities it is perhaps more appropriate to view these processes on a sliding scale of potential plasticity for a given circuit. We know that for both LTP and LTD there are absolute maxima and minima that can be reached as a result of the type, intensity, duration and timecourse of CS used.

1.7.9. Impact of Sex Hormones on Plasticity

Sex hormones are produced both systemically by the gonads and locally in the hippocampus and can influence structural and synaptic plasticity. As progesterone, androgens and estrogens are interrelated particularly in their biosynthesis investigations

of the impact of these hormones on receptor function and subsequent plasticity can be challenging without the use of synthesis and degradation enzyme inhibitors. Estradiol classically acts through its two intracellular receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), but can also impact receptor function at the synapse through NMDAR, mGluR and g-protein-coupled estrogen receptors (Ooishi et al., 2012).

The vast majority of the field has focused on the impact of these hormones on CA1 synaptic plasticity, and most prominently on LTP; however emerging evidence has implicated a role for sex hormones on DG LTP (Frick et al., 2015). Exogenous estradiol is known to enhance NMDAR-dependent LTP (Cordoba Montoya et al., 1997; Foy, 2001, 2011) in both males and females, and a similar facilitation of CA1 LTP is seen in the naturally-cycling female rat (Warren et al., 1995). This effect is thought to be mediated through the ability for estradiol to increase glutamate binding to the NMDAR (Woolley et al., 1997). Furthermore the effect of estradiol appears to be differentially mediated based on its binding to either ERα or ERβ. In the juvenile male DG LTP is facilitated by estradiol's actions through ERα whereas plasticity is suppressed through the actions on ERβ (Tanaka and Sokabe, 2013) however evidence in adult males has shown that ERβ facilitates DG LTP (Kramár et al., 2009). Juvenile castrated males treated with testosterone and normally-developing adult males display CA1 LTD following tetanic stimulation that would produce normal LTP in castrated males treated with testosterone at adulthood, and in castrated, untreated males (Hebbard et al., 2003) indicating an age-dependent effect on synaptic plasticity.

The study of the impact of sex hormones on hippocampal LTD is complicated by the age-dependent nature of this form of plasticity (see (Pinar et al., 2017) for review). While there is evidence that estradiol has a similar effect on LTD as it does on LTP, variance in animal age, dosage and duration of exogenous hormone treatment and LFS protocols used have yielded inconsistent results. For example estradiol treatment in ovariectomized female rats rescued LTD in some studies (Day and Good, 2005; Desmond et al., 2000) but not in others (Sharrow et al., 2002). Furthermore LTD was enhanced following pre-treatment with estradiol in adult males (Mukai et al., 2007b,

2007a; Murakami et al., 2015) although this effect has not been seen consistently (Vouimba et al., 2000). In contrast one clear role for estradiol is in the suppression of LTD in aged male and female offspring (Foster and Kumar, 2007; Foy et al., 2008; Norris et al., 1996) where it is thought that estradiol may protect against age-related memory decline at least in part through its actions on LTD.

Evidence for the involvement of estrogens in synaptic plasticity is paralleled by the influence of these hormones on structural plasticity. In the CA1, there is clear evidence that spine densities on pyramidal neurons fluctuate with the estrus cycle in females, with densities being highest during the proestrus phase when estradiol is similarly at its peak concentration (Li et al., 2004; Oberlander and Woolley, 2016; Woolley et al., 1990; Woolley and McEwen, 1993, 1994). While estradiol is associated with increased neurogenesis in the DG (see (Galea, 2008; Triviño-Paredes et al., 2016) for reviews), evidence for estradiol-mediated changes in spine densities have yet to be uncovered in granule cells (Woolley et al., 1990).

1.8. The PNEE Hippocampus

The hippocampus is particularly vulnerable to the teratogenic effects of ethanol both structurally and functionally (see (Berman and Hannigan, 2000; Fontaine et al., 2016; Gil-Mohapel et al., 2010b; Medina, 2011) for review). While these effects vary by PNEE model used, BEC achieved, timing of PNEE, postnatal age of interest, sex and hippocampal subregion there are some emerging trends in the effects of PNEE on the hippocampus.

1.8.1. Structure

The most consistent reports are of reduced pyramidal cell numbers and densities in the CA1 region using a variety of PNEE models and timings of exposure (Bonthius and West, 1990, 1991b). Even in similar liquid diet models of exposure, as is used in the current dissertation, there are discrepancies in the effects on cell numbers and densities in the hippocampal subregions that may be a result of stereological counting method and

age of offspring. In juveniles following liquid diet PNEE CA1 cell densities are reduced (Miller, 1995; Wigal and Amsel, 1990) although the effects on DG cell densities are mixed with reports of reductions when ethanol was delivered throughout gestation and the early postnatal period (Wigal and Amsel, 1990) but not when exclusively either gestational or postnatal unless the postnatal BECs were high (Miller, 1995). When examining later in the lifespan reductions in CA1 pyramidal neurons are maintained, though there is little to no effect on DG granule cells despite the use of postnatal PNEE paradigms with high BACs (Barnes and Walker, 1981; Bonthius and West, 1991b; Tran and Kelly, 2003). Given that most DG granule cells are generated during this early postnatal period it is unsurprising that gestational PNEE has not yielded significant changes in DG granule cells, and it is possible that continued neurogenesis in the DG can restore normal cell densities by adulthood. Even when ethanol exposure was restricted to the later postnatal period (P10-15) there was no effect on either DG granule cells nor CA1, CA2 nor CA3 pyramidal cells at either P16 or P30, with a significant effect only being seen in a decrease in cells of the hilus of the DG (Miki et al., 2000b, 2000a, 2003). Critically, these data support that PNEE leads to a permanent loss of pyramidal cells in the CA1 region. There is also some evidence that PNEE causes changes in cell proliferation, neurogenesis and survival in the DG across the lifespan (see (Gil-Mohapel et al., 2010a) for review).

1.8.2. Function; Learning & Memory

Learning and memory impairments are common features of FASD in the clinical population (Mattson et al., 2011; Rasmussen et al., 2006, 2008). The use of animal models has also provided evidence that there are learning and memory deficits following PNEE; and in particular in spatial learning and memory, behavioural domains known to involve the hippocampal formation.

The Morris water maze (MWM) is commonly used to study learning and memory in animals, and a virtual version of this task has recently shown impairments in young children with FAS (Hamilton et al., 2003). Similarly, while children with FAS are able to recall a series of objects immediately, their memories for these objects decays more

quickly after a delay than non-FAS subjects. These children are also deficient in reproducing spatial arrangement of shapes and in correctly arranging numbers and shapes on the face of a drawn clock, indicating possible hippocampal dysfunction but also damage to other high-order structures of the brain (Uecker and Nadel, 1996). In a longitudinal study of youth exposed to prenatal ethanol memory problems were some of the most highly-correlated measures with teratogen exposure (Streissguth et al., 1994). The use of imaging tools have helped uncover some possible underlying functional changes in learning and memory-related brain structures such as the hippocampus. In young people (aged 10-18 years) exposed to heavy prenatal ethanol impaired spatial working memory was associated with elevations in BOLD responses as measured by fMRI while undergoing the task (Spadoni et al., 2009). Learning and memory deficits appear to persist into adulthood and are associated with long-term reductions in hippocampal volume (Coles et al., 2011). Animal models of PNEE have been invaluable in uncovering specific learning and memory impairments and their potential mechanistic underpinnings in a way that imaging studies in humans cannot (Berman and Hannigan, 2000). Behaviourally, the impact of PNEE on spatial learning and memory has been well-documented on tasks such as the MWM, in radial arm mazes, and in various forms of object recall.

The MWM is likely the best-studied spatial learning and memory task used in rodents and performance on this task is impaired in young weanling animals following PNEE. Specifically, these animals have longer latencies to find a hidden platform, and have poor memory for the spatial location of the platform on probe trials (Blanchard et al., 1987; Goodlett et al., 1987). Sex differences in learning and memory in the MWM task have also been reported with pubertal (P36+) female offspring being more severely impacted than males by PNEE (An and Zhang, 2013). When delivering ethanol during the early postnatal period (P7-9), as juveniles, male offspring experienced significant deficits in place learning that were not evident during this period of exposure in females (Goodlett and Peterson, 1995; Johnson and Goodlett, 2002a). In older animals following PNEE it is possible that at low and moderate BECs the effects on spatial learning and memory may be more subtle, as deficits are not consistently reported at this age (Abel,

1979; Christie et al., 2005; Gianoulakis, 1990; Kelly et al., 1988; Kim et al., 1997). This has led to the use of more complex forms of the task by either extending the delay between training or testing (Matthews and Simson, 1998), or studying the ability of the animals to adapt to changing spatial locations of the platform (Savage et al., 2002). Others have found that despite a lack of evident learning and memory impairment in the MWM, adult PNEE guinea pigs employed an alternative search strategy which involved increased thigmotaxic behaviour around the edge of the pool prior to navigating to the platform (McAdam et al., 2008), although this could be a result of an altered stress response or increased anxiety characteristic of PNEE animals (Hellemans et al., 2010; Osborn et al., 1998; Weinberg et al., 2008).

Aside from the use of the classic MWM task, other evaluations of spatial learning and memory have been used to uncover deficits in PNEE offspring such as shock-avoidance (Abel, 1979), radial arm mazes (Reyes et al., 1989), object learning and many others. A recent study from our laboratory found that PNEE resulted in deficits in temporal ordering behaviours but not in metric change at adulthood which are considered to be CA and DG-dependent tasks, respectively (Patten et al., 2016a). While this study did not find sex differences in learning and memory, other studies have begun examining sex as a biological variable in the context of PNEE.

Despite the various methods of PNEE and learning and memory tasks there are some emerging trends and interests from the field in general that have guided the current study. First, that juvenile PNEE offspring appear to experience greater deficits than their adult counterparts. This hypothesis is supported by the view that PNEE results in a developmental delay that could be overcome with age, possibly through postnatal neurogenesis in the hippocampus, nutritional support or some other mechanism of ‘catch-up’. Second, an emerging interest in the field is to examine sex as a biological variable in the context of FASD. Both clinical and pre-clinical studies have laid a foundation to support that both sexes do not display identical patterns of disruption by PNEE.

1.8.3. Function; Synaptic Plasticity

Hippocampal synaptic plasticity can be impaired by PNEE across the lifespan; the degree of deficits varies by the PNEE model used, type of electrophysiological recordings and hippocampal subregion examined (see (Fontaine et al., 2016; Pierrefiche and Olivier, 2017; Pinar et al., 2017) for review).

1.8.3.1 CA1 LTP

Synaptic plasticity in the CA1 region, specifically LTP, by far is the most studied in the context of PNEE. Likely as a result of the sources of variability described above despite being best studied, the impact of PNEE on CA1 LTP is inconsistent (*no effect*: (Bellinger et al., 1999; Byrnes et al., 2004; Krahl et al., 1999; Tan et al., 1990); *reduction in magnitude*: (An et al., 2013; Izumi et al., 2005; Kervern et al., 2015; Richardson et al., 2002; Swartzwelder et al., 1988)). Given the scope of the present dissertation relevant studies of LTP completed in juvenile animals will be discussed further. *In vivo* LTP was found to be impaired by PNEE (gavage 20% v/v EtOH throughout gestation) in juvenile (P36) male offspring, whereas in females PNEE led to enhanced LTP (An and Zhang, 2013).

1.8.3.2 CA1 LTD

The effect of PNEE on CA1 LTD is emerging through increasing interest in this form of plasticity. In one study, PNEE (drinking water; 10% v/v EtOH throughout gestation, lactation) led to a facilitation of NMDAR-dependent LTD in young adults (P55) induced by both single pulse LFS (600 x 1Hz) and paired pulse LFS (900 x 1Hz; 200ms inter-pulse-intervals) *in vitro* (Kervern et al., 2015). The single pulse LFS was insufficient to elicit LTD in control slices, indicating that PNEE may reduce the threshold for LTD and thus facilitate its induction. This facilitation was accompanied by an increase in the expression of the GluN2B subunit of the NMDAR in the synapse, and suggests that there may be a PNEE-induced facilitation of NMDAR function in the slice that may contribute to the enhancement of LTD. This study, however was conducted using only male offspring. In a study from our laboratory, PNEE (liquid diet gestational)

did not cause significant differences in LTD (LFS: 900 x 3Hz) *in vivo* (Titterness and Christie, 2008).

Some recent studies have begun to investigate bidirectional plasticity following PNEE. *In vivo* recordings in CA1 have also uncovered that PNEE leads to enhanced magnitudes of depotentiation (induced by 900 x 1Hz LFS following established LTP) in males at P36 following PNEE (gavage 20% v/v EtOH throughout gestation)(An et al., 2013). In a follow-up of this study, the same result was obtained in male offspring of the same age, however in female offspring there was an reduction in the magnitude of depotentiation as a result of PNEE (An and Zhang, 2013). This study of bidirectional plasticity in the CA1 following PNEE showed a sex-specific effect of PNEE in adolescent animals, where the balance of synaptic plasticity is changed such that in males the balance is shifted towards depotentiation and away from potentiation whereas in females the opposite is true.

1.8.3.3 DG LTP

The effect of PNEE on DG LTP appears to be fairly consistent between studies, although differences in the type of HFS, PNEE paradigm, offspring age and type of electrophysiology can impact the experimental outcome. Generally, the magnitude of LTP in the male DG is reduced by PNEE in adolescents and adults (Christie et al., 2005; Patten et al., 2013a; Sickmann et al., 2013; Sutherland et al., 1997; Titterness and Christie, 2012; Varaschin et al., 2010, 2014) *in vivo*. In adult (P105-140) male offspring following PNEE impairments in LTP were only observed when a sub-optimal HFS was used (3 tetanus trains), but not when stronger HFS (10 tetanus trains) was used as the CS *in vivo* (Varaschin et al., 2010). While the specific mechanisms of these two types of LTP were not investigated in this study, the administration of an antagonist of H_3 histamine receptors reversed the LTP deficits. These data indicate that PNEE may lead to subtle changes in plasticity, and in this case specifically to the threshold for the induction of LTP. This study and the present dissertation support that examining ranges of synaptic plasticity shed more light on the full impact of PNEE on plasticity and on learning and memory impairments characteristic of this neurodevelopmental disorder. There is also

evidence that the activity of $mGluR_5$s support the expression of sub-optimal LTP (Naie and Manahan-Vaughan, 2005; Raymond et al., 2000; Raymond and Redman, 2002), and the number and function of these receptors have been shown to be impaired by PNEE (Galindo et al., 2004).

There is also emerging interest and evidence of sex differences in DG LTP as a result of PNEE. A study from our laboratory found that PNEE (liquid diet gestational) reduced the magnitude of LTP in males but enhanced the magnitude of LTP in females (Titterness and Christie, 2012) in agreement with previous findings in the *in vivo* CA1 (An and Zhang, 2013). Interestingly, when a similar study was conducted using an identical PNEE paradigm in adult offspring (P55-70) there was so significant effect of PNEE on LTP in female offspring however the reduction in magnitude in males persisted (Patten et al., 2013a; Sickmann et al., 2013), which suggests that perhaps there are some compensatory mechanisms that exist in females either during puberty or through normal aging in females that does not exist in males. In these studies, a relationship was established between oxidative stress and the amount of the major antioxidant in the brain, glutathione, and the PNEE-induced LTP deficits in males, however the same could not be established in females. The use of a compound that acutely reduces glutathione in the brain reduced the magnitude of LTP in control males to the level seen in PNEE-exposed offspring, however it had no effect in the females (Patten et al., 2013a). Given these sex differences, ovariectomies were performed to control for female sex hormones, but had no effect on LTP and could not explain the apparent lack of differences as a result of PNEE, although it does not account for hippocampal synthesis of sex hormones in these animals.

Despite the consistent reports of sex differences in the impact of PNEE on LTP *in vivo*, investigations of LTP *in vitro* have yielded different results. Using the same paradigm of PNEE as the studies from the Christie laboratory described above (liquid diet gestational), the magnitude of NMDAR-dependent LTP was reduced in adult offspring of both sexes regardless of whether theta-burst stimulation (TBS) or HFS was used as the CS (Helfer et al., 2012). Interestingly, the impact of PNEE on LTP *in vitro* is

trimester-dependent as PNEE restricted to either the first trimester (GD 1-11) or third-trimester (P 4-9)-equivalents had no effect, whereas PNEE during the second trimester (GD 11-21)-equivalent was sufficient to affect the magnitude of LTP (Helfer et al., 2012). The discrepancies in the findings *in vitro* and *in vivo* likely result from some combination of the need to block $GABA_A$Rs *in vitro* for the induction of LTP and the fact that in the slice preparation hippocampal afferents and efferents are severed.

1.8.3.4 DG LTD

To date there have been no published investigations of the effect of PNEE on DG LTD. Unpublished dissertation data (Dr. Jennifer Helfer) from the Christie laboratory in adult offspring of both sexes applied prolonged LFS (LFS_{1800}) to induce LTD and found no effect of PNEE. The LFS_{1800} in this study may be too prolonged to visualize the potentially subtle effects of PNEE on LTD however in these adults, the typical LFS (900 x 1Hz) was insufficient in inducing any synaptic depression. Interestingly this LTD was also unaffected by pharmacological blockade of either the NMDAR or the $mGluR_5$ when applied separately. It is possible that in the DG with prolonged LFS, both NMDAR and mGluR-LTD pathways could be activated and therefore blockade of the LTD may require combined blockade of both NMDARs and mGluRs. Given the findings of imbalanced bidirectional synaptic plasticity in the CA1 by PNEE in males and females, the present dissertation aims to uncover whether the dynamic range of plasticity in the DG is affected by PNEE in a sex-specific manner in juvenile offspring.

1.9 Current Project Aims

This dissertation aims to examine the impact of PNEE on glutamatergic synaptic plasticity in the juvenile DG of both sexes. Some of the key aspects of this work and their experimental rationales are described below. Throughout this dissertation we are comparing DG synaptic plasticity of control and ethanol-exposed offspring within sex. A small sample of pair-fed control experiments were also conducted as is standard to this field of study and the plasticity in these animals are compared to control offspring in Appendix B.

The previous experimental evidence from our laboratory using the liquid diet model of PNEE has suggested male-specific vulnerability in DG LTP at adulthood and during puberty *in vivo* (Patten et al., 2013a, 2013b; Titterness and Christie, 2012), however LTP is one single form of plasticity in the DG and it is possible that female offspring are indeed affected although perhaps in some other form. While we were able to establish a relationship between oxidative stress and impaired LTP in males, a similar mechanism could not be identified in the females at adulthood, which may have been due to some protective effects of sex hormones or that perhaps in female offspring the unimpaired LTP may come at a physiological cost to other plasticity mechanisms such as in LTD. In an effort to control for sex hormones in our previous work we ovariectomized females at puberty however this does not control for locally-synthesized sex hormones in the hippocampus. As a result, the present dissertation examines offspring of **both sexes prior to the onset of puberty** and sexual maturity. Furthermore, this pre-pubertal age range is particularly interesting as some of the CNS effects of PNEE become most apparent in the classroom such as impaired attention, decision-making, learning and memory and academic performance in general which can help identify children for diagnostic investigation. Specific to the present area of study, in order to assess the dynamic range of plasticity we must also examine LTD, whose magnitude has long been recognized as being age-dependent in that during development (prior to adulthood) and in aged offspring it is most easily elicited by standard LFS (900 x 1Hz). Additionally, we will investigate whether the dynamic range of plasticity is affected by PNEE by examining maxima and minima of synaptic efficacy in our slice preparation in these animals. This work expands upon our recent work by examining the dynamic range of bidirectional plasticity in young offspring of both sexes following PNEE. We hypothesize that synaptic plasticity in the DG of young animals is impacted differently between the sexes and as such the sexes have been evaluated separately.

Objective 1: To determine whether PNEE affects DG synaptic plasticity in juvenile male and female offspring.

Objective 2: To uncover whether PNEE restricts or shifts the dynamic range for bidirectional DG synaptic plasticity.

Objective 3: To examine whether PNEE changes the capacity to shift between forms of synaptic plasticity.

Objective 4: To identify mechanisms that underlie DG synaptic plasticity, which could be impaired by PNEE.

2.0 MATERIALS & METHODS

2.1 Animals & Breeding

2.1.1 Animals

All procedures involving animals were performed in accordance with the University of Victoria Institutional Animal Care Committee following the standards set by the Canadian Council for Animal Care (Animal Use Protocol Number 2014-012/2018-012).

2.1.2 Breeding

Adult male and virgin female Sprague dawley rats were obtained from Charles River Laboratories (Quebec, Canada). Upon arrival and prior to breeding females were housed in pairs and males were housed individually in clear polycarbonate cages with corn cob bedding. The room was maintained on a 12-h light:dark cycle (lights on at 4am) with constant humidity and temperature. Following acclimation for at least one week, individual virgin females were paired with a breeding male. Vaginal smears using 0.9% sodium chloride (NaCl) were performed at the beginning of each light cycle to determine pregnancy. An upright light microscope was used to detect the presence of sperm. Following the detection of sperm, the female was immediately re-housed individually in a clear polycarbonate cage with nesting material and assigned to one of two prenatal diet groups, and this day was defined as GD 1. As depicted visually in Figure 11.

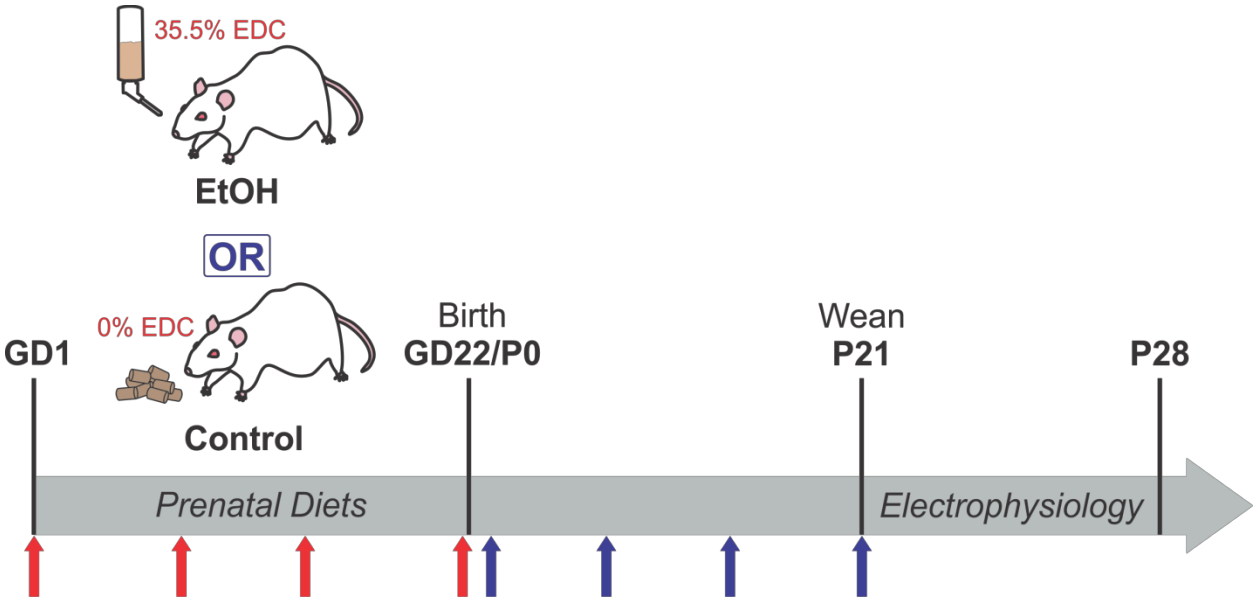

Figure 11. Experimental timeline

Dams were assigned to either the Ethanol (EtOH) or control prenatal diet conditions from gestational day (GD)1-22. The EtOH diet contained 35.5% EtOH-derived calories (EDC). During gestation dam weights were recorded on GD1, 7, 14 and 21 (red arrows). Pup and dam weights were then weighed on postnatal day (P)1, 3, 7, 14 and 21 (blue arrows). On P21 pups were weaned into same-sex cages in triplet or pairs and were used for juvenile in vitro electrophysiology between P21 and P28.

2.2 Prenatal Diet Treatments

Ethanol – *Ad libitum* access to a nutritionally fortified liquid diet (Weinberg/Keiver high protein liquid diet-experimental, no. 710324, Dyets Inc, Bethlehem, PA, USA) containing 35.5% ethanol-derived calories (EDC) throughout gestation (GD1-21). Dams were gradually introduced to the liquid diet over a three-day period. On GD1 one third of the ethanol diet was combined with two thirds of a similar liquid diet containing no ethanol (liquid control diet as is administered for pair-feeding; Described in Appendix B) on GD2 two thirds of the ethanol diet was combined with one third of the pair-fed diet and finally from GD3-21 dams were supplied only with the ethanol diet. On GD22, the typical day of birth, dams were re-supplied with standard solid chow, which was provided throughout parturition. Dams in this condition typically achieve BECs of 80-180mg/dl (Christie et al., 2005; Patten et al., 2013a, 2013b, 2016b; Uban et al., 2010).

Liquid diets were provided to animals approximately 2 hours prior to the beginning of each dark phase for each day of gestation. The daily consumption of the diet was recorded and the dam body weights were recorded on GDs1, 7, 14 and 21.

Control – *Ad libitum* access to standard solid rat chow throughout pregnancy and parturition. The dam body weights were recorded on GD1, 7, 14 and 21.

2.3 Litters & Weaning

On GD 22 dams gave birth and this was considered P0. The offspring were culled to 12 pups on P1 to 6 pups of each sex, where possible. In the case of litter sizes over 12 pups that did not have milk spots or those that were smallest were culled. The dam weight and total weight of male and female pups were recorded on P1, 3, 7, 14 and 21. On P21 pups were weaned into same-sex triplets or pairs and were used for experimentation as juveniles between P21 and P28.

2.4 Electrophysiology

The method for tissue preparation, slicing and electrophysiological recordings are described below and depicted visually in Figure 12.

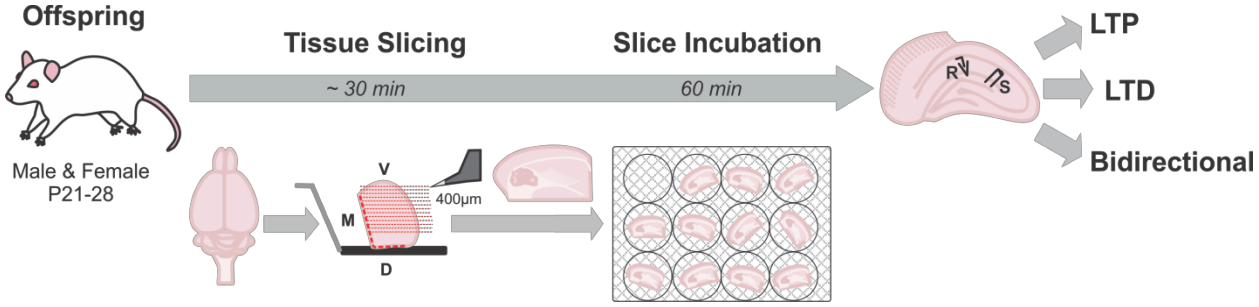

Figure 12. In vitro slice electrophysiology preparation timeline

Juvenile male and female rats (postnatal day (P) 21-28) following either control or ethanol diet exposure were anesthesized, rapidly decapitated and their brains quickly excised, dissected and fixed with the medial (M), dorsal (D) and ventral (V) surfaces arranged according to the diagram above. Over the course of 30 minutes the tissue is

sliced in 400μM thick slices with a vibratome from V to D and transferred into a chamber where they were incubated for at least 60 minutes. Individual slices were then placed on electrophysiology rigs and recording (R) and stimulating (S) electrodes lowered into the dentate gyrus as depicted (right) and randomly assigned to a variety of recordings, either long-term potentiation (LTP), long-term depression (LTD) or bidirectional plasticity in the form of depotentiation.

2.4.1 Slice Preparation

Between P 21-28 male and female offspring from each prenatal condition were used for *in vitro* electrophysiology studies of DG synaptic plasticity. During this time no female offspring displayed vaginal opening associated with sexual maturation. Animals were deeply anesthetized with inhaled isofluorane USP (AVP, Fresnius Kabi) and rapidly decapitated upon loss of toe and tail pinch reflexes. Brains were quickly excised and submerged in cold artificial cerebrospinal fluid (aCSF; 125mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4, 25mM $NaHCO_3$, 2mM $CaCl_2$, 1.3mM $MgCl_2$ and 1.4mM Dextrose) that was equilibrated with carbogen (95% O_2/5% CO_2). Transverse hippocampal slices (400μm) were cut in aCSF using a vibrating blade (Feather razor blades, Ted Pella; Pelco 100 Vibratome Sectioning System, Ted Pella). The slices were then left undisturbed for a minimum of 1 hour in warmed (32°C), carbogenated aCSF prior to being transferred to the recording chamber. The use of the transverse hippocampal slice for *in vitro* slice electrophysiology was first introduced by Knut Krede and Rolf Westgaard and is now most commonly used as it provides access to the hippocampus and fibre alignment that are ideal for studying synaptic plasticity (Skrede and Westgaard, 1971).

2.4.2 Recordings

Following a minimum of 1 hour of recovery slices were transferred to the recording chamber continuously perfused with aCSF maintained at 30°C for all electrophysiology experiments. Slices were visualized using an upright microscope (Olympus BX5OWI) where extracellular field potentials were recorded using a glass pipette (made from 0.86 inner diameter, 1.5mm outer diameter borosilicate glass pulled by a Model P-1000 Flaming/Brown micropipette puller, Sutter Instruments) filled with aCSF and placed in the dendritic field of DG granule cells (with a resistance of between

0.5-1 MOhm). A concentric bipolar stimulating electrode (FHC) was lowered into the MPP input to the DG (see Figure 13 for representative placement) and delivered test pulses (0.12ms pulse width) in order to optimize placement and the resulting fEPSP. Resulting fEPSPs were optimized such that the maximal amplitude of the response was at least 0.8mV in an effort to ensure that healthy responses, with the capacity to increase and decrease in fEPSP slope were included in this study.

Electrophysiological data were recorded with a Multiclamp 700B (Molecular Devices) and acquired with pClamp 10.5 (Axon Instruments).

Following placement, maximal responsiveness was determined by increasing the stimulus strength, which was then set at the intensity required to elicit either 50% (for LTP) or 70% (for LTD) of the maximum for each individual slice. During pre-conditioning recordings, a single pulse was delivered every 15s at this determined stimulus intensity until the slope of the resulting fEPSP was stable for at least 20 minutes (80 traces). This step is critical as it is a baseline measurement against which the magnitudes of plasticity are evaluated. As such, all slices that are included for analysis have met the inclusion criteria described below during this baseline period. PP and input-output (I/O) curve experiments were then briefly conducted in order to evaluate whether PNEE altered presynaptic neurotransmitter release probability (PP ratio) or responsiveness to increasing stimuli (I/O). The paired pulse (PP) experiment consisted of the delivery of pairs of pulses (50 ms inter-pulse interval) five times with 15s between pairs of pulses. I/O curves were generated by gradually increasing the stimulation pulse width with the stimulation intensity kept constant (30-300 μs pulse width; 15s intervals).

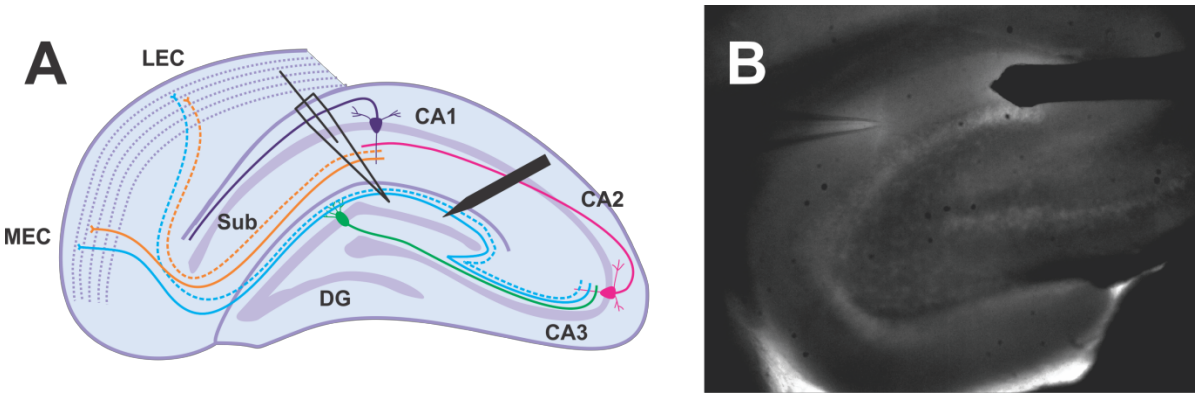

Figure 13. Electrode placement in the juvenile dentate gyrus for *in vitro* electrophysiological recordings.

(**A**) Simplified diagram of the trisynaptic hippocampal circuit with the approximate placement of the recording pipette (left) and stimulating electrode (right) in the suprepyramidal blade of the dentate gyrus (DG). The lateral entorhinal cortex (LEC) and medial entorhinal cortex (MEC) project to the DG forming the lateral perforant path (dotted blue line) and the medial perforant path (solid blue line) and synapse on DG granule cells (green). The granule cells project to the cornu ammonis 3 (CA3) pyramidal cells (pink) which then project to CA1 pyramidal neurons (purple). The CA1 pyramidal neurons project to the subiculum (Sub). It should be noted that the LEC and MEC can also project directly to other regions of the CA including the CA1 (yellow dotted and solid lines). (**B**) Photomicrograph of an in vitro hippocampal slice showing the placement of the recording pipette (left) and the stimulating electrode (right) in the medial perforant path.

2.4.3 Conditioning Stimulus Protocols

Following the assessment of basal synaptic transmission, slices were randomly subjected to the appropriate conditioning stimulus (CS) protocols for LTP or LTD. For LTP HFS was delivered as 4 trains of 50 pulses at 100Hz with a 30 second inter-train-interval (0.24 ms pulse width) in the presence of a $GABA_A$ inhibitor, bicuculline methiodide (BIC, 10μM, MJS Biolynx Inc). For LTD, low frequency stimulation (LFS) was delivered at 900 (LFS_{900}) or 1800 (LFS_{1800}) pulses at 1Hz for 15 or 30 minutes respectively (0.24ms pulse width).

2.4.4 Drug Information

In order to determine underlying mechanisms for the LTP and LTD measured in these studies a number of inhibitors were used. Pharmacological inhibitors were dissolved and aliquoted in dH_2O unless otherwise specified and stored at -20°C until use

at which time they were thawed and diluted in aCSF to the appropriate concentrations described below. Drug aCSF was bath applied following establishment of 20 minutes of stable pre-conditioning recording for 10 minutes of additional pre-conditioning recording, throughout the PP and I/O experiments and throughout the desired CS (see Figure 14). For all recordings the post-conditioning recordings were all done under bath application of normal aCSF. Antagonist drugs used in these studies consist of DL-2-Amino-5-phosphonopentanoic acid (DL-APV; 50μM; Tocris Bioscience), 2-Methyl-6-(phenylethynyl)pyridine (MPEP; 10μM; Tocris Bioscience) and nimodipine (NIMO; 20μM, Tocris Bioscience) and N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 4μM, Tocris Bioscience) and Tat-GluA2$_{3Y}$ (10μM; Generously donated by the laboratory of Yu Tiang Wang, University of British Columbia). DL-APV is a competitive NMDAR antagonist that binds to the glutamate binding site on the receptor. MPEP is a potent and selective non-competitive antagonist to the mGluR$_5$. NIMO is an L-type calcium channel blocker. AM251 is a potent, selective inverse agonist for the CB1 receptor and was dissolved in 15% DMSO (final concentration in aCSF: 0.075% DMSO). Tat-GluA2$_{3Y}$ is a synthetic peptide that has been used to prevent phosphorylation of the tyrosine residues on the GluA2 subunit which in turn prevents endocytosis of the receptor, which is classically associated with LTD (Ahmadian et al., 2004; Dong et al., 2012; Fox et al., 2007). Given that the actions of this compound are on the intracellular c-termini of AMPARs, the drug was washed over the slice for 20 minutes of pre-conditioning recordings, during LFS and for the initial 30 minutes of post-conditioning recordings.

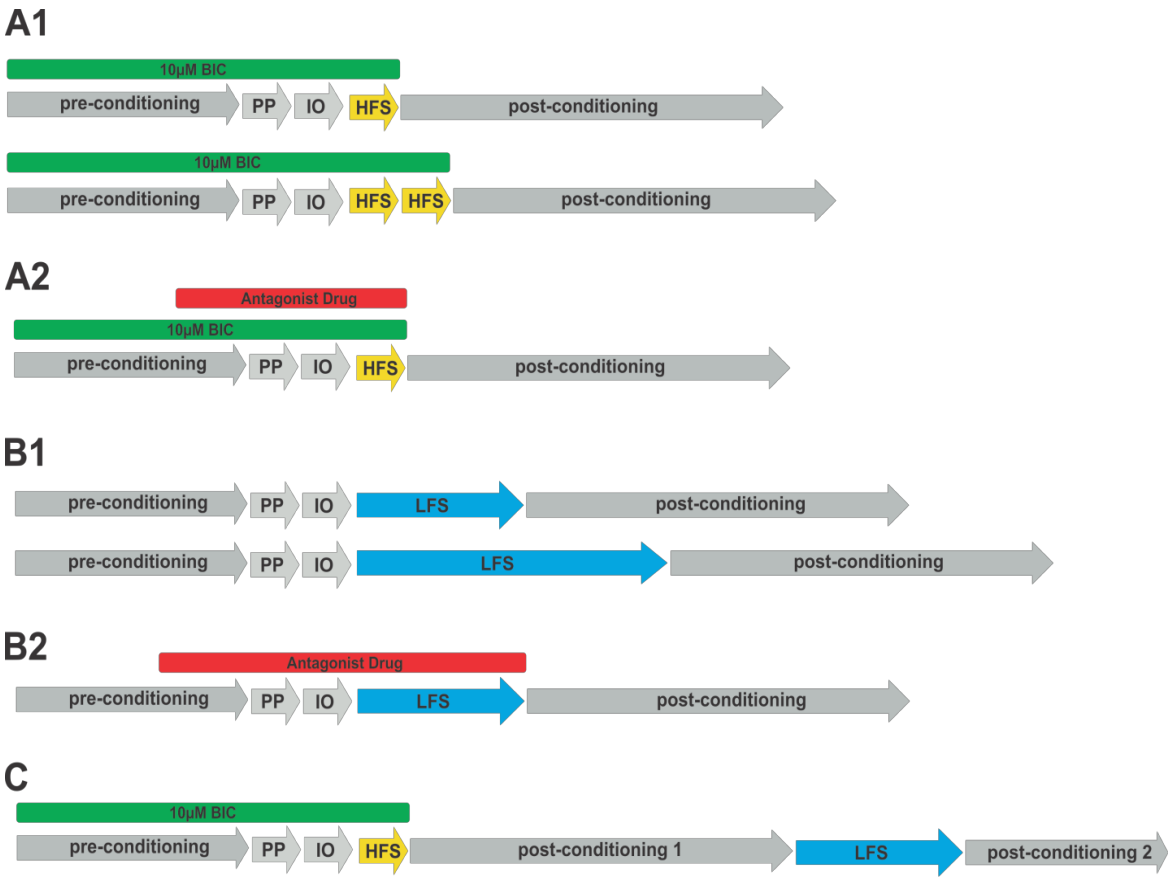

Figure 14. Electrophysiology protocol timelines.

Pre-conditioning recordings consist of 20 minutes of stability prior to administration of other protocols such as PP, IO and the conditioning stimuli (CS). Following either CS (HFS or LFS) post-conditioning recordings take place for 60 minutes. (A1) Protocol timelines for LTP for single HFS (top) and multiple HFS (bottom) recordings. (A2) Protocol timeline for LTP mechanism experiments where drug delivery occurs for 10 additional minutes of pre-conditioning, during PP and IO and the HFS. (B1) Protocol timelines for LTD for 900x (top) and 1800x (bottom) 1Hz LFS. (B2) Protocol timeline for LTD mechanism experiments where drug or drug cocktail delivery occurs for 10 additional minutes of pre-conditioning, during PP and IO and the LFS. (C) Protocol timeline for depotentiation experiments where following 60 minutes of post-conditioning 1 LFS is delivered and followed by a 30-minute post-conditioning 2 recording. Abbreviations: BIC: bicuculline methiodide; IO: input-output; HFS: high-frequency stimulation; PP: paired pulse; LFS: low-frequency stimulation.

2.4.5 Data &Statistical Analyses

Data Analyses Software: Electrophysiological data were initially analyzed using Clampfit 10.5 (Axon Instruments), basic functions in Microsoft Excel 2010 and statistical comparisons were made using R Studio. Graphs and tables were assembled in Microsoft Excel 2010 and CorelDraw X6.

Paired-Pulse Measurements: In PP experiments, the initial slope of the average second fEPSP were compared to that of the first fEPSP and used to generate a ratio of the second pulse relative to the first.

Input/Output (I/O) curve analysis: I/O curves were generated by comparing the initial slopes of the fEPSPs relative to the stimulation pulse width (from 30-300 μs). All data are represented as the mean ± the standard error of the mean (SEM).

Synaptic Plasticity Analysis: For synaptic plasticity experiments, the initial slope of the fEPSPs in the pre-conditioning recording were determined and used to generate an average pre-conditioning slope to which all slope measurements were compared as a percentage of change from that average value. The initial slope of the fEPSPs in the post-conditioning recordings were determined and expressed as a percentage of change from the average pre-conditioning slope value. The average percentage of change of the fEPSP slope over the course of one minute (4 traces) was calculated and expressed graphically for both pre-conditioning and post-conditioning recordings. To quantify short-term plasticity and long-term plasticity, the average percentage of change in the post conditioning from 0-1 minutes and 55-60 minutes respectively, were compared.

Inclusion Criteria: In order to make appropriate statistical comparisons for potential changes in synaptic plasticity, it is critical to begin with a stable baseline recording period. Responses from slices were considered stable when (1) the fEPSP slope value did not differ by any more than ± 10% of the average fEPSP slope value and (2) there was no drift in the fEPSP slope value (i.e. the slope of the line of best fit for the baseline period did not exceed | ± 0.5 |. Additionally, in order to draw meaningful conclusions regarding any potential changes to magnitudes of synaptic plasticity, the fEPSP slopes during the post-conditioning period of analysis (minutes 55-60) must also be relatively stable. Unstable pre- or post-conditioning recordings during the time windows of analysis can mean that average values may not be truly representative of the

sample, and may not reflect the actual stability or magnitude of plasticity and could indicate instability in the experimental setup. As such, slices included for analysis in this dissertation maintained a stable fEPSP slope from minutes 55-60 where the slope of a line of best fit drawn for these values did not exceed | ± 1.5 |. All data are represented as the mean ± SEM.

Cumulative Probability Analysis: For cumulative probabilities, average magnitudes of plasticity (either LTP or LTD) are ordered from least to greatest, and are graphed against the actual frequency of the distribution of the data. The accompanying actual frequencies can be used to determine what proportion of the dataset fall at or below the specified value. These graphics can be helpful in illustrating the distribution of a dataset in order to better understand how experimental groups (in this case two diet conditions) may or may not be statistically significantly different.

Statistical tests: Comparisons between control and PNEE groups are analyzed separately by sex as a result of *a priori* hypotheses that the sexes are differentially affected by the teratogen exposure. As such, for comparisons of magnitudes of plasticity between the control and EtOH conditions, a two-tailed student's t-test was used. Where statistical significance was reached using the t-tests ($p < 0.05$), the effect size was calculated using Cohen's d.

3.0 RESULTS

Throughout this dissertation *a priori* hypotheses regarding the effects of PNEE on both sexes, results from male and female offspring are statistically analyzed separately. All data are represented as the mean ± the SEM.

3.1 Developmental Data

Dams randomly assigned to either the control or EtOH condition were weighed throughout gestation until the day before birth (GD 21). Both control and EtOH-exposed dams consistently gained weight over the course of 22 days of gestation (Control: 51.80 ± 8.03 %; EtOH: 46.46 ± 2.38 %; Table 2). There were two dam mortalities in each condition related to dystocia, or complications during the birthing process which typically involve pups becoming lodged in the birth canal.

Table 2. Maternal Weight Gain During Gestation

Pregnant Sprague-Dawley rats were weighed on the day that sperm was found (designated as gestational day (GD) 1) and then once weekly on GD 7, 14 and 21 prior to giving birth on GD 22. Average weights are expressed ± the standard error of the mean (SEM).

Average	**Control**	**Ethanol**
Weight at **GD 1** (g)	230.24 ± 11.96	232.63 ± 10.70
Weight at **GD 7** (g)	250.51 ± 12.76	244.31 ± 12.41
Weight at **GD 14** (g)	276.71 ± 13.43	270.66 ± 12.12
Weight at **GD 21** (g)	344.71 ± 12.08	341.09 ± 17.66
% Weight Gain	**51.80 ± 8.03 %**	**46.46 ± 2.38 %**

Both control and EtOH-exposed dams gave birth on average on GD 22. Prenatal diets had no effect on neither the size of the litter nor on the number of male or female pups born ($p_{litter\ size} = 0.562$; $p_{males} = 0.461$; $p_{females} = 0.723$; Table 3). The offspring were weighed regularly on P1, P3, P7, P14 and P21 (see Table 4). Over the course of early postnatal development all pups gained weight (see Figure 15), although the percentage of pup weight gain was not significantly different between groups nor between groups when separated by sex ($p_{totalgain} = 0.982$; $p_{malegain} = 0.808$; $p_{femalegain} = 0.586$). There was a transient effect of the ethanol prenatal diet treatment where at P1 and P3 there was a significant reduction in the weight of male offspring ($p_{P1} = 0.0066^{*}$; $p_{P3} = 0.014^{\Delta}$), and there was a trend toward significance in females at P7 ($p = 0.052$) although there were no other significant effects of prenatal diet on offspring weights in either sex.

Table 3 Offspring Numbers

Litter size and number of male and female offspring were evaluated on the day after birth (P1). PNEE had no significant effect on the number of pups in each litter nor on the number of male or female pups in each litter. Following this, litters were culled to 12 total pups and to 6 pups of each sex where possible. The average numbers are expressed below ± the SEM. A two-tailed student's t-test was used to compare the outcomes between the prenatal diet conditions. Statistical significance was considered when $p < 0.05$.

Average	**Control**	**Ethanol**
Litter Size	12.00 ± 0.60	11.50 ± 0.60
N_{males}	6.40 ± 0.64	5.63 ± 0.80
$N_{females}$	5.60 ± 0.52	5.88 ± 0.58

Table 4. Offspring Weight Gain

Average offspring weight gain separated by sex starting on the day after birth (P1) to weaning an experimental use (P21). On P1 litters were culled to 12 pups consisting of 6 pups of each sex where possible. A two-tailed student's t-test was used to evaluate the effect of prenatal diet treatment within sex and age. PNEE had no effect on offspring weight at any age in female pups, however it significantly reduced male pup weights at P1 and P3 but at no other age. The average weights are expressed below ± the SEM. Statistical significance was considered when $p < 0.05$

and is indicated using distinct symbols (* or $^{\Delta}$), indicating statistical significance from the values indicated with the identical symbol within this table.

Weight	Average Control Pup Weight (g)		Average Ethanol Pup Weight (g)	
	Male	Female	Male	Female
P1	8.13 ± 0.16*	6.93 ± 0.49	7.20 ± 0.24*	6.58 ± 0.29
P3	10.69 ± 0.36$^{\Delta}$	8.94 ± 0.63	9.29 ± 0.35$^{\Delta}$	8.79 ± 0.37
P7	16.83 ± 0.67	16. 45 ± 0.55	15.48 ± 0.83	14.15 ± 0.91
P14	29.71 ± 2.57	30.85 ± 1.39	29.70 ± 1.37	28.18 ± 1.30
P21	55.52 ± 3.72	48.69 ± 5.31	48. 34 ± 2.53	44.43 ± 2.41

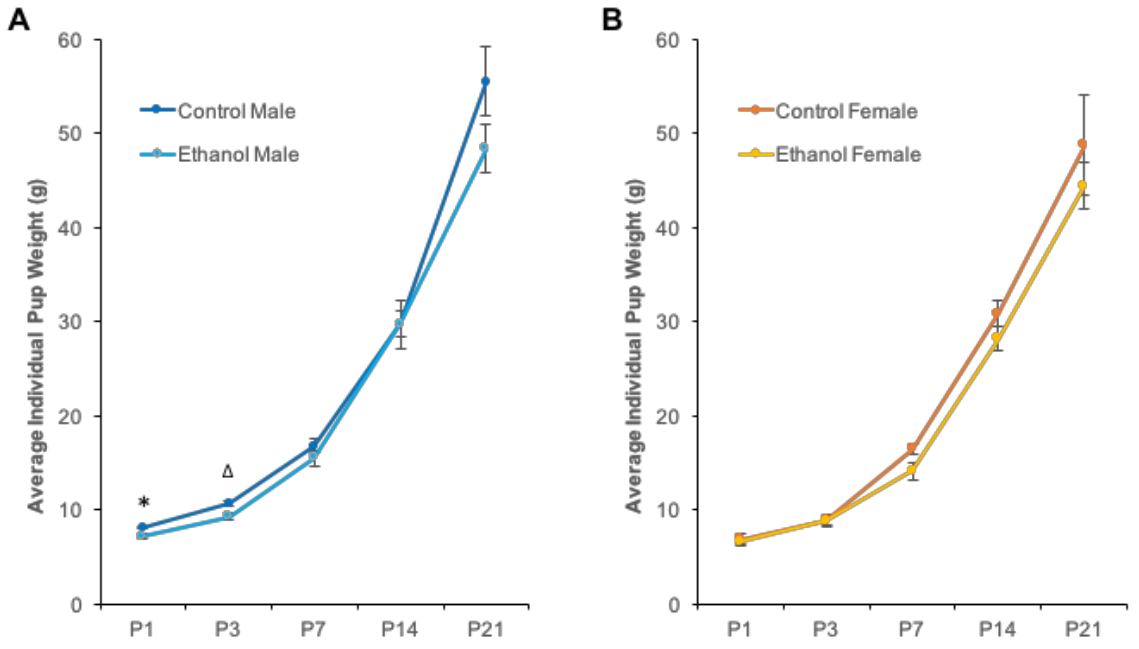

Figure 15. Average offspring weight gain

Average offspring weights for males (A) and females (B) following consumption of either solid chow control or ethanol-containing liquid diets from postnatal day (P) 1 - 21. Points are the average individual offspring weights by condition and error bars represent the standard error of the mean. * and $^{\Delta}$ indicate significant differences ($p < 0.05$) in weight between the ethanol and control conditions at a particular age.

3.1.1 Ethanol Liquid Diet Consumption

Dams assigned to the EtOH condition were provided with *ad libitum* access to an EtOH-containing liquid diet in the place of standard solid rat chow. Dams consumed on average 66.25g ± 3.75g of the liquid diet daily, with variability in volumes from 48.59g and 85.70g per day. Animals in this condition consumed on average 13.57 ± 0.39 g/kg/day ethanol. There was variability in the average daily data with ethanol consumption ranging from 11.58 g/kg/day to 14.96 g/kg/day between dams.

3.2 Basic Electrophysiological Parameters.

In order to determine whether PNEE affects NT release or postsynaptic responsiveness we examined PP plasticity and I/O curves respectively. These experiments were conducted prior to measurements of long-term plasticity (LTP or LTD). The results of these experiments can aid in the interpretation of potential mechanisms by which PNEE alters synaptic communication and plasticity. As such and as described by the methodological timelines in Figure 14 PP plasticity and I/O curves were examined under regular aCSF conditions (as in LTD recordings) and under the influence of $GABA_A$ inhibition by BIC. Our results indicate that PNEE does not significantly change PP plasticity in either sex or when $GABA_A$Rs are inhibited by BIC (Figure 16). In males, the PPR in EtOH-exposed offspring (0.997 ± 0.0646) and in controls (1.027 ± 0.0315) was not found to be statistically significantly different by a two-tailed student's t-test ($p = 0.568$; Figure 16 A1). Similarly during BIC wash-in PPRs in EtOH-exposed (1.199 ± 0.0496) males and controls (1.238 ± 0.0470) were not significantly different from one another ($p = 0.572$; Figure 16 B1). In females, PPRs in EtOH (1.044 ± 0.0271) and control (1.112 ± 0.0206) were not significantly different ($p = 0.0647$; Figure 16 A2) nor were they different when exposed to BIC (EtOH: 1.265 ± 0.0512; Control: 1.193 ± 0.0562; $p = 0.367$; Figure 16 B2).

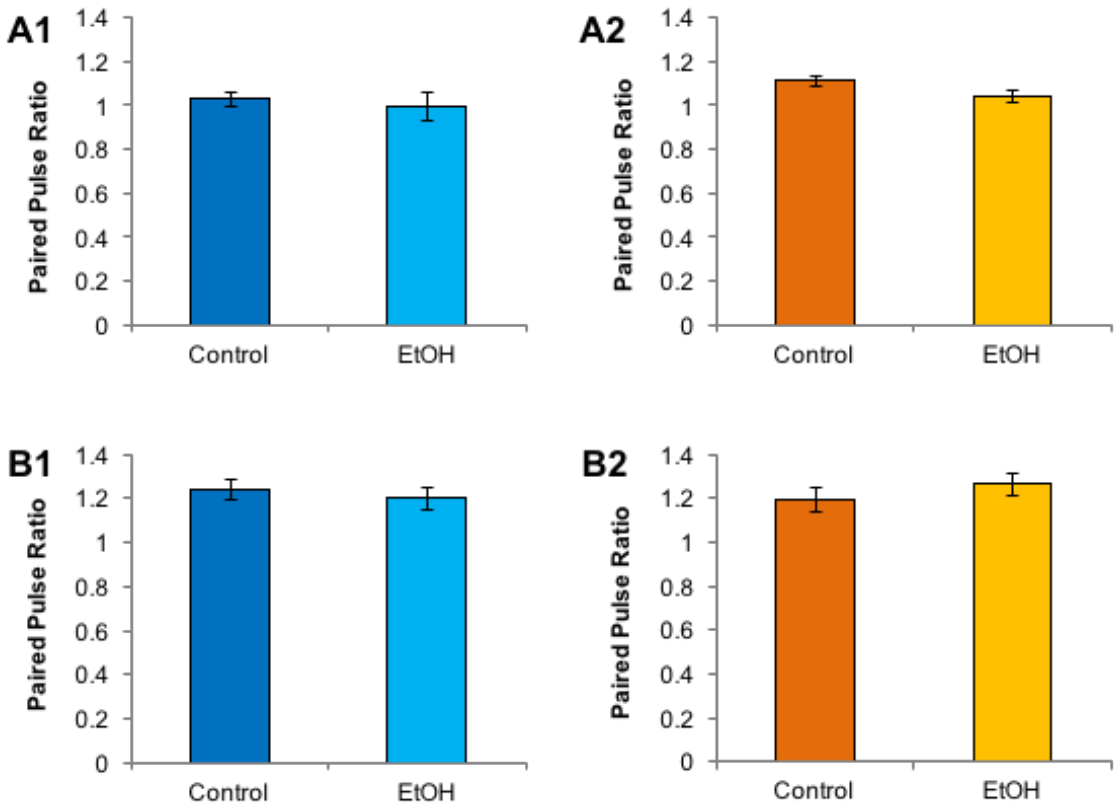

Figure 16. Paired Pulse Plasticity is Unaffected by PNEE.

The ratios of the slopes of the second pulse relative to the slopes of the first pulses are unaffected by PNEE in males (**A1**) nor in females (**A2**) nor when $GABA_A$Rs are blocked by BIC as in LTP recordings in males (**B1**) or females (**B2**). Bars represent average paired pulse ratios and error bars represent standard error of the mean (SEM).

In order to assess whether PNEE changes postsynaptic responsiveness to our stimuli we constructed an I/O curve of increasing pulse widths and the resulting fEPSP slopes (Figure 17). Given that for our LTP recordings we used BIC to inhibit $GABA_A$ we also constructed I/O curves in the presence of BIC. In males without BIC, PNEE resulted in significantly reduced fEPSP slopes on average at 90 ($p = 0.0292$), 120 ($p = 0.005$), 150 ($p = 0.0035$), 180 ($p = 0.005$), 210 ($p = 0.0023$), 240 ($p = 0.0032$), 270 ($p = 0.0046$) and 300 µs ($p = 0.0053$) pulse widths. Under no other conditions was the I/O curve significantly affected by PNEE.

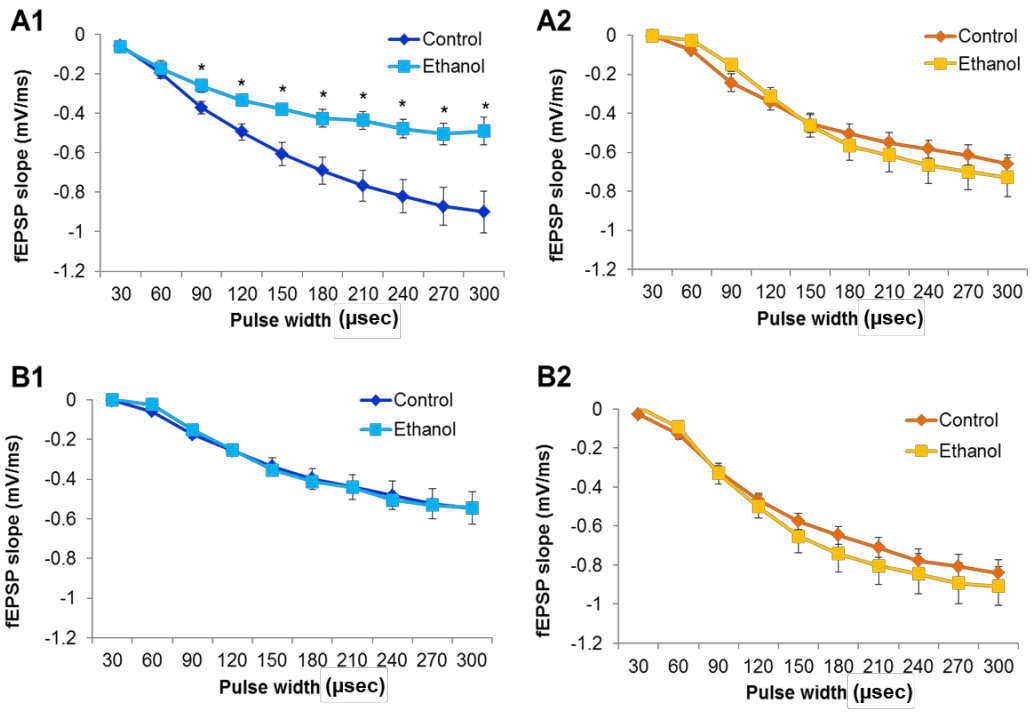

Figure 17. PNEE Results in Changes to the Input-Output Curve only in Males Without $GABA_A$ Blockade.

Postsynaptic responsiveness to increasing pulse widths is reduced by PNEE in males (**A1**) but not in females (**A2**) when $GABA_A$ is not inhibited by bicuculline methiodide (BIC) prior to LTD recordings. Under BIC conditions, the input-output curve is unaffected by PNEE in both males (**B1**) and females (**B2**). Points represent average fEPSP slopes by group and error bars represent the standard error of the mean (SEM). * represent $p < 0.05$ relative to control levels at the same pulse width.

3.3 Long-Term Potentiation

Measures of short and long-term potentiation were evaluated following the delivery of a CS in the form of HFS (4 trains 50 x 100Hz) in the presence of BIC (10µM). PTP was determined as the average percentage of change in the fEPSP slope relative to the baseline for minutes 0-1. LTP was determined as the average percentage of change in the fEPSP slope relative to the baseline for minutes 55-60.

3.3.1 Long-Term Potentiation in Males

HFS led to an immediate increase in the percentage change of the fEPSP slope in both the Control (PTP: 148.04 ± 13.60 %; n = 8 slices, 5 animals, 3 litters) and EtOH

(PTP: 112.36 ± 14.54 %; n = 8 slices, 5 animals, 3 litters) males, but this PTP was not statistically significantly different between diet conditions as per a two-tailed student's t-test (p = 0.0948; Figure 18 A, D).

We hypothesized that, as per our previous findings, PNEE would significantly reduce the magnitude of LTP in males and therefore ran a one-tailed student's t-test to compare the magnitude of LTP in the present study. EtOH males (LTP: 47.76 ± 11.76 %; n = 8 slices, 5 animals, 3 litters) had significantly reduced magnitudes of LTP (p = 0.00670) as compared to their control male (LTP: 91.81 ± 17.18 %; n = 8 slices, 5 animals, 3 litters) counterparts (Figure 18 B, C, D). The effect of PNEE on the magnitude of LTP in males was found to be large (d = 1.06).

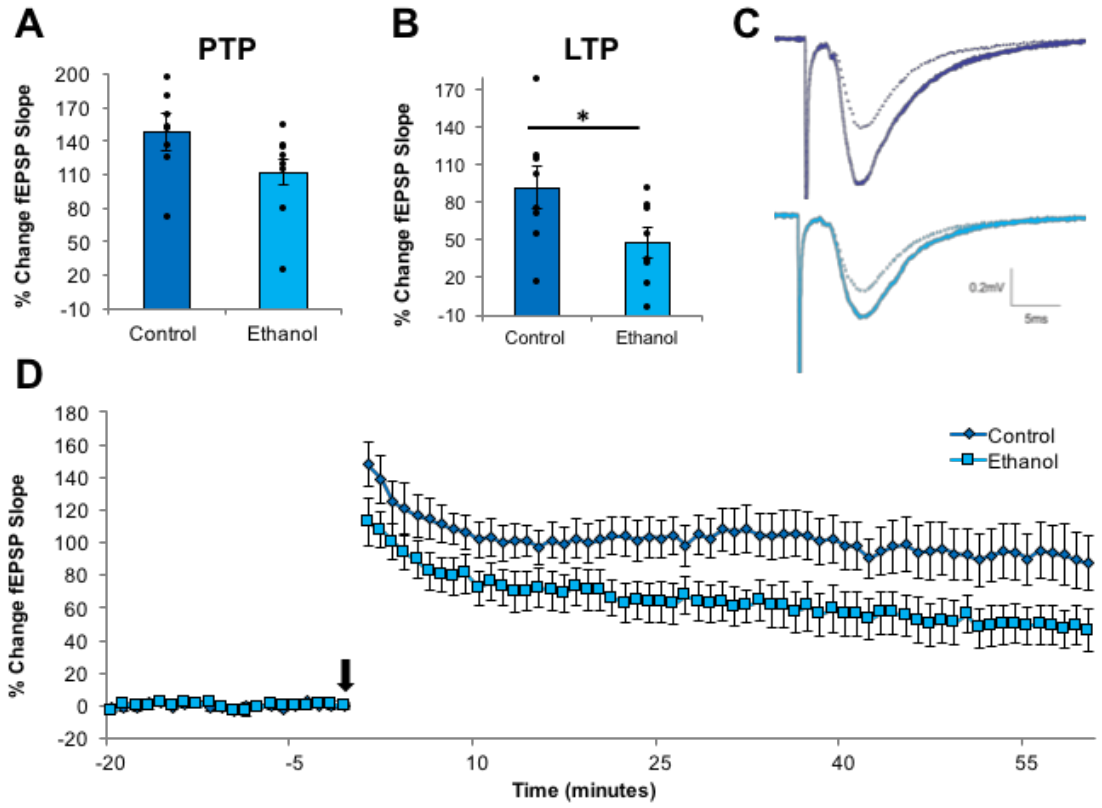

Figure 18. Short- and Long-Term Potentiation in Males Following PNEE

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrow in D). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative

to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. (**C**) Representative traces of the average fEPSPs in the control (dark blue, above) and ethanol (light blue; below) for the baseline recordings (dotted lines) and average LTP (solid line). The scale bar represents 0.2mV by 5msec. (**D**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 8 slices, 5 animals, 3 litters; N_{EtOH} = 8 slices, 5 animals, 3 litters * $p < 0.05$.

3.3.2 Long-Term Potentiation in Females

PTP was measured as the average percentage of change in the fEPSP slope for minutes 0-1 following HFS, and was elevated above baseline in both Control (PTP: 123.83 ± 17.75 %; n = 7 slices, 5 animals, 2 litters) and EtOH-exposed (PTP: 95.11 ± 13.77 %; n = 11 slices, 5 animals, 3 litters) females, although there were no statistically significant differences between conditions ($p = 0.224$; Figure 19 A,D).

HFS led to a long-term increase in the average percentage of change in the fEPSP slope in both conditions, measured as LTP from time 55-60 minutes after the HFS. In controls (LTP: 74.82 ± 7.53 %; n = 7 slices, 5 animals, 2 litters) significantly greater magnitudes of LTP were elicited than in EtOH-exposed (LTP: 29.67 ± 7.66 %; n = 11 slices, 5 animals, 3 litters) female offspring ($p = 0.000756$; Figure 19 B, C, D). The effect of PNEE on the magnitude of LTP in females was found to be large ($d = 1.92$).

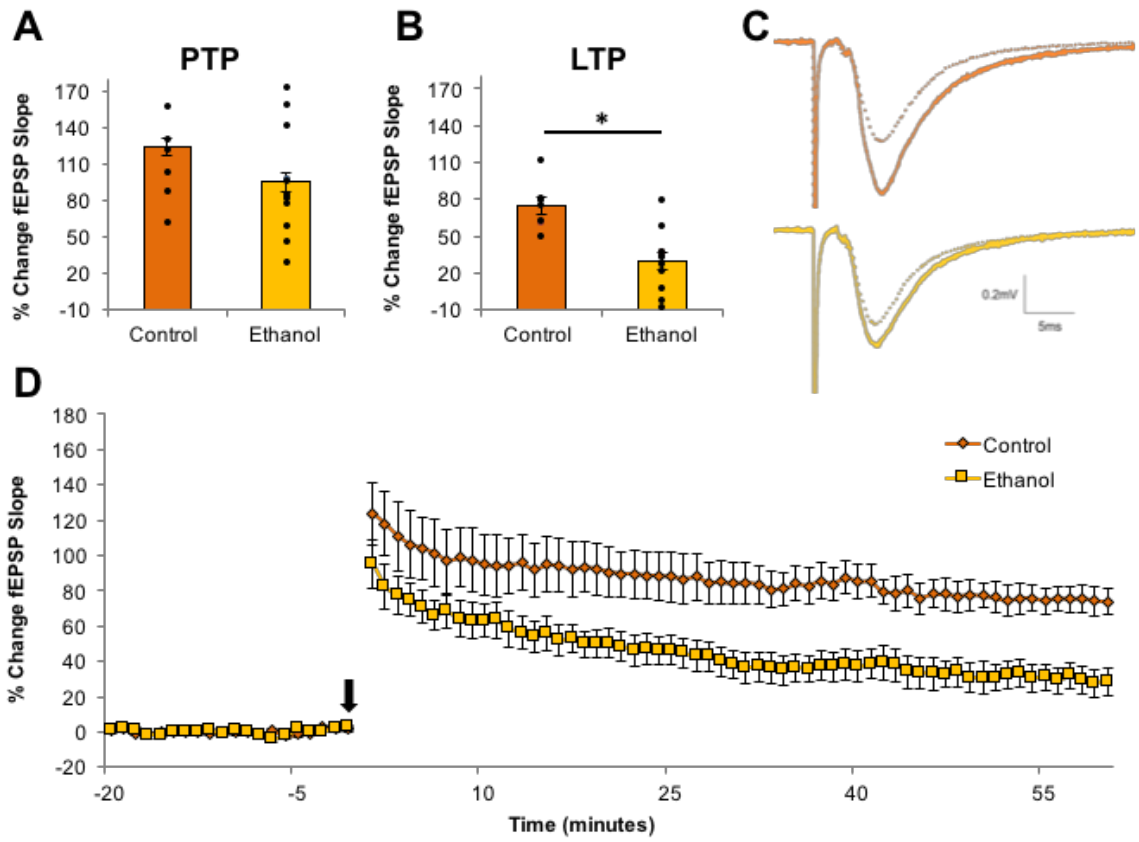

Figure 19. Short- and Long-Term Potentiation in Females Following PNEE.

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrow in D). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. (**C**) Representative traces of the average fEPSPs in the control (orange, above) and ethanol (yellow; below) for the baseline recordings (dotted lines) and average LTP (solid line). The scale bar represents 0.2mV by 5msec. (**D**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$. $N_{control} = 7$ slices, 5 animals, 2 litters; $N_{EtOH} = 11$ slices, 5 animals, 3 litters.

3.3.3 Mechanism of LTP

As there are many mechanisms for a variety of forms of LTP, it was important to characterize the underlying receptors whose function may be impaired by PNEE. These exaperiments can help identify therapeutic targets for overcoming PNEE-induced deficits. Previous work from our laboratory using this HFS has been found to be

NMDAR-dependent thus we used the competitive antagonist DL-APV (50µM) for the last 10 minutes of the pre-conditioning recording and during the delivery of the HFS in control male and female offspring (see Figure 14 for schematic timeline).

The NMDAR antagonist DL-APV significantly attenuated both PTP (Control: 148.04 ± 13.60 %; n = 8 slices, 5 animals, 3 litters; DL-APV: 28.20 ± 4.04 %; n = 3 slices, 2 animals, 1 litter; Figure 20; $p = 2.79 \times 10^{-5}$) and blocked LTP (Control: 91.81 ± 17.18 %; n = 8 slices, 5 animals, 3 litters; DL-APV: -4.23 ± 2.24 %; n = 3 slices, 2 animals, 1 litter; Figure 20; p = 0.000774).

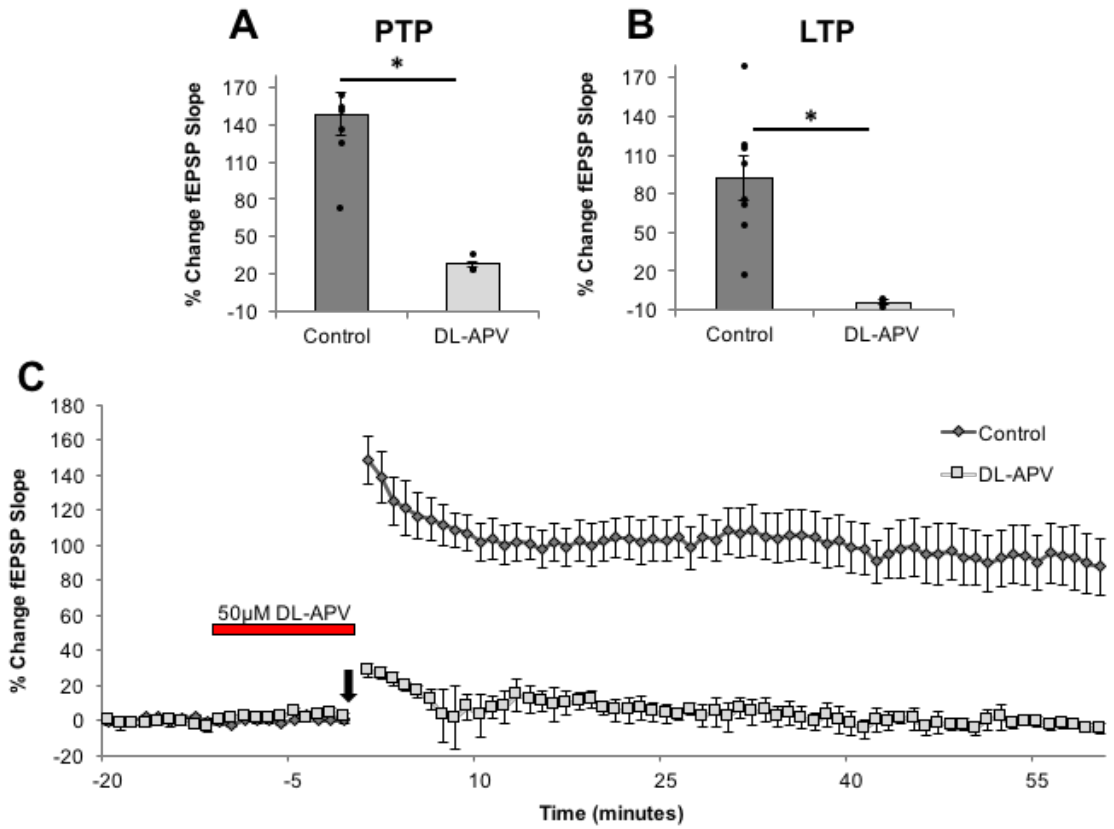

Figure 20. The Impact of NMDAR Blockade on LTP

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrow in D). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. (**C**) Average LTP recordings from

the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the NMDAR antagonist DL-APV (50μM). * $p < 0.05$. $N_{control} = 8$ slices, 5 animals, 3 litters; $N_{DL\text{-}APV} = 3$ slices, 2 animals, 1 litter.

3.3.4 Cumulative Probability of the Impact of PNEE on Long-Term Potentiation

Cumulative probability graphs are effective for illustrating potential differences between an experimental manipulation (such as prenatal diet in this dissertation) and the proportion of samples that fall within certain magnitudes of plasticity. In both males (Figure 21 A) and females (Figure 21 B) it is clear that there is a leftward shift in cumulative probability such that PNEE yields lower magnitudes of LTP in both sexes. In order to compare sexes, a one-way analysis of variance (ANOVA) was conducted and yielded a significant main effect ($p = 0.0019$). A Tukey HD post-hoc text was conducted and revealed significant sex differences only between control males and ethanol females ($p = 0.0406$). No other specific comparisons between sexes were significantly different.

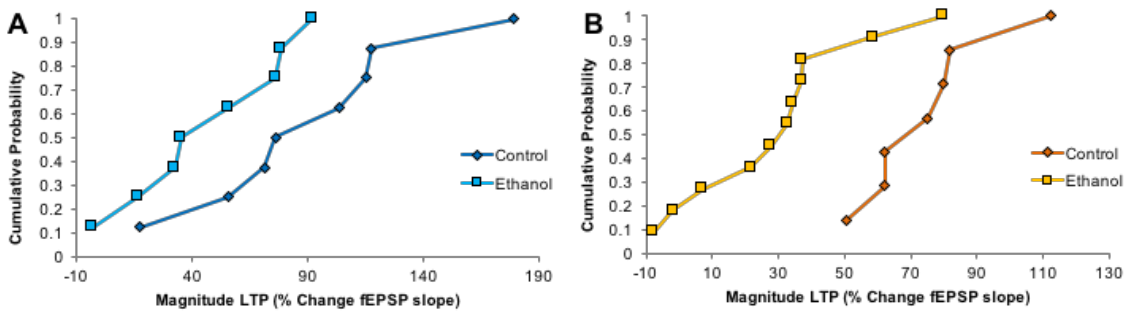

Figure 21. Cumulative Probability for LTP in Males and Females Following PNEE

Actual frequencies of the distribution of the magnitudes of LTP in both (A) male and (B) female offspring following either a control or EtOH prenatal diet as measured by the percentage of change in the field excitatory postsynaptic potential (fEPSP) slope.

3.3.5 Maximizing Long-Term Potentiation

It is possible that PNEE leads to an increase in the threshold necessary to induce normal magnitudes of LTP such that a single bout of HFS (1xHFS) is insufficient to recruit or activate the second messenger cascades necessary to support LTP, thus we

administered two bouts of HFSs (2xHFS; 2 x 4 x 50 pulses at 100Hz) to maximize the LTP in these slices in both sexes.

Unsurprisingly, in male PNEE offspring 2xHFS did not have any effect on the magnitude of PTP as compared to that elicited by 1xHFS (1xHFS: 112.36 ± 14.54 %; n = 8 slices, 5 animals, 3 litters; 2xHFS: 139.24 ± 19.21 %; n = 13 slices, 7 animals, 3 litters; p = 0.342; Figure 22 A,C), as PTP was not affected by PNEE in general. However, 2xHFS did yield significantly greater magnitudes of LTP than did 1xHFS (1xHFS: 47.76 ± 11.76 %; n = 8 slices, 5 animals, 3 litters; 2xHFS: 94.76 ± 12.43 %; n = 13 slices, 7 animals, 3 litters; p = 0.0230; Figure 22 B,C) in male PNEE offspring. The effect size on the magnitudes of LTP in males was found to be large (d = 1.15). See Appendix C for cumulative probabilities.

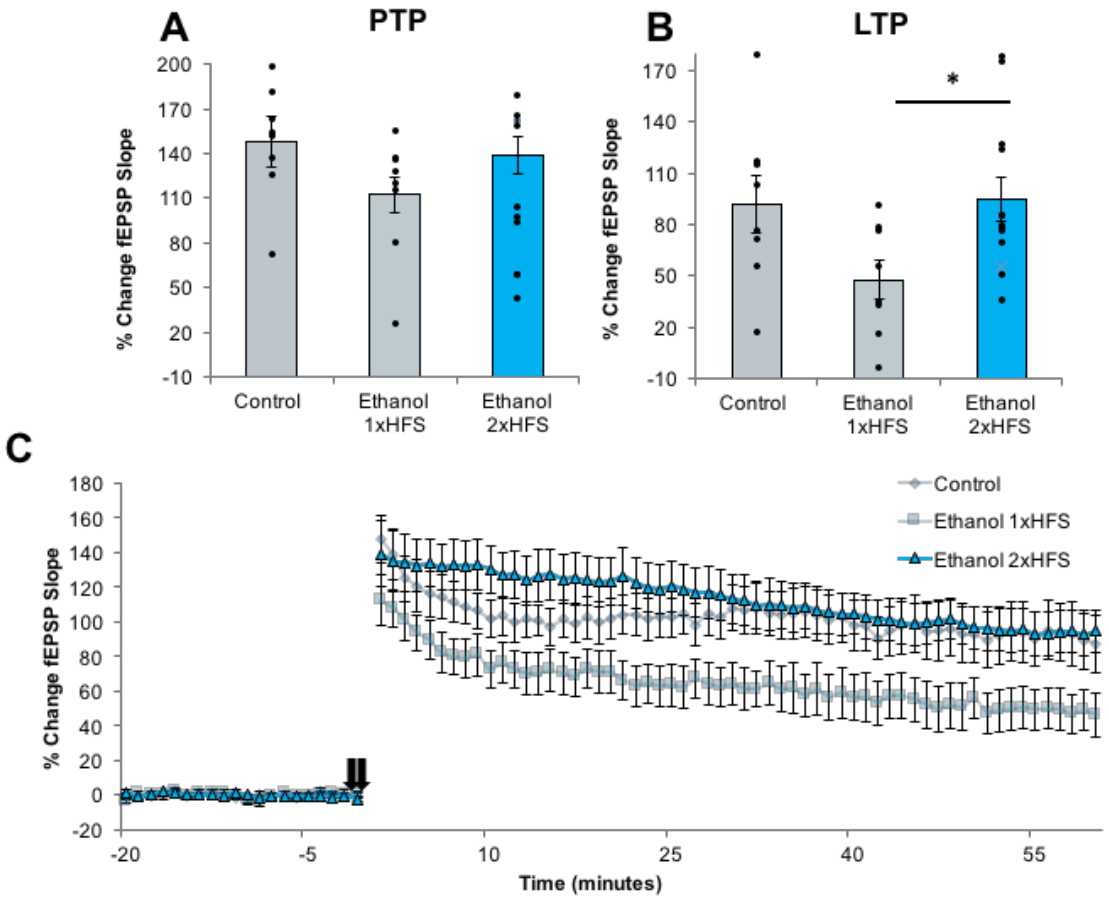

Figure 22. Multiple High Frequency Stimulation-Induced Long-Term Potentiation in Males Following PNEE.

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrows in C). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. Semi-transparent bars represent the magnitudes of PTP and LTP elicited by 1xHFS in control and EtOH offspring. (**C**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Semi-transparent plots represent the average LTP recordings from the beginning of baseline to the end of post-conditioning recording in control and EtOH offspring following 1xHFS. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$. $N_{control}$ = 8 slices, 5 animals, 3 litters; $N_{EtOH\ 1xHFS}$ = 8 slices, 5 animals 3 litters; $N_{EtOH\ 2xHFS}$ = 13 slices, 7 animals, 3 litters.

In female PNEE offspring, a similar effect was observed in that PTP was unaffected by 2xHFS (1xHFS: 95.11 ± 13.77 %; n = 11 slices, 5 animals, 3 litters; 2xHFS: 119 ± 11.54

%; n = 16 slices, 5 animals, 2 litters; p = 0.603; Figure 23 A,C). Despite having no effect on PTP, 2xHFS significantly increased the magnitude of LTP in female PNEE offspring (1xHFS: 29.67 ± 7.66 %; n = 11 slices, 5 animals, 3 litters; 2xHFS: 63.59 ± 7.92 %; p = 0.0051; n = 16 slices, 5 animals, 2 litters; Figure 23 B,C). The size of this effect on the magnitudes of LTP in females was found to be large (d = 2.17). See Appendix C for cumulative probabilities.

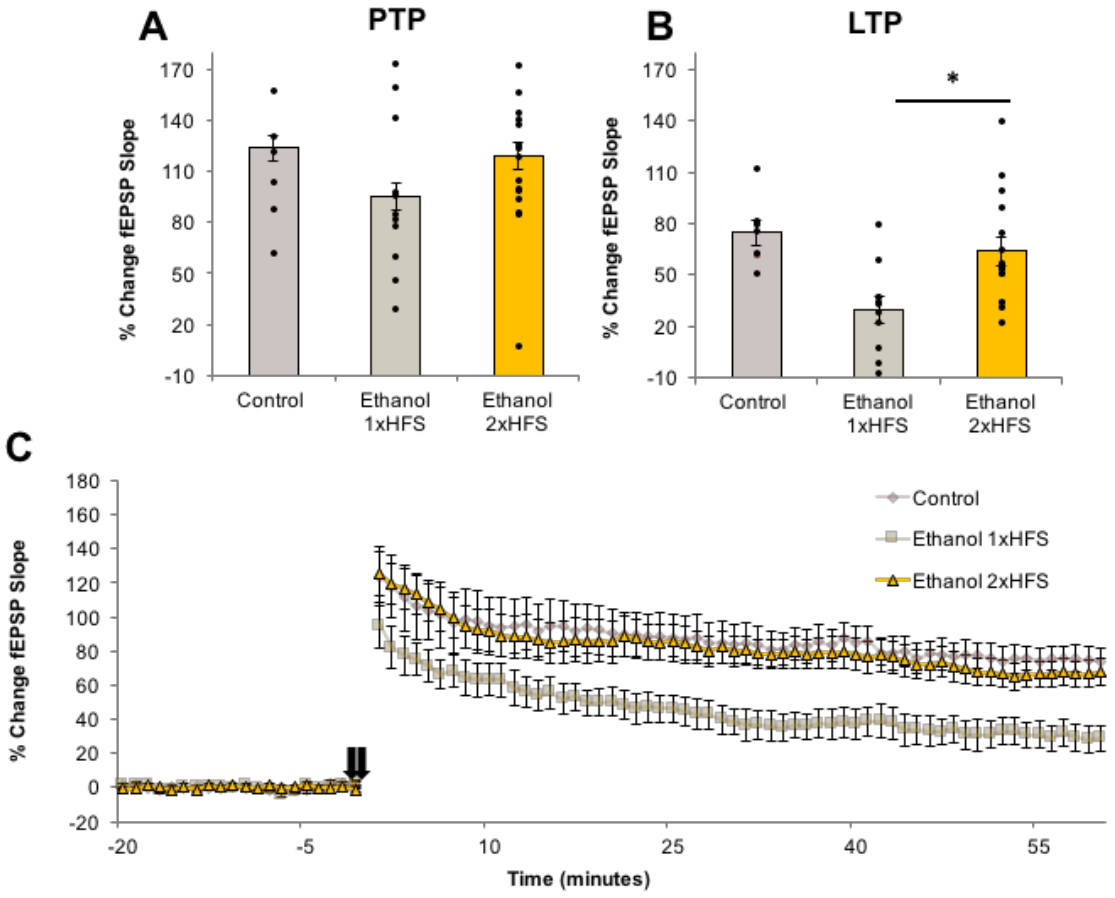

Figure 23. Multiple High Frequency Stimulation-Induced Long-Term Potentiation in Females Following PNEE.

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrows in C). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. Semi-transparent bars represent the magnitudes of PTP and LTP elicited by 1xHFS in control and EtOH offspring. (**C**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Semi-

transparent plots represent the average LTP recordings from the beginning of baseline to the end of post-conditioning recording in control and EtOH offspring following 1xHFS. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$. $N_{control} = 7$ slices, 5 animals, 2 litters; $N_{EtOH\ 1xHFS} = 11$ slices, 5 animals, 3 litters; $N_{EtOH\ 2xHFS} = 16$ slices, 5 animals, 2 litters.

3.4 Long-Term Depression

Next, we examined the 'flip-side' of LTP in order to identify the negative component of the dynamic range of synaptic plasticity. Given the LTP deficits observed in this dissertation, a shift in this dynamic range could manifest itself as an enhancement in LTD, whereas a restriction in this range would appear as an impairment in the magnitude of LTD as a result of PNEE. LTD was elicited either by a standard LFS (900x 1 Hz) (LFS_{900}) or by a maximizing LFS consisting of 1800x 1 Hz (LFS_{1800}) to determine whether PNEE impacts the dynamic range of LTD in juvenile male and female offspring. Furthermore, in order to assess whether PNEE alters short term plasticity as a result of LFS, STD was evaluated from 0-1 minutes after delivery of the CS although the precise mechanisms underlying this process are not well-defined.

3.4.1 Long-Term Depression Elicited by LFS_{900} in Males

The magnitude of STD, the first minute of recordings following LFS_{900}, was similar between the control (STD: -38.73 ± 4.29 %; n = 12 slices, 5 animals, 2 litters) and EtOH-exposed (STD: -40.30 ± 6.91 %; n = 11 slices, 5 animals, 3 litters) male offspring when compared by a two-tailed student's t-test ($p = 0.851$; Figure 24).

LFS_{900} induced stable LTD from 55-60 minutes in the post-conditioning recordings. Interestingly, the magnitude of LTD was significantly reduced in EtOH (LTD: -10.83 ± 3.33 %; n = 11 slices, 5 animals, 3 litters) males relative to controls (LTD: -24.89 ± 4.76 %; n = 12 slices, 5 animals, 2 litters; $p = 0.0257$; Figure 24). The effect of PNEE on the magnitude of LTD by LFS_{900} in males was found to be large ($d = 0.999$).

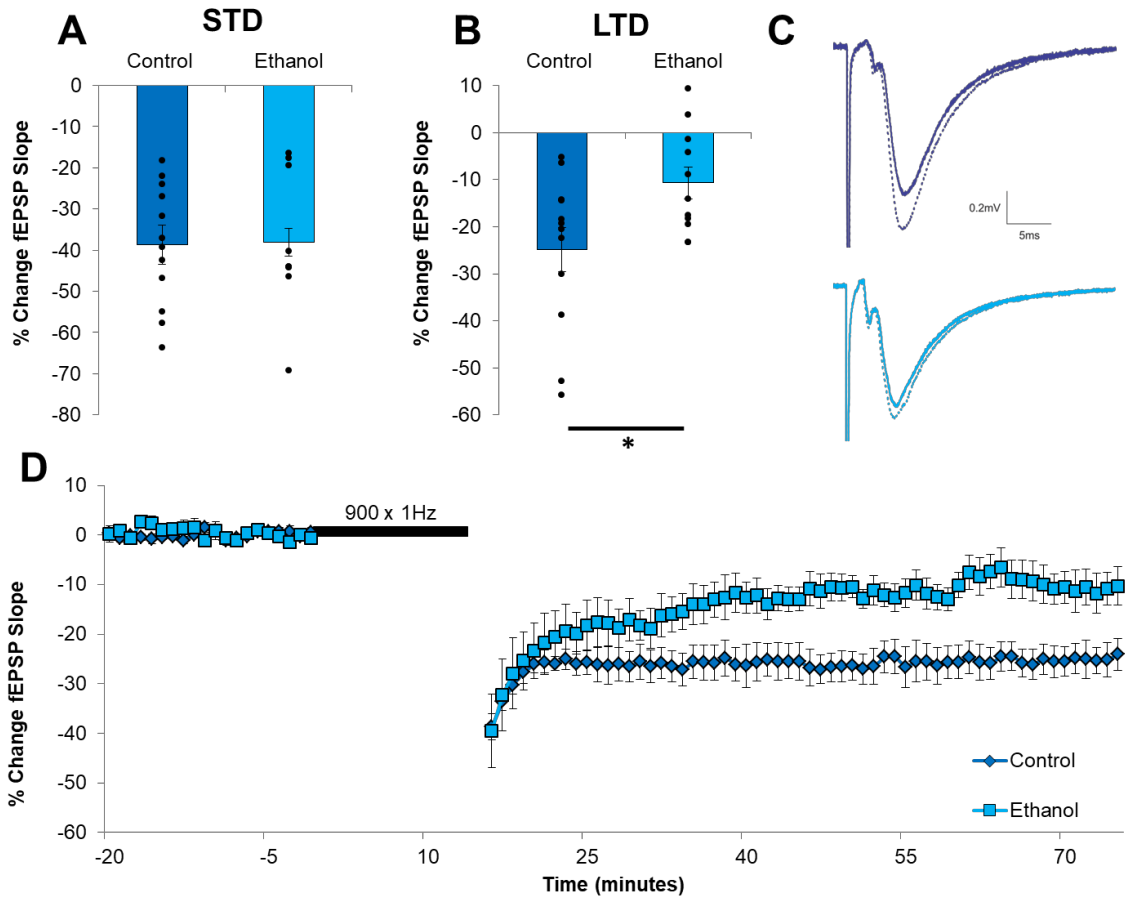

Figure 24. Short- and Long- Term Depression in Males Following PNEE

(**A**) Short-term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the low frequency stimulation (LFS; 900x1Hz; black bar in D). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Representative traces of the average fEPSPs in the control (dark blue, above) and ethanol (light blue; below) for the baseline recordings (dotted lines) and average LTD (solid line). The scale bar represents 0.2mV by 5msec. (**D**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$. $N_{control}$ = 12 slices, 5 animals, 2 litters; N_{EtOH} = 11 slices, 5 animals, 3 litters.

3.4.2 Long-Term Depression Elicited by LFS_{900} in Females

In females the magnitude of STD was not significantly different between control (STD: -52.44 ± 4.86 %; n = 14 slices, 6 animals, 2 litters) and EtOH-exposed (STD: -47.89 ± 3.86 %; n = 9 slices, 7 animals, 3 litters) female offspring as per a two-tailed student's t-test (p = 0.472; Figure 25).

Unlike what we found in males, the magnitude of LTD induced by LFS_{900} was not different between female control (-28.46 ± 3.56 %; n = 14 slices, 6 animals, 2 litters) and EtOH (-27.90 ± 5.46 %; n = 9 slices, 7 animals, 3 litters) offspring (p = 0.932; Figure 25).

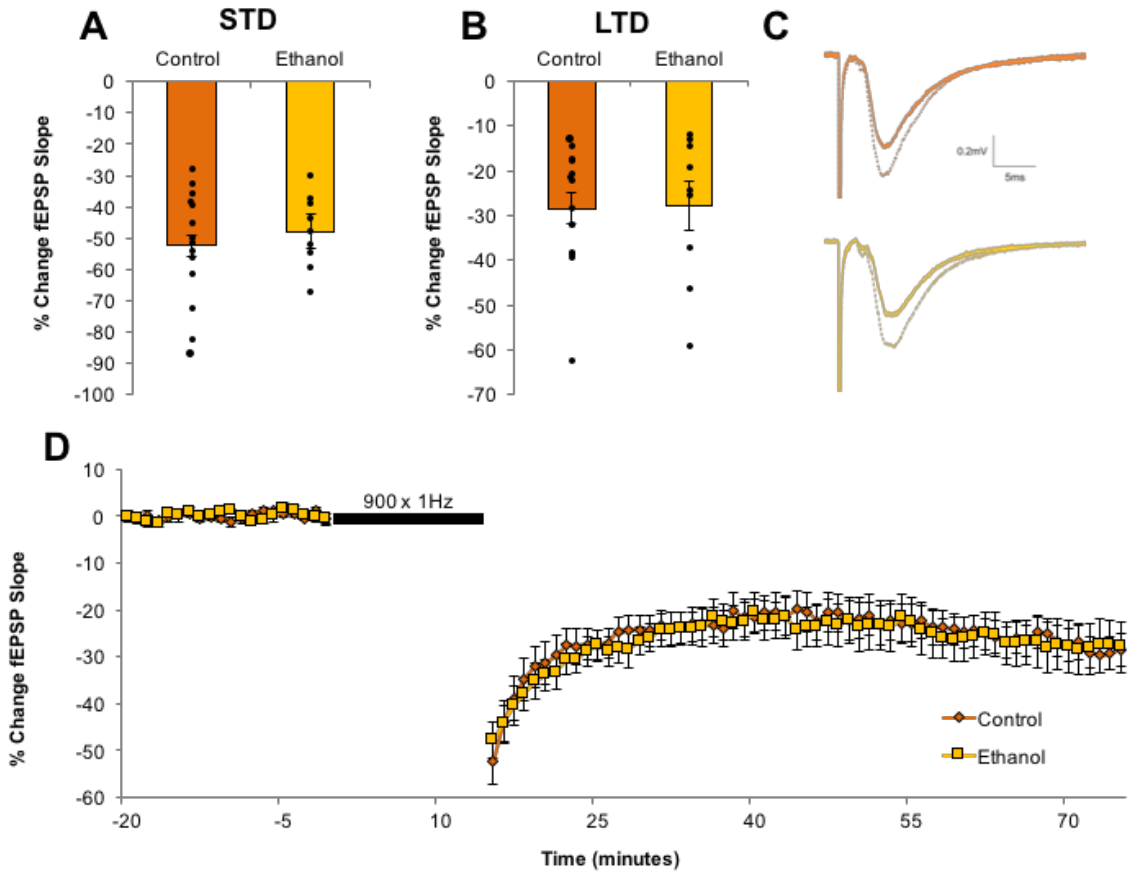

Figure 25. Short- and Long- Term Depression in Females Following PNEE

(**A**) Short-term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of the low frequency stimulation (LFS; 900x1Hz; black bar in D). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Representative traces of the average fEPSPs in the control (orange, above) and ethanol (yellow; below) for the baseline recordings (dotted lines) and average LTD (solid line). The scale bar represents 0.2mV by 5msec. (**D**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 14 slices, 6 animals, 2 litters; N_{EtOH} = 9 slices, 7 animals, 3 litters.

3.4.3 Cumulative Probability of the Impact of PNEE on Long-Term Depression

Cumulative probabilities are another method by which the distribution of the magnitudes of synaptic plasticity within a dataset can be illustrated. In this type of analysis the proportion of data that falls within specific magnitudes of plasticity are depicted. In Figure 26 A it is clear that in males there is overlap between the EtOH-exposed and control offspring at the lower magnitudes of LTD (0 to -20%), and the differences between the diet groups become more apparent at the higher magnitudes of LTD (-30 to -55%) that were not observed in the EtOH group which likely underlie the significant differences between the diet conditions for LTD. In contrast, in the females depicted in Figure 26 B there is clear overlap between the EtOH and control groups throughout the complete range of LTD supporting the lack of a significant effect of teratogen exposure in females. In order to compare sexes, a one-ANOVA was conducted and yielded a significant main effect ($p = 0.0184$). A Tukey HSD post-hoc text was conducted and revealed significant sex differences between control females and ethanol males ($p = 0.02$) and between ethanol males and ethanol females ($p = 0.0437$).

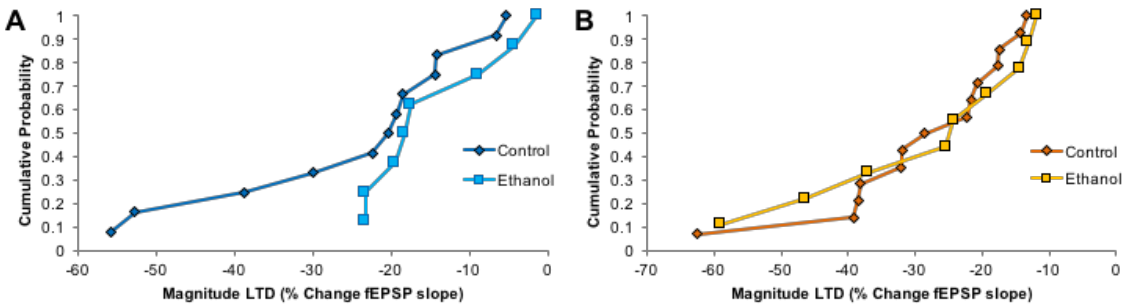

Figure 26. Cumulative Probability for LTD in Males and Females Following PNEE

Actual frequencies of the distribution of the magnitudes of LTD in both (A) male and (B) female offspring following either a control or EtOH prenatal diet.

3.4.3 The Search for the Mechanism of Long-Term Depression Induced by LFS_{900}

In order to understand the potential underlying mechanisms of the LTD in the present study induced by LFS_{900} we examined a variety of receptor and channel blockers that have historically been shown to play roles in certain types of LTD. Specifically we

used DL-APV to inhibit NMDARs, MPEP to inhibit $mGluR_5$s, NIMO to inhibit L-type voltage-gated calcium channels or AM251 to inhibit CB1 receptors. Additionally, we used a peptide Tat-$GluA2_{3Y}$ which prevents phosphorylation of tyrosine residues on this AMPAR subunit thought to play a role in AMPAR endocytosis classically associated with LTD. Unless otherwise noted, the inhibitor or drug cocktail was delivered for the last 10 minutes of the pre-conditioning recording and throughout the LFS_{900} and these experiments were conducted in control male and female offspring and were compared to the magnitude of LTD induced in male and female controls combined.

Surprisingly, the NMDAR antagonist DL-APV (50μM) did not cause significant changes to either STD (Control: -46.11 ± 3.49%; n = 26 slices, 11 animals, 4 litters; DL-APV: -34.99 ± 5.69 %; n = 6 slices, 3 animals, 1 litter; Figure 27; p = 0.129) or LTD induced by LFS_{900} (Control: -26.80 ± 2.88%; n = 26 slices, 11 animals, 4 litters; DL-APV: -26.68 ± 5.11; n = 6 slices, 3 animals, 1 litter; Figure 27; p = 0.983). Using an increased concentration of DL-APV (100μM) also had no effect on the magnitude of LTD induced by LFS (data not shown).

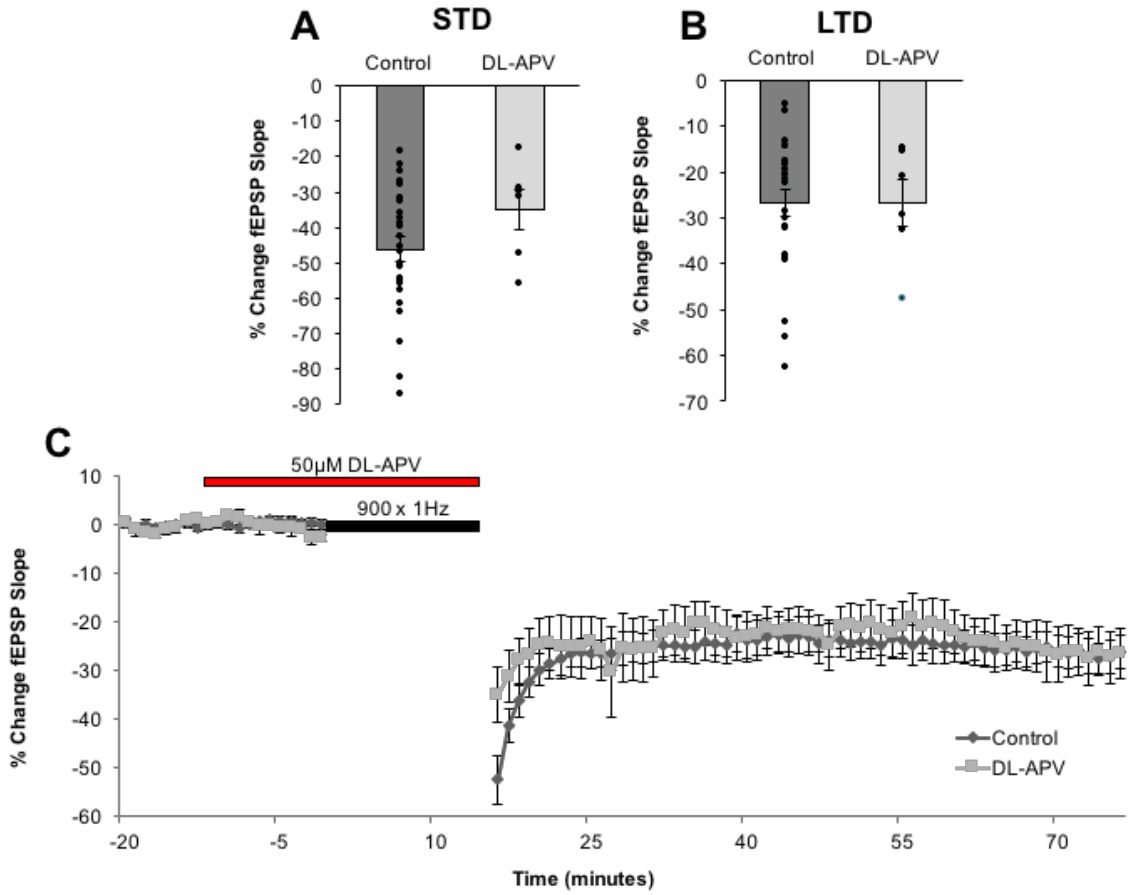

Figure 27. The Impact of NMDAR Blockade on LTD

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the competitive NMDAR antagonist DL-APV (50µM). $N_{control}$ = 26 slices, 11 animals, 4 litters; $N_{DL\text{-}APV}$ = 6 slices, 3 animals, 1 litter.

Given that there are large concentrations of $mGluR_5$s on DG granule cells and that LTD dependent on these receptors has been observed experimentally in other studies, we used the non-competitive $mGluR_5$ antagonist MPEP (10µM) (Faas et al., 2002; Volk et al., 2006) to inhibit their function. Unexpectedly, MPEP blocked neither STD (Control: -46.11 ± 3.49%; n = 26 slices, 11 animals, 4 litters; MPEP: -38.38 ± 9.12 %; n = 6 slices, 4 animals, 1 litter; Figure 28; p = 0.456) nor LTD (Control: -26.80 ± 2.88%; n = 26

slices, 11 animals, 4 litters; MPEP: -20.15 ± 6.40 %; n = 6 slices, 4 animals, 1 litter; Figure 28; p = 0.374).

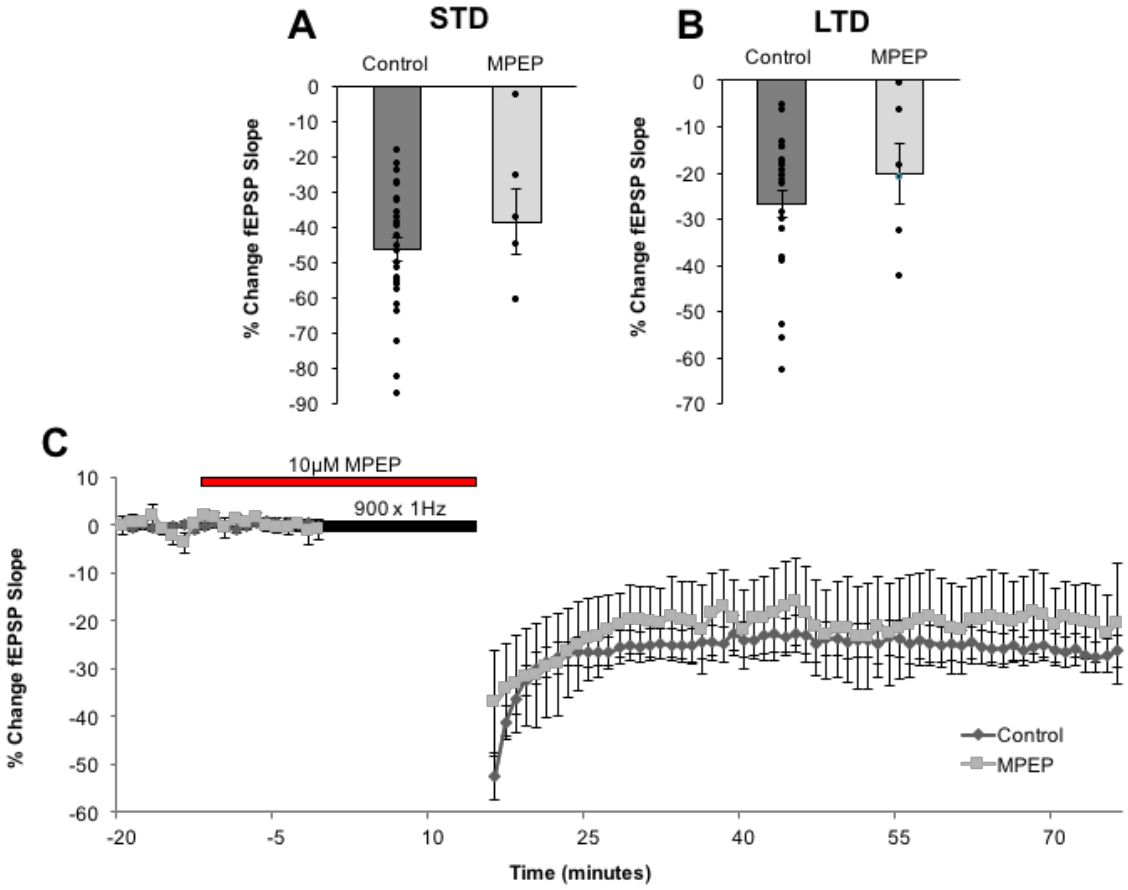

Figure 28. The Impact of mGluR$_5$ Blockade on LTD

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the non-competitive mGluR$_5$ antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP; 10μM). $N_{control}$ = 26 slices, 11 animals, 4 litters; N_{MPEP} = 6 slices, 4 animals, 1 litter.

L-type calcium channels have been implicated in hippocampal LTD (Christie et al., 1997; Coussens et al., 1997) and therefore were the next mechanistic target for the

present dissertation. The L-type calcium channel blocker NIMO (20µM) (Wickens and Abraham, 1991) did not significantly affect either STD (Control: -46.11 ± 3.49%; n = 26 slices, 11 animals, 4 litters; NIMO: -58.92 ± 18.14 %; n = 3 slices, 2 animals, 1 litter; Figure 29; p = 0.555) or LTD (Control: -26.80 ± 2.88%; n = 26 slices, 11 animals, 4 litters; NIMO: -30.15 ± 8.20 %; n = 3 slices, 2 animals, 1 litter; Figure 29; p = 0.731).

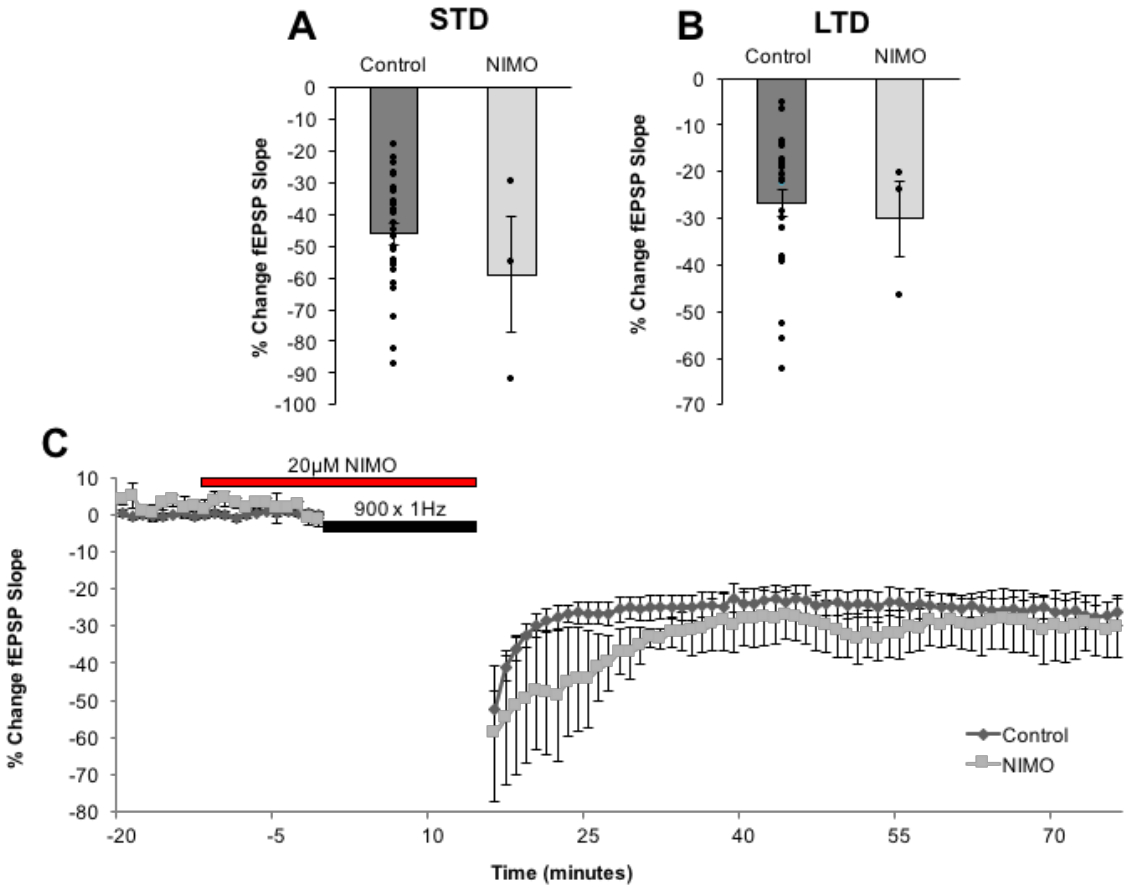

Figure 29. The Impact of LTCC Blockade on LTD

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the L-type calcium channel (LTCC) blocker Nimodipine (NIMO; 20µM). $N_{control}$ = 26 slices, 11 animals, 4 litters; N_{NIMO} = 3 slices, 2 animals, 1 litter.

The function of the above-mentioned receptors, particularly NMDARs and mGluRs may be interrelated and it is possible that the coupled function of multiple receptor types are necessary to support this form of LTD. If this is true, then pharmacological inhibition of one single receptor type may be insufficient to block this LTD. As such, we used a cocktail of the aforementioned compounds in an effort to block LTD. A cocktail of DL-APV (50µM) , MPEP (10µM) and NIMO (20µM) similarly was unable to block STD (Control: -46.11 ± 3.49%; n = 26 slices, 11 animals, 4 litters; Cocktail: -37.74 ± 3.64; n = 3 slices, 2 animals, 1 litter; Figure 30; p = 0.142) or LTD (Control: -26.80 ± 2.88%; n = 26 slices, 11 animals, 4 litters; Cocktail: -37.50 ± 10.09 %; n = 3 slices, 2 animals, 1 litter; Figure 30; p = 0.402).

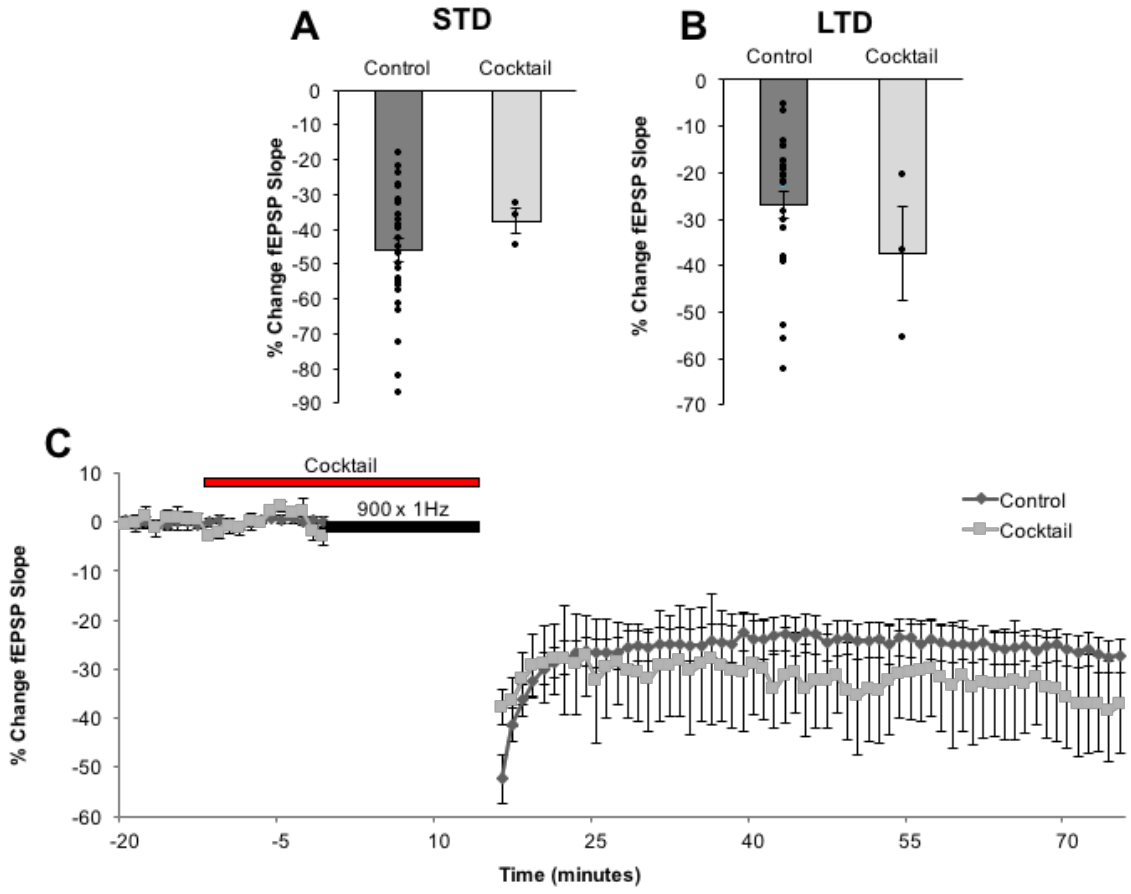

Figure 30. The Impact of Simultaneous Blockade of NMDARs, mGluR$_5$s and LTCCs on LTD

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the drug cocktail. $N_{control}$ = 26 slices, 11 animals, 4 litters; $N_{cocktail}$ = 3 slices, 2 animals, 1 litter.

Endocytosis of the AMPAR is classically associated with LTD and recent work has found that phosphorylation of tyrosine residues on the GluA2 is associated with LTD in the hippocampus by using a synthetic peptide that blocks phosphorylation at this site (Ahmadian et al., 2004; Man et al., 2000). Thanks to the donation from Yu Tian Wang at the University of British Columbia, we used a pilot sample of 10μM Tat-$GluA2_{3Y}$ that was dissolved in aCSF and washed onto the slice for 20 minutes of pre-conditioning, throughout LFS and for 30 minutes of the post-conditioning recording in order to ensure entry of this compound into the postsynaptic cell to access the intracellular c-terminus of the GluA2 subunit of the AMPAR. Unexpectedly this exposure to Tat-$GluA2_{3Y}$ had no effect on LTD (-22.79 ± 1.63 %; n = 6 slices, 4 animals, 1 litter; Figure 31; p = 0.235) nor on STD (-37.22 ± 4.00 %; n = 6 slices, 4 animals, 1 litter; Figure 31; p = 0.12).

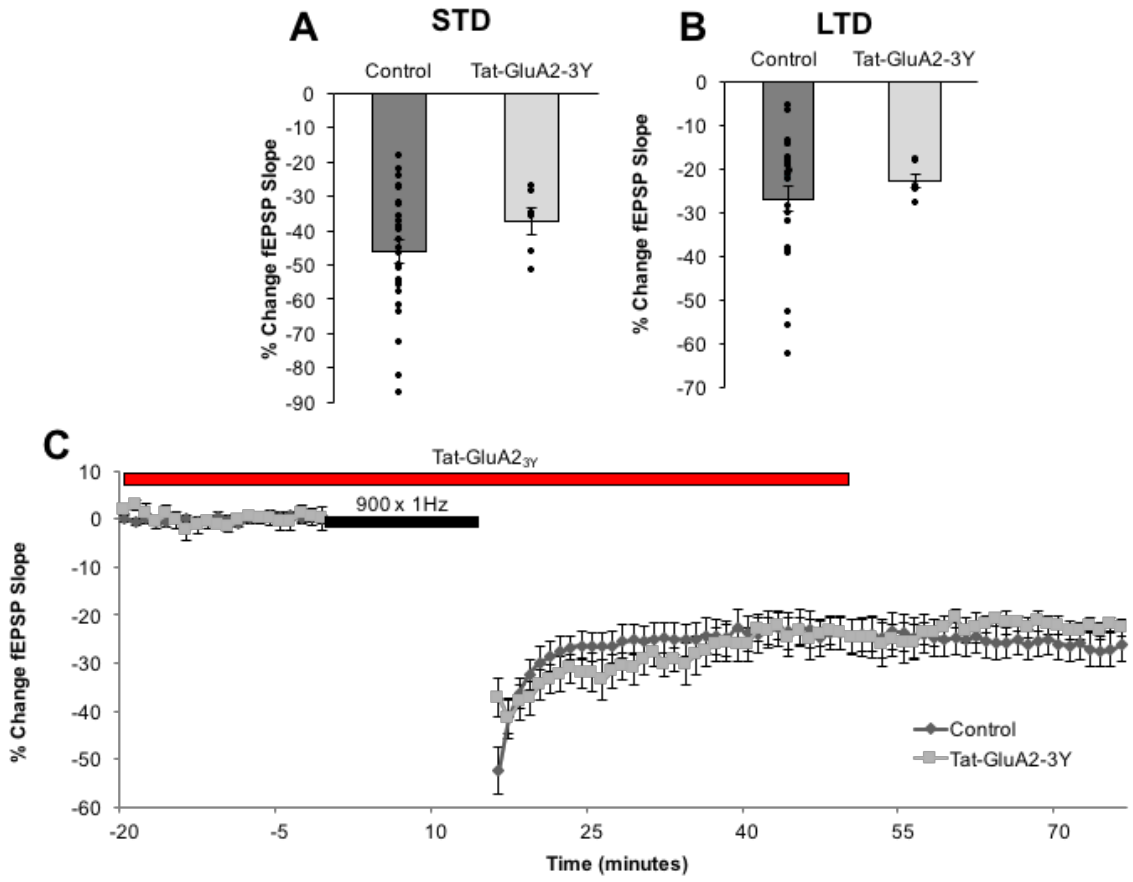

Figure 31. The Impact of Tat-GluA2$_{3Y}$ on LTD.

(A) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS$_{900}$; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS$_{900}$. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the peptide Tat-GluA2$_{3Y}$ (10μM) that blocks phosphorylation of select tyrosine residues on the GluA2 subunit of the AMPAR which is associated with LFS-induced AMPAR endocytosis. $N_{control}$ = 26 slices, 11 animals, 4 litters; N_{tat} = 6 slices, 4 animals, 1 litter.

A slightly different form of LFS (10Hz for 10 minutes) induces a CB1 receptor-dependent form of LTD in the DG, thus it is possible that the LTD examined in the present study may share some overlapping mechanisms. The inverse agonist AM251 (4μM) (Pedro Grandes, Manuscript submitted) was used to inhibit CB1 receptors which led to a significant reduction in the magnitude of STD (Control: -46.11 ± 3.49%; n = 26

slices, 11 animals, 4 litters; AM251: -28.24 ± 3.09%; n = 17 slices, 8 animals, 1 litter; Figure 32; p = 0.0025). The inhibition of these receptors also led to a significant attenuation of LTD (Control: -26.80 ± 2.88%; %; n = 26 slices, 11 animals, 4 litters; AM251: -15.83 ± 6.24%; n = 17 slices, 8 animals, 1 litter; Figure 32; p = 0.050). The effect size of this attenuation of LTD by AM251 was found to be medium (d = 0.613).

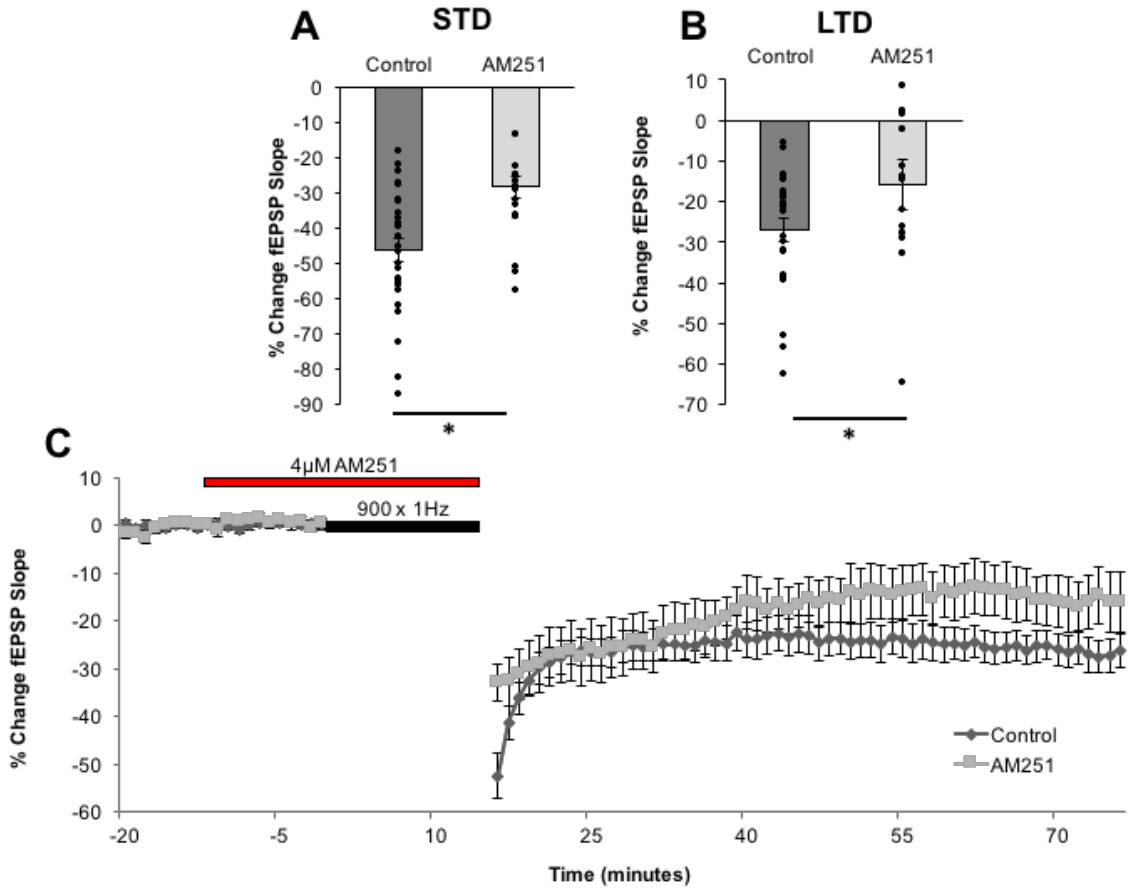

Figure 32. The Impact of CB1 Receptor Blockade on LTD.

(A) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the cannabinoid type 1 (CB1) receptor inverse agonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 4μM). * $p < 0.05$. $N_{control}$ = 26 slices, 11 animals, 4 litters; N_{AM251} = 17 slices, 8 animals, 1 litter.

LTD that is eCB-dependent in the DG requires the activity of both CB1 receptors and mGluRs. Recent work (Pedro Grandes, manuscript submitted) has found that this LTD (10Hz for 10 min) could be blocked by pharmacological inhibitors of either CB1 receptors by AM251, group 1 mGluRs by DHPG as well as inhibitors of $mGluR_1$ by CPCCoEt or $mGluR_5$ by MPEP. Futhermore, this eCB-LTD was unaffected by delivery of nimodipine (LTCCs) or by DL-APV (NMDARs) as we have observed the present experiments. Given the significant attenuation of LTP by AM251 (Figure 32), and the apparent relationship between CB1 receptors and group 1 mGluRs, we delivered a cocktail of available compounds AM251 and MPEP in an effort to determine whether the LTD evaluated in the present dissertation was in fact dependent on the coincident function of these two receptor signalling systems. A cocktail of 10μM MPEP and 4μM AM251 was washed onto the slice for the last 10 minutes of pre-conditioning recordings and throughout the LFS followed by a post-conditioning recording in regular aCSF. This cocktail had no effect on STD (-46.66 ± 6.97 %; n = 10 slices, 6 animals, 1 litter; Figure 33; p = 0.864) however it significantly reduced the magnitude of LTD (-0.821 ± 4.14 %; n = 10 slices, 6 animals, 1 litter; Figure 33; $p = 4.69 \times 10^{-5}$; d = 1.82, large effect size).

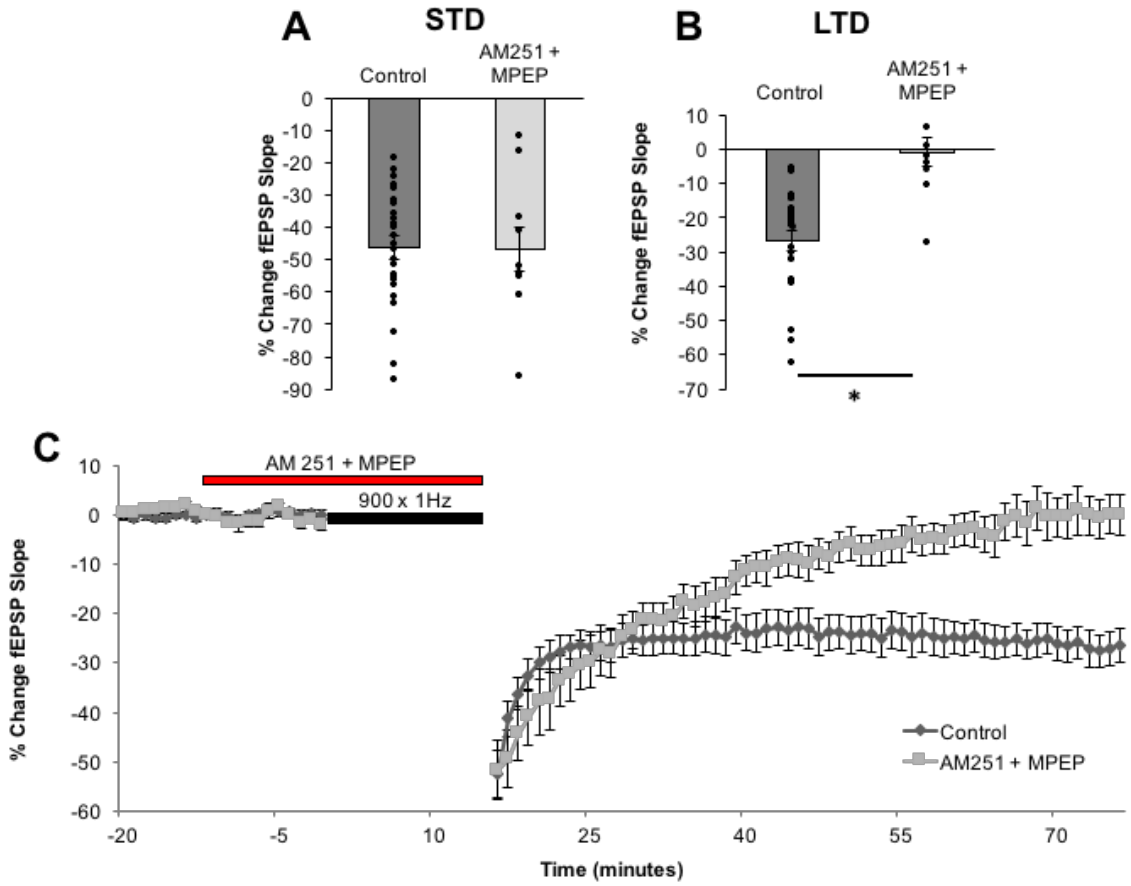

Figure 33. The Impact of Blockade of Both CB1 Receptors and mGluR$_5$s on LTD.

(**A**) Short-term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. The red bar represents the duration of exposure to the cannabinoid type 1 (CB1) receptor inverse agonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 4μM) and to the non-competitive $mGluR_5$ antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP; 10μM). * $p < 0.05$. $N_{control}$ = 26 slices, 11 animals, 4 litters; $N_{AM251+MPEP}$ = 10 slices, 6 animals, 1 litter.

3.4.4 Long-Term Depression Elicited by LFS_{1800} in Males

In order to understand how PNEE impacts the range of DG LTD, the magnitude of LTD was maximized by delivering double the amount of pulses in the LFS. It is possible that although LFS_{900} is insufficient in initiating the cascades necessary to support LTD, additional CS may overcome this deficit in males. Prolonged LFS to 1800 x 1 Hz (LFS_{1800}) led to a significant STD in both control (STD: -65.93 ± 8.58 %; n = 8 slices, 4 animals, 2 litters) and EtOH males (STD: -60.44 ± 15.78 %; n = 5 slices, 3 animals, 3 litters), which was not statistically significantly different between groups ($p = 0.769$; Figure 34).

LFS_{1800} in the EtOH-exposed males yielded a greater magnitude of LTD than did LFS_{900} ($p = 0.0280$). The magnitude of LTD after prolonged LFS was not significantly different between the control (LTD: -36.82 ± 5.12 %; %; n = 8 slices, 4 animals, 2 litters) and EtOH (LTD: -32.66 ± 6.86 %; n = 5 slices, 3 animals, 3 litters) condition ($p = 0.639$; Figure 34).

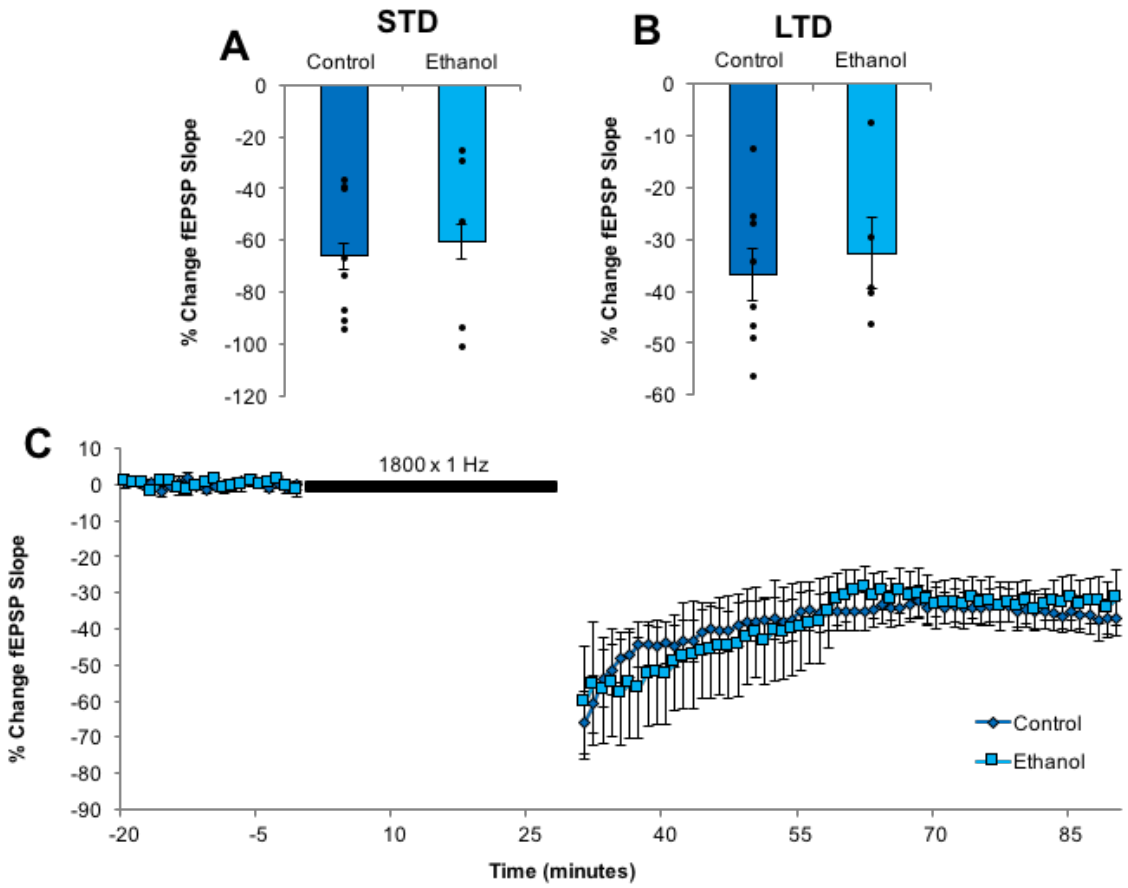

Figure 34. Prolonged Low Frequency Stimulation-Induced Long-Term Depression in Males Following PNEE

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{1800}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{1800}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 8 slices, 4 animals, 2 litters; N_{EtOH} = 5 slices, 3 animals, 3 litters.

3.4.5 Long-Term Depression Elicited by LFS_{1800} in Females

Despite the fact that there were no differences in LFS_{900}-induced LTD between the diet conditions in female offspring, we administered LFS_{1800} to determine whether PNEE impacts the maximal magnitude of DG LTD. It is conceivable that while the LTD induced by LFS_{900} was not different between prenatal diet conditions, perhaps the range

for LTD could be pushed to greater magnitudes in controls than in PNEE offspring. LFS_{1800} yielded STD that was not significantly different between the control (STD: -52.10 ± 5.28 %; n = 14 slices, 6 animals, 4 litters) and EtOH (STD: -60.73 ± 10.11 %; n = 7 slices, 5 animals, 3 litters) conditions in female offspring (p = 0.468; Figure 35).

Similarly, the magnitude of LTD induced by LFS_{1800} was not significantly different between the control (LTD: -36.64 ± 5.44 %; n = 14 slices, 6 animals, 4 litters) and EtOH-exposed (LTD: -28.83 ± 5.67 %; n = 7 slices, 5 animals, 3 litters) female offspring (p = 0.337; Figure 35).

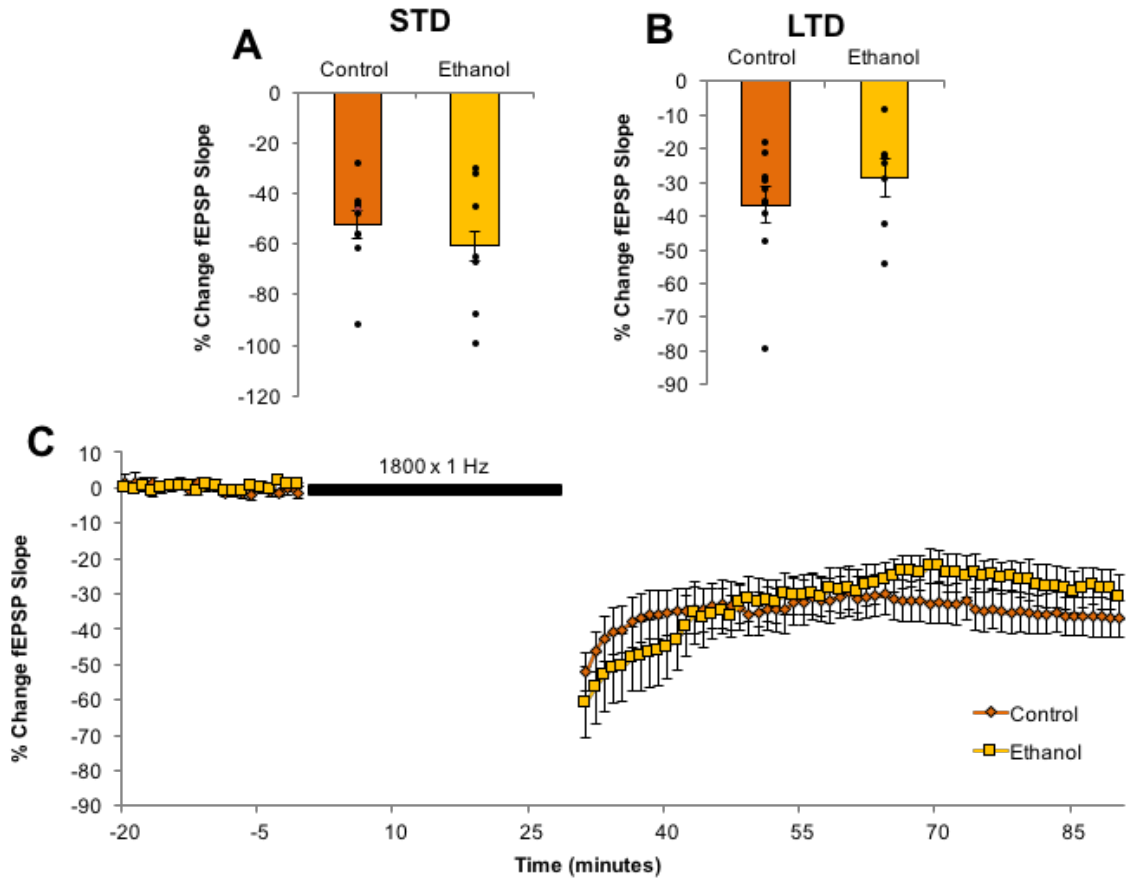

Figure 35. Prolonged Low Frequency Stimulation-Induced Long-Term Depression in Females Following PNEE

(**A**) Short term depression (STD) was measured as the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{1800}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative

to baseline for minutes 55-60 following delivery of the LFS_{1800}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 14 slices, 6 animals, 4 litters; N_{EtOH} = 7 slices, 5 animals, 3 litters.

3.5 Depotentiation

Depotentiation is distinct from the *de novo* forms of LTD discussed above and has not been investigated in the context of PNEE in the DG. In the CA1 region, PNEE resulted in enhanced magnitudes of depotentiation in males and reduced magnitudes of depotentiation in females at P36 (An et al., 2013; An and Zhang, 2013). Given that we observed impairments in bidirectional synaptic plasticity as a result of PNEE we sought to determine whether depotentiation was also affected by teratogen exposure. This form of bidirectional plasticity was induced by delivery of LFS_{900} following the establishment of 60 minutes of post-conditioning recordings of LTP caused by HFS. Depotentiation was measured as the difference in the average percentage of change in the fEPSP slope between minutes 55-60 and minutes 100-105 of the post-conditioning recording in order to control for the differences in the magnitude of LTP.

The magnitude of depotentiation was unchanged by PNEE in either males (Control: 56.39 ± 9.65 %; n = 6 slices, 4 animals, 2 litters ; EtOH: 48.04 ± 6.89 %; n = 8 slices, 6 animals, 3 litters; Figure 36; $p = 0.501$) or females (Control: 45.19 ± 11.94 %; n = 7 slices, 6 animals, 2 litters; EtOH: 37.68 ± 8.81 %; n = 9 slices, 8 animals, 2 litters; Figure 37; $p = 0.622$). Interestingly LFS_{900} yielded greater depression from a previously potentiated state than the same stimulus delivered from a baseline condition in males ($p = 0.0261$) but not in females ($p = 0.221$).

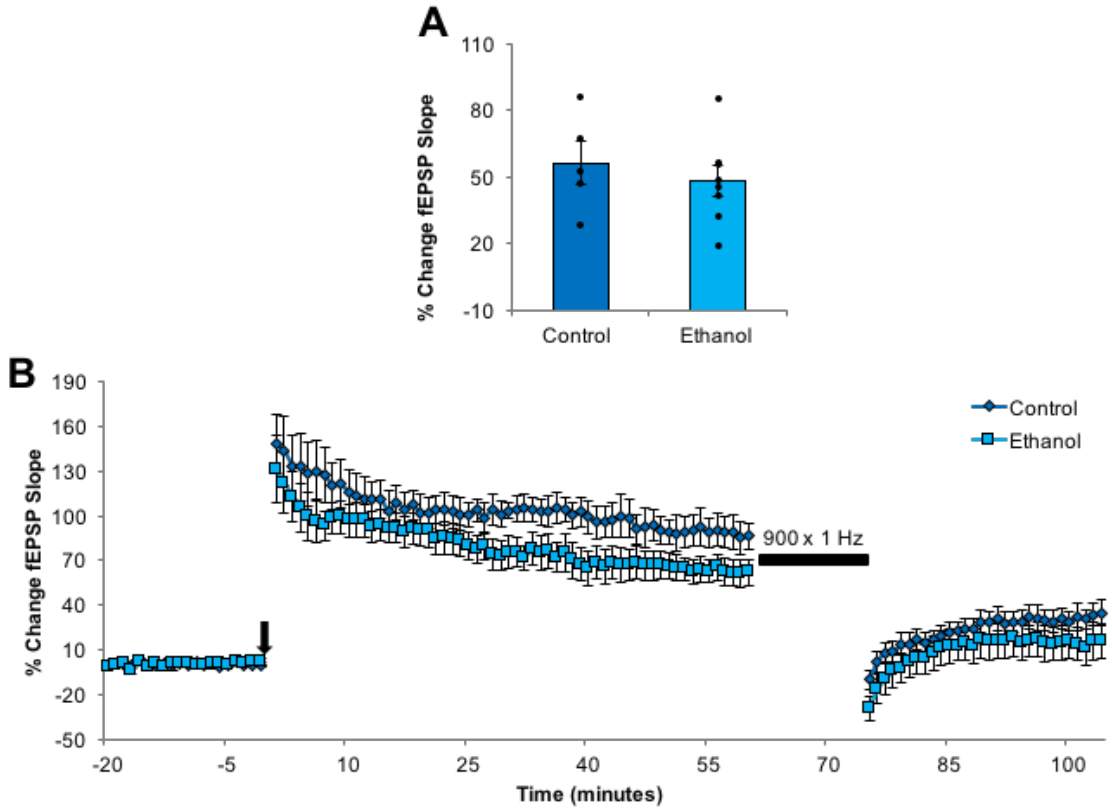

Figure 36. Depotentiation in Males Following PNEE

(**A**) Magnitude of depotentiation in control and ethanol-exposed male offspring. Bars represent the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope calculated as the average percentage change in the fEPSP slope of minutes 100-105 subtracted from that of minutes 55-60, defined as the magnitude of depotentiation. Points represent the average magnitude of depotentiation for each individual slice in this dataset. (**B**) Average depotentiation recordings from the beginning of baseline to the end of the post-conditioning recordings. The black arrow indicates the delivery of high-frequency stimulation and the black bar represents the low-frequency stimulation (900 x 1Hz). Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 5 slices, 4 animals, 2 litters; N_{EtOH} = 8 slices, 6 animals, 3 litters.

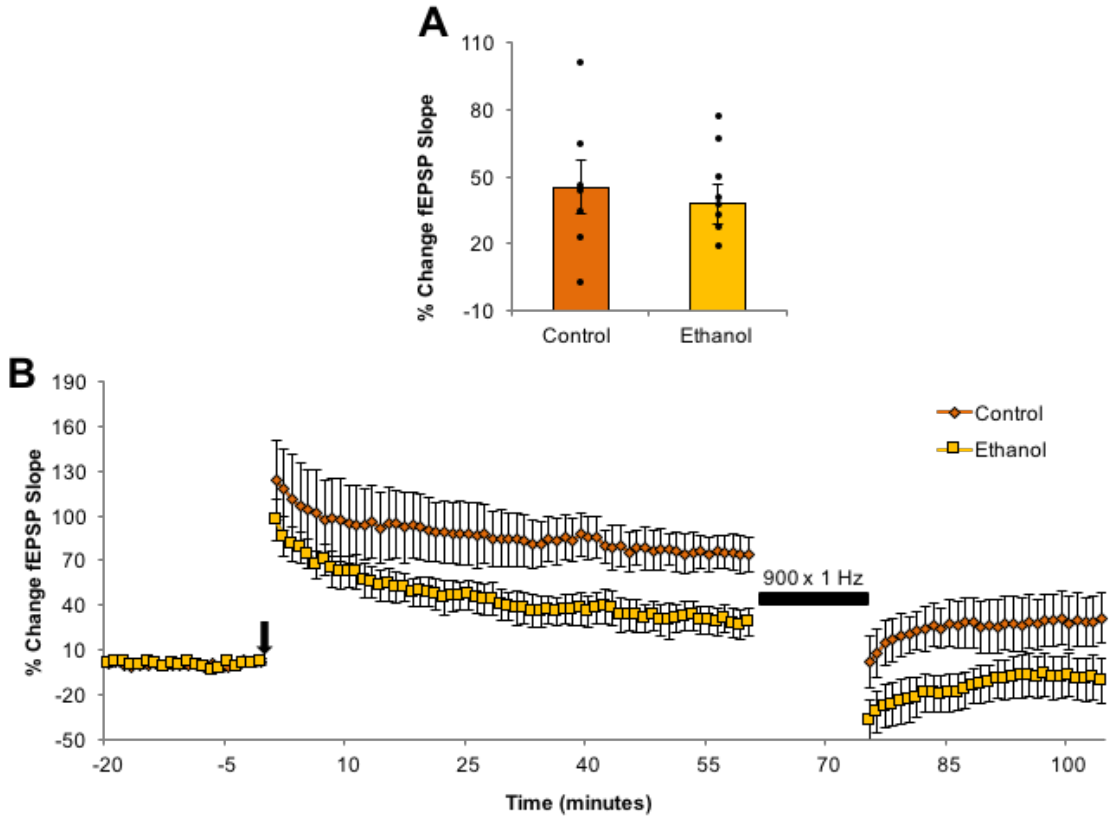

Figure 37. Depotentiation in Females Following PNEE

(**A**) Magnitude of depotentiation in control and ethanol-exposed female offspring. Bars represent the average percentage of change in the field excitatory postsynaptic potential (fEPSP) slope calculated as the average percentage change in the fEPSP slope of minutes 100-105 subtracted from that of minutes 55-60, defined as the magnitude of depotentiation. Points represent the average magnitude of depotentiation for each individual slice in this dataset. (**B**) Average depotentiation recordings from the beginning of baseline to the end of the post-conditioning recordings. The black arrow indicates the delivery of high-frequency stimulation and the black bar represents the low-frequency stimulation (900 x 1Hz). Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. $N_{control}$ = 7 slices, 6 animals, 2 litters; N_{EtOH} = 9 slices, 8 animals, 2 litters.

3.6 The Impact of PNEE on the Dynamic Range of Synaptic Plasticity

PNEE restricts the range of bidirectional plasticity in males and females, as described throughout this dissertation and summarized in Figure 38. A percentage of plasticity was calculated by comparing the magnitudes of LTP or LTD in ethanol-exposed males and females to the magnitudes of the plasticity in the respective controls, which were set to 100%. In males, there is a reduction to both LTP (52.02% of control) and LTD (39.65% of control; Figure 38 C) whereas in females there is a specific

reduction in the magnitude of LTP (58.56% of control) with no impact on LTD (98.02% of control; Figure 38 C).

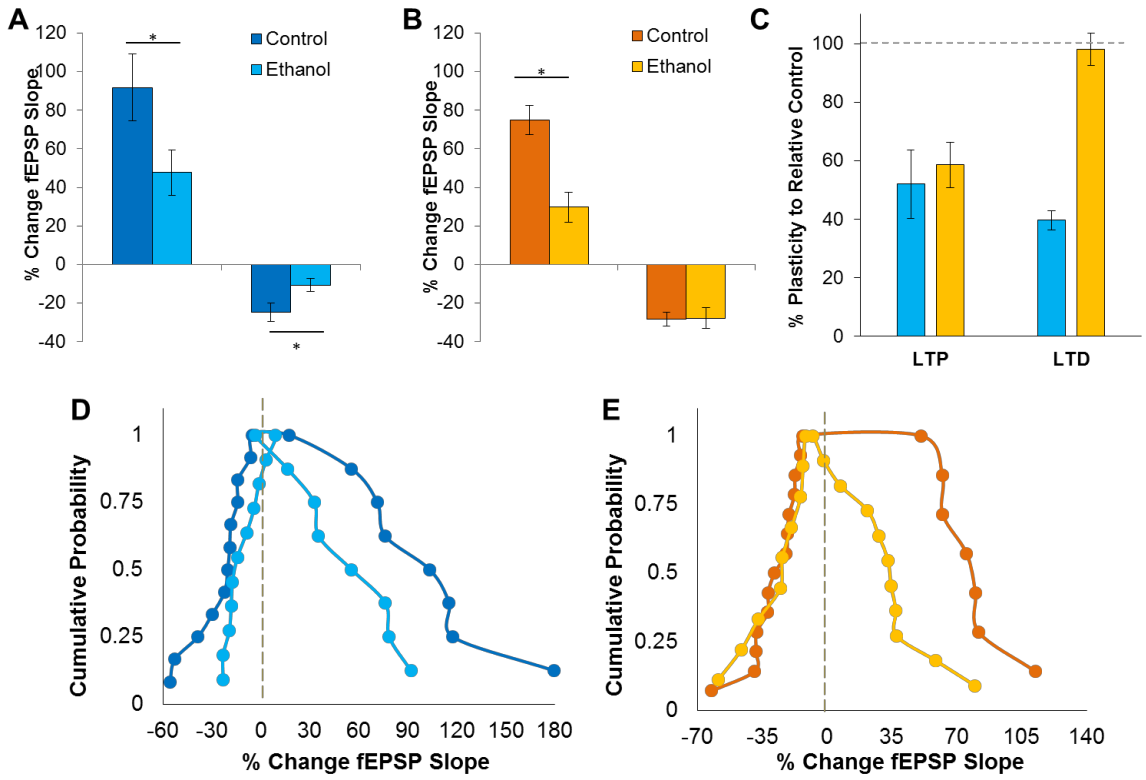

Figure 38. Summary of Impairments to Bidirectional Synaptic Plasticity Following PNEE in Males and Females

(**A**) The average magnitudes of long-term potentiation (LTP; left) and long-term depression (LTD; right) in control and ethanol-exposed male offspring. (**B**) The average magnitudes of LTP (left) and LTD (right) in control and ethanol-exposed female offspring. (**C**) The percentage of plasticity for each ethanol group was compared to the relative control in each condition and used to generate a percentage of the magnitudes of each type of plasticity in control males or females (bars). Light blue bars represent ethanol-exposed males and the yellow bars represent the ethanol-exposed females. The grey dashed line represents 100%, or the control level of plasticity in each group. (D) Cumulative probabilities for bidirectional synaptic plasticity in control (dark blue) and ethanol-exposed (light blue) males. The dashed grey line represents 0% change in the fEPSP slope. (E) Cumulative probabilities for bidirectional synaptic plasticity in control (dark orange) and ethanol-exposed (yellow) females. The dashed grey line represents 0% change in the fEPSP slope. Error bars represent the SEM. * $p < 0.05$.

Increasing the duration of the CS (either HFS or LFS) achieved control levels of LTP and LTD in ethanol-exposed offspring of both sexes indicating that the impairments to bidirectional plasticity may be due to changes in the thresholds of stimulation

necessary to induce synaptic plasticity. Despite the significant reductions in the magnitudes of LTP in both sexes and LTD in males, these impairments can be overcome with further stimulation and highlight the need for therapeutic agents that can facilitate the induction of normal bidirectional plasticity.

4.0 DISCUSSION

4.1 Summary of Major Findings

This study is the first of its kind to examine the dynamic range of synaptic plasticity in the juvenile DG of both sexes following PNEE. First, our initial I/O experiments indicated that in males, but not in females, PNEE significantly reduced the fEPSP slope to greater pulse widths of stimulation, which was normalized by $GABA_A$ inhibition (Figure 17). This suggests that there may be a male-specific reduction in excitability of the DG to MPP stimulation. We found that unlike our previous findings *in vivo* the magnitude of NMDAR-dependent LTP was attenuated in both sexes by PNEE (Figures 18 and 19). Furthermore, we revealed for the first time an impairment by PNEE to $mGluR_5$ and CB1 receptor-dependent LTD in juvenile males but not in females in the DG (Figures 24, 25 and 33). This LTD required coincident activity of $mGluR_5$s and CB1 receptors and occurred independently of NMDARs, LTCCs and possibly phosphorylation of Tyr residues on the GluA2 subunits of the AMPAR. Despite the PNEE-induced differences in *de novo* LTD (from a baseline condition) and in the magnitude of LTP in males we found no effect of PNEE on depotentation (Figure 36). Similarly, depotentation was unaffected by PNEE in females (Figure 37) where only the magnitude of LTP was reduced. The impairments in LTP and LTD can, however, be overcome by the delivery of a second HFS (Figure 22, 23) or prolonging of the LFS (Figure 34, 35) indicating that the impairments caused by PNEE may change the threshold of stimulation necessary to induce this plasticity rather than causing long-term irreparable damage to plasticity machinery.

4.2 The Effect of PNEE on Input-Output Curves

In the present dissertation we found a male-specific impairment in the slope of the fEPSP to increasing pulse widths of stimulation under normal aCSF conditions prior to our LTD recordings (Figure 17). This effect was not observed in female offspring when recorded under the same conditions. Interestingly, in our LTP recordings, where $GABA_A$ is inhibited by BIC we found no effect of PNEE in either sex on the recorded I/O

relationship. In our previous studies of DG plasticity in both sexes we did not observe such differences in the I/O relationship, which may be because these recordings were all of LTP, which in slices, would be examined during $GABA_A$ inhibition rather than under LTD recording conditions where normal inhibitory control is not manipulated. The method by which we analyse potential differences in the I/O curve typically involved normalizing the data to some central point, to account for potential differences in the responses between slices. When we applied the same normalization to the data in the present dissertation, it masked the reduced fEPSP slopes represented in Figure 17. Alternatively, it is possible that the impact on the I/O curve observed in this dissertation could also be a function of animal age. Following ethanol gavage (6g/kg/day) from GD8-20 there is a report of a similar reduction in the I/O relationship in young (P25-32) male PNEE rat offspring (Krahl et al., 1999) in the CA1 region. In this study, there was no significant effect of PNEE at a lower ethanol dose (4g/kg/day), nor in adult (P63-77) animals. Curiously, the altered I/O relationship was not accompanied by a LTP deficit in the slices generated from young male offspring. A similar result was obtained in the CA1 region of adult male guinea pig offspring after PNEE (gavage ethanol at 4g/kg/day from GD2-67) where increasing stimulus intensities resulted in reductions in the resulting fEPSP amplitude *in vivo* (Richardson et al., 2002). In this study however PNEE prevented the induction of CA1 LTP. The effect in males in normal aCSF has significant implications for the rest of this body of work because it suggests that DG slices from male PNEE offspring may be less responsive, or less excitable to increasing stimulus durations.

4.3 Male & Female LTP Impaired by PNEE

In the present dissertation we found that PNEE reduced the magnitude of DG LTP in both sexes during the juvenile period. These results are in line with previous data from the Christie laboratory *in vitro* (Helfer et al., 2012) but not *in vivo* (Patten et al., 2013a) at adulthood.

Interestingly, using the same model of PNEE a previous study from our laboratory found that the magnitude of DG LTP was enhanced by ethanol in adolescent females (P 30-35) induced by Theta Burst Stimulation (TBS) *in vivo* (Titterness and Christie, 2012). Although this enhancement in LTP in the females may be due to the fact that sex differences were found in the control group, with females exhibiting less LTP than males. These inherent sex differences in LTP in young rats have also been found in other studies (Maren, 1995; Maren et al., 1994). One study also reported that the reduced LTP in control females was paralleled by reduced NMDAR activation compared to that observed in males (Maren, 1995). Methodological differences likely play some role in explaining why these sex differences were not apparent in the present dissertation such as the age ranges used, the strain of rat, the CS used and the fact that these recordings from Maren and colleagues and those of Titterness and Christie were all made *in vivo*. Indeed previous findings from our laboratory have not found significant sex differences between controls *in vitro* (Helfer et al., 2012) although these recordings were conducted in slices from adult offspring. Direct comparisons between results obtained using these two electrophysiological techniques (*in vivo* & *in vitro*) can be challenging due to the inherent differences in recording from hippocampal slices where afferent and efferent connections are severed and the temperature of the brain is manipulated versus the intact brain in the whole animal. In contrast with the present study, two injections of 2.9 g/kg ethanol on GD 8 resulted in a decrease in the threshold for DG LTP in P45 male offspring (Gómez et al., 1992). The use of various methods of PNEE, timing and dosages of ethanol, ages of examination of offspring, hippocampal subregions and stimulus protocols used lead to variability between studies as we have recently reviewed (Fontaine et al., 2016).

4.3.1 Damage to the NMDAR by Prenatal Ethanol

The effect of PNEE on NMDAR structure and function has been repeatedly investigated by various research groups (see (Costa et al., 2000) for review). The present study found an impairment in NMDAR-dependent LTP in both males and females as a result of PNEE, similar to what has been described elsewhere. Perhaps surprisingly, there

are few reports of changes to the NMDAR structure in the hippocampus by PNEE during the juvenile period of interest in the present dissertation.

The effects of PNEE on NMDAR structure have generally appeared to be transient in the hippocampus during the early postnatal period, and studies have sought to identify changes to specific subunits of the receptor. For example there are reports of decreased amounts of the GluN2B subunit at P1 (Hughes et al., 1998), reduced GluN2D at P7 (Naassila and Daoust, 2002) and increases in GluN2A at P10 (Nixon et al., 2004) in the hippocampi of PNEE offspring that return to control levels by P21. A recent study isolated synaptic membrane fractions in an effort to associate the structure of NMDARs at the synapse with synaptic efficacy and found that PNEE offspring at adulthood had increased amounts of the obligatory GluN1 subunit and of GluN3A coupled with decreased GluN2B and these changes were associated with reduced magnitudes of LTP in the DG (Brady et al., 2013). It is possible that using this tissue sampling technique may provide more specific resolution to examine receptors that are likely to be involved in synaptic function.

The more consistent effects of PNEE on the NMDAR in the literature are related rather to NMDAR function. Across these studies NMDAR function is decreased as a result of PNEE in the hippocampus by examining the effect of NMDA-sensitive ^{3}H-glutamate binding (Savage et al., 1991, 1992) or NMDA-dependent Ca^{2+} influx (Lee et al., 1994; Spuhler-Phillips et al., 1997; Weaver et al., 1993). Likely related to the observed decreases in Ca^{2+} flux by PNEE through the NMDAR, there is also evidence of reduced activation of the second messenger signalling cascades initiated by acute wash of NMDA onto hippocampal slices. While there was no observed impact of PNEE on PKA or PKC-dependent ERK activation, there was a significant decrease in ERK2 activation in slices from adult PNEE offspring (Samudio-Ruiz et al., 2010).

4.4 Sex-Specific Impairment in Threshold for LTD

Hippocampal LTD in general is understudied and perhaps more so in the DG and especially following PNEE. Using a common LFS paradigm (LFS_{900}; 900 x 1Hz) as was

used in the seminal study of LTD (Dudek and Bear, 1993) we uncovered a sex-specific reduction in the magnitude of LTD in males that was overcome when a greater LFS was used (LFS_{1800}; 1800 x 1Hz). These data indicate that PNEE may in fact increase the threshold for LTD in males only and that exposure to this teratogen does not lead to damage to the essential machinery underlying this form of plasticity, and may instead impair some aspects of receptor function despite a lack of difference in presynaptic NT release (Figure 17). In males following PNEE, it is possible that LFS_{900} is insufficient in recruiting receptors and subsequent signalling cascades that underlie the LTD observed in control males and in both diet groups in females. This LTD is dependent on coincident activity of $mGluR_5$s and CB1 receptors, where at least one component of this signalling cascade is affected in males by PNEE and are discussed below.

4.4.1 NMDAR-Independent LTD in the DG

There are many forms of LTD that have been induced using a variety of electrical and pharmacological LFS in the hippocampus. In the present dissertation we found that the LTD elicited by LFS_{900} in the juvenile DG was dependent on the synergistic activity of $mGluR_5$s and CB1Rs, but not on activity of NMDARs, LTCCs or $mGluR_5$s alone. We also found that the use of a peptide that blocks phosphorylation of tyrosine residues on the GluA2 subunit of the AMPAR, which blocks endocytosis of the AMPAR through this mechanism, had no effect on this LTD. This result in particular is surprising however it could mean one of two things, that our LTD does not involve AMPAR endocytosis through this mechanism though perhaps endocytosis could be triggered through some other pathway or that we did not have sufficient penetration of the peptide into the postsynaptic cell. To our knowledge this study is the first to describe LFS_{900}-induced LTD in the DG of young rats that is dependent on both CB1 receptors and $mGluR_5$s.

Independence of LTD from NMDARs is supported by other studies in the DG *in vitro* (Trommer et al., 1996; Wang et al., 1997). The characterization of LTD in the juvenile DG that perhaps related most to the present work is that of Trommer, Liu & Pasternak who found an age dependent effect on the magnitude of LTD elicited by LFS_{900} and found that this LTD was not affected by pharmacological blockade of

NMDARs, group 1 mGluRs, LTCC nor by chelation of postsynaptic calcium by BAPTA (Trommer et al., 1996). This LTD was reversible and was saturable by the delivery of three LFS_{900} and was found to be dependent on a change in the voltage in the postsynaptic cell, although the mechanism behind the necessary postsynaptic depolarization in unknown. Similarly, in older rats (weighing 80-120g) LFS_{900} yielded LTD that was NMDAR-independent but dependent on a rise in postsynaptic calcium levels from both extra- and intracellular sources (Wang et al., 1997). An NMDAR-independent form of LTD has been observed in the DG *in vivo*, which also existed independent of LTCCs as in the present dissertation and this persistent LTD was also maintained independent of protein synthesis (Pöschel and Manahan-Vaughan, 2007). This LTD also persisted *in vivo* in the DG for over 24 hours. As previously discussed, eCB-LTD (LFS: 10Hz for 10min) is also not dependent on LTCCs nor on NMDARs but rather requires activation of either CB1Rs, $mGluR_1$ or $mGluR_5$ to support synaptic depression in the DG (Pedro Grandes, Manuscript submitted).

In addition, there is well-documented evidence for the involvement of postsynaptic calcium in the induction of LTD in the hippocampus. *In vitro* studies have confirmed that this LTD requires activation of LTCCs and release of calcium from internal stores in other to initiate the second-messenger signalling cascades to support LTD (Christie et al., 1996, 1997; Cummings et al., 1996; Reyes and Stanton, 1996).

4.4.2. Involvement of eCBs in DG LTD

The identification of cannabinoids and their endogenous receptors were discovered relatively recently and as such, the understanding of the function of this system is still in its infancy. In fact, the active component of cannabis compounds were only first determined in the mid-1960s which subsequently led to the formal identification of the CB1 receptor, its primary receptor expressed throughout the brain, including the hippocampus (Iverson, 2005; Matsuda et al., 1993; Tsou et al., 1998, 1999). It was not until the 1990s that the endogenous cannabinoids, anandamide and 2-arachidonoylglycerol (2-AG) were first identified (Devane et al., 1992; Mechoulam et al., 1995). Unlike classic NTs, eCBs are not stored but rather are synthesized on demand and

in neurons they are synthesized and released in response to depolarization and Ca^{2+} influx (Di Marzo et al., 1998). CB1 receptors in the hippocampus are localized to GABAergic terminals, astrocytes and to a lesser extent glutamatergic neurons (Gutiérrez-Rodríguez et al., 2018; Tsou et al., 1999).

In the hippocampus, eCBs have been associated with forms of synaptic plasticity. Specifically, eCBs can be synthesized in response to activation of group I mGluRs and subsequently suppress NT release from the presynaptic terminal by acting as retrograde signals (Varma et al., 2001). Depolarization-induced suppression of inhibition in CA1, a form of retrograde plasticity where depolarization of glutamatergic neurons leads to suppression of GABA release from interneurons, is dependent on the activity of both $mGluR_5$s and CB1 receptors, although synthesis of eCBs may occur via either mGluR activation or postsynaptic depolarization (Varma et al., 2001). DHPG (group 1 mGluR agonist)-induced LTD in the CA1 is associated with a decrease in presynaptic NT release and the STD but not LTD, was dependent on CB1 receptor function (Rouach and Nicoll, 2003). These data suggest that while DG LTD may be triggered by postsynaptic activity, its locus may in fact be presynaptic, as is classically associated with CB1 receptor involvement. The putative mechanism by which $mGluR_5$-induced eCB synthesis and subsequent activity could affect the DG LTD measured in this dissertation is illustrated in Figure 39, as follows. mGluR activation leads to the formation of DAG and IP_3 (due to activation of the G_q-coupled receptor). DAG is then hydrolyzed by DAG lipase-α into 2-AG, which diffuses into the synaptic cleft and binds to presynaptic CB1 receptors. CB_1 activation leads to inhibition of adenylate cyclase and therefore inhibition of the accumulation of cAMP through coupling of the receptor to $G_{i/o}$ proteins. Thus, when located at presynaptic sites, CB1 receptor activation can attenuate NT release most likely through inhibition of VGCCs or possibly through another mechanism involved in vesicle fusion and NT release (Chevaleyre et al., 2006; Doherty and Dingledine, 2003; Katona and Freund, 2012; Mato et al., 2008; Wilson et al., 2001; Wilson and Nicoll, 2002). Classically this mGluR-mediated signaling results in suppression of presynaptic NT release (whether acting at GABAergic or glutamatergic terminals) (Freund et al., 2003; McAllister and Glass, 2002) and subsequently reduced excitatory postsynaptic currents

(Maejima et al., 2001). There is an example of postsynaptically-mediated CB1 receptor-dependent synaptic plasticity in the cerebellum (Safo and Regehr, 2005). Inhibitory interneurons in the hippocampus express significantly greater proportions of CB1 receptors than do glutamatergic neurons, although there is clear expression of CB1 receptors on presynaptic terminals in DG (Katona et al., 2006). It is also possible that this eCB-LTD in the DG also involves activation of these receptors and involvement of the inhibitory network in the DG in this form of synaptic plasticity.

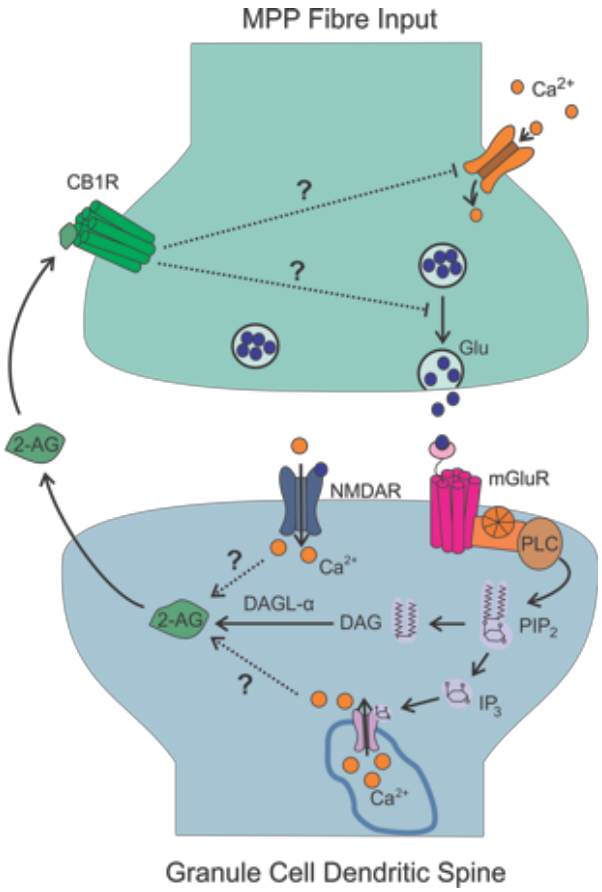

Figure 39. Putative mechanism for long-term depression in the juvenile DG

Low-frequency stimulation of medial perforant path (MPP) fibres leads to opening of voltage-gated calcium (Ca^{2+}) channels (orange) and subsequently to vesicle binding and glutamate (Glu) release (dark blue circles) which binds to postsynaptic glutamate receptors located on DG granule cells such as NMDARs and mGluRs as well as AMPARs (not pictured). $mGluR_5$ activation results in G_q second messenger signalling, involving activation of PLC and generation of IP_3 and DAG from cleavage of PIP_2. While IP_3 generation leads to Ca^{2+} release from intracellular stores, DAG is hydrolyzed by DAGL-α into the endocannabinoid 2-AG. This endocannabinoid diffuses across cell membranes into the synaptic cleft where it binds to CB1 receptors on the presynaptic

terminal. These CB1 receptors are coupled to $G_{i/o}$ proteins, whose activation reduces NT release possibly by inhibiting voltage-gated calcium channels or by inhibiting some of the steps involved in vesicle fusion and NT release.

Based on the experimental data presented in this dissertation we can begin to unravel the mechanism for LTD in the juvenile DG. While this LTD is not occluded by blockade of NMDARs, $mGluR_5$s nor LTCCs independently or together, LTD was impaired by blockade of CB1 receptors and completely blocked by combined blockade of $mGluR_5$s and CB1 receptors. The synthesis of 2-AG is accomplished primarily by activation of $mGluR_5$s (Jung et al., 2005; Lafourcade et al., 2007; Maccarrone et al., 2008; Uchigashima et al., 2007) although $mGluR_1$ activation can also lead to the synthesis of this eCB (Maejima et al., 2001; Melis et al., 2004). Curiously, independent inhibition of $mGluR_5$s alone by MPEP had no effect on the magnitude of LTD whereas independent inhibition of CB1 receptors by AM251 caused a significant reduction in the magnitude of STD and LTD. These data clearly indicate an integral role for eCBs in this LTD but begs the question as to what initiates eCB synthesis and its associated signalling cascade if not the $mGluR_5$s. As described above, 2-AG synthesis can also be initiated by rises in intracellular Ca^{2+}, which could be the result of various receptor functions including $mGluR_1$s, NMDARs or GluA2-lacking AMPARs and could potentially compensate for the blockade of $mGluR_5$s in the MPEP alone experiments to support eCB-LTD. What remains unknown is whether the locus for this LTD is indeed presynaptic, as is classically associated with CB1 receptor activation, in addition to the potential presynaptic mechanism by which CB1 receptor activation results in reduced fEPSP slopes.

Furthermore, the sex-specific mechanism by which PNEE impairs DG LTD in males but not in females is unknown although there is emerging evidence of sex differences in hippocampal eCB activation. There is a relationship between estradiol, $mGluR_1$s and eCB activity where acute estradiol causes suppression of inhibitory currents in the CA1 region of females but not of males and there is evidence of an estradiol-independent effect in females where blocking the breakdown of the eCB anandamide

significantly suppressed inhibitory transmission, an effect that was not observed in males (Huang and Woolley, 2012; Tabatadze et al., 2015). In the hypothalamus the gonadal status of animals can also affect the density of eCB receptors being expressed at a given time (de Fonseca et al., 1994). Together these data support possible sex differences in CB1 receptor activation, which may be involved in the sex differences measured in this dissertation as well as highlighting the need for further study of both sexes in hippocampal eCB-dependent plasticity.

4.4.3. Damage to the eCB System by Ethanol

Emerging evidence has demonstrated a clear relationship between ethanol and the function of the eCB system which has led to increased study of this system in the context of FASD (Basavarajappa, 2015). This system is thought to play a role in many brain functions but of particular interest to the present work is the relationship between eCBs, ethanol and learning and memory. This system blocks ethanol-induced potentiation of GABA release and suppresses ethanol-induced glutamatergic transmission (Basavarajappa et al., 2008; Talani and Lovinger, 2015). Ethanol administration causes long-term changes to the eCB signalling system, decreasing CB1R mRNA expression, as well as receptor densities, functionality and can lead to increases in the levels of eCBs themselves (Basavarajappa et al., 1998; Mitrirattanakul et al., 2007; Ortiz et al., 2004). There are also reports of reduced expression of $mGluR_5$s at adulthood as a result of PNEE in the DG, which could account for the impairments in LTD observed in the present dissertation (Galindo et al., 2004). There is a report of PNEE-induced (gavage ethanol at either 3 or 6 g/kg/day from GD 8-20) impairments in excitatory eCB-LTD in dopaminergic VTA neurons at 4-10 weeks of age in male offspring, which was accompanied by a PNEE-induced downregulation of presynaptic CB1 receptors (Hausknecht et al., 2017). This recent study provides additional rationale that PNEE can lead to long-term dysregulation of the eCB system in the brain.

Other possible hints to eCB dysfunction as a result of PNEE is the fact that this system is thought to play a role in alcoholism and addiction (Colombo et al., 2005; Hungund et al., 2002; Maldonado et al., 2006; Parsons and Hurd, 2015). Humans with

FASD are reported to show a greater attraction to the smell and taste of alcohol (Middleton et al., 2009; Youngentob and Glendinning, 2009), and there are reports that these individuals are at greater risk for becoming alcoholics (Streissguth et al., 1996; Yates et al., 1998) and to potentially become parents to children with FASD. Clearly there is a link between the eCB system and ethanol consumption which may play a role in the consequences of alcoholism, withdrawal and in the case of the present study, in FASD. This exciting new evidence of the interplay between ethanol and eCBs warrants further investigation and also raises the question as to the effects of simultaneous exposure of these two teratogens to the developing fetus. This latter point is of paramount importance for the field of teratogenesis moving forward with the relaxation of laws surrounding cannabis use.

4.4.4 Depotentiation unaffected by PNEE

In the present work there is no evidence of an effect of PNEE on the magnitude of depotentiation, or the reversal of LTP, which may be related to behavioural and cognitive flexibility. These findings are in contrast with those of An and colleagues who found sex differences in the balance between LTP and depotentiation (An et al., 2013; An and Zhang, 2013). Given the apparent lack of an effect of PNEE on the magnitude of depotentiation, it is possible that the mechanism underlying this apparent reversal of LTP are different from those underlying de novo LTD. If these mechanisms overlapped, one might expect to see a similar impairment in male offspring in depotentiation as was observed in *de novo* LTD. In the CA1 region depotentiation has been associated with the activity of mGluRs but not dependent on NMDARs, LTCCs or the retrograde signal NO (Bashir and Collingridge, 1994). Future pharmacological experiments should determine the receptors involved in depotentiation, starting with confirmation that this bidirectional form of plasticity does not involve CB1 receptors or $mGluR_5$s nor NMDARs as dysfunction of these receptor signalling pathways may underlie the LTD and LTP deficits caused by PNEE respectively as demonstrated in this dissertation.

4.5 Proposed Mechanism for Bidirectional Synaptic Plasticity Deficits Following PNEE

In an effort to synthesize and explain the results obtained in this dissertation we propose that PNEE could result in a male-specific imbalance in excitation/inhibition in the DG that results in a net decrease in excitability (as shown in the I/O curves) in these slices, resulting in potentially less glutamate release at the MPP-granule cell synapse. Reduced glutamate release during the LFS_{900} would likely then result in reduced $mGluR_5$ activation, and less intracellular Ca^{2+} which would mean reduced 2-AG synthesis and reduced presynaptic CB1 receptor activation. This effect of reduced excitation can be overcome by extending the LFS (to 1800x), which could be reaching a ceiling for postsynaptic $mGluR_5$ activation, resulting in no effect of PNEE on the magnitude of LTD achieved as compared to control. Coupled with a potential excitation/inhibition imbalance, our LTP data (where the influence of the E-I imbalance may be negated by $GABA_A$ inhibition) suggests a NMDAR dysfunction, although this impairment does not appear to be sex-specific as NMDAR-LTP is blocked in both sexes by PNEE. The combination of these two factors could offer some explanation for the significant impairments in DG LTP *in vivo* that were not seen in females, as in this preparation inhibition is not blocked as it is *in vitro* for LTP recordings. Should the LTD measured in this dissertation have been NMDAR-dependent we may have observed impaired LTD in both sexes which we would expect to have been exacerbated in males as compared to females, if there is indeed E-I imbalance as is suggested by the I/O data. The concept of E-I dysfunction is not novel in the field of FASD (see (Sadrian et al., 2013) for review) although the current understanding of E-I imbalance associated with FASD suggests that PNEE leads to hyperexcitability possibly due to reduced inhibition caused by impairments in interneuron proliferation by ethanol. It is possible that our proposed imbalance may be specific to the DG, although this warrants further investigation.

4.6 Why does PNEE Result in Sex Differences in DG Synaptic Plasticity?

Theoretically developing male and female offspring have an equal likelihood to be exposed to ethanol in the gestating female rat, which raises the question as to why we observe different effects of PNEE on bidirectional synaptic plasticity between the sexes as juveniles. As described in the introduction of this dissertation, there are differences between the sexes in circulating sex hormones during early development and sex

hormones can be synthesized in a variety of brain regions, including the hippocampus (see (McCarthy, 2008, 2009) for review). It is possible that the aromatization of testosterone to estradiol involved in masculinizing the male brain may play a role in vulnerability to EtOH-exposure during this time. In immature neurons in the developing brain GABA plays a dramatically different role than it does in mature neurons of the adult brain. Due to high chloride levels caused by greater amounts of NKCC1, the transporter that moved chloride into these cells, than KCC2, the transporter that removes chloride from the cell, the chemical gradient drives chloride ions outwards, leading to depolarization of the membrane and opening of voltage-gated receptors such as those for calcium, when GABA receptors are activated (Rivera et al., 1999). Estradiol has long been thought to have neuroprotective actions, due to at least in part its ability to dampen potential glutamate-mediated excitotoxicity, potentially by downregulating mGluRs and reducing calcium release from the endoplasmic reticulum in hippocampal neurons (Hilton et al., 2006), although this idea is still an area of intense study. Considering that activation of GABA receptors can lead to membrane depolarization and influx of calcium, it is possible that over-activation of these receptors during development also has the capacity to cause damage to the brain. Estradiol in fact enhances these GABA responses by increasing calcium flux through voltage-gated LTCCs, which is important in normal neural development for cell differentiation and synaptic integration (Ganguly et al., 2001). In the context of this dissertation, EtOH is a positive allosteric modulator of the GABA receptor (specifically of $GABA_A$), and is delivered throughout the gestation period. This raises the question as to whether it is possible that to coincidence of the actions of estradiol and EtOH on GABA receptors in the male brain in particular could contribute to the apparent vulnerability of this sex to impairments in bidirectional synaptic plasticity in the DG and possibly reduced excitability of this area as suggested by the work contained in this dissertation. Direct agonism of $GABA_A$ by muscimol combined with estradiol has been shown to lead to increased hippocampal cell death, an effect which could be prevented by blockade of LTCCs (Nuñez et al., 2003; Nunez and McCarthy, 2003). The effects of PNEE on hippocampal cell counts are subtle as compared to the described widespread damage in the aforementioned study, although this could be due to the fact that musicmol is itself an agonist of GABA receptors, whereas

EtOH is a positive allosteric modulator and alone is not capable of activating the receptor.

4.7 The Problem With Pair-Feeding

The purpose of a pair-fed control group when using a liquid diet model of PNEE is to control for any potential confounds in administering a liquid diet. In this model of PNEE pair-fed dams are weight-matched to an ethanol dam and the amount of diet provided to the pair-fed dam is yoked to the amount consumed by the ethanol partner dam. An ethanol dam is provided *ad libitum* access to an ethanol-containing diet which can be consumed throughout the dark and following light cycle until it is replenished. All experimental diets are replenished daily and provided within 2 hours of the beginning of the dark cycle. In the present study ethanol dams consume on average 66.25g of diet per day. In the case of the pair-fed dams they are provided with specific amounts of diet according to the consumption volume of their ethanol partner dam and this is typically consumed within a few hours of replenishment then the dam is effectively food deprived until replenishment the following day (Gallo and Weinberg, 1981). While this strategy may accurately account for the volume of diet consumed it does not control for differences in the pattern of consumption. This altered and restricted pattern of diet consumption likely lead to some form of prenatal stress which affects hippocampal processing. Furthermore this diet does not account for ethanol-induced impairments in intestinal micro- and macronutrient absorption, damage to intestinal mucosal epithelium and inflammation of the gut (Bode and Bode, 2003; Bujanda, 2000; Weinberg, 1984). As such, pair-feeding can be considered a separate experimental group rather than an appropriate control to be used to evaluate the effect of PNEE on hippocampal synaptic plasticity. This group was not included in the principal experiments outlined in this dissertation but basic LTP and LTD comparisons in both sexes are made to the solid chow control group in Appendix B.

4.8 Putative Therapies for FASD

A number of therapies have been proposed and demonstrated to restore a number of functions across the various neurobehavioural domains that are commonly associated with FASD (Bertrand, 2009). While specific pharmacological treatments have been administered in conjunction with ethanol during gestation it is likely that the most effective and most likely to be used treatments in humans may be administered postnatally, long after the exposure has ceased.

Ethanol is known to increase ROS in the cell and has been shown to cause increased oxidative stress in offspring following PNEE (Guerri et al., 1994; Montoliu et al., 1995, 2002). As such, much work has investigated the potential use of a variety of antioxidant dietary supplements or pharmacological treatments to attenuate some of the various effects of teratogen exposure on brain structure and function. Treatment with other antioxidants such as vitamin E (Heaton et al., 2000, 2002; Mitchell et al., 1999; Siler-Marsiglio et al., 2005), and astaxanthin (Zheng et al., 2014) among many other candidate compounds. Previous findings from our laboratory have supported the use of omega-3 fatty acid diet supplementation to ameliorate hippocampal synaptic plasticity (Patten et al., 2013b). Omega-3 fatty acids have beneficial effects on the brain in general as a result of their anti-apoptotic , anti-inflammatory and antioxidant properties (Dyall et al., 2007; Dyall and Michael-Titus, 2008; Gómez-Pinilla, 2008; Mori and Beilin, 2004). Specifically, omega-3 supplementation in the diets of male PNEE offspring restored normal DG LTP *in vivo* at adulthood.

The ethanol-induced deficiency in zinc in the developing brain has been considered a putative mechanism by which this teratogen damages the CNS (Keen et al., 2010) and as such dietary supplementation with Zinc during gestation has been used to overcome PNEE-induced spatial learning and memory deficits (Summers et al., 2008). Other substances such as vitamin A and folate have also been proposed as dietary therapies to counteract the adverse effects of PNEE (Ballard et al., 2012; Ojeda et al., 2009).

Choline is an emerging therapeutic agent that has been used in both animal models of PNEE and in human cases of FASD and shows great promise in mitigating the structural and functional damage to the brain. This essential nutrient counteracts some of the damage caused to basic outcomes of offspring including the common low birth weights and developmental delays in some physical measures and behavioral functions when delivered either prenatally or postnatally (Monk et al., 2012; Ryan et al., 2008; Thomas et al., 2009, 2010). There have been a small number of clinical trials conducted in human children and these results have indicated that choline treatment for young children is feasible and tolerable (Wozniak et al., 2013) and may have potential usefulness in improving cognition (Kable et al., 2015; Wozniak et al., 2015). In the latter Wozniak study, there appeared to be an age-dependent effect of choline on memory, with greatest benefits occurring in younger children.

Finally, behavioural interventions such as exercise and environmental enrichment have regularly been used to counteract the impact of ethanol on the developing offspring. Previous evidence from our laboratory has demonstrated the efficacy of exercise in overcoming PNEE-induced deficits in hippocampal LTP, spatial memory and neurogenesis (Boehme et al., 2011b; Brocardo et al., 2012; Christie et al., 2005).

4.9 Limitations

While it was surprising that the Tat-GluA2$_{3Y}$ was unable to block DG LTD, it is possible that this exposure may have resulted in insufficient penetration of the peptide into the postsynaptic cells to effectively block phosphorylation on the tyrosine residues of the GluA2 subunit. Furthermore, it is also possible that if AMPAR endocytosis is indeed involved in the expression of this form of LTD that perhaps it does not require phosphorylation at these sites on the GluA2. In order to confirm a true lack of dependence of this LTD on phosphorylation of the tyrosine residue(s) on the GluA2 subunit of the AMPAR, a further set of experiments must be performed using a perforated whole cell patch clamp technique where the peptide can be delivered directly into the granule cell during LFS$_{900}$ of MPP fibres.

In the field of PNEE the definition of experimental n's can be controversial given the method by which the subjects are generated. In this dissertation, experimental manipulation in the form of the different diets is applied to a single subject (the dam), and as such it can be viewed that all offspring from a single litter are considered an n of one rather than of a potential 12. The potential confound of such 'litter effects' can be attributed to dam variability such as in the case of this work in diet consumption, health status and in maternal care, the confound of litter effects have been reviewed elsewhere (Champagne et al., 2003; Holson and Pearce, 1992; Jensh et al., 1970; Zorrilla, 1997). As such, the experimental n's for each experiment contained within this dissertation are described in number of slices, animals and litters. Future studies should comprise the use of additional offspring from additional litters in order to ensure that the variability in our experimental samples is representative.

4.10 Future Directions

4.10.1 Exploring the Impact of PNEE on Hippocampal Plasticity

The data from this dissertation are an initial foray into the various forms of plasticity that may be impacted by PNEE in the juvenile DG and provide rationales for studying other types of both LTP and LTD in this unique area.

One of the first priorities for future experiments is to further characterize this novel juvenile DG LTD in order to begin identifying the mechanism by which this plasticity is impaired by PNEE in males. First, we must identify which eCB is primarily responsible for this signalling. It is likely that the retrograde signalling molecule is in fact 2-AG, however washing over a 2-AG degradation enzyme during LFS_{900} in addition to MPEP should block the observed LTD if this the putative mechanism described in Figure 39 is representative of our experimental data. Additionally, pharmacological inhibition of DAGL or PLC during LFS_{900} will also help further characterize the molecular underpinnings of this LTD by washing over the drugs RHC-80267 or U73122 respectively. Finally, while we have identified a role for $mGluR_5$s in this LTD, it is

possible that the other group I mGluR ($mGluR_1$) could be involved as they share common second messenger cascades, and the potential role for this receptor should be determined.

Given that we found a sex-specific impairment in a form of LTD that involves activation of CB1 receptors, it would be interesting to investigate whether PNEE also affects another form of eCB-LTD. To this end, there is accumulating evidence of a relationship that exists between cannabinoids (endocannabinoids or drugs of abuse) and ethanol. For example, cannabinoid administration in the neonatal brain appears to prime the brain to be more vulnerable to the apoptotic effects of ethanol (Hansen et al., 2008). In fact, the impact of ethanol consumption on the eCB system is thought to underlie some aspects of ethanol addiction. CB1-dependent LTD is induced by LFS in the form of 10 minutes of stimulation at 10 Hz in the DG and is also dependent upon the function of $mGluR_5$s, which are known to be impaired by PNEE (Galindo et al., 2004). Given the previous findings that DG eCB-LTD impaired by chronic ethanol consumption could be restored by pharmacological blockage of the eCB synthesis enzyme MAGL, it would be worthwhile to examine whether this same pharmacological treatment could restore normal DG LTD in male PNEE offspring. It would be particularly interesting to investigate whether there are sex-specific impairments in this form of LTD as was found in the present dissertation as a result of PNEE. Furthermore, in order to distinguish whether PNEE affects one of either CB1 receptor or $mGluR_5$ dependent forms of plasticity, it would be necessary to induce pharmacological forms of LTD by washing DHPG onto hippocampal slices to induce mGluR-LTD in slices of both male and female offspring. Further to this, given that PNEE significantly reduced the magnitude of NMDAR-LTP it would be possible to investigate NMDAR-LTD by directly washing NMDA onto *in vitro* hippocampal slices. Given the independence of this LTD of NMDAR and LTCC activity, the use of a drug such as thapsigargin to reduce the amount of calcium available to be released from intracellular stores may give a more complete picture of some of the essential mechanisms for this LTD.

If PNEE does indeed result in an imbalance between excitation and inhibition we could begin examining this electrophysiologically. It is possible that inhibitory circuitry

in the DG could be participating in the mGluR5-CB1 LTD, as there are significantly more CB1 receptors on interneurons than there are on glutamatergic terminals, and the I/O curves in males indicate either reduced excitability or enhanced inhibition, either of which could impact LTD. One initial method by which we can test this is by pharmacologically inhibiting $GABA_A$ receptors during the LFS in control and PNEE males to determine whether the same effect of reduced magnitudes of LTD could be observed. If altered, or potentially enhanced GABAergic transmission is involved in or has direct impact on LTD in PNEE offspring then we might expect BIC treatment to enhance LTD, possibly to control levels, in males.

In order to further examine the deficits in NMDAR-LTP in these offspring, the use of alternative electrophysiological and autoradiographic techniques may be necessary. First, by using whole-cell patch clamp techniques we are able to examine the ion flux through the NMDARs themselves to assess whether the LTP impairments may be due to reduced current passing through these receptors. Similar to those studies completed by Weaver and colleagues and Lee and colleagues (Lee et al., 1994; Weaver et al., 1993), we could measure the intracellular rise in Ca^{2+} in response to NMDA wash-over on the slice and compare this rise in control and EtOH offspring to determine specifically whether, as per the aforementioned authors, reduced Ca^{2+} entry and subsequently reduced second messenger signalling cascades could account for the lower magnitudes of LTP following PNEE. Another potential method that could be used to compliment this prospective study would be to conduct calcium imaging in the DG granule cells in response to MPP stimulation, although this method may not provide the resolution to dissociate subtle PNEE-induced changes in Ca^{2+} dynamics.

In addition to the electrophysiological experiments described above it will be important to identify how PNEE leads to the impairment in $mGluR_5$-CB1 receptor-dependent LTD and in NMDAR-dependent LTP. Given that there is evidence that EtOH exposure can impact receptor number and function as well as ligand concentrations, these factors will have to be evaluated in the DG of male and female offspring following PNEE. Furthermore, the effect of PNEE on the number of $mGluR_5$s and NMDARs must

also be evaluated using a technique such as western blotting. When assessing the number of these synaptic receptors following PNEE the use of synaptic fractions of DG tissue will be key to relate back to the plasticity findings of this dissertation. Furthermore, given that CB1 receptors are located on excitatory and inhibitory terminals, confirming the potential localization of any changes to receptor numbers to particular regions of the DG can allow us to infer, based on location or potential co-localization with another set of markers, whether there is a cell-type specific change in receptor number using immunohistochemistry.

4.10.2. Associating Plasticity Deficits with Behavioural Impairment

Further validation of the changes in DG synaptic plasticity in juveniles of both sexes can be conducted through a series of behavioural studies. The function of the DG has been associated with a number of spatial learning and memory tasks including the MWM, novel object recognition and the metric change task among others (Patten et al., 2016b; Richter-Levin et al., 1994, 1995; Straube et al., 2003). Given that DG LTP has been associated with exploration of novel environments and DG LTD has been involved in exploration of large directional objects (Kemp and Manahan-Vaughan, 2008) employing tasks that incorporate these separate aspects may help in dissociating the sex-specific LTD impairments in males from the LTP impairments in both sexes following PNEE during the juvenile developmental period.

In this dissertation we found that the delivery of additional CSs was able to maximize and overcome PNEE-induced plasticity deficits. These results could loosely be associated with spatial memory behavioural experiments where there are groups that receive variable amounts of training on the task where animals receiving the standard amount of training trials, for example in a MWM task, may show PNEE-induced deficits and a group being overtrained would likely show no differences between control and PNEE offspring. While this is not directly related to, for example the delivery of additional HFS to overcome LTP deficit these experiments may provide additional evidence that PNEE may impair the function of plasticity machinery but in a way that can be overcome to restore normal plasticity and perhaps memory function. This hypothetical

set of experiments may actually help align the discrepancies observed in the effect of PNEE on, for example, MWM learning and memory as many of these studies use variable training paradigms which could in some cases involve overtraining PNEE offspring and potentially washing out the subtle effects of PNEE on hippocampal processing. Furthermore, it would be worthwhile to examine how juvenile PNEE offspring of both sexes are affected in reversal learning in a spatial memory task such as the MWM, where the hidden platform is moved to a new location and the animal must 'edit' the original spatial memory to acquire the new location. When the platform location is rotated to a new place each day, adult PNEE offspring show delays in learning the new location (Savage et al., 2002).

4.10.3. Impact of PNEE on Non-Hippocampal Learning

While there is a wealth of literature examining how the hippocampus is affected by PNEE there are other areas of the brain capable of supporting other forms of learning and memory that are also likely to be affected by PNEE.

Of particular interest is the impact of PNEE on the main olfactory system, which plays essential roles in all aspects of rodent development and behaviour. Dysfunction of the olfactory system has recently been used as an early indicator for various neurodevelopmental and neurodegenerative conditions (see (Wilson et al., 2014) for review). An early pathological study described significant damage to the olfactory bulb (OB) and olfactory stalks of children and fetuses heavily exposed to ethanol prenatally (Peiffer et al., 1979). Physical damage to structures of the olfactory system have also been reported in animal models of FASD (Akers et al., 2011; Bonthius and West, 1991a; Parnell et al., 2009). Children and adolescents exposed to ethanol in utero have been reported to have impaired olfactory identification abilities for common household odours and have been considered to have blunted responses to both smell and taste (Bower et al., 2013; Hansen and Jirikowic, 2013). Despite this, offspring that have been exposed to ethanol in utero have heightened responses to the smell and taste of ethanol itself, perhaps due to some associations formed by the smell and taste of ethanol in the amniotic fluid (Faas et al., 2000; Schaal et al., 2000), which may play a role in the susceptibility of

these individuals to alcoholism later in life (Spear and Molina, 2005; Youngentob and Glendinning, 2009).

There are several similarities between the hippocampus and the two key areas of the olfactory system, the OB and the olfactory or piriform cortex (PC). Like the DG, the OB supports adult neurogenesis in rodents as well as glutamatergic synaptic plasticity at the first synapse for olfactory system entering the brain (olfactory nerve – mitral cell synapse). The PC also shares characteristics of the hippocampus in that it is also a three-layered, paleocortical structure and is plays a role in pattern completion and separation of odour mixtures similar to the hippocampus for spatial and object pattern completion and separation. The PC itself also projects to the EC, providing access for the hippocampus to olfactory information. The PC also supports glutamatergic synaptic plasticity and is essential for olfactory associative learning, and in particular for early odor preference learning (EOPL) (Yuan et al., 2014). Furthermore, PNEE causes significant changes to genes important for synaptic plasticity in olfactory areas of juvenile rats (Middleton et al., 2009). An initial study reported EOPL deficits in young offspring exposed to ethanol during gestation (Barron et al., 1988) although these deficits in associative olfactory memory were not apparent at adulthood. In support of a possible age-dependent deficit in olfactory learning, a recent study found that while odour associative memories were intact at adulthood, fine odour discrimination was impaired by PNEE (Akers et al., 2011). These fascinating anatomical and behavioural deficits related to the olfactory system have, as yet, been associated with functional impairments in the circuitry of either the OB or PC and thus these areas are ripe for dissociating the mechanism for damage by PNEE.

4.11 Conclusions

The experiments contained within this dissertation shed light on the potential ways in which PNEE can impair neural communication, synaptic plasticity and ultimately learning and memory. We showed that PNEE alters the threshold for induction of bidirectional synaptic plasticity in the DGs in juvenile males and in the threshold for NMDAR-dependent synaptic potentiation only in juvenile female offspring. To our knowledge this is the first study to examine de novo LTD in the DG of juvenile rats

following PNEE. These data suggest that in this model of FASD, additional stimulation is necessary to recruit necessary receptors and initiate their downstream second messenger signaling cascades in order to achieve control-level bidirectional synaptic plasticity. This piece of the puzzle that is FASD is important and rarely reported in the literature due to few studies investigating plasticity beyond LTP or LTD induced by a single conditioning stimulus train. Facilitating function or recruitment of these signaling systems could be leveraged by therapeutic treatments in the future. In addition to addressing our research question, this work also found a novel form of LTD in the juvenile rat DG that was dependent on the synergistic function of both CB1receptors and $mGluR_5$s. The findings of this work offer important insights into the juvenile DG of both males and females and identify signalling cascades that can be targeted therapeutically for critical early intervention for young children suffering from FASD.

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Appendix A – Slice Ns

The number of slices used in each set of electrophysiological experiments are outlined in the following tables for control (Table 5) and ethanol (Table 6) offspring as well as for the pharmacological manipulations to block either LTP or LTD (Table 7).

Table 5. Number of control offspring slices

Multiple slices from individual animals were used across multiple experiments throughout the work described in this dissertation. Below is the number of individual slices included for analysis in the final datasets. Only one recording was obtained from an individual slice.

CONTROL		
	Male	**Female**
LTP	8	7
LTD_{900}	12	14
LTD_{1800}	8	14
Depotentiation	5	7

Table 6. Number of ethanol offspring slices

Multiple slices from individual animals were used across multiple experiments throughout the work described in this dissertation. Below is the number of individual slices included for analysis in the final datasets. Only one recording was obtained from an individual slice.

ETHANOL		
	Male	**Female**
LTP	8	11
LTP_{2xHFS}	13	16
LTD_{900}	11	9

LTD_{1800}	5	7
Depotentiation	8	9

Table 7. Number of slices used for pharmacological blockade experiments

Multiple slices from individual animals were used across multiple experiments throughout the work described in this dissertation. Below is the number of individual slices included for analysis in the final datasets. For these experiments slices from males and females were combined and used equally across drug groups. Only one recording was obtained from an individual slice.

DRUG BLOCKADE	
	N
DL-APV LTP	3
Control LTD	26
DL-APV LTD	6
MPEP LTD	6
NIMO LTD	3
COCKTAIL LTD	3
TAT-GLUA2-3Y LTD	6
AM251 LTD	17
AM251 + MPEP LTD	10

Appendix B - The Impact of Pair-Feeding on DG LTP and LTD

In the study of PNEE it is common to have an additional control group, particularly in diet forms of PNEE. In the liquid diet model of PNEE, a pair-fed control is typically always included in the evaluation of the effect of the EtOH-containing liquid diet on any given measure. Pair-fed animals receive a similar liquid diet as the EtOH group except that the EDCs are replaced with calories from maltose-dextrin, a common food additive found in baby formula and other products readily available to consumers. The original inclusion of this group was to control for the effect of the liquid diet and dietary restriction in general however in practice, pair-feeding does not meet these experimental goals as an appropriate control group. The EtOH dams do indeed have their diet altered in that they no longer have access to solid chow, but rather ~150g of the EtOH-containing liquid diet, which they may consume *ad libitum*. Despite the restriction to ~150g of diet, no dams in this dissertation consumed all of this diet. The amount of diet provided to the pair-fed dams is yoked to the amount of diet consumed by a weight-matched EtOH dam, thus there is a true restriction in consumable diet for the pair-fed dams. Generally, the pair-fed dams consume this diet shortly after presentation of the bottle and then have no other dietary supplementation until 24 hours later, which is likely a source of stress for these animals and can have long-term impact on the offspring as a prenatal stressor. This control is imperfect as discussed above. For these reasons the PNEE group was compared to the *ad libitum* solid chow control throughout this dissertation, however the impact of pair-feeding on DG synaptic plasticity was examined nonetheless and compared to the solid-chow control group.

Methods

All procedures involving animal acquisition and breeding were conducted as described in the methods section of the main body of this dissertation.

Pair-fed Diet – Controlled access to a nutritionally fortified liquid diet (Weinberg/Keiver high protein liquid diet-control, no. 710109, Dyets Inc, Bethlehem,

PA, USA) identical to the ethanol diet with the EDC isocalorically substituted with maltose-dextrin. In order to control for the stress associated with the consumption of a liquid diet in the ethanol group, dams assigned to the pair-fed condition received the same amount of food in g/kg/day as their weight-matched ethanol-exposed dams. Pair-fed dams were supplied with the pair-fed liquid diet from GD1-21, where on GD22, the liquid diet was replaced with standard solid rat chow which was provided throughout parturition.

Liquid diets were provided to animals approximately 2 hours prior to the beginning of each dark phase for each day of gestation. The daily consumption of the diet was recorded and the dam body weights were recorded on GDs1, 7, 14 and 21.

Treatment of offspring and dams after birth is consistent with what is described in the main body of this dissertation. *In vitro* electrophysiology in the DG was carried out as described in transverse hippocampal slices from P21-28 in male and female offspring.

Results

Developmental Data

The measures of prenatal dam weight gain (Table 8) and postnatal offspring weights (Table 9) are described below compared to controls. Pair-fed dams gained on average 58.65 ± 4.51% of their body weight over the course of gestation. Pair-fed dams consumed, on average 73.03g of the pair-fed diet daily throughout gestation with individual dams consuming daily averages ranging from 66.33g to 77.35g of the pair-fed diet.

Table 8. Maternal weight gain during pair-feeding

Pregnant Sprague-Dawley rats were weighed on the day that sperm was found (designated as gestational day (GD) 1) and then once weekly on GD 7, 14 and 21 prior to giving birth on GD 22. Average weights are expressed ± the standard error of the mean (SEM).

Average	**Control**	**Pair-Fed**
Weight at **GD 1** (g)	230.24 ± 11.96	220.47 ± 9.74
Weight at **GD 7** (g)	250.51 ± 12.76	235.07 ± 11.20
Weight at **GD 14** (g)	276.71 ± 13.43	271.73 ± 9.24
Weight at **GD 21** (g)	344.71 ± 12.08	348.90 ± 5.20
% Weight Gain	**51.80 ± 8.03 %**	**58.65 ± 4.51 %**

There were no statistically significant differences between the average offspring weights of control and pair-fed litters at any age as shown in Table 9 and depicted graphically in Figure 40.

Table 9. Postnatal offspring weights after pair-feeding

Average offspring weight gain separated by sex starting on the day after birth (P1) to weaning an experimental use (P21). On P1 litters were culled to 12 pups consisting of 6 pups of each sex where possible. A two-tailed student's t-test was used to evaluate the effect of prenatal diet treatment within sex and age. The average weights are expressed below ± the SEM. Statistical significance was considered when $p < 0.05$.

Average	**Control**	**Pair-Fed**
Litter Size	12.00 ± 0.60	11.67 ± 0.88
N_{males}	6.40 ± 0.64	8.67 ± 0.67

$N_{females}$	5.60 ± 0.52	4.33 ± 1.33
Weight **$P1_{male}$**	8.13 ± 0.16	7.58 ± 0.56
Weight **$P1_{female}$**	6.93 ± 0.49	7.62 ± 1.01
Weight **$P3_{male}$**	10.69 ± 0.36	9.42 ± 0.38
Weight **$P3_{female}$**	8.94 ± 0.63	9.01 ± 0.90
Weight **$P7_{male}$**	16.83 ± 0.67	14.72 ± 1.22
Weight **$P7_{female}$**	16. 45 ± 0.55	14.40 ± 1.16
Weight **$P14_{male}$**	29.71 ± 2.57	26.26 ± 2.27
Weight **$P14_{female}$**	30.85 ± 1.39	25.46 ± 2.66
Weight **$P21_{male}$**	55.52 ± 3.72	41.40 ± 5.61
Weight **$P21_{female}$**	48.69 ± 5.31	40.31 ± 5.53

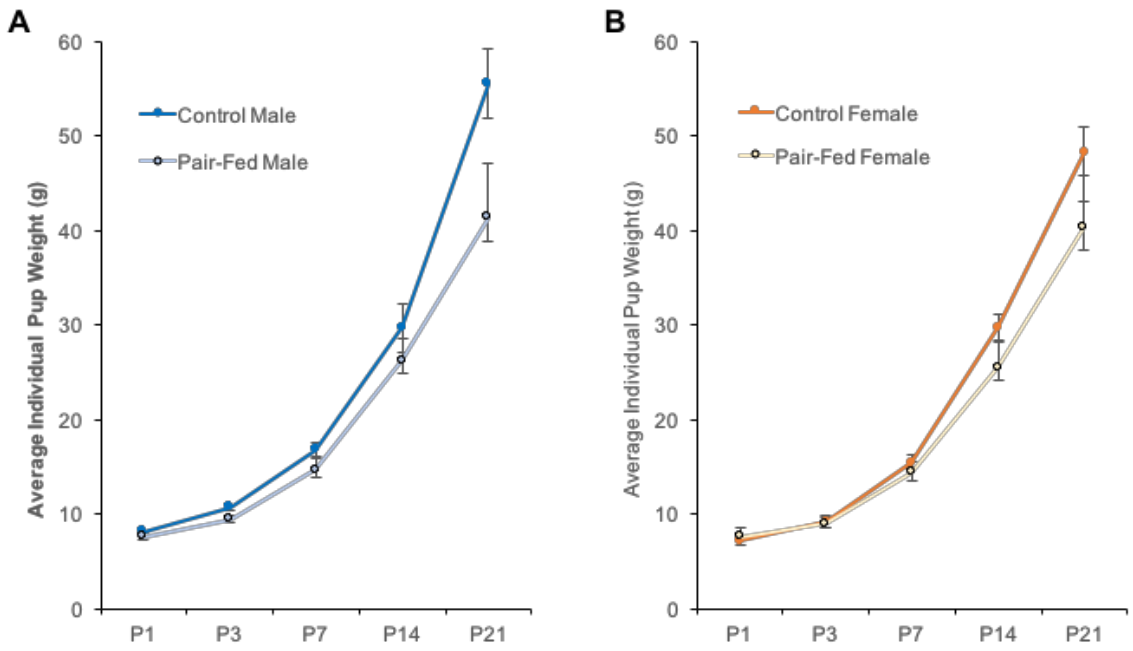

Figure 40. Average offspring weight gain following pair-feeding

Average offspring weights for males (A) and females (B) following consumption of either solid chow control or pair-feeding liquid diets. Points are the average individual offspring weights by condition and error bars represent the standard error of the mean.

Synaptic Plasticity

Both LTP and LTD were evaluated in male and female pair-fed offspring. LTP was induced by a single episode of HFS and LTD was induced by LFS_{900}. HFS did not induce significantly less PTP in Pair-Fed male offspring (PTP: 113.19 ± 9.36 %) as compared to controls (PTP: 148.04 ± 13.60 %; p = 0.055; Figure 41 A, C). The magnitude of LTP was significantly reduced by pair-feeding in males (Control LTP: 91.81 ± 17.18 %; Pair-Fed LTP: 42.38 ± 8.34 %; p = 0.0266; Figure 41 B, C). Similarly, pair-feeding had no impact on PTP in female offspring (PTP: 135.57 ± 15.05 %) but

caused a significant reduction in the magnitude of LTP (LTP: 32.30 ± 8.05 %; p = 0.0036; Figure 42 B,C).

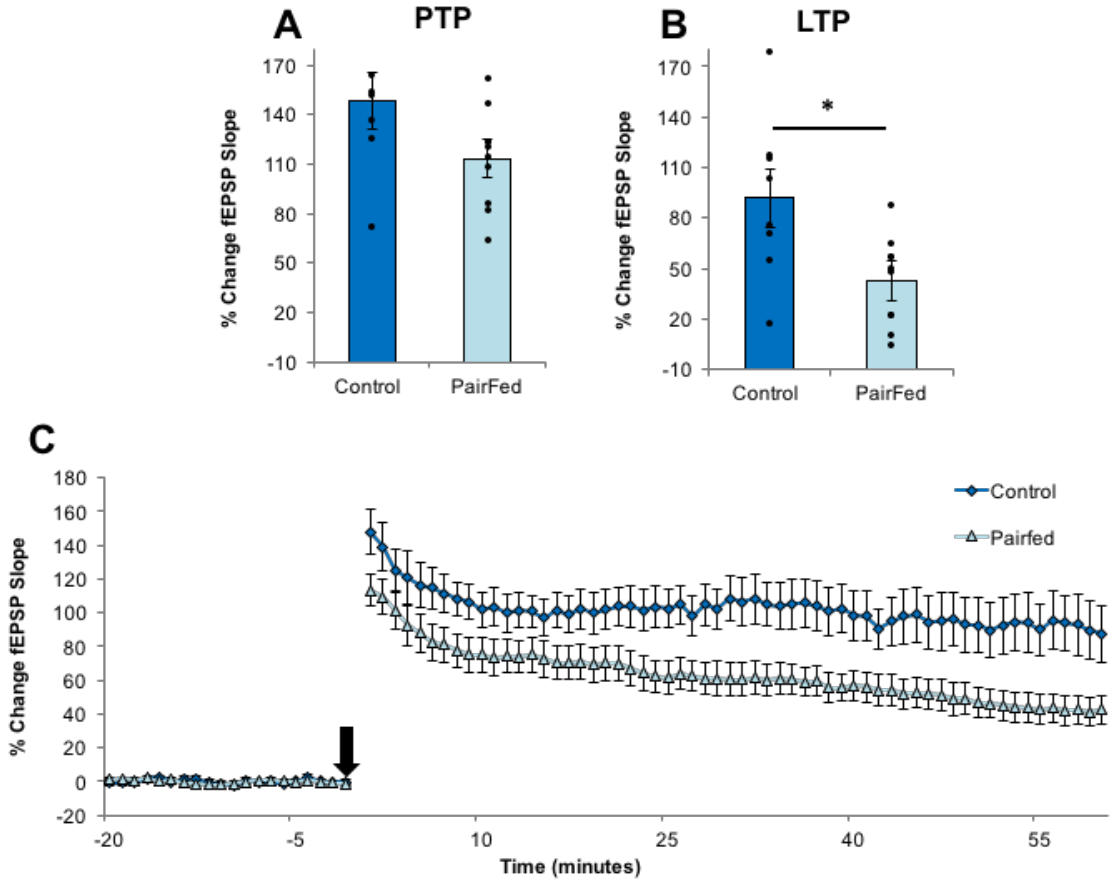

Figure 41. Short- and Long-Term Potentiation Following Pair-Feeding in Males

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrow in D). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. (**C**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$.

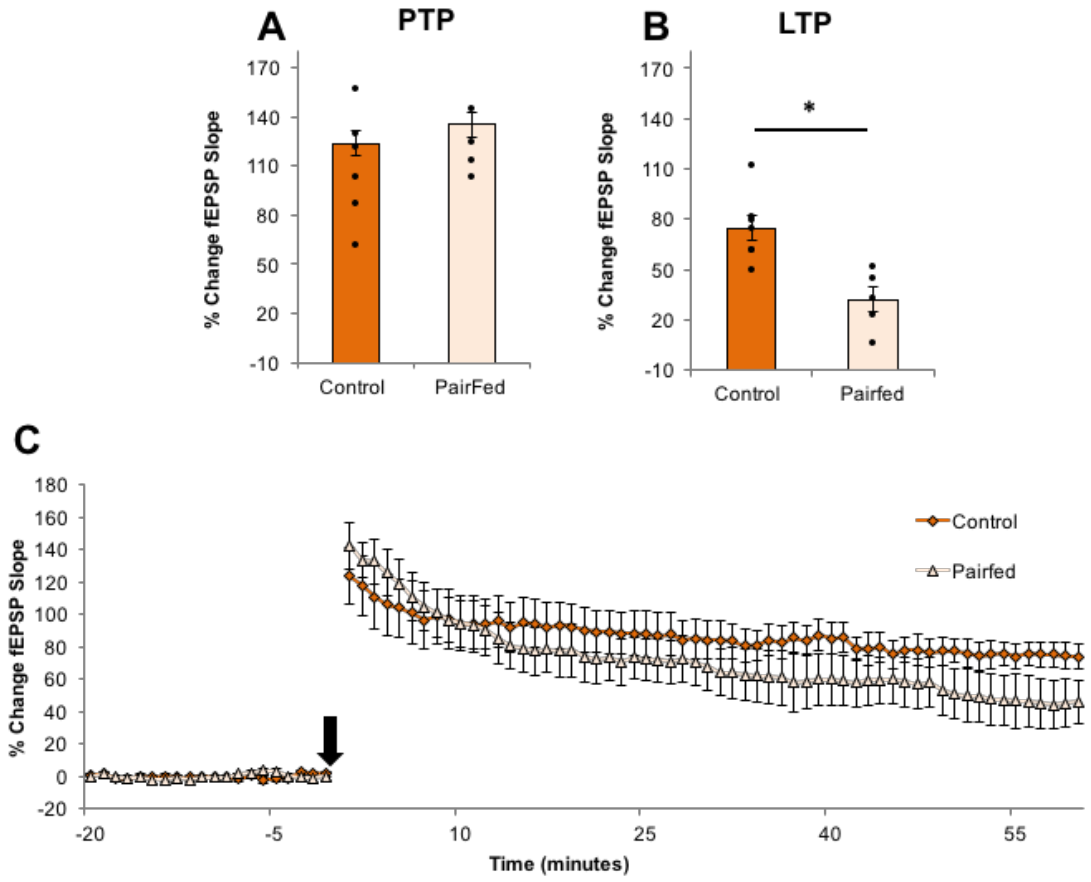

Figure 42. Short- and Long-Term Potentiation Following Pair-Feeding in Females

(**A**) Post-tetanic stimulation (PTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 0-1 following delivery of the high frequency stimulation (HFS; black arrow in D). (**B**) Long-term potentiation (LTP) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the HFS. Bars represent the average percentage of change in the fEPSP slope for PTP (A) and LTP (B) with points representing the average PTP and LTP for each individual slice in this dataset. (**C**) Average LTP recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM. * $p < 0.05$.

Neither the magnitudes of STD nor LTD were significantly affected by pair-feeding in male offspring (see Table 10 for averages and SEMs; Figure 43; STD: $p = 0.557$; LTD: $p = 0.194$).

Table 10. Average STD and LTD in Pair-Fed and Control Male Offspring

The average magnitudes of STD and LTD were evaluated in the first minute and in the last 5 minutes (55-60) of the post-conditioning recordings following delivery of LFS_{900} in both groups. These are expressed as averages ± the standard error of the mean. The effect of prenatal diet was evaluated statistically using a student's t-test where statistical significance was considered to be $p < 0.05$. Pair-feeding had no impact on either STD or LTD in male offspring compared to controls.

	STD	LTD
Control	-38.73 ± 4.29 %	-24.89 ± 4.76 %
Pair-Fed	-41.67 ± 4.19 %	-18.30 ± 2.33 %

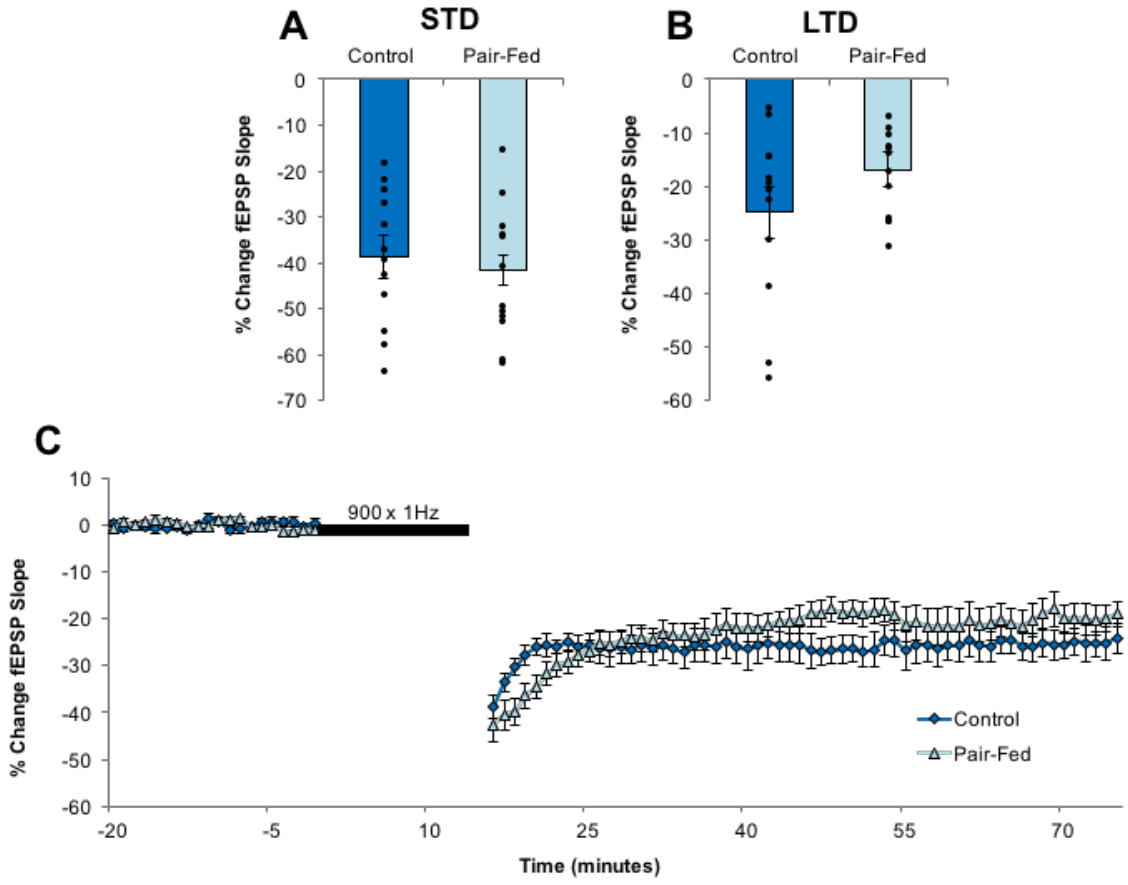

Figure 43. Short- and Long-Term Depression Following Pair-Feeding in Males

(**A**) Short term depression (STD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in

the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM.

Similarly, neither magnitudes of STD nor LTD were significantly impacted by pair-feeding in female offspring (see Table 11 for averages and SEMs; Figure 44; STD: $p = 0.375$; LTD: $p = 0.0758$).

Table 11. Average STD and LTD in Pair-Fed and Control Female Offspring

The average magnitudes of STD and LTD were evaluated in the first minute and in the last 5 minutes (55-60) of the post-conditioning recordings following delivery of LFS_{900} in both groups. These are expressed as averages ± the standard error of the mean. The effect of prenatal diet was evaluated statistically using a student's t-test where statistical significance was considered to be $p < 0.05$. Pair-feeding had no impact on either STD or LTD in female offspring compared to controls.

	STD	LTD
Control	-52.44 ± 4.86 %	-28.46 ± 3.56 %
Pair-Fed	-46.23 ± 4.86 %	-20.96 ± 1.82 %

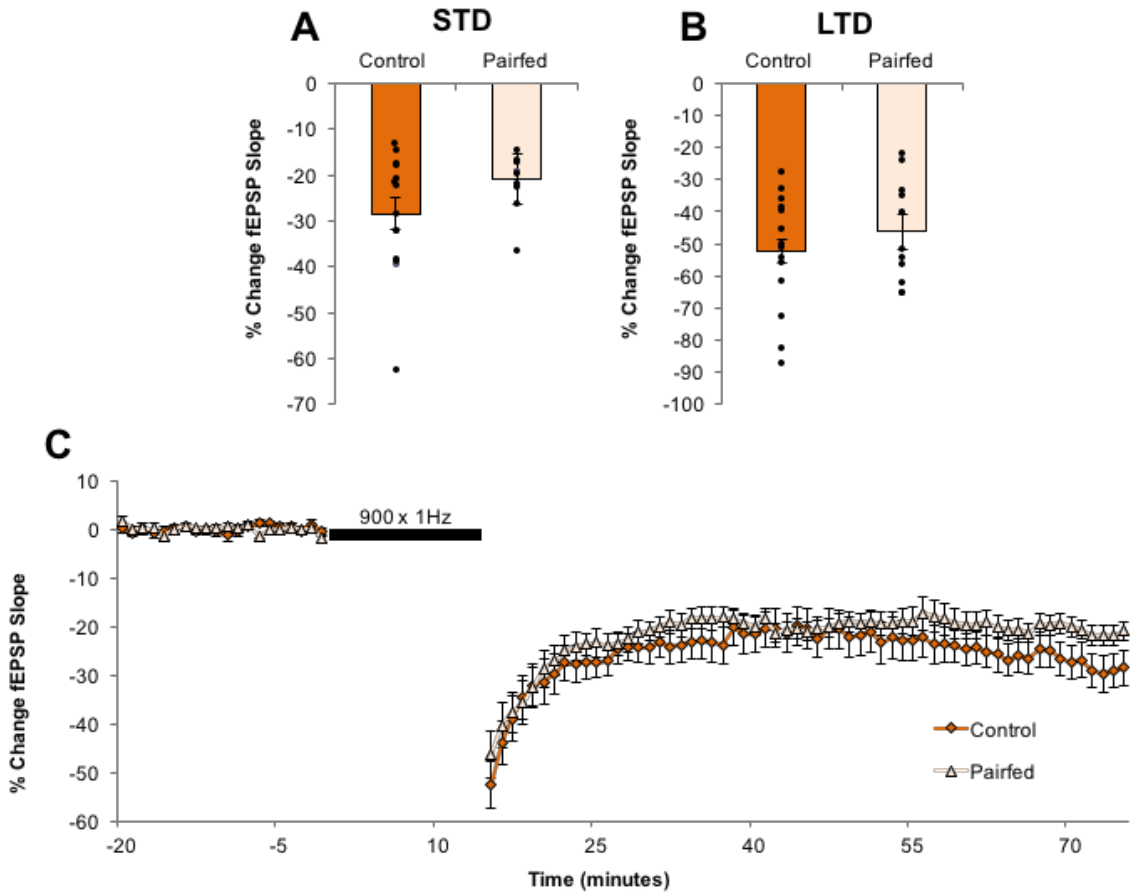

Figure 44. Short- and Long-Term Depression Following Pair-Feeding in Females

(**A**) Short term depression (STD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 0-1 following delivery of low frequency stimulation (LFS_{900}; black bar in C). (**B**) Long-term depression (LTD) was measured as the average percentage of change in the fEPSP slope relative to baseline for minutes 55-60 following delivery of the LFS_{900}. Bars represent the average percentage of change in the fEPSP slope for STD (A) and LTD (B) with points representing the average STD and LTD for each individual slice in this dataset. (**C**) Average LTD recordings from the beginning of baseline to the end of the post-conditioning recording. Dots represent the average percentage of change in the fEPSP slope relative to baseline and the error bars represent the SEM.

Table 12. Slice Ns in pair-fed experiments

Multiple slices from individual animals were used across multiple experiments throughout the work described in this dissertation. Below is the number of individual slices included for analysis in the final datasets. Only one recording was obtained from an individual slice.

PAIR-FED		
	Male	**Female**
LTP	10	5
LTD_{900}	12	11

Discussion

Pair-feeding led to impaired LTP in both male and female offspring, without affecting LTD in either sex. These data indicate that pair-feeding causes a deficit in NMDAR-dependent synaptic plasticity in a non-sex-specific manner, and that this NMDAR-LTP, but not LTD may be sensitive to prenatal dietary restriction stress in both sexes.

Appendix C – Supplementary Cumulative Probabilities of Synaptic Plasticity

Cumulative probabilities can often be effective is displaying the range of data encompassed in a synaptic plasticity dataset, particularly in identifying possible sources of differences between groups. As such, cumulative probabilities were calculated for all plasticity experiments as described in the main body of this dissertation. Cumulative probabilities for the mechanisms of LTP are shown in Figure 45, while multiple HFS experiments are displayed in Figure 46. The cumulative probabilities for LTD blockade experiments are displayed in Figure 48 and for LFS_{1800} experiments in Figure 47.

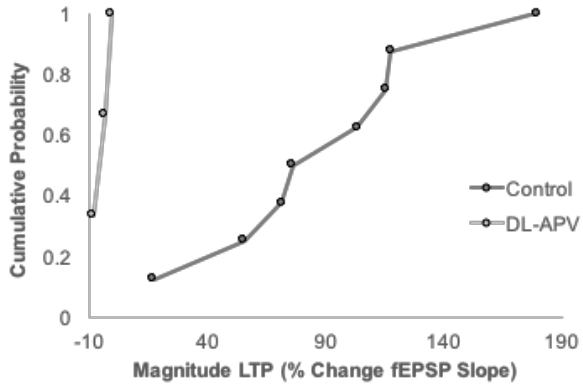

Figure 45. Cumulative Probability for LTP Blockade by DL-APV

Actual frequencies of the distribution of the magnitudes of LTP in controls and under NMDAR blockage by 50μM DL-APV.

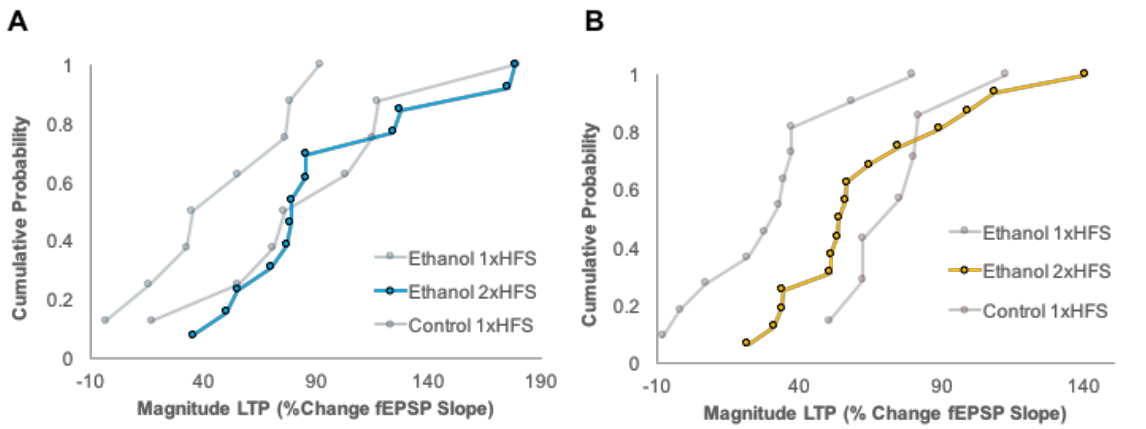

Figure 46. Cumulative Probabilities for Multiple HFS LTP

Actual frequencies of the distribution of the magnitudes of LTP in both (A) male and (B) female offspring following either a control or EtOH prenatal diet. Semi-transparent points and lines represent the cumulative probabilities for the magnitudes of LTP induced by 1xHFS.

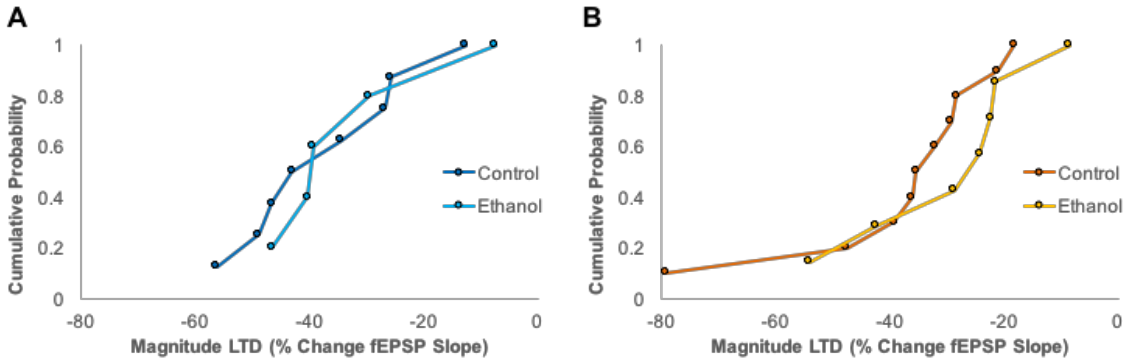

Figure 47. Cumulative Probabilities for LFS_{1800} LTD

Actual frequencies of the distribution of the magnitudes of LTD induced by LFS_{1800} in both (A) male and (B) female offspring following either a control or EtOH prenatal diet.

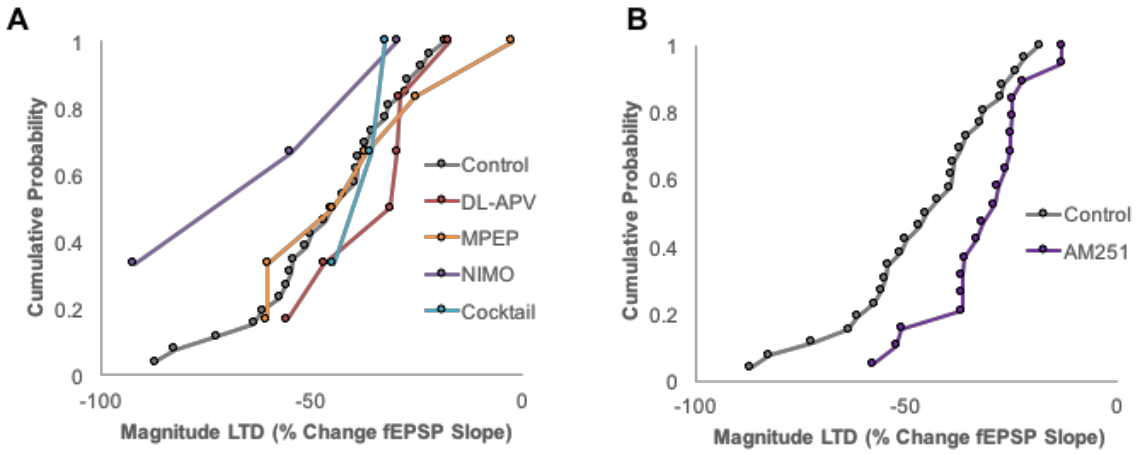

Figure 48. Cumulative Probabilities for LTD Blockade Experiments

Actual frequencies of the distribution of the magnitudes of LTD under drug blockade conditions. (A) Cumulative probabilities for compounds that had no significant effect on the magnitude of LTD. (B) Cumulative probabilities for AM251, which had a significant effect on the magnitude of LTD compared to control slices.