SEX-SPECIFIC EFFECTS OF PRENATAL ETHANOL EXPOSURE ON HIPPOCAMPAL SYNAPTIC PLASTICITY IN ADOLESCENT RATS

by

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Abstract

The hippocampus is a brain region intimately involved with learning and memory. Long-term depression (LTD) and long-term potentiation (LTP) are putative mechanisms behind learning and memory. Prenatal and postnatal events that alter LTP and/or LTD also impair hippocampal-dependent learning and memory. Prenatal stress and prenatal ethanol exposure (PNEE) can both independently reduce LTP in male offspring; acute postnatal stress enhances LTD in males. It remains to be determined how these events alter synaptic plasticity in adolescent females.

Stress-induced changes to LTD might be more pronounced following PNEE due to a heightened stress response in ethanol-exposed offspring. In Chapter 2, it was found that acute stress was required for the expression of LTD in control males but blocked LTD in females. Acute stress was required for LTD in males following PNEE but LTD was not apparent in females.

The experiments in Chapter 3 were designed to investigate how combined exposure to stress and ethanol *in utero* affect LTP in the hippocampus of adolescent males and females. PNEE reduced LTP in males but enhanced LTP in females. In animals not exposed prenatally to ethanol, prenatal stress significantly reduced LTP in males but not females. On the other hand, LTP was reduced in males exposed both ethanol and stress *in utero* but the magnitude of LTP was not significantly different from that of ethanol-exposed males. In females, however, prenatal stress reduced the ethanol-induced enhancement of LTP.

These findings indicate that synaptic plasticity in adolescent males and females is differentially affected by prenatal and postnatal events. Putative mechanisms behind the observed plasticity will be discussed in Chapter 4. Specifically, PNEE can delay the onset of puberty, which might alter the influence of the depressant effect of estradiol on synaptic plasticity in adolescent females. PNEE has previously been shown to "masculinize" the female brain and "feminize" the male brain, which might influence synaptic plasticity during adolescence. The expression of the placental barrier to CORT is also altered by prenatal stress and PNEE and might also contribute to observed changes in synaptic plasticity. Potential pitfalls of the experiments and future directions will be presented.

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Abbreviations

11β-HSD- 11 beta-hydroxysteroid dehydrogenase
ACTH- adrenocorticotropin releasing hormone
ADH- alcohol dehydrogenase
ADX- adrenalectomy
AL- ad libitum
AMH- anti-müllerian hormone
AMPAR- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA- analysis of variance
AR- androgen receptor
BEC- blood ethanol concentration
CA- cornu ammonis
Ca^{2+}- calcium
CaMK- calcium calmodulin-dependent kinase
CNS- central nervous system
CORT - corticosterone
CPP- (±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
CRE- cyclic-AMP response element
CREB- cyclic-AMP binding protein
CRH- corticotropin releasing hormone
DG- dentate gyrus
DHT- dihydrotestosterone
E- ethanol
EDC- ethanol-derived calories
E-LTP- early long-term potentiation
EPSC- excitatory posty-synaptic current
EPSP- excitatory post-synaptic potential
ER- estrogen receptor
FAS- fetal alcohol syndrome
FASD- fetal alcohol spectrum disorder
FSH- follicle stimulating hormone
GABAR- γ-Aminobutyric acid receptors
GCL- granule cell layer
GD- gestation day
GluR- glutamate receptor
GnRH- gonadotropin releasing hormone
GR- glucocorticoid receptor
HFS- high frequency stimulation
HPA- hypothalamic-pituitary-adrenal
HPG- hypothalamic-pituitiary gonadal
K^{+}- potassium
L-LTP- late long-term potentiation
LFS- low frequency stimulation
LH- luteinizing hormone
LPP- lateral perforant path
LTD- long-term depression
LTP- long-term potentiation
MAPK- mitogen-activated protein kinase
Mg^{2+}- magnesium
MPP- medial perforant path
MR- mineralocorticoid receptor
mRNA- messenger ribonucleic acid
MWM- Morris water maze
Na^{2+}- Sodium
NMDAR- N-methyl-D-aspartate receptor
NS- non-stress
NSF- N-ethylmaleimide-sensitive fusion protein
OVX- ovariectomy
PCL- pyramidal cell layer
PF- pair-fed
PKC- protein kinase c
PND- postnatal day
PNEE- prenatal ethanol exposure
POMC- proopiomelanocortin
PP1- protein phosphatase 1
PP2- protein phosphatase 2
PPF- paired-pulse facilitation
PS- prenatal stress
PSD-95- post-synaptic density 95
PTP- post-tetanic potentiation
S stress
SD-POA- sexually dimorphic nucleus of the preoptic area
SL-M- stratum laconosum-moleculare
SO- stratum oriens
SR- stratum radiatum
Sry- sex-determining region of the Y chromosome
STP- short-term potentiation
TBS- theta-burst stimulation
Thy- H(3)-thymadine
VDCC- voltage-dependent calcium channel

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Dedication

To my wonderful family

Co-Authorship Statement

Chapter 2 is a revised manuscript co-authored with Dr. Brian Christie at the University of British Columbia. As the first author, I was in charge of all aspects of the project including literature review, generating the animals, performing all experiments, data collection, data analysis, drafting the manuscript and generating the figures.

Chapter 3 is a revised manuscript co-authored with Dr. Brian Christie at the University of British Columbia and the University of Victoria. As the first author, I was in charge of all aspects of the project including literature review, generating the animals, performing all experiments, data collection, data analysis, drafting the manuscript and generating the figures.

1 General Introduction

1.1 Fetal Alcohol Syndrome

A brief review of the diagnostic criteria for fetal alcohol syndrome will be given followed by an overview of drinking patterns of pregnant women. Brain regions affected by gestational ethanol exposure will be discussed as well as the contribution of animal models to our understanding of the teratogenic effects of ethanol. Finally, ethanol metabolism during pregnancy will be discussed.

1.1.1 Diagnostic Criteria for Fetal Alcohol Syndrome

Fetal alcohol syndrome (FAS) is the leading cause of preventable mental retardation (Pulsifer, 1996) and is caused by maternal consumption of alcohol during pregnancy. Approximately 6-22 babies are born each day with FAS (Lupton et al., 2004) and the lifetime cost of care for such an individual can reach $1.4 million (Lupton et al., 2004). Diagnosis for FAS requires confirmed or suspected alcohol use during pregnancy as well as deficits in the following three categories: 1) Facial abnormalities such as a smooth philtrum, small palpebral fissure and/or thin upper lip; 2) growth deficits defined as both height and weight in the lower 10th percentile when adjusted for factors such as race, socioeconomic status, etc.; and 3) central nervous system (CNS) deficits that include neurological impairments (i.e., coordination problems), functional deficits (i.e. decreased IQ, behavioral and mood disorder) and structural deficits (i.e., smaller head circumference, reduced size of brain structures) (Bertrand et al., 2005), see **Figure 1.1**. As not all *in utero* alcohol exposure produces deficits that are robust enough for FAS

diagnosis (Larkby and Day, 1997) or generate all qualifying characteristics, such as having a lowered IQ but no facial dysmorphia (Mattson et al., 1997), a commonly used umbrella term for any insults that result from prenatal alcohol exposure is fetal alcohol spectrum disorder (FASD).

Individuals with FAS/D can have a high co-morbidity of conditions such as attention deficit hyperactivity disorder, learning disorders, speech and language disorders and sensory impairments (Burd et al., 2003). Some characteristics of FAS/D, such as craniofacial abnormalities, can become less apparent over time although deficits in height, weight and IQ can persist into adulthood (Mattson et al., 1997; Spohr et al., 1993; Streissguth et al., 1991).

1.1.2 Drinking Patterns during Pregnancy

Pivotal to the development of FAS/D is the amount and duration of alcohol exposure during pregnancy. Despite increased awareness that consumption of alcohol during pregnancy is harmful to the fetus (Tough et al., 2006), women often continue to drink during pregnancy. Drinking patterns among pregnant women indicate that approximately 12.8% consumed any alcohol during pregnancy (i.e., at least 1 drink) while 2.7% reported binge-drinking (i.e. $\geq$ 5 drinks per sitting) and 3.3% reported frequent drinking (i.e., $\geq$ 7 drinks per week or $\geq$5 drinks on any occasion) (2002). Low, sporadic alcohol consumption during pregnancy can increase the risk of FAS-associated disorders (Martinez-Frias et al., 2004) and even moderate exposure to ethanol *in utero* can affect IQ levels (Streissguth et al., 1990). A comprehensive examination of experimental studies, however, suggests that the fetus is most at jeopardy when exposed to large amounts of alcohol (Hatfield, 1985).

It remains to be determined why women continue to drink upon recognition of pregnancy. Caucasian women with higher income and a college education are at risk of drinking during pregnancy (Floyd et al., 1999; Tough et al., 2006) suggesting that "education" does not promote abstinence while pregnant. Another risk factor for drinking during pregnancy is partner violence. Women are also more likely to consume alcohol during pregnancy if there is a history of physical abuse by their partner (Grimstad et al., 1998) or if their partner has a drinking problem (Bresnahan et al., 1992; Muhajarine and D'Arcy, 1999). Women in abusive relationships can also have reduced social support and this reduce support can increase alcohol consumption (Stephens, 1985). Taken together, these studies indicate a complex relationship between social constructs and alcohol consumption during pregnancy.

1.1.3 Brain Structures Affected in Humans with FAS

Brain abnormalities can be central to FAS/D and much attention has focused on morphological alterations that result from prenatal alcohol exposure (Mattson et al., 2001; Riley and McGee, 2005). The basal ganglia are a collection of brain structures that are collectively involved with eye and body movement (Basso et al., 2005; O'Driscoll et al., 2000). The size of the basal ganglia is reduced by gestational exposure to alcohol (Archibald et al., 2001; Mattson et al., 1992; Mattson et al., 1994; Mattson et al., 1996) which might contribute to poor eye-tracking in individuals with FAS/FASD (Green et al., 2009; Green et al., 2007). The shape and size of the corpus callosum is altered in FAS/FASD (Bookstein et al., 2002a; Bookstein et al., 2002b; Mattson et al., 1992; Riley et al., 2004; Swayze et al., 1997). The corpus callosum is implicated with interhemispheric transfer of information (Bloom and Hynd, 2005; Hoptman and

Davidson, 1994) and changes in callosal structure induced by alcohol exposure might account for poor performance on tasks that require interhemispheric transfer of information (Roebuck et al., 2002; Roebuck-Spencer et al., 2004). Changes in callosal structure as a result of prenatal alcohol exposure might also explain behavioral deficits in executive and motor function present in FAS individuals (Bookstein et al., 2002b) as well as impaired bimanual coordination (Schapiro et al., 1984). Cerebellar volume is reduced in FAS (Archibald et al., 2001; Mattson et al., 1994; Mattson et al., 1996) possibly accounting for impaired performance on eye-blink conditioning tasks (Coffin et al., 2005). Pertinent to the current thesis are the effects of gestational alcohol exposure on the hippocampus. The hippocampus is a bilateral brain region intimately involved with learning and memory, discussed in more detail below. Although the structural deficits induced by alcohol are equivocal (Archibald et al., 2001; Autti-Ramo et al., 2002; Geuze et al., 2005; Riikonen et al., 1999), spatial impairments in individual with FAS (Uecker and Nadel, 1996; Uecker and Nadel, 1998) suggest that the functional integrity of the hippocampus is compromised. These studies indicate that global abnormalities in the structure and function of the brain are affected by exposure to alcohol *in utero* and that that hippocampal function might be impaired in individuals with FAS/D.

1.1.4 Rodents as a Model of FAS/D

Several animal models of FAS/D have been developed to better characterize the abnormalities that result from prenatal ethanol exposure (PNEE). A variety of species have been used including monkeys (Astley et al., 1999; Bonthius et al., 1996), sheep (Falconer, 1990; Gleason and Hotchkiss, 1992), chick (Cartwright and Smith, 1995a; Cartwright and Smith, 1995b), zebrafish (Bilotta et al., 2004), drosophila (Giesel and

Niemann, 1985) and guinea pigs (Iqbal et al., 2004; Richardson et al., 2002) but the most common species utilized is the rodent. The relatively short gestation period, large litters, plethora of apparati and manipulations available to assess behavior and learning, the advent of transgenic mouse lines and the similarity of specific rodent behavioral tasks to humans (such as the Morris water maze) (Hamilton et al., 2003) make rats and mice an attractive species in which to study the effects of PNEE. The diagnostic criteria for FAS in humans are growth deficits, CNS disorders and facial abnormalities (Chudley et al., 2005) and many of these characteristics have been recapitulated in rodent models of FAS/FASD. Growth deficits (e.g., decreased weight gain) have consistently resulted in rodents following PNEE (Christie et al., 2005; Fernandez et al., 1983; Gallo and Weinberg, 1986; Hannigan et al., 1993; Redila et al., 2006) and these deficits can persist into adulthood (Middaugh et al., 1988). Consumption of 35% ethanol-derived calories (EDC) throughout gestation can decrease brain, liver, heart and kidney weight (Gallo and Weinberg, 1986). Central nervous system deficits are commonly observed in rodents following PNEE and consist of a decrease in the density of the cerebellar molecular layer (Lancaster and Samorajski, 1987), incomplete development of the splenium of the corpus callosum (Moreland et al., 2002), a narrowing of the cortex (Schapiro et al., 1984), and functional and structural alterations to the hippocampus (Barnes and Walker, 1981; Christie et al., 2005).

1.1.5 Timing of Ethanol Exposure

A drawback of using rodents to study FAS/D, however, is that the brain grows at various rates in different species (**Figure 1.2)**, which can influence whether the region will be exposed to ethanol. The human hippocampus, for example, predominantly develops

during the latter part of the third trimester but the rodent hippocampus continues to develop during the early postnatal period (Rahimi and Claiborne, 2007; Seress, 2007). As a result, if the fetuses are exposed to alcohol (i.e., *in utero*) then developmental exposure of the hippocampus to ethanol will be different in rodents and humans. Within the rodent literature distinct differences in hippocampal morphology can result when ethanol is administered *in utero* or by combined *in utero* and postnatal exposure (Maier, 1999). Additionally, alcohol exposure does not produce uniform deficits (discussed below), which should be taken into account when studying animal models of FAS.

The timing of ethanol exposure during development can produce distinct effects on offspring. Exposure to ethanol throughout gestation can impair orienting ability (Gallo and Weinberg, 1982) and postural reflexes (Lehotzky et al., 1988), and an inability to inhibit responding (Becker and Randall, 1989; Fernandez et al., 1983; Mihalick et al., 2001). Animals exposed to ethanol throughout gestation or via binge-exposure have been shown to exhibit increased ambulation in an open field maze (Becker and Randall, 1989; Fernandez et al., 1983) and were hyperactive (Lehotzky et al., 1988). The timing of ethanol exposure, however, can influence whether spatial impairments will be apparent. For example, Goodlett and Peterson (1995) found that males and females were equally impaired on a spatial learning and memory task if ethanol exposure occurred during postnatal day 4-9 (PND4-9), but exposure to ethanol during PND7-9 only impaired performance in males (Goodlett and Peterson, 1995). Subsequently, Minetti (1996) found that ethanol exposure on gestation day 8 (GD8) impaired the retention of a spatial memory task only in adult females (Minetti et al., 1996). Exposure to ethanol throughout gestation, however, can also impair spatial learning (Blanchard et al., 1987; Christie et

al., 2005; Kim et al., 1997; Neese et al., 2004; Reyes et al., 1989; Zimmerberg and Weston, 2002) suggesting that the functional integrity of the hippocampus might be particularly sensitive to gestational and/or early prenatal ethanol exposure.

1.1.6 Ethanol Metabolism during Pregnancy

As reviewed by Ferreira and Willoughby (2008), ethanol is mainly metabolized in hepatocytes by the cytoplasmic oxidation of ethanol into acetaldehyde by the enzyme alcohol dehydrogenase (ADH). ADH captures reducing equivalents $NADH+H^+$ by the coenzyme NAD^+. Alternatively, ethanol can be metabolized in the smooth endoplasmic reticulum of the hepatocyte by cytochrome P450 monooxygenase; the by-products of this reaction are acetaldehyde, water and FADH. Acetaldehyde is metabolized in mitochondria by acetaldehyde dehydrogenase using coenzyme NAD^+ forming acetate and $NADH+H^+$ (Ferreira and Willoughby, 2008).

Ethanol consumption during pregnancy can have rapid, direct and long lasting effects on the placenta and fetus (reviewed by Burd et al., 2007). In humans, maternal ethanol consumption can rapidly increase ethanol concentrations in the amniotic fluid and fetus (Brien et al., 1983; Idanpaan-Heikkila et al., 1972) and the blood ethanol concentration (BEC) in the fetus is similar to the mother (Espinet and Argiles, 1984; Guerri and Sanchis, 1985; Hill et al., 1983). After maternal BEC levels have normalized and ethanol is no longer present in maternal blood, ethanol can still be detected in the amniotic fluid (Brien et al., 1983) due to the slow rate of amniotic ethanol metabolism (Brien et al., 1983). Therefore, the fetus can be exposed to ethanol well after maternal cessation of drinking. Ethanol also rapidly promotes placental vasoconstriction (Acevedo et al., 1997; Burd et al., 2007), an effect that endures for the total time that ethanol is in

the body (Acevedo et al., 2001; Kay et al., 2000), and umbilical spasms can be induced with a dose of alcohol equivalent to one drink (Savoy-Moore et al., 1989). Altered umbilical and placental function can ultimately impair oxygen and nutrient transport to the fetus. Although ethanol metabolism is increased in pregnant rats (Badger et al., 2005) a common side-effect of ethanol consumption during pregnancy is reduced food intake (Abel, 1978; Ludena et al., 1983). Maternal under nutrition can subsequently increase fetal toxicity from ethanol (Shankar et al., 2007).

Previous studies indicate that a byproduct of alcohol, acetaldehyde, is also toxic to the developing fetus (Ali and Persaud, 1988; Hard et al., 2001; Lee et al., 2005b). The goal of the studies described in this thesis was not to distinguish whether ethanol or acetaldehyde produced the changes in offspring described below. It should be noted, however, that we cannot rule out the possibility that acetaldehyde may have contributed to some of the deficits observed.

1.2 Anatomy of the Hippocampus

The hippocampus was the structure of focus in the current thesis and as such it is necessary to review the anatomy of the hippocampus. Traditional nomenclature will first be discussed and then brief descriptions of laminar organization and cell structures for the CA1 and dentate gyrus will be given.

1.2.1 Nomenclature

The hippocampus (Greek for sea horse) is a bilateral structure that runs along the dorsal/ventral axis of the rodent brain. The hippocampal formation is composed of distinct subregions that include the fascia dentata (dentate gyrus; DG), cornu ammonis (CA), subiculum, presubiculum, parasubiculum, and entorhinal cortex. For clarity, the

term “hippocampus” in this thesis refers to the CA and DG regions shown in **Figure 1.3**. The rodent hippocampus is proportionally larger than the human hippocampus and, as such, extends across the septal (dorsal) and temporal (ventral) poles of the rodent brain. The CA is divided into three fields: CA1, CA2, and CA3. The CA1 is separated from the DG by a fissure and the CA3 extends into the region of the DG. The DG is a V-shaped structure. The portion of the V that abuts the hippocampal fissure (below CA1) is referred to as the suprapyramidal blade; the opposite portion is the infrapyramidal blade. The apex of the V is referred to as the crest. For clarification, the CA and/or DG are considered the target structure when discussing afferent and efferent connections. Projections from a structure to the CA, for example, are considered afferent connections and projections from the CA to different brain structures are considered efferent connections.

An anatomical review of the hippocampal formation and comparative analysis between the rodent and human hippocampus are beyond the scope of this thesis. Anatomy of the rodent CA1 and DG, however, will be discussed in detail and a few differences distinctions between the human and rodent hippocampus will be highlighted. Hippocampal anatomy is discussed in detail in The Hippocampus Book (Andersen, 2007) and is briefly reviewed below.

1.2.2 Cornu Ammonis

1.2.2.1 Laminar Organization of CA1

The CA is divided into distinct regions (CA1, CA2, CA3) and its principal cell layer is composed of pyramidal cells. For brevity, the CA1 will be described in the most detail, as this region was studied in the current thesis. The laminar organization of the CA1, shown in **Figure 1.4**, contains many different layers, the most superficial of which is the alveus.

The alveus is a collection of afferent and efferent axons (including axons from the principle cells of the CA1). Immediately ventral to the alveus is the stratum oriens (SO), which abuts the pyramidal cell layer (PCL). The CA1 SO is ~50-100 μm thick and contains basal dendrites of the pyramidal cells. Ventral to the PCL is the stratum radiatum (SR) and the stratum laconosum-moleculare (SL-M) is ventral to the SR. Apical dendrites of CA1 pyramidal cells extend across the SR and SL-M (Ishizuka et al., 1995) and terminate just dorsal to the hippocampal fissure.

1.2.2.2 Principle Cells of the CA1

The principle cell of CA1 region is the pyramidal cell. Pyramidal cells in the CA1 have a cell diameter of ~15 μm and the cell bodies are located in the PCL. Pyramidal cells in the CA1 are more densely packed than pyramidal cells in either the CA2 or CA3. Pyramidal cells send a single apical dendrite into the SR that can terminate in both the SR and SL-M (Ishizuka et al., 1995). Dendritic length is quite extensive and there are approximately 11.5 spines/10 μm on apical dendrites in the SL-M apical and approximately 8 spines/10 μm on basal dendrites in the SO (Gould et al., 1990). Spines are small protrusions from the dendrite that mediate the majority of excitatory contact in the brain (McKinney, 2010; Megias et al., 2001; von Bohlen Und Halbach, 2009). Axons from pyramidal cells project to different brain regions via the alveus.

1.2.2.3 Afferent and Efferent Connections of CA1

The CA1 receives afferent projection from different brain regions and projects to many regions (Meibach and Siegel, 1977a; Swanson, 1977; Swanson and Cowan, 1977). However, only afferent connections from the entorhinal cortex and CA3 will be described.

1.2.2.3.1 Entorhinal Cortex

Pyramidal cells in layer III of the entorhinal cortex send afferents to the CA1 via the perforant path and the alveus. In the more temporal aspect of the CA1, afferents from layer II of the entorhinal cortex follow a similar trajectory as the perforant path projection to the DG (described 1.2.3.3.1) but are more lateral to the dentate perforant path. This fiber bundle perforates the subiculum and terminates in the SL-M of the CA1 (Deller et al., 1996). The septal pole of the CA1 receives fibers from the entorhinal cortex via the alveus. The fibers perforate through the SO, PCL and SR to terminate in the SL-M (Deller et al., 1996).

Efferents from the CA1 project to many brain regions, including the entorhinal cortex via the alveus. Within the alveus, CA1 axons bifurcate with a branch that extends rostrally through the fornix and a branch that extends toward the entorhinal cortex (Cenquizca and Swanson, 2007).

1.2.2.3.2 CA3

The CA1 receives excitatory input from ipsilateral and contralateral CA3 via Schaffer Collatoral/Commissural projections (Blackstad, 1956; Fricke and Cowan, 1978). These fibers terminate on distal apical dendrites located in the SR and on basal dendrites in the SO.

1.2.3 Dentate Gyrus

1.2.3.1 Laminar Organization of the DG

The DG consists of three layers. The stratum granulosum (granule cell layer (GCL)) contains the principle cells of the DG (granule cells) and gives the DG the distinctive v-

shape. The GCL is the width of approximately 4-8 granule cell somata. The dorsal portion of the GCL is referred to as the suprapyramidal blade and the opposing blade is the infrapyramidal blade; the apex of the V-shape is referred to as the crest. The laconosum moleclare (molecular layer) abuts the GCL and extends ~250 μm to the hippocampal fissure. The molecular layer contains the dendrites of granule cells and afferent projections from layer II of the entorhinal cortex. The final region of the DG is the polymorphic cell layer located within the confines of the GCL. The polymorphic layer (also known as the hilus) contains excitatory and inhibitory interneurons and axons from DG granule cells that project to the CA3. The laminar organization of the DG is illustrated in **Figure 1.5**.

1.2.3.2 Principle Cells of the DG

Granule cells are the principal cells of the DG and give the DG the distinctive V-shape. The body of granule cells is approximately 10 μm in diameter and 18 μm in height (Claiborne et al., 1990) and ~4-8 granule cell somata compose the GCL. Dendrites from granule cells project into the molecular layer, but these projections can vary as a function of granule cell location within the GCL. For example, granule cells located deep within the GCL (closest to the hilus) send a single dendritic shaft through the GCL and have a more limited dendritic field. Granule cells in the more superficial region of the GCL (closer to the molecular layer) have a single dendritic shaft but a more distributed dendritic field (Seress and Pokorny, 1981). These two types of cells are present in the adult and neonate dentate gyrus with more superficial cells classified as more mature (Seress and Pokorny, 1981). Granule cell dendrites on the suprapyramidal blade have 1.6 spines/μm (Desmond and Levy, 1985) indicating there could be as many as 5600 spines

on a single granule cell neuron in the suprapyramidal blade. Unmyelinated axons of DG granule cells project into the polymorphic layer and send extensive collaterals that terminate in the polymorphic layer and the stratum lucidum of the CA3.

1.2.3.3 Afferent and Efferent Connections of DG

1.2.3.3.1 Entorhinal Cortex

The major excitatory afferent to the DG arises from layer II of the entorhinal cortex. Afferents from layer II of the entorhinal cortex project to the DG in a bundle of fibers (angular bundle) that perforate the subiculum and terminate in the outer two-thirds of the molecular layer. The lateral and medial portions of layer II afferents to the DG terminate in distinct regions of the molecular layer (Van Hoesen and Pandya, 1975); projections from the lateral perforant path (LPP) terminate in the outer third of the molecular layer while medial perforant path (MPP) projections terminate in the middle third of the molecular layer.

The MPP and LPP also exhibit distinct physiological characteristics. The waveform shown in **Figure 1.6** is an example of an extracellular recording obtained by recording from the hilar region of the DG while stimulating the MPP. The positive going deflection of the signal represents the excitatory post-synaptic potential (EPSP), which represents a brief depolarization of the postsynaptic membrane in response to stimulation of the MPP. The downward deflection superimposed on the waveform represents the near synchronous firing of DG granule cells and is called the population spike (Lomo, 1971). As the stimulating electrode is moved from the medial to lateral entorhinal cortex, several characteristics of the waveform change. Specifically, there is a larger delay in the onset of the EPSP and the half-width of the signal increases when moving from the medial to

lateral entorhinal cortex (McNaughton and Barnes, 1977). Additionally, stimulating the MPP results in a population spike that is located on the rising phase of the EPSP but LPP stimulation results in a population spike that is located on the falling phase of the EPSP, indicating that the onset latency of the pop spike changes depending on which pathway is stimulated (McNaughton and Barnes, 1977).

1.2.3.3.2 Commissural Projections of the Polymorphic Layer

Mossy cells of the polymorphic layer send contralateral and ipsilateral projections that terminate in the inner third of the molecular layer (Buckmaster et al., 1992; Frotscher et al., 1991; Laurberg and Sorensen, 1981). The hilus also receives commissural input from the contralateral DG (Hjorth-Simonsen and Laurberg, 1977). These associational/commissural projections are thought to arrive from mossy cells located in the polymorphic layer (Ribak et al., 1985). Stimulation of the commissural projection promotes inhibition of granule cells via GABA-A receptors (Douglas et al., 1983; Steward et al., 1990).

1.2.3.3.3 Mossy Fiber Afferents to the CA3

Granule cells send unmyelinated axons through the hilus to the ipsilateral CA3 referred to as the mossy fiber projection. These fibers terminate just above and below the pyramidal cell layer (Blackstad et al., 1970; Claiborne et al., 1986; Gaarskjaer, 1978); the region above the pyramidal cell layer is referred to as the stratum lucidum. The mossy fibers have en passant presynaptic terminals called mossy fiber expansions (Amaral and Dent, 1981), which form attachments with spines on the CA3 dendrites called thorny excrescences.

1.2.4 Tri-synaptic Circuit of the Hippocampus

Many brain regions project to the DG and CA1 but unilateral connections between the entorhinal cortex, DG, CA3 and CA1 (referred to as the "tri-synaptic circuit") (Anderson et al., 1971) is of importance to the current thesis. As illustrated in **Figure 1.7**, the first connection of the tri-synaptic circuit consists of ipsilateral afferent projections from layer II of the entorhinal cortex, referred to as perforant path projections, to the molecular layer of the DG (synapse 1). Mossy fiber afferents from the DG terminate on the proximal dendrites of ipsilateral pyramidal cells located in the stratum laconosum of the CA3 (synapse 2). Schaffer/collaterals from the CA3 bilaterally innervates CA1 pyramidal cells in the stratum radiatum (synapse 3). The effect of prenatal ethanol on Schaffer/collateral projections to the CA1 and perforant path connections to the DG was investigated in the experiments described below.

1.3 Functional Attributes of Hippocampus

The following section contains a brief overview of hippocampal function followed by evidence that distinct regions of the hippocampus differentially contribute to spatial learning and memory.

1.3.1 Overview of Hippocampal Function

The centrality of hippocampal function to memory was most evident following the publication of several case studies of individuals that suffered memory loss as a result of hippocampal damage (Scoville and Milner, 1957). One patient in particular, HM, shed light on the pivotal importance of the hippocampus for the episodic and spatial learning and memory (reviewed by Corkin, 2002). Hippocampal damage in early life can impair

long-term recall, spatial navigation and episodic memory (or memory for events) but IQ was in the low to average range (Vargha-Khadem et al., 1997) suggesting that the hippocampus is instrumental for declarative memory and spatial memory.

A given environment is composed of objects that are located within distinct regions of space. The objects have certain characteristics (e.g., size, shape, color, texture, etc.) that make the objects unique. Populations of cells (place cells) exhibit location-specific firing in a given environment (Eichenbaum et al., 1989), an observation that led to the formulation of the "cognitive map theory" of hippocampal function (O'Keefe and Nadel, 1978). According to this theory, spatial navigation through an environment is possible due to the construction and maintenance of spatial maps within the hippocampus. This is certainly feasible since hippocampal damage can lead to impaired spatial navigation and taxi drivers, who require good spatial navigation skills, have larger anterior hippocampi than non-taxi drivers (Maguire et al., 2000).

To more thoroughly probe the role of the hippocampus with spatial learning and memory, Maquire *et al.* (2006) performed an experiment that recruited an individual (TT) who had been a professional taxi driver in London for 37 years, until he suffered from limbic-encephalitus and was no longer able to work. Macquire and colleagues tested TT on his ability to recall spatial information and to navigate his way through the streets of London by using a virtual maze. TT could recognize landmarks, could approximate proximal relationships between objects, and could successfully orient himself within an environment (Maguire et al., 2006). However, TT's navigational skills in a simulated drive through London were impaired compared to control taxi drivers and TT was not able to navigate to the home where he had moved after his illness (Maguire et al., 2006).

Together, these studies indicate that the hippocampus is important not only for the acquisition of new spatial relationships (i.e., remember one's way to a new home) but also for successful navigation through a previously learnt environment. It was not until recently, however, that subregions of the hippocampus were found to differentially contribute to aspects of spatial learning and memory (Rolls and Kesner, 2006) as discussed below.

1.3.1.1 Subregion-specific Contribution to Hippocampus Function

1.3.1.1.1 CA1

The CA1 has been implicated with temporal processing in an environment. In 2001, Gilbert and colleagues investigated how lesions to the CA1 affected temporal processing of a spatial environment. The task consisted of an octagonal platform with 8 arms radiating from each side of the platform. A door located at the end of the arm that was closest to the platform prevented the animal from entering any of the arms. Training consisted of placing the animal in the platform with all the doors closed to each arm. The door of each arm was systematically opened across training trials enabling non-lesioned animals to enter each arm and retrieve the food reward. In the first trial, for example, the door to arm 1 was opened and the non-lesioned animal navigated to the end of the arm, retrieved the food reward and returned to the platform. The door of arm 1 was closed and the door for arm 4 was opened for the next trial. The same procedure was performed for all of the arms of the maze. The test trial occurred immediately after the presentation of the last arm. During the test trial, two doors were opened simultaneously and the animal was to enter the arm that had been presented first during training. In the above example, the correct choice would be to enter arm 1 and the wrong choice would be to enter arm 4.

Animals were trained to criterion on this task and then underwent surgery to lesion the CA1. Lesions to the CA1 impaired performance on this task but animals with lesions to the DG were not impaired. This indicates that the CA1 is important for temporal processing of spatial environments, which has been supported by other studies (Huerta et al., 2000; Tonegawa et al., 1996; Tsien et al., 1996).

1.3.1.1.2 Dentate Gyrus

An important aspect of spatial navigation is the location of objects within the environment. If two objects were proximally located then navigation in relation to those objects would be quite different than if 5 meters separated the objects. The DG might be preferentially recruited to process these metric attributes (Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2008). Novelty in the metric location of objects also involves the DG and CA3. For example, Lee *et al*. (2005) tested animals in a spatial-novelty detection task following lesions to CA1, CA3 or DG. Animals were given several trials to explore an environment that contained 5 objects. The location of one of the objects was moved following the exploration trials and the animal was then reintroduced to the environment. Intact animals and animals with lesions to the CA1 spent significantly more time exploring the displaced object. Lesions to either the DG or CA3, however, reduced exploration of the displaced object (Lee et al., 2005a). This indicates that the DG and CA3 are important for detecting novel spatial locations of previously familiar objects.

The role of the DG in spatial processing was further investigated using a spatial pattern separation task (Gilbert et al., 2001). In this study, rodents were exposed to a large, circular cheeseboard environment. An object was placed over a hole that was baited with food and the animal was to displace the object to retrieve the food reward. On

subsequent trials, the same food well was baited and an object was placed over the well. A second identical object was placed in the environment at a predetermined distance from the first object; the distance between the two objects was systematically altered across training trials. Once the animal learnt which object covered the food well, the DG was lesioned and animals were again tested on the task. Performance on this task was significantly impaired following lesions to the DG when the two objects were located close together in the environment; when the objects were spatially restricted within the environment, animals with DG were not impaired. CA1 lesions did not impair performance on this task regardless of the spatial distance between the objects. These findings suggest that the DG contributes to pattern separation of an environment.

1.4 Hippocampal Synaptic Plasticity

> "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."
>
> Donald Hebb, 1949

This postulate, put forth by Donald Hebb in 1949 (Hebb, 1949), was the formal description of the notion that neural organization was the basis of information storage in the brain (reviewed by Zielinski, 2006). The first experimental evidence that a population of cells can persistently influence the activity of a different population of cells was obtained in 1973. While recording from the rabbit DG, Bliss and Lomo (1973) found that brief, high frequency stimulation (HFS) of the entorhinal cortex lead to a persistent increase in synaptic efficacy of dentate gyrus granule cells in the rabbit (Bliss and Lomo, 1973). This discovery, subsequently coined long-term potentiation (LTP) (Douglas and

Goddard, 1975), spawned a field of neuroscience devoted to the elucidation of the mechanisms behind synaptic plasticity and the functional contribution of synaptic plasticity to information storage in the brain. The following is a brief overview of the mechanisms behind LTP and long-term depression (LTD) within the hippocampus. The first section will review the characteristics of LTP that make it an attractive model for a mechanism behind learning and memory and then the phases of LTP will be discussed. Within the descriptions of LTP phases will be an overview of the alleged mechanisms that contribute to each phase of LTP. Finally, putative mechanisms behind LTD will be reviewed.

1.4.1 LTP as a Mechanism behind Learning and Memory

Since the discovery that HFS can induce changes in the activity of a population of cells (Bliss and Lomo, 1973), many characteristics of LTP have been found supporting the notion that synaptic plasticity might contribute to learning and memory (Bliss and Collingridge, 1993). For example, LTP was originally found to persist for days (Bliss and Gardner-Medwin, 1973) but has recently been shown to last at least up to one year in the rat (Abraham et al., 2002) indicating that LTP can be long lasting. LTP also exhibits *input specificity* (Andersen et al., 1977): pathways that received HFS will be potentiated but neighboring pathways will not. In order for potentiation to occur, however, a minimum level of stimulation is required (McNaughton et al., 1978) suggesting that stimulation must be sufficiently strong to induce long lasting changes in synaptic efficacy, a principle known as *cooperativity* (McNaughton et al., 1978). Although LTP is input specific, potentiation of a neighboring pathway can occur under certain circumstances. Potentiation of a weakly stimulated pathway can occur if the stimulation

is paired (or active) at the same time as a more strongly stimulated convergent pathway, a phenomenon known as *associativity* (Levy and Steward, 1979; McNaughton et al., 1978). These findings indicate that a minimum threshold of excitation must be achieved (*cooperativity*) to elicit long lasting changes in the synaptic efficacy of a specific population of neurons (*input specificity*) and paired activity of two convergent pathways can promote LTP in a weakly stimulated path (*associativity*).

1.4.2 Phases of LTP

The change in EPSP slope that results following HFS goes through distinct phases that are illustrated in **Figure 1.8**. The following is a description of those phases and the mechanisms associated with each phase. It is first important, however, to briefly review two types of receptors that have been largely implicated with hippocampal synaptic plasticity, namely the N-methyl-D-aspartate receptor (NMDAR) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR).

1.4.2.1 NMDAR

The NMDAR is a cation permeable, heteromeric receptor predominantly located on the postsynaptic membrane. NMDARs are composed of two obligatory NR1 subunits and a combination of NR2 and NR3 subunits (Cull-Candy et al., 2001; Dingledine et al., 1999; Hollmann et al., 1994). Each NMDAR subunit contains three transmembrane domains with a single re-entrant loop that forms the pore of the channel (Dingledine et al., 1999). The NR2 class of subunits contains four variants (NR2A, 2B, 2C and 2D) but attention will be focused on NR2A and NR2B subunits. NR3 subunits (NR3A and 3B) have only recently been discovered (Nishi et al., 2001; Sun et al., 1998). Subunit composition of the NMDAR largely determines the functional characteristics of the receptor (Cull-Candy et

al., 2001). High conductance channels are composed of NR2A and NR2B subunits while low conductance channels are largely composed of either NR2C or NR2D subunits (Stern et al., 1992). NR2A-containing NMDARs also have a faster deactivation rate than NR2B-containing NMDARs (Cull-Candy et al., 2001).

NMDAR "activation" requires several events. Current flux through NMDARs increases with depolarization of the postsynaptic membrane due to the voltage-dependent blockade of NMDARs by magnesium (Mg^{2+}) (Nowak et al., 1984). Glycine must first be bound to the NR1 subunit before glutamate is able to activate NMDARs (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Glycine potentiates the response of NMDARs (Johnson and Ascher, 1987) and it has been suggested that this augmentation might arise from activity-dependent release of glycine (Li et al., 2009). That NMDARs require postsynaptic depolarization and binding of two co-agonists makes it an attractive coincidence detector: coordinated pre and postsynaptic activity (i.e., glutamate/glycine release and postsynaptic depolarization) promotes calcium influx through NMDARs (Wigstrom and Gustafsson, 1986). These properties of the NMDAR might allow for input specificity and cooperativity that are central components of LTP.

1.4.2.2 AMPAR

AMPA receptors are composed of four different subunits (glutamate receptor-GluR1-4) (Ozawa et al., 1998) and are permeable to sodium (Na^{+}) and potassium (K^{+}). Depending on subunit composition, however, AMPARs are also permeable to calcium (Ca^{2+}). AMPARs are impermeable to Ca^{2} if the GluR2 subunit is present (Dingledine et al., 1999; Hollmann et al., 1991; Jonas et al., 1994) and GluR1-2 and/or Glur2-3 subunits are highly expressed in adult hippocampal pyramidal cells (Martin et al., 1993; Wenthold et

al., 1996). Glutamate binds to AMPARs (Monaghan et al., 1985; Stein et al., 1992) and AMPARs deactivate quickly following the clearance of glutamate from the synapse (Colquhoun et al., 1992). The high open probability of AMPARs (Jonas et al., 1993) makes it an ideal receptor to mediate basal synaptic transmission (Jonas, 1993).

1.4.2.3 Post-tetanic Potentiation

Immediately following tetanic stimulation, the EPSP can significantly increase from baseline for a short period of time and is referred to as post-tetanic potentiation (PTP). This initial increase in EPSP slope is independent of NMDAR activation and decays over a short period of time (within two minutes following HFS) (Stevens et al., 1994; Volianskis and Jensen, 2003). During HFS, calcium can accumulate in the presynaptic terminal. Blocking mitochondrial efflux of calcium blocked PTP (Tang and Zucker, 1997) suggesting that PTP results from the clearance of calcium from the presynaptic terminal (Tang and Zucker, 1997).

1.4.2.4 Short-term Potentiation

Following the decay of PTP, a short-lasting potentiation can be observed called short-term potentiation (STP). A presynaptic mechanism underlies STP (Volianskis and Jensen, 2003). Following HFS, STP quickly decays following basal stimulation. If HFS is applied and basal stimulation is not resumed for a period of 6 hours, however, STP will not have decayed (Volianskis and Jensen, 2003) but will rapidly decay with stimulation. These findings suggest that, like PTP, STP results from a presynaptic mechanism. Unlike PTP, STP decays predictably following stimulation: a faster decay results from the application of more test stimuli (Volianskis and Jensen, 2003).

1.4.2.5 Early Long-term Potentiation

Induction of early long-term potentiation (E-LTP) depends on the complex coordinated activity of pre-and post-synaptic components. LTP depends on synaptic transmission (Dunwiddie et al., 1978) and induces the passage of current through NMDARs (Wigstrom and Gustafsson, 1986) indicating a coordinated relationship between presynaptic neurotransmitter release and subsequent NMDAR activation. Stimulation patterns that are sufficiently strong to depolarize the postsynaptic membrane result in the release of Mg^{2+} block and allow calcium to influx through NMDARs (Dingledine, 1983; Frank et al., 1989; Melchers et al., 1988) ultimately activating a variety of downstream pathways necessary for the expression and maintenance of LTP.

E-LTP is expressed by recruiting several kinases such as protein kinase c (PKC) (Asztely et al., 1990; Wang and Feng, 1992), calcium-calmodulin-dependent kinase (CaMK) (Malenka et al., 1989; Miyamoto and Fukunaga, 1996; Reymann et al., 1988) and mitogen-activated protein kinase (MAPK) (Giovannini, 2006; Miyamoto, 2006; Waltereit and Weller, 2003). For example, application of a PKC inhibitor blocked E-LTP but PTP and STP remained intact (Lovinger et al., 1987); similar results have been obtained for CaMKII (Malinow et al., 1988). Alpha CamKII expression transiently increased in the soma following HFS with a more persistent increase found in the dendrites (Thomas et al., 1994). Insertion of a constitutively active form of CamKII into hippocampal neurons induced LTP and prevented potentiation by a HFS (Pettit et al., 1994) indicating that CamKII is both necessary and sufficient to generate LTP (Lledo et al., 1995). Since multiple protein kinase inhibitors reduce E-LTP it is therefore possible that multiple kinases are recruited during E-LTP but that some more potently block E-

LTP than others (Hvalby et al., 1994). Induction of LTP can also increase the activation of src tyrosine kinase (Lu et al., 1998) indicating that multiple intracellular pathways can be activated during the induction of LTP. These results indicate that PTP and STP do not recruit kinase activity whereas kinase activation is central to E-LTP.

The actions of different protein kinases on downstream targets are complex but kinases can promote changes that underlie LTP. PKC can phosphorylate the NR1, 2A and 2B subunit of NMDARS (Grosshans and Browning, 2001; Tingley et al., 1993) effectively increasing NMDAR-mediated responses (Urushihara et al., 1992). Activation of NMDARs can, in turn, promote AMPAR insertion into the post-synaptic membrane (Lu et al., 2001). Activation of src tyrosine kinase can enhance AMPA- and NMDA-mediated excitatory postsynaptic currents (EPSC) (Yu et al., 1997b) possibly by reducing tonic inhibition of NMDARs imposed by zinc (Zheng et al., 1998). Src-mediated enhancement of NMDAR EPSCs (Yu et al., 1997a; Yu et al., 1997b; Yu and Salter, 1999) is fitting with evidence that AMPA- and NMDA-mediated responses are enhanced following HFS (O'Connor et al., 1995). CamKII is closely associated with NMDARs (Leonard et al., 1999) and autophosphorylation of CaMKII promotes direct binding of CaMKII to the NR2B subunit of NMDARs (Strack and Colbran, 1998). There is also evidence that CaMKII can phosphorylate NR2A subunits (Caputi et al., 1999). LTP increases tyrosine phosphorylation of NR2B subunits in vivo (Rosenblum et al., 1996) which might contribute to the maintenance of LTP (Rostas et al., 1996). Together these findings indicate that multiple kinase systems can be recruited following HFS and perhaps the coordinated activity of these kinases promotes the transition of E-LTP into L-LTP.

1.4.2.6 Late Long-term Potentiation

1.4.2.6.1 Protein Transcription

Evidence that transcription might be involved in late long-term potentiation (L-LTP) came from studies showing an increase in the expression of immediate early genes following HFS (Abraham et al., 1993; Cole et al., 1989; Correia et al., 2008; Demmer et al., 1993). Protein transcription inhibitors can block L-LTP (Frey et al., 1996; Nguyen and Kandel, 1996) providing further support that protein transcription is central to L-LTP. Phosphorylation of cyclic-AMP binding protein (CREB) is increased during L-LTP (Deisseroth et al., 1996; Schulz et al., 1999; Segal and Murphy, 1998) as is cyclic-AMP response element (CRE)-mediated transcription (Impey et al., 1996). Two peaks of CREB phosphorylation occur during LTP with the first increase at 30 minutes post-HFS and the second at 2 hours post-HFS (Schulz et al., 1999). This pattern of CREB phosphorylation indicates that LTP-inducing stimulation promotes fast and delayed nuclear signaling. Nuclear CaMKIV has been found to phosphorylate CREB (Bito et al., 1996; Ho et al., 2000; Kang et al., 2001) and it has been proposed that CaMKIV and MAPK contribute to the rapid and delayed changes in CREB phosphorylation, respectively, following HFS (Wu et al., 2001).

1.4.2.6.2 Protein Translation

The first evidence that LTP was accompanied by protein synthesis came from Duffy and colleagues in 1981. They found that LTP was associated with an increase in the secretion of membrane bound proteins (Duffy et al., 1981), a finding supported by subsequent studies (Krug et al., 1984; Stanton and Sarvey, 1984). Application of a broad spectrum inhibitor of protein translation during HFS did not block E-LTP but potentiation decayed

3-4 hours after the HFS (Krug et al., 1984). This suggests that protein translation is pivotal to L-LTP but not E-LTP. Blocking messenger ribonucleic acid (mRNA) synthesis (e.g., actinomycin D), however, did not affect L-LTP (Otani and Abraham, 1989) and dendritic protein synthesis can contribute to L-LTP (Steward and Schuman, 2001). Taken together, these studies indicate that the protein synthesis from existing mRNA in dendrites might contribute to the expression of L-LTP. Surprisingly, application of protein synthesis inhibitors prior to HFS impaired the expression of LTP past 6 hours (Otani and Abraham, 1989) indicating that the proteins responsible for this expression are synthesized during E-LTP and that the time window for L-LTP is approximately 6 hours post HFS.

1.4.2.7 Temporal Aspect of the Mechanisms that Contribute to L-LTP

Although different mechanisms can contribute to the phases of LTP, it is likely that successful passage through one phase of LTP is required before subsequent phases of LTP can exist. That is, E-LTP cannot be expressed until STP has expired and L-LTP will not be expressed in the absence of E-LTP. Protein kinase activity might only be required for a specific period of time before other mechanisms take over for the expression of late phase LTP (Huber et al., 1995), for example. Application of a broad spectrum protein kinase inhibitor before or during HFS did not alter the expression of late-phase LTP yet kinase inhibition immediately following HFS did reduced late-phase LTP (Huber et al., 1995). This suggests that protein synthesis-dependent LTP does, at some level, require kinase activation.

1.4.2.8 AMPAR Trafficking Following HFS

Both NMDA and AMPA receptors have putative roles in the induction of LTP (Izumi et al., 1987) although subsequent studies indicate that NMDARs more directly contribute to the induction of LTP while non-NMDARs (i.e., AMPARs) are more instrumental for the expression of LTP (Davies and Collingridge, 1989; Muller et al., 1988). Blocking NMDARs can prevent LTP (Morris et al., 1986). Since NMDARs are calcium permeable, blocking NMDAR activity during HFS might prevent the activation of several downstream cascades that contribute to LTP, discussed above. Another mechanism through which NMDARs might contribute to LTP is via the trafficking of AMPARs, which is important for LTP (Malinow, 2003; Malinow and Malenka, 2002). Activation of NMDARs can promote AMPAR insertion into the post-synaptic membrane (Lu et al., 2001; Pickard et al., 2001) from recycling endosomes (Brown et al., 2007; Park et al., 2004). Phosphorylation of GluR1 subunits following HFS contributes to synaptic trafficking of AMPARs (Boehm and Malinow, 2005) and newly inserted AMPARs are not calcium permeable (Adesnik and Nicoll, 2007; Gray et al., 2007). PKA signaling is required for AMPAR insertion following HFS (Yang et al., 2008) and promotes AMPAR insertion by phosphorylating Ser845 on the GluR1 subunit (Roche et al., 1996). Therefore, downstream cascades activated via NMDARs can contribute not only to the induction but also the expression of LTP.

1.4.3 Theta-burst Stimulation

Within the hippocampus, stimulation patterns that mimic theta-activity can induce stable LTP. Theta activity in the hippocampus results from populations of cells that exhibit burst firing at approximately 6-10 Hz and this pattern of activity is present under urethane

anesthesia (Kramis et al., 1975). The first indication that theta-patterned stimulation can induce LTP was obtained in 1984 when Bawin and colleagues found that 5 Hz stimulation of the CA1 induced stable LTP (Bawin et al., 1984). This finding was supported by subsequent research (Larson et al., 1986; Staubli and Lynch, 1987) and was even extended to the DG (Pavlides et al., 1988). Theta-burst stimulation (TBS) consists of a series of bursts and each burst can contain a number of pulses that are delivered at specific frequencies (e.g., 4 pulses at 100 Hz). Application of 4 pulses at 100 Hz does not induce LTP unless the pulses are delivered in bursts separated by 200 ms (Larson et al., 1986) coinciding with the positive phase of theta (Pavlides et al., 1988).

Application of TBS induces LTP through the coordinated activity of NMDARs and γ-Aminobutyric acid receptors (GABARs). Two types of GABA receptors mediate the majority of inhibitory signaling: $GABA_A$ and $GABA_B$ receptors (Dutar and Nicoll, 1988a; Dutar and Nicoll, 1988b). Application of TBS in the presence of a $GABA_B$ receptor antagonist blocked LTP (Brucato et al., 1996; Mott and Lewis, 1991) indicating that $GABA_B$-mediated signaling is important for TBS LTP. $GABA_B$ receptors also induce burst firing in the hippocampus (Mott et al., 1989) and $GABA_B$ antagonists block the burst activity seen during TBS (Mott and Lewis, 1991). Application of stimuli spaced 200 ms apart coincides with the peak decay of $GABA_B$ –mediated inhibitory current (Mott et al., 1993), which might contribute to the prolongation of the NMDAR-mediated EPSP when bursts of stimuli are delivered 200ms apart (Larson and Lynch, 1989; Mott and Lewis, 1991). Taken together, it has been suggested that GABA release during the first stimulus of the TBS train activates $GABA_B$ receptors that then disinhibit NMDARs

resulting in an influx of calcium and activation of downstream cascades that promote LTP.

1.4.4 Long-term Depression

Thus far the discussion about synaptic plasticity has revolved around LTP, but an equally important form of synaptic plasticity is long-term depression (LTD). First, it is important to mention that two forms of LTD exists, namely heterosynaptic depression and homosynaptic depression. Stimulation of one pathway that is of sufficient strength and intensity to induce LTP can result in long-term depression of a neighboring pathway (Abraham et al., 1985; Abraham and Goddard, 1983; Alger et al., 1978) collectively referred to as heterosynaptic depression. Homosynaptic depression, on the other hand, is a reduction of the EPSP slope in response to prolonged periods of low frequency stimulation of a single pathway and was first described by Dudek and Bear in 1992 (Dudek and Bear, 1992). LTD can depend on NMDARs (Dudek and Bear, 1992), which is surprising given the involvement of NMDARs with LTP. However, the differential contribution of NMDARs to LTP and LTD lies not only in the possible contribution of different NMDAR subunits but also with AMPAR regulation and the dynamic interplay between protein kinase and phosphatase activity. The following is a brief discussion of the involvement of LTD in learning and memory as well as the putative mechanisms that underlie LTD in the hippocampus.

1.4.4.1 LTD as a Model of Learning and Memory

Acute stress can reduce LTP but enhance LTD (Shors and Thompson, 1992; Xiong et al., 2004; Xu et al., 1998) and impaired spatial performance following acute stress has been attributed to enhanced LTD (Kim et al., 1996; Wong et al., 2007). It is possible, however,

that bidirectional synaptic plasticity is recruited during hippocampal-dependent learning and memory. For example, the detection of novelty within an environment can induce LTD in the CA1 (Manahan-Vaughan and Braunewell, 1999). Not only does LTD contribute to spatial learning (Duffy et al., 2008) but the magnitude of LTD is significantly correlated with spatial performance (Nakao et al., 2002). There is also evidence that spatial learning can actually reduce CA1 LTP (Makhracheva-Stepochkina et al., 2008). Interestingly, exploration of specific components of an environment such as novel objects or a novel empty environment can promote CA1 LTD and LTP, respectively, (Kemp and Manahan-Vaughan, 2004) suggesting that bidirectional synaptic plasticity can differentially encode specific aspects of the environment (Kemp and Manahan-Vaughan, 2008). Taken together, these studies indicate that LTD is just as important as LTP for spatial learning and memory and if the capacity for bidirectional synaptic plasticity is altered (i.e., shifted toward LTD following acute stress) then the overall functionality of the hippocampus might be impaired.

1.4.4.2 Protein Phosphatase Involvement in LTD

The removal of a phosphate group from proteins is accomplished by protein phosphatases and this process is intimately involved in the induction and expression of LTD (Mulkey et al., 1993). After this discovery, the mechanisms behind LTD were quickly elucidated. Following low frequency stimulation (LFS) calcineurin (a protein phosphatase) is activated that, in turn, dephosphorylates inhibitor-1 (Mulkey et al., 1994). Through this inactivation of inhibitor-1, protein phosphatase 1 (PP1) is activated (Mulkey et al., 1994) and activity of protein phosphatase 2A (PP2A) rapidly increases following LFS that is sufficient to induce LTD (Thiels et al., 1998).

The relative activity of either protein kinases or phosphatases can influence whether LTP or LTD will result. Stimulation frequencies greater than 25 Hz results in LTP while low stimulation between 1-5 Hz results in LTD; frequencies in between these ranges (e.g., 10 Hz) does not induce LTP or LTD (Dudek and Bear, 1992). However, LTD could be induced following 10 Hz stimulation if protein kinases are blocked and/or extracellular calcium levels were reduced (Coussens and Teyler, 1996). Furthermore, inhibition of protein phosphatases and/or increased extracellular calcium facilitated LTP following 10 Hz stimulation (Coussens and Teyler, 1996). These data indicate that the overall activity levels of protein kinases and phosphatases might influence whether LTP or LTD can be induced. Calcineurin has a greater affinity for calcium than CaMKII (Klee et al., 1979; Schulman and Lou, 1989; Stewart et al., 1983) suggesting that phosphatases might be preferentially activated with lower levels of calcium than kinases. Therefore, during low frequency stimulation or when intracellular calcium concentrations are relatively lower (i.e., compared to HFS) then preferential activation of phosphatases might occur and thus result in LTD.

1.4.4.3 AMPA and NMDA Receptor Involvement in LTD

LTD in the hippocampus can be NMDAR-dependent (Dudek and Bear, 1992) indicating that the NMDARs are intimately involved with both LTP and LTD in the hippocampus. The differential contribution of these receptors to bidirectional synaptic plasticity lies not only in the downstream cascades that are recruited following stimulation but also with differential AMPAR trafficking (reviewed by Malenka, 2003; Sheng and Kim, 2002). Several studies have reported that clathrin-mediated endocytosis of AMPARs mediates the expression of LTD (Man et al., 2000; Wang and Linden, 2000) indicating that

removal of AMPAR from the postsynaptic membrane is required for LTD. Soon after these initial discoveries it was found that NMDAR-mediated activation of calcineurin can contribute AMPAR internalization (Beattie et al., 2000; Gellerman et al., 1997). The GluR2 subunit of AMPARs is associated with N-ethylmaleimide-sensitive fusion protein (NSF) and this association promotes AMPAR stabilization at the membrane (Lee et al., 2002). The GluR2 subunit also contains a binding site for AP2, a protein involved with clathrin-mediated endocytosis (Kirchhausen, 1999), overlaps with NSF (Lee et al., 2002) and blocking the actions of AP2 impairs LTD (Lee et al., 2002). Overall, these studies suggest that NMDA-induced calcineurin activation following LFS promotes AP2/clathrin associations with GluR2 subunits, thus promoting AMPAR internalization (Sheng and Kim, 2002).

1.4.4.4 NMDAR Subunit Contribution to Bidirectional Synaptic Plasticity

Previous studies have shown that specific NMDAR subunits differentially contribute to bidirectional synaptic plasticity (Hrabetova et al., 2000). Application of antagonists specific to NR2A-containing NMDARS blocked LTP in the CA1 region of the hippocampus but left LTD intact. On the other hand, antagonists specific for NR2B-containing NMDARs blocked LTD but left LTP intact (Liu et al., 2004). These findings have received mixed support within the literature (Berberich et al., 2005; Fox et al., 2006; Hendricson et al., 2002; Morishita et al., 2007). NR2A-containing NMDARs can target AMPARs to the membrane while activation of NR2B-containing NMDARs promotes AMPAR internalization (Kim et al., 2005; Tigaret et al., 2006) further supporting the notion of subunit specific contribution to synaptic plasticity. Subunit contribution to LTP within the DG, however, might be different. For example, LTP in the DG increases

tyrosine phosphorylation of NR2B subunits (Rosenblum et al., 1996) and specifically increases NR2B subunit expression (Thomas et al., 1996; Williams et al., 1998). These studies suggest a complex and region specific contribution of NMDAR subunits to bidirectional synaptic plasticity in the hippocampus.

1.5 Sexual Differentiation

The first portion of this section describes sexual development during gestation followed by a description of the hypothalamic-pituitary-gonadal (HPG) axis. The aromatization hypothesis is briefly discussed in terms of sexual dimorphism of the hippocampus. Although peripubertal animals were used in the current thesis, a description of the rodent estrous cycle is given as well as a brief discussion on hormonal changes that occur during puberty. Finally, the activational effects of estradiol and testosterone are discussed in order to highlight possible influences of pubertal hormones on the plasticity observed in the current studies.

1.5.1 Gonadal and Genital Development

The sequence of events that contribute to sexual differentiation in mammals will be briefly described in the current section but a more thorough account can be found in Wilson, 1978. Chromosomal sex determination occurs the moment of ovum fertilization. A single ovum contains 22 autosomes and one X chromosome while spermatozoa contain 22 autosomes and either an X or a Y chromosome. If the fertilizing sperm has an X-chromosome, then the zygote will have 46 chromosomes with an XX composition and will thus be genetically female; 46 chromosomes with an XY composition is genetically male. Early in development, the gonads of an XX and XY fetus are indistinguishable and are often referred to as indifferent organs (**Figure 1.9**). The presence of a gene located on

the short arm of the Y chromosome, called the sex-determining region of the Y chromosome (Sry) (Sinclair et al., 1990), will determine if the indifferent organ will develop into testes (male) or ovaries (female) (Berta et al., 1990). In the presence of Sry, the indifferent gonads will develop into testes whereas ovaries will develop in the absence of Sry.

Testicular secretion of hormones contributes to the masculinization of the gonads. The indifferent gonads of XX and XY fetuses have two ducts connecting the gonads to the body wall, namely the Müllerian duct and the Wolfian duct, shown in **Figure 1.9**. Secretion of anti-Müllerian hormone (AMH) by the testes causes the Müllerian duct to regress. Ovaries do not secret AMH and in the absence of AMH the Müllerian duct will develop into fallopian tubes, uterus and inner portion of the vagina. Testicular secretion of testosterone promotes the virilization of the Wolfian duct by the development of epididymis, vas deferens and seminal vesicles. An enzyme in the genital skin (5-α-reductase) amplifies the masculinization process by converting testosterone into dihydrotestoserone (DHT) thus promoting the development of the penis and scrotum.

1.5.2 Hypothalamic-Pituitary-Gonadal Axis

The HPG axis contributes to the synthesis of sex hormones, of which testosterone and estradiol will be the focus of this thesis. Activation of the preoptic area of the hypothalamus can promote the release of gonadotropin releasing hormone (GnRH) into the bloodstream at the median eminence. GnRH travels through the hypophyseal portal system to the anterior pituitary where it binds to GnRH receptors on gonadotrophs located in the anterior pituitary to stimulate the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In males, LH acts on leydig cells

located in the testes to stimulate the production of testosterone, while LH and FSH work in concert to promote the maturation of spermatozoa. In females, LH stimulates the production of estradiol and progesterone in the ovaries. FSH contributes to the development of the ovarian follicle but both FSH and LH are required for estradiol secretion from the follicle. It is important to note that although testosterone and estrogens are typically denoted as the "male" and "female" hormone, respectively, estradiol is present in males and testosterone is present in females (Armstrong et al., 1975; Dorrington and Armstrong, 1975; Erickson and Ryan, 1976; Fortune and Armstrong, 1977; Moor, 1977; Younglai, 1972), albeit in much lower levels than the opposite sex.

1.5.3 Aromatization Hypothesis

Testosterone and estradiol can promote the organization and activation of different brain regions. Organizational effects are considered permanent or long lasting changes that occur following hormonal exposure during the perinatal peiord. Activational effects, on the other hand, persist only in the presence of the hormone during adolescence and adulthood. During neonatal development, estradiol can exert organizational influence on brain regions to promote sexual differentiation. A potent estrogen binding protein, α-fetoprotein, is present in liver and yolk sack (Gitlin and Boesman, 1967) of fetal rats. Maternal estradiol is bound by α-fetoprotein (Attardi and Ruoslahti, 1976; Keel and Abney, 1984; Vannier and Raynaud, 1975) effectively protecting against masculinization and defeminization of the female brain (Bakker et al., 2006). Fetal ovaries begin to synthesize estradiol around PND5 (Weniger, 1993; Weniger and Zeis, 1987; Weniger et al., 1993) but are not active *in utero* (Csernus, 1986; Weniger, 1993; Weniger et al., 1993) indicating that circulating estradiol derived from a neighboring female fetus does

not largely contribute to the masculinization of the male brain by estradiol. Testosterone, however, is not bound by α-fetoprotein and can be aromatized to estradiol. Testicular secretion of testosterone can occur as early as GD17 (Csernus, 1986), which coincides with a period of brain sexual differentiation. Aromatase activity is significantly higher in males than females, particularly in the hypothalamus (Beyer et al., 1993; George and Ojeda, 1982), a brain region that exhibits distinct sexual dimorphism.

Interestingly, estradiol levels gradually increase in the brain of male and female fetuses during embryonic days 15-19. Brain aromatase activity does not increase during this period (MacLusky et al., 1985) but maternal estradiol levels do steadily increase toward the end of gestation (Shaikh, 1971; Taya and Greenwald, 1981). Changes in fetal levels of α-fetoprotein toward the end of gestation might also facilitate fetal exposure to maternal estradiol. Levels of α-fetoprotein drop dramatically after birth and continue to decrease until PND 6-8 and remain constant throughout adulthood (Gitlin and Boesman, 1967; Masseyeff et al., 1975). During PND 1-3, aromatase activity is significantly higher in the male brain than the female brain (MacLusky et al., 1985) and, since female ovaries do not synthesize estradiol until PND5 (Weniger, 1993; Weniger and Zeis, 1987; Weniger et al., 1993), the male brain is once again exposed to higher levels of estradiol than the female brain. These findings suggest that there are two periods during perinatal development where the male brain is exposed to higher levels of estradiol than the female brain through the aromatization of testosterone to estradiol.

1.5.4 Sexually Dimorphic Nucleus of the Preoptic Area

Evidence in favor of the aromatization hypothesis for the masculinization of the male brain originated from studies conducted on the sexually dimorphic nucleus of the pre-

optic area (SDN-POA) (Raisman and Field, 1973). Although not the brain region of focus within the current thesis, the effects of aromatized testosterone on the SDN-POA will be discussed to highlight how this process of aromatized testosterone can contribute to the sexual differentiation of the brain. The possible involvement of this process in the sexual differentiation of the hippocampus will then be discussed.

Inactivation of the SDN-POA in males can reduce copulatory behavior (Hurtazo et al., 2008) indicating that this region contributes to sexual behavior in the adult rat. The SDN-POA is significantly larger in males than females but removal of the testes (orchidectomy) on PND1 reduced the size of the SDN-POA in males by 50% (Jacobson and Gorski, 1981). Neonatal treatment of females with testosterone propionate (an aromatizable form of testosterone) significantly increased the size of the SDN-POA to a size similar to control males (Dohler et al., 1982; Jacobson et al., 1981) and subsequently masculinized sexual behavior in adult females (Sachs and Thomas, 1985). Gestational exposure to testosterone can also influence the morphology of the SDN-POA. Exposure to testosterone on GD17, but not 21, can masculinize the SDN-POA in females (Ito et al., 1986). Unlike postnatal exposure, however, gestational exposure to testosterone did not masculinize sexual behavior in the adult female rat (Ito et al., 1986). These studies suggest that sex differences in brain structure and sexual behavior can be influence by the aromatization of perinatal testosterone into estradiol.

Testosterone-induced changes to cell proliferation and survival within the SDN-POA might influence the size of the SDN-POA. By injecting pregnant rats with H(3)-thymidine (thy) on the latter part of gestation, Jacobson and Gorski (1981) investigated the development of the SDN-POA in fetal rats (Jacobson and Gorski, 1981). Thy is

incorporated into the DNA of cells undergoing mitosis (cell division) (Schultze and Oehlert, 1960). On GD17, the female SDN-POA contained more thy-positive cells than males, but this difference reversed favor of males by GD17 (Jacobson and Gorski, 1981). Increased apoptosis (or cell death) in the female SDN-POA between PND7-10 may further reduce the size of the SDN-POA (Davis et al., 1996). Neonatal treatment of females with testosterone propionate abolished the sex difference in apoptosis (Davis et al., 1996). Interestingly, estradiol might actually promote apoptosis in the SDN-POA in females (Tsukahara et al., 2008) but reduces apoptosis in males (Vancutsem and Roessler, 1997). The level of pro-apoptotic proteins are increased by estradiol in neonatal females (Tsukahara et al., 2008) whereas estradiol-induced increases in the protein levels of NR1 can minimize apoptosis in the male SDN-POA (Hsu et al., 2001). These studies suggest that perinatal androgen exposure can produce distinct effects on brain development.

1.5.5 Putative Role of Perinatal Androgens in Hippocampal Sexual Differentiation

Although not studied in as much detail as the SDN-POA, there is evidence that gonadal hormones can influence the differentiation of the hippocampus. Within the DG, males have significantly more granule cells than females (Roof and Havens, 1992; Severi et al., 2005) and the volume of the GCL is greater in males than females (Tabibnia et al., 1999). There have also been reports of left/right asymmetry in the male hippocampus but not in the female hippocampus (Roof and Havens, 1992; Tabibnia et al., 1999), although this effect might be specific to the type of strain studied (Tabibnia et al., 1999). Granule cells in the neonatal male hippocampus exhibit greater dendritic branching than in the female hippocampus and this sex difference persists throughout puberty (Bartesaghi et al., 2003).

Additionally, it has been reported that the CA1 SO is larger in males compared to females (Lavenex et al., 2000). Sex-differences in granule cell size and spatial processing were abolished in females that were treated neonatally with testosterone propionate (Roof and Havens, 1992) but testosterone itself can also directly masculinize the hippocampus (Zhang et al., 2008). It is therefore possible that perinatal androgen exposure can masculinize the hippocampus, although this needs to be further investigated.

1.5.6 Rodent Estrous Cycle

The rodent estrous cycle lasts 4-5 days and consists of four distinct phases: proestrus, estrus, metestrus and diestrus (Haim et al., 2003; Long and Evans, 1922). Each phase of the cycle is characterized by specific vaginal cytology as the cells of the vaginal wall desquamate (**Figure 1.10**). Proestrus lasts for 12-14 hours and is characterized by round nucleated cells. Estrus lasts for 25-27 hours and is characterized by irregularly shaped, un-nucleated cells. Metestrus lasts 6-8 hours and is characterized by an increase in leukocytes. Finally, diestrus lasts 55-57 hours and is characterized by the presence of mainly leukocytes but nucleated cells can also be present. The highest levels of testosterone and estradiol are present on the afternoon of proestrus but rapidly decline toward the end of proestrus (Haim et al., 2003). Progesterone levels peak during the night of proestrus and are transiently elevated during the dark phase of metestrus (Haim et al., 2003). Testosterone and estradiol levels begin to rise again during the light phase of diestrus to peak again during proestrus (Haim et al., 2003). Hormonal changes during the estrous cycle might be due to fluctuations in gonadotrophin levels. LH and FSH are highest on the afternoon of proestrus (Gay et al., 1970), possibly stimulating estradiol.

Thereafter, FSH gradually decreases while LH levels over the following three days with minimal LH present during the dark phase of the light cycle (Gay et al., 1970).

1.5.7 Gonadal Hormone Receptor Localization in the Hippocampus

Estrogen receptors (ERs) are expressed in the hippocampus (Loy et al., 1988; Maggi et al., 1989; Shughrue et al., 1997) and have region and cell specific expression. Two types of ERs (ERα and ERβ) are expressed in the hippocampus and can have distinct effects on hippocampal function (Toran-Allerand, 2005; Walf and Frye, 2008). Within the hippocampus, ER density is higher in the dorsal hippocampus than the ventral (Weiland et al., 1997) and the greatest density of ERs is in the hilus of the DG and SR of the CA1 (Weiland et al., 1997). Although ERα and ERβ levels in the male and female hippocampus are similar (Shughrue et al., 1997; Weiland et al., 1997) ERβ is expressed in higher levels than ERα (Shughrue et al., 1997). Early studies found that ERα is localized in nuclei of CA1 pyramidal cells while ERβ has both nuclear and cytoplasmic localization in CA1 pyramidal cells (Azcoitia et al., 1999; Kalita et al., 2005). Within the DG, however, ERβ immunoreactivity is predominantly localized to glial cells (Azcoitia et al., 1999). ERs are also located on inhibitory neurons (Su et al., 2001) and the majority of ERα in the dorsal hippocampus is co-localized with cells that express the enzyme that converts glutamate into GABA (Hart et al., 2001). Within the hippocampus, ERs levels fluctuate across the estrous cycle with ERβ mRNA lowest during proestrus ERα mRNA does not fluctuate across the estrous cycle (Szymczak et al., 2006).

Androgen receptors (AR) are also present in the hippocampus although predominantly localized to the CA1 (Clancy et al., 1992; Kerr et al., 1995; Sar et al., 1990). ARs are present in the female hippocampus but also fluctuate across the estrous

cycle. In particular, the only time when AR levels change is during estrus, where there is a dramatic reduction of ARs in the hippocampus of females compared to the other phases of the estrous cycle (Feng et al., 2010). Overall, however, males have significantly more AR+ cells in the hippocampus than females (Feng et al., 2010).

1.6 Adolescence and Puberty

1.6.1 Behavioral Changes

Adolescence and puberty are overlapping periods of development that are often difficult to define. Broadly speaking, adolescence is a transition period between childhood and adulthood that encompasses physical, emotional and cognitive development whereas puberty is defined in terms of sexual maturation (Sisk and Zehr, 2005). In rodents, behavioral changes are an indication of the transition into, and out of, adolescence. Peak levels of play behavior are present during adolescence but gradually decrease with further maturation (Thor and Holloway, 1984). Allogrooming, or social cleaning, is the predominant social interaction in early development but is gradually replaced by play fighting behavior that emerges around PND20 (Pellis and Pellis, 1997). Play fighting in males becomes progressively more adult-like throughout puberty (Meaney and Stewart, 1981; Takahashi and Lore, 1982) but play-fighting behaviors do not change as females progress through puberty (Pellis and Pellis, 1990). These sex-specific changes in play behavior can result from perinatal androgen exposure (reviewed by Pellis, 2002) again suggesting that androgens can be instrumental for successful sexual differentiation of play behavior during adolescence.

1.6.2 Hormonal Changes

Behavioral changes can be an outward indication that a rodent is progressing through adolescence, but underlying hormonal changes can dictate the onset of puberty, or sexual maturation. Hormonal control of the onset of puberty is complex and poorly understood (Roa et al., 2009) yet the following is a brief review of hormonal changes that accompany puberty. Although ovaries can synthesis estradiol by PND5 (Weniger, 1993; Weniger and Zeis, 1987; Weniger et al., 1993) and serum estradiol levels in the young female rat are high (Cheng and Johnson, 1974), more than 99% of circulating estradiol is bound by α-fetoprotein between PND5-18 (Puig-Duran et al., 1979). Estradiol levels precipitously drop around PND21 and remain low until the first preovulatory rise at approximately PND30 (Cheng and Johnson, 1974; Ewing et al., 1966; Germain et al., 1978). The ovarian surge in estradiol synthesis might promote a surge of LH-FSH release from the anterior pituitary (Sarkar and Fink, 1979) and the surge in LH-FSH often occurs in the afternoon the day before vaginal opening (Sarkar and Fink, 1979). Direct actions of estradiol on target tissue can promote vaginal opening (Gitlin, 1974), although the exact mechanism behind this is poorly understood.

In males, relative testicular weight begins to increase around PND20 (Ewing et al., 1966) but testosterone secretion is low during this early postnatal period (de Jong and Sharpe, 1977). Testosterone secretion increases around PND40 (de Jong and Sharpe, 1977) and mature spermatozoa are present around PND50 (de Jong and Sharpe, 1977), even though relative testicular weight does not plateau until PND40-80 (Ewing et al., 1966).

Animals used in the current study were used in slightly different developmental stages. Offspring used in the experiments described in Chapters 2 and 3 were 30-35 days old, a time range in which vaginal opening may or may not be present (McGivern et al., 1984; McGivern et al., 1987; Sliwowska et al., 2008). It is therefore possible estradiol levels in females between PND30-35 are similar to adult females. In males, however, testosterone levels have not yet reached adult levels by PND30-35 (de Jong and Sharpe). Therefore, between PND30-35, hippocampal synaptic plasticity in males and females will be differentially exposed to pubertal hormones. We chose to not use the males at an older age (e.g., PND40-45) and similar developmental stage as females in order to standardize the age range. Therefore, offspring were used for experimentation between PND30-35 to allow for a one-week adaptation period following weaning and to encompass a period where vaginal opening is not always present in females.

1.7 Activational Effects of Estradiol and Testosterone

Gonadal hormones can modulate hippocampal synaptic plasticity. LTP in the CA1 is significantly higher during proestrus than during estrus or met/diestrus (Good et al., 1999; Warren et al., 1995). Although estradiol can reduce the threshold for the induction of LTD (Zamani et al., 2000), thereby enhancing LTD (Desmond et al., 2000), the predominant effect of estradiol might be to attenuate LTD (Day and Good, 2005; Good et al., 1999; Shiroma et al., 2005). Several mechanisms have been proposed accounting for the effects of estradiol on synaptic plasticity. Blocking NR2B-containing NMDARs prevents the potentiating effect of estradiol on LTP (Smith and McMahon, 2006) indicating that estradiol might enhance LTP via NMDARs. Enhanced NMDAR-mediated currents by estradiol (Foy et al., 1999; Shiroma et al., 2005) can increase the

concentration of postsynaptic calcium (Shiroma et al., 2005) and increase the activity of several downstream cascades implicated in LTP (e.g., CamKII and MAPK) (Kim et al., 2002; Sawai et al., 2002; Shiroma et al., 2005). Estradiol can also potentiate NMDAR-dependent LTP via src tyrosine/MAPK pathway (Bi et al., 2000) and this pathway can contribute to the enhanced LTP observed during proestrus (Bi et al., 2001).

Specific ER subtypes can regulate hippocampal synaptic plasticity. Activation of ERα and ERβ can increase post-synaptic density-95 (PSD-95) and the GluR1 subunit of AMPA receptors in the CA1 (Waters et al., 2009). ERβ activation can increase the expression GluR2 subunits (Waters et al., 2009) indicating that both ER subtypes can positively regulate the expression of proteins that contribute to LTP. Recently, the role of ER subtypes in LTP has been investigated in transgenic mice that lack functional ERβ receptors. CA1 LTP is significantly reduced in ERβ knock-out mice compared to controls (Day et al., 2005); since functional ERα receptors were present in these transgenic mice, this suggests that ERα might depress hippocampal synaptic plasticity. Application of ERβ agonists can increase hippocampal LTP as well as PSD-95 and the GluR1 subunit of AMPARs (Liu et al., 2008) suggesting that the potentiating effect of estradiol on synaptic plasticity might be mediated via ERβ. Interestingly, ERβ is not expressed in the adolescent rodent hippocampus (Orikasa et al., 2000) perhaps accounting for the depressive effect of estradiol on CA1 LTP in the adolescent hippocampus (Ito et al., 1999).

Although not investigated as thoroughly as estradiol, testosterone can also modulate hippocampal synaptic plasticity. CA1 LTP induced by high frequency stimulation was not affected by castration (Sakata et al., 2000); LTP was reduced,

however, in castrated males following primed burst stimulation (Sakata et al., 2000) suggesting that the effect of testosterone on LTP might depend on the stimulation protocol employed. Pubertal castration enhanced CA1 LTP in adulthood an effect that was abolished by treating castrated animals with testosterone (Harley et al., 2000). In the adolescent hippocampus, LTD was induced by activation of ARs following application of a conditioning protocol that normally elicits LTP (Hebbard et al., 2003). These studies suggest that testosterone depresses synaptic plasticity, but this remains to be further investigated.

1.8 Stress

1.8.1 Neurobiology of the Stress Response

1.8.1.1 Hypothalamic-Pituitary-Adrenal Axis

Stressors (real or perceived, intrinsic or extrinsic forces) can threaten the equilibrium (homeostasis) of an organism. Stress is a state of real or perceived threat to homeostasis (Evangelia et al., 2005) and the stress response is mediated by the hypothalamic-pituitary-adrenal (HPA) system (De Kloet et al., 1998; Herman et al., 1996). Stimulation of the paraventricular region of the hypothalamus promotes the initial synthesis of proopiomelanocortin (POMC), which is cleaved to form corticotropin releasing hormone (CRH) and β-endorphin. CRH stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary, which then targets the adrenal cortex to stimulate the release of cortisol (humans) or corticosterone (CORT-rodent). Corticosterone binging globulin (CBG) is protein that binds CORT to reduce the levels of freely circulating CORT. CGB binding activity is greater in females than males (Gala and

Westphal, 1965) and this sex-difference is first apparent around PND30 (Gala and Westphal, 1965). It has been proposed that this sex-difference in CBG activity is due to the suppressive effects of testosterone on CBG activity (Gala and Westphal, 1965).

The actions of CORT on target tissue are mediated by two receptors, Type I receptors (mineralocorticoid receptors-MR) and Type II (glucocorticoid receptors-GR). MRs are predominantly localized in the hippocampus and have a 5-10-fold higher affinity for CORT than GRs (Reul and de Kloet, 1985; Reul and de Kloet, 1986). GRs are more widely localized throughout the brain (Reul and de Kloet, 1985; Reul and de Kloet, 1986) with relatively high expression in the hippocampus compared to other brain regions (Reul and de Kloet, 1985). CORT levels naturally fluctuate throughout the day and, for nocturnal mammals like rats, CORT peaks right before the onset of the dark phase of the light cycle and levels steadily decrease to reach nadir toward the start of the light cycle (Allen-Rowlands et al., 1980; Takahashi et al., 1979). MRs are predominantly occupied during the circadian trough (Reul and Kloet, 1985), which is sufficient to mediate tonic feedback on the HPA axis (Bradbury et al., 1994). During periods of elevated CORT, such as the diurnal peak of CORT or during periods of stress, MRs and GRs are both occupied by CORT (Reul and de Kloet, 1985) and negative feedback on the HPA axis in this situation is mediated by both MRs and GRs (Bradbury et al., 1994).

1.8.1.2 Role of the Hippocampus in the Stress Response

The hippocampus exerts regulatory control over HPA activity. MRs and GRs are highly localized within the hippocampus (Chao et al., 1989; Van Eekelen and De Kloet, 1992), suggesting that the hippocampus may contribute to HPA activity (Herman et al., 1996). Hippocampal stimulation can reduce glucocorticoid secretion (Dunn and Orr, 1984;

Sapolsky et al., 1991) a function predominantly mediated by the CA1 (Herman et al., 1996). The hippocampus does not directly project to the hypothalamus (Swanson, 1977) but CA1 projections can terminate in the septum and the bed nucleus of the stria terminalis (Swanson, 1977) a region that then innervates the hypothalamus (Meibach and Siegel, 1977a; Meibach and Siegel, 1977b; Swanson and Cowan, 1977). Therefore, through indirect projections to the paraventricular region of the hypothalamus, CA1 output can regulate HPA activity (Bouille and Bayle, 1978; Casady and Taylor, 1976; Sapolsky et al., 1984; Sapolsky et al., 1991).

1.8.2 Glucocorticoid Receptor Expression in the Hippocampus

An exhaustive review of MR and GR function and regional distribution is beyond the scope of this thesis. However, a brief description of MR and GR localization within the hippocampus will be given. CA1 pyramidal cells and DG granule cells express MRs and GRs (Eekelen et al., 1988; Han et al., 2005; Van Eekelen et al., 1988), but MR density is highest in the CA1 pyramidal cell layer and GR density is highest in the DG (Reul and de Kloet, 1985). MRs and GRs exhibit cytoplasmic and nuclear localization (Pekki et al., 1992; van Steensel et al., 1996). Within the nucleus, MRs and GRs are largely targeted to separate and distinct compartments (van Steensel et al.; van Steensel et al., 1996), although MR/GR co-localization can occur within the nucleus (van Steensel et al., 1996). Occupied and unoccupied GRs can be localized within the cell nucleus (Pekki et al., 1992) but nuclear localization of GRs is increased following an acute stress or at the diurnal peak in cort concentration (Kitchener et al., 2004). Hippocampal MR and GR expression varies across development and, initially, GR and MR levels are significantly higher in males than females during the first postnatal week (Ordyan et al., 2008). In

females, GR expression is low during the second postnatal week but dramatically increases by the third week of life (Ordyan et al., 2008); adult levels of hippocampal MRs are reached by PND7 (Sarrieau et al., 1988).

1.8.3 Hippocampal Synaptic Plasticity Following Exposure to a Stressor

Activation of the HPA axis can alter hippocampal synaptic plasticity. Exposure to an acute stress can reduce LTP and enhance LTD in the CA1 of males (Artola et al., 2006; Constantine et al., 2002; Foy et al., 1987; Kim et al., 1996; Krugers et al., 2005; Pavlides et al., 2002; Xiong et al., 2004; Xu et al., 1997; Xu et al., 1998). Application of an NMDAR antagonist during exposure to the stressor can prevent stress-induced changes to LTP and LTD (Kim et al., 1996) possibly via NR2B-containing NMDARS (Wong et al., 2007). Acute stress increases NMDAR-invoked endocytosis of GluR2-containing AMPARs (Martin et al., 2009). Interestingly, the depressive effect of acute stress on synaptic plasticity is also mediated by GR (Xu et al., 1998), possibly through GR-induced increases in GluR2 surface expression (Martin et al., 2009). Taken together, these findings implicate multiple receptor systems in the effects of acute stress on synaptic plasticity.

It is important to note that the type of stressor can dramatically influence whether there will be impairments in hippocampal function. For example, Xiong (2003) exposed male rats to two types of acute stressors (a one-time exposure to an elevated platform for 30 minutes or a one-time exposure to three pulses of electric shock separated by 1 or 5 seconds) and two types of sub-acute stressors (two, 30 minute exposures to the elevated platform for 5 days or 10 sets of footshocks across 5 days). They found that CA1 LTD was significantly enhanced by both acute and sub-acute footshock stress; LTD was

enhanced by elevated platform stress but not when the stressor was sub-acute (Xiong et al., 2003). Interestingly, these stressors had opposite effects on learning and memory. Specifically, only acute foot shock stress impaired the acquisition of a spatial learning task but sub-acute footshock enhanced retrieval (Xiong et al., 2003). Furthermore, the perceived threat of the stressor can influence whether hippocampal function will be impaired. For example, Woodson (2003) found that elevations in CORT levels were equivalent in a male rat exposed to either a sexually receptive female or a cat (Woodson et al., 2003) indicating that both stressors equally activated the HPA axis. However, memory impairments were only apparent following exposure to the cat (Woodson et al., 2003). These findings indicate that the nature of the stressor can dramatically influence the functional integrity of the hippocampus.

1.8.4 HPA Activity following PNEE

PNEE can induce long-lasting changes to HPA activity. Humans exposed to ethanol *in utero* exhibit heightened basal and stress-induced changes to CORT (Haley et al., 2006; Jacobson et al., 1999). Furthermore, this heightened HPA activity in ethanol-exposed infants was dissimilar between males and females (Haley et al., 2006) indicating that HPA activity is differentially affected by prenatal ethanol exposure in males and females, a notion that is supported using animal models of FAS/D (Gabriel et al., 2001; Gabriel et al., 2000; Glavas et al., 2001; Sliwowska et al., 2008; Weinberg et al., 1996). Heightened HPA activity in response to a stressor, however, is not always apparent. In particular, Weinberg et al. 1982 observed that CORT levels in ethanol-exposed male and female offspring were similar to controls following exposure to a stressor at postnatal day 39

(Weinberg and Gallo, 1982), a finding that extended upon within Chapter 2 of the current thesis.

1.8.5 Pregnancy and HPA Activity

HPA activity changes across gestation. In terms of CORT, maternal CORT levels peak between GD14-18 (Dupouy et al., 1975; Williams et al., 1999a) and remain elevated for the remainder of gestation (Dupouy et al., 1975). Diurnal variations in CORT are still present in pregnant rodents, although the peak CORT levels are significantly lower than non-pregnant female rats (Atkinson and Waddell, 1995). By GD18, however, the diurnal peak in CORT was significantly greater in pregnant females than non-pregnant females (Koehl et al., 1999). Fetal HPA activity is also present during gestation. ACTH and CORT are present in fetal serum beginning on GD16 and peak on GD19 (Boudouresque et al., 1988). Maternal adrenalectomy (ADX) on GD12 completely abolished maternal CORT levels when assessed on GD14 (Cohen et al., 1990). However, CORT levels were not depleted if ADX was performed on GD14 (Cohen et al., 1990). The CORT present in ADX dams on GD14 was attributed to fetal CORT (Cohen et al., 1990) since fetal CORT can be transported into maternal circulation (Dupouy et al., 1975; Milkovic et al., 1973).

Fetal exposure to maternal CORT is regulated by the placental enzyme 11 beta-hydroxysteroid dehydrogenase (11β-HSD) (reviewed by Yang, 1997). Maternal CRH and ACTH do not cross the placenta (Dupouy et al., 1980; Williams et al., 1999b) but CORT readily crosses the placenta (Macdonald and Matt, 1984). The rodent placenta contains mRNA that codes for two different 11β-HSD enzymes: 11β-HSD1 and 11β-HSD2 (Burton and Waddell, 1999) that have distinct functions. 11β-HSD1 is a bidirectional enzyme that predominantly acts as an oxidoreductase to form active corticosterone but

11β-HSD2 is a unidirectional enzyme that inactivates corticosterone (Krozowski, 1999; Krozowski et al., 1999). Therefore, 11β-HSD2 protects the fetus from exposure to maternal CORT. Interestingly, maternal CORT can actually decrease the 11β-HSD2 protein levels throughout gestation (Staud et al., 2006), which might contribute to a steady increase in fetal CORT during the latter part of gestation (Boudouresque et al., 1988). Fetal uptake of CORT is significantly higher in females than males (Montano et al., 1993) which might be countered by significantly higher 11β-HSD2 mRNA in the female fetus than the male fetus (Wilcoxon et al., 2003).

1.9 Summary and Objectives

A major goal of the current thesis was to elucidate how hippocampal synaptic plasticity is affected by PNEE in adolescent male and female offspring. Previous studies have shown that PNEE reduces LTP in males (Christie et al., 2005; Richardson et al., 2002; Swartzwelder et al., 1988) but this has yet to be investigated in adolescent females despite evidence of basal sex differences in synaptic plasticity (Maren, 1995; Maren et al., 1994). Furthermore, PNEE can alter HPA activity in a sexually dimorphic manner (Weinberg et al., 2008b; Weinberg et al., 1996) suggesting that the effects of acute stress on hippocampal synaptic plasticity might be exaggerated in ethanol-exposed offspring. In Chapter 2, we investigated the effect of acute stress on CA1 LTD in male and female offspring following PNEE. Acute stress can enhance CA1 LTD (Wong et al., 2007; Xiong et al., 2004; Xiong et al., 2003; Xu et al., 1998) via GR activation (Xu et al., 1998). Ethanol-exposed offspring can have an exaggerated CORT response to a stressor (Gabriel et al., 2001; Gabriel et al., 2000; Glavas et al., 2007; Haley et al., 2006; Jacobson et al., 1999; Kim et al., 1999; Weinberg, 1988; Weinberg and Gallo, 1982;

Weinberg et al., 2008a; Weinberg et al., 1996) suggesting that stress-induced changes to synaptic plasticity might be more pronounced in ethanol-exposed offspring. We hypothesized that ethanol-exposed male and female offspring would have significantly more LTD in the CA1 region following exposure to an acute stress. The results of the study presented in Chapter 2 revealed a complex relationship among sex, prenatal food deprivation and PNEE on stress-induced changes to CA1 LTD.

The experiments in Chapter 3 were designed to determine if PNEE and prenatal stress synergistically alter DG LTP. Ethanol consumption can increase CORT levels non-pregnant humans and rodents (Rivier, 1993; Rivier, 1996; Wand and Dobs, 1991) and reduces 11β-HSD2 levels in the placenta of female fetuses (Wilcoxon et al., 2003) possibly exposing ethanol females to abnormally high levels of CORT. Maternal ADX can rescue some of the deleterious effects imposed by PNEE on the offspring (Redei et al., 1993; Taylor et al., 2002; Wilcoxon et al., 2003). Prenatal stress and PNEE have independently been shown to reduce hippocampal LTP in male offspring (Christie et al., 2005; Gi Hoon et al., 2006; Richardson et al., 2002; Son et al., 2006; Sutherland et al., 1997; Swartzwelder et al., 1988; Yaka et al., 2007; Yang et al., 2006) making it difficult to dissociate the effects of gestational CORT or ethanol on plasticity in adult offspring. Previous studies have shown that adolescent females have reduced DG LTP compared to adolescent males (Maren et al., 1994) suggesting that PNEE and/or prenatal stress might have sexually dimorphic effects on synaptic plasticity. It was therefore hypothesized that DG LTP would be significantly reduced in offspring exposed to either stress or ethanol *in utero* but offspring exposed to both stress and ethanol *in utero* would exhibit the greatest reduction in LTP.

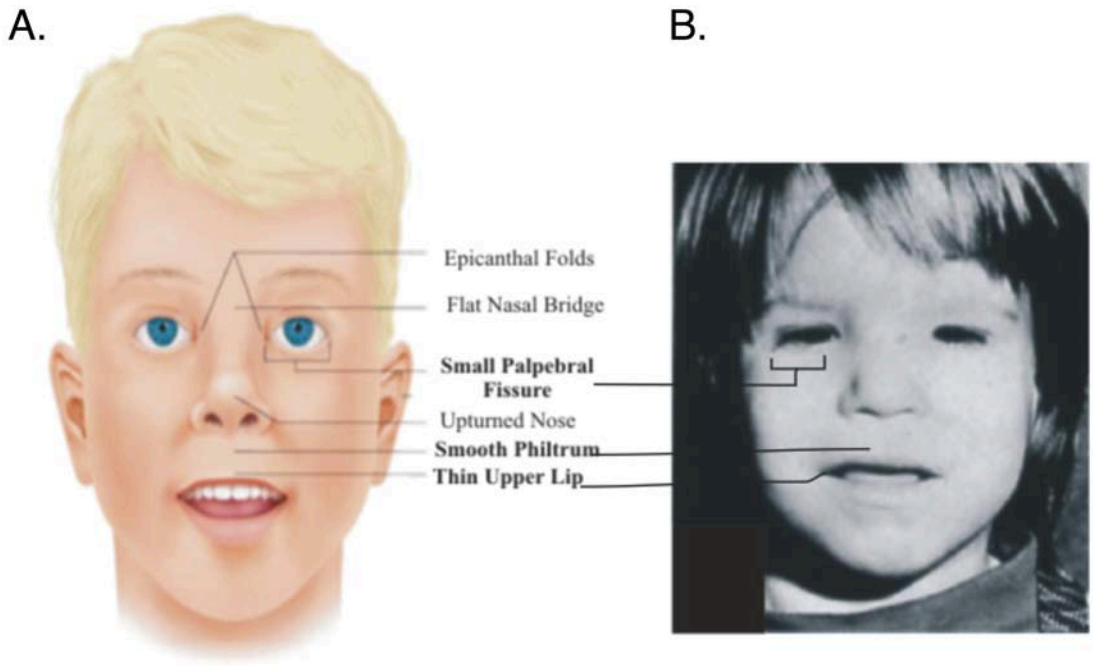

Figure 1.1 Identifying facial abnormalities in fetal alcohol syndrome.

A. Illustration of common facial abnormalities that result from exposure to ethanol in utero. B. Caucasian female with FAS. More characteristic abnormalities are highlighted in bold and include a small palpebral fissure, smooth philtrum and thin upper lip (modified from Sulik, 2005; Wattendorf and Muenke, 2005).

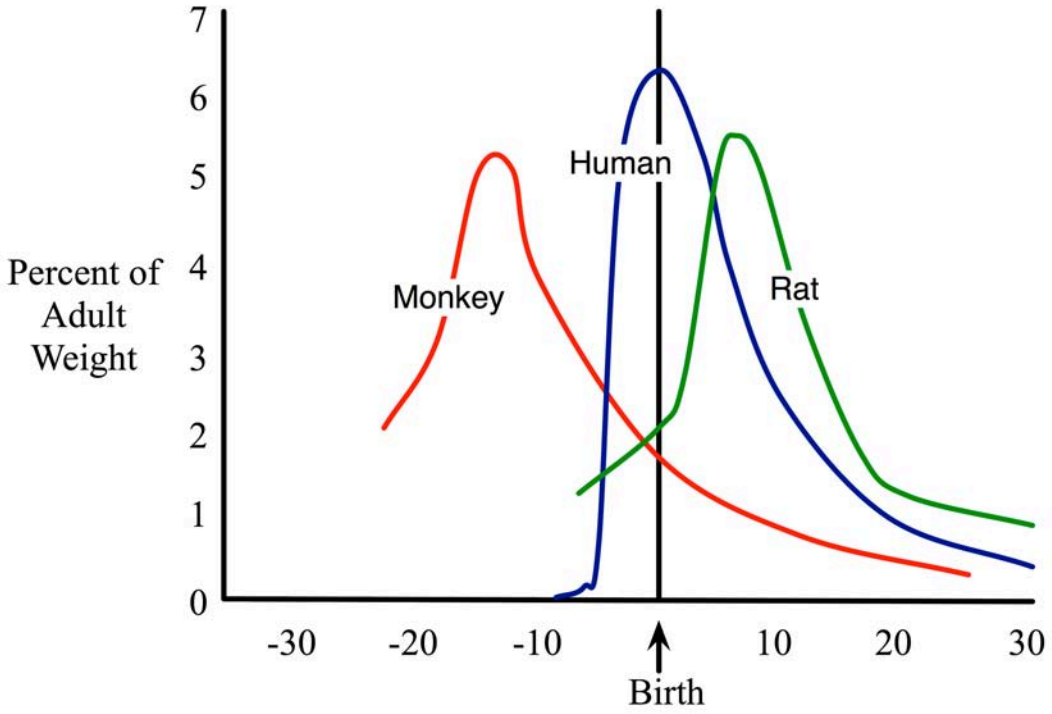

Figure 1.2 Diagram of brain growth velocities for different mammalian species.

Curves are expressed as rates of brain weight changes across time. Units are expressed as follows: rhesus monkey, 4 days; humans, months; rat, days. Growth velocities indicate that the monkey, human and rat brain develop at different rates relative to birth (modified from Cudd, 2005).

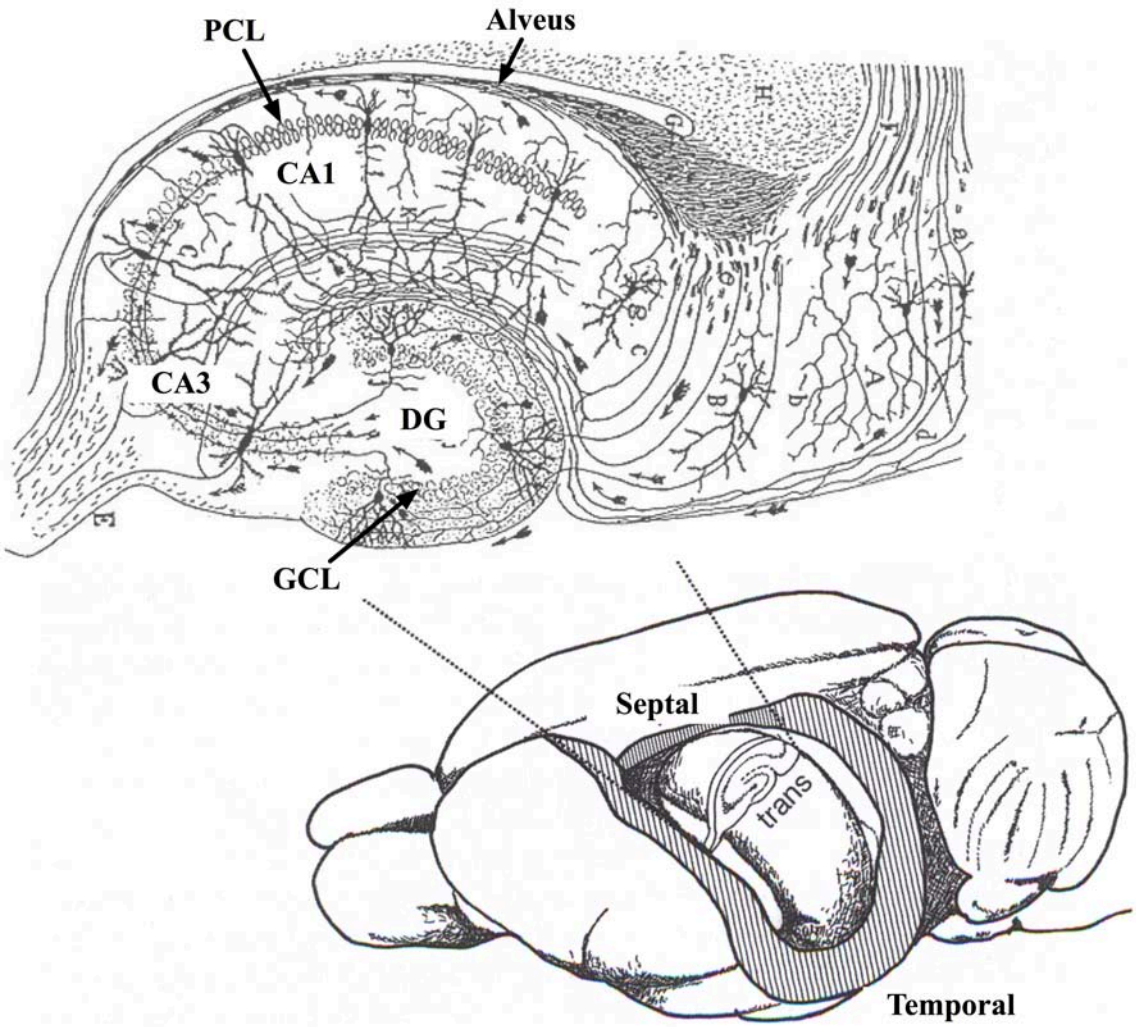

Figure 1.3 Diagram of the rodent hippocampus.

A rodent brain is depicted in the lower portion of the figure with the cortex removed to expose the underlying hippocampus. The banana-shaped hippocampus extends across the septo-temporal poles of the brain. A blow-up of a cross-section of the hippocampus is shown above the rodent brain. The coronal section illustrates the different regions of the hippocampus. CA: cornu ammonis, DG: dentate gyrus, GCL: granule cell layer, PCL: pyramidal cell layer (modified from O'Keefe and Nadel, 1978; y Cajal, 1909).

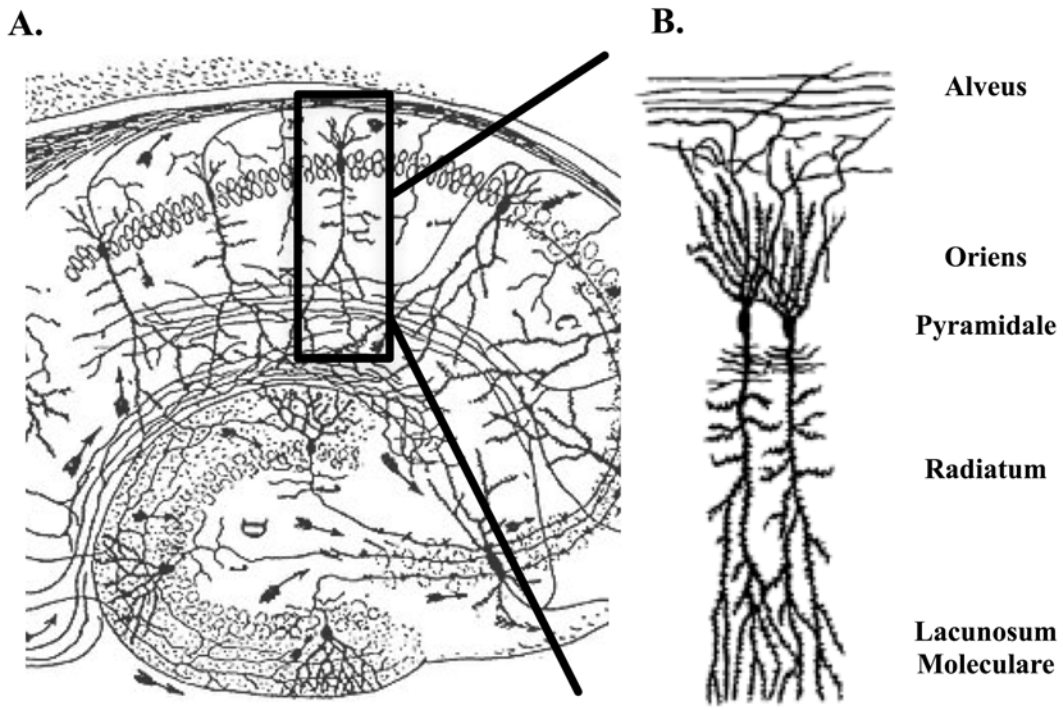

Figure 1.4 Laminar organization of the CA1.

A. Coronal section of the rodent hippocampus. B. Magnification of the CA1 with the different layers listed. (modified from O'Keefe and Nadel, 1978; y Cajal, 1909).

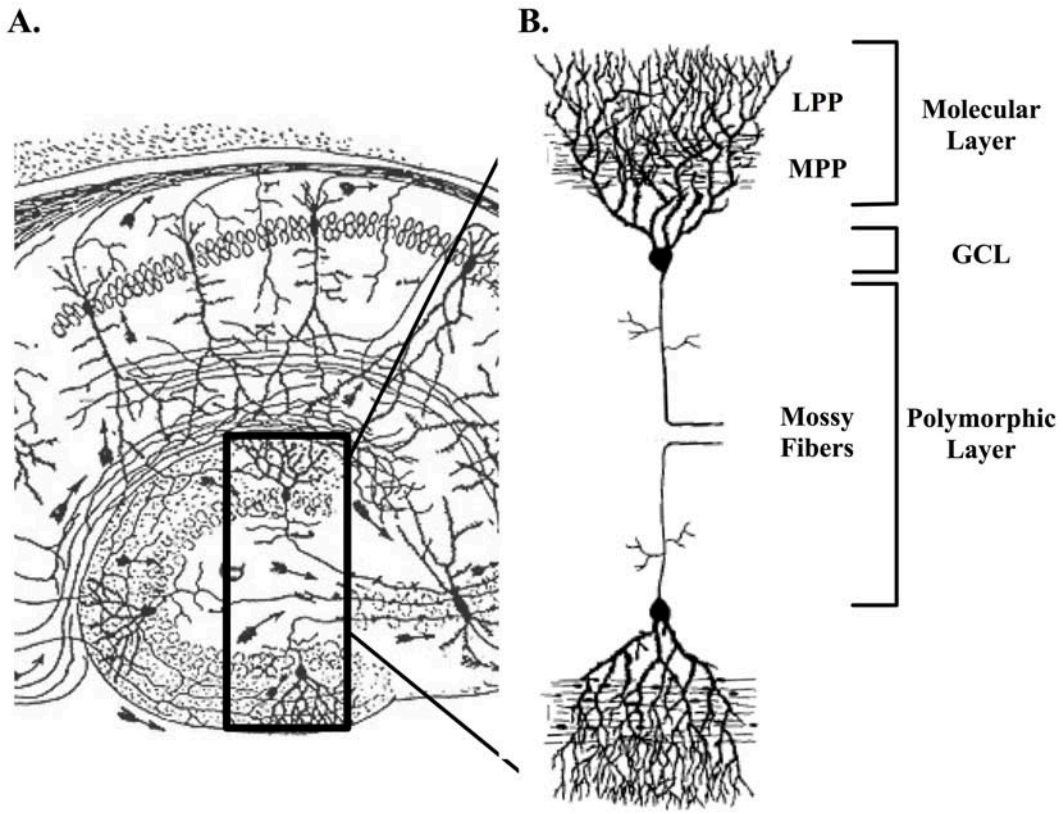

Figure 1.5 Laminar organization of the dentate gyrus.

A. Coronal section of the rodent hippocampus. B. Magnification of the dentate gyrus with the different layers listed. Mossy fiber output is also indicated. GCL: granule cell layer, LPP: lateral perforant path, MPP: medial perforant path (modified from Lomo, 1971; y Cajal, 1909).

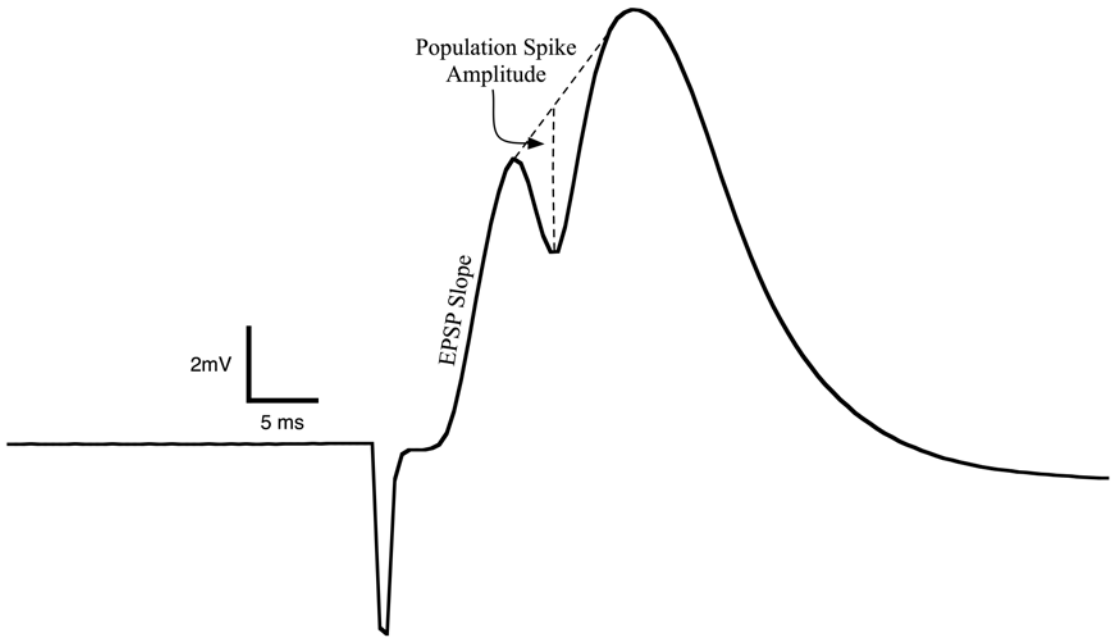

Figure 1.6 Sample waveform from the dentate gyrus.

The initial positive going deflection is the EPSP that is immediately followed by a downward deflection that represents the population spike. The population spike represents the near synchronous firing of a population of cells (i.e., granule cells).

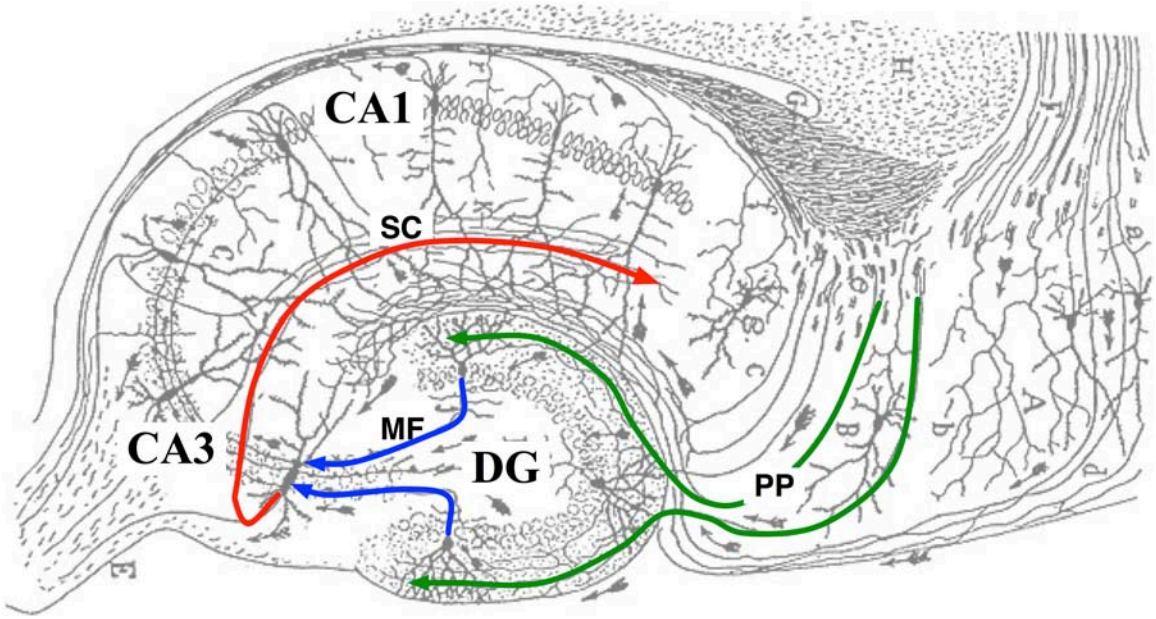

Figure 1.7 Hippocampal tri-synaptic circuit.

The entorhinal cortex sends projections to the dentate gyurs via the perforant path (synapse 1). Granule cells in the DG connect to CA3 via mossy fiber projections (synapse 2). CA3 projects to CA1 through Schaffer collaterals (synapse 3). CA: corno ammonis, DG: dentate gyrus, MF: mossy fiber, PP: perforant path, SC: Schaffer collateral. (modified from y Cajal, 1909)

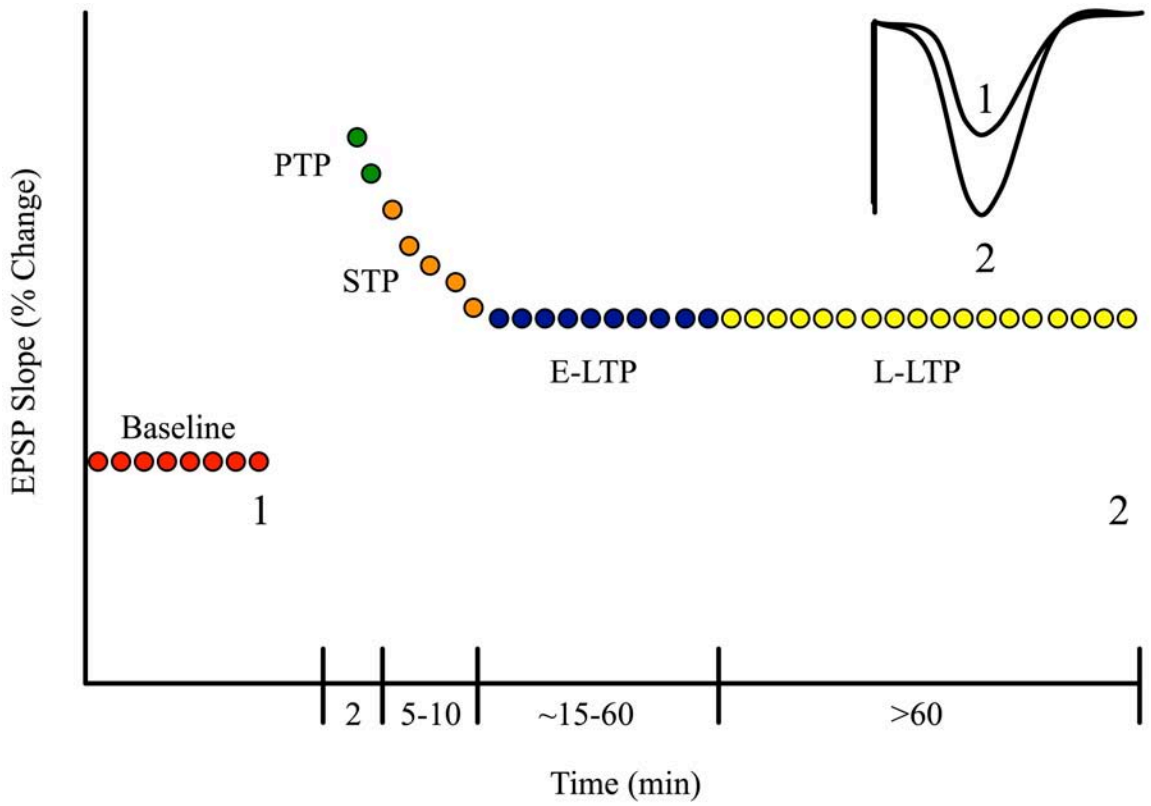

Figure 1.8 Stages of long-term potentiation.

Diagrammatic representation of the phases of long-term potentiation (LTP). Basal stimulation is applied for a specified period of time (e.g., 15 minutes) and once the baseline is stable a conditioning stimulation is applied (e.g., TBS). Immediately after conditioning stimulation, basal stimulation is resumed. Post-tetanic potentiation lasts for approximately 2 minutes following the termination of the conditioning stimulation. The decay of short-term potentiation depends on the rate of stimulation but is depicted as lasting 5-10 minutes in the figure. Approximately 15 minutes following the termination of the conditioning stimulus, early long-term potentiation can be apparent; E-LTP lasts for approximately 60 minutes. The onset of late long-term potentiation varies between an hour the three hours after the conditioning stimulus and can persist for up to a year. Sample traces taken at the time of baseline (1) and at 60 minutes post-conditioning stimulation (2) illustrate the change in EPSP slope. E-LTP: early long-term potentiation, EPSP: excitatory post-synaptic potential, L-LTP: late long-term potentiation, PTP: post-tetanic potentiation, STP: short-term potentiation.

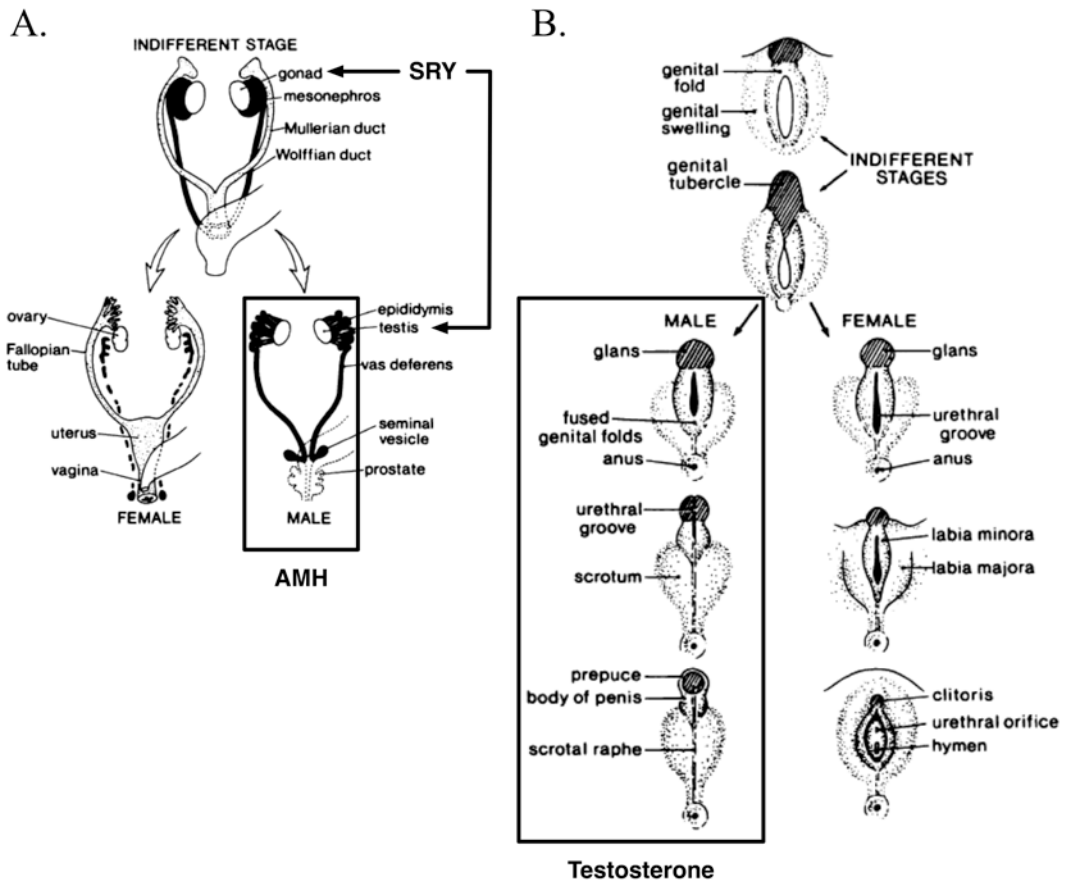

Figure 1.9 Diagram of the indifferent organ.

A. The male and female fetus each contains an identical organ that will differentiate in to male and female genitalia. Due to the actions of Sry (located on the Y-chromosome), the gonad differentiates into the testes. Secretion of AMH by the testes causes the Müllerian duct to regress; testicular secretion of testosterone promotes development of epididymis, vas deferens and seminal vesicles. In the absence of Sry, ovaries develop and the indifferent organ differentiates into the fallopian tubes, uterus and vagina. B. Representation of external genitalia. In the early stages of development, external genitalia looks identical between the male and female fetus. Secretion of AMH and testosterone from the testes promotes the differentiation into stereotypic male external genitalia. AMH: anti-Müllerian hormone. (modified from Wilson et al., 1980).

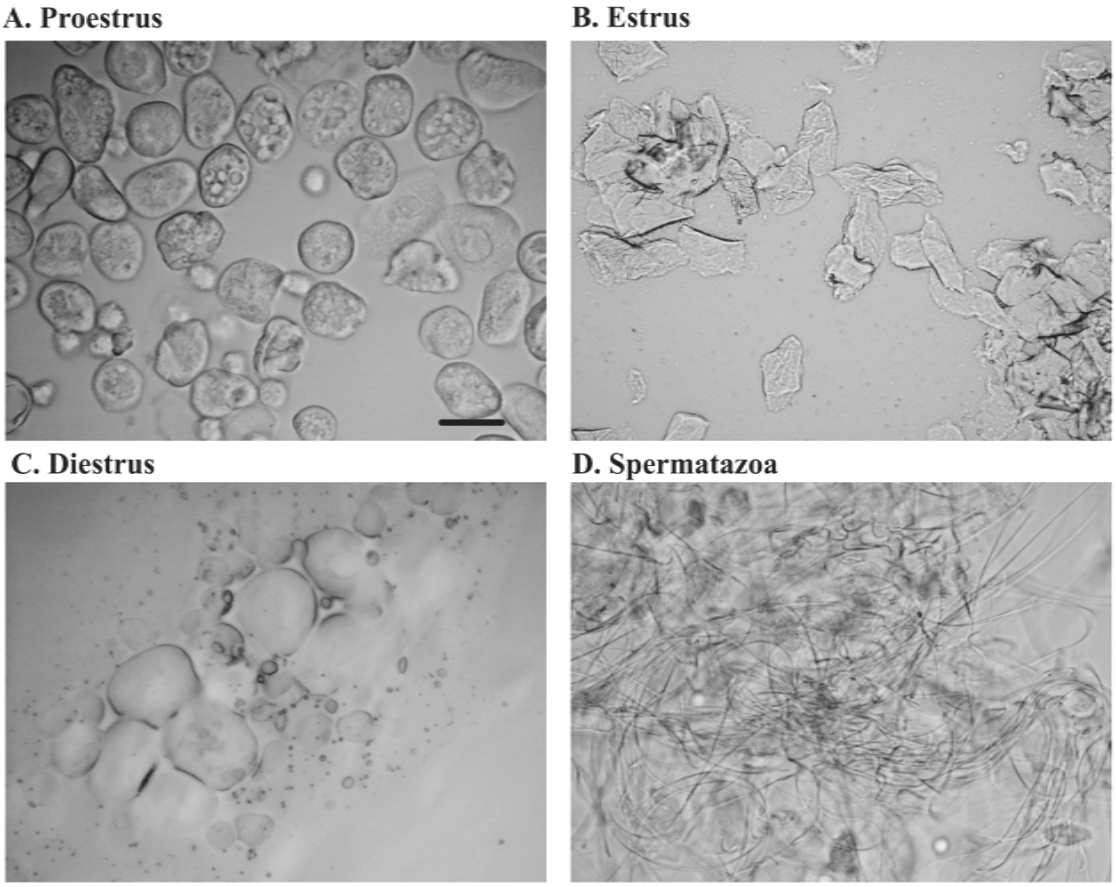

Figure 1.10 Cell cytology across the estrous cycle.

A. Round, nucleated cells are the predominant cell type in a vaginal smear taken from a female rat in proestrus. B. Thin, sheet-like cells predominate during estrus. C. Leukocytes and non-nucleated cells are found in a vaginal smear taken at diestrus. D. Sample of spermatozoa in a vaginal smear. Metestrus and Diestrus have similar cell cytology so only diestrus was shown in the figure. Scale bar: 200μm. (modified from Haim et al., 2003)

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2 Long-term Depression *in vivo*: Effects of Sex, Stress, Diet and Prenatal Ethanol Exposure. [1]

Long-term depression of synaptic efficacy is reliably induced with low-frequency stimuli (LFS) in the hippocampus *in vitro* (Christie et al., 1996; Christie et al., 1997; Doyere et al., 1996; Dudek and Bear, 1993) and has also been observed *in vivo* (Heynen et al., 1996), though with less reliability than it is observed *in vitro* (Fox et al., 2006; Staubli and Scafidi, 1997). Interestingly, prior exposure to acute stress enhances the capacity of LFS to induce LTD (Xiong et al., 2004; Xiong et al., 2003; Xu et al., 1998) while simultaneously reducing the capacity for LTP in the hippocampus (Xiong et al., 2004; Xu et al., 1997; Xu et al., 1998). Thus, it may be that stress shifts the induction threshold for synaptic plasticity away from LTP to favor LTD. If this is the case, it would suggest that an exaggerated stress response might further enhance the capacity for LTD.

In humans and animals, fetal alcohol syndrome and fetal alcohol spectrum disorder (FAS and FASD, respectively) can encompass a variety of physiological abnormalities and deficits, including mental retardation (Abel and Sokol, 1986; Marcus, 1987; Spohr et al., 1993; Wattendorf and Muenke, 2005). Human and animal studies also indicate that *in utero* ethanol exposure negatively impacts hippocampal structure and function (Berman and Hannigan, 2000) resulting in impaired hippocampus dependent learning, as well as a reduced capacity for long term potentiation (LTP) (Christie et al., 2005; Richardson et al., 2002; Sutherland et al., 1997; Swartzwelder et al., 1988). Animals with prenatal ethanol

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exposure (PNEE), an animal model of FAS/FASD, can exhibit a heightened stress response (Bertrand et al., 2005; Gabriel et al., 2001; Kim et al., 1999a; Streissguth and O'Malley, 2000; Taylor et al., 1982; Weinberg, 1988; Weinberg and Gallo, 1982; Weinberg and Petersen, 1991), suggesting that stress-induced changes to synaptic plasticity might be more apparent in these animals.

The current study will first investigate the capacity for both juvenile male and female animals to exhibit LTD in the hippocampal CA1 region *in vivo* following acute exposure to stress. In addition, the effects of prenatal ethanol exposure on LTD in male and female animals will also be investigated.

2.1 Methods

2.1.1 Animals and Mating

All animals used in this study were generated in the animal care colony of the Department of Psychology at the University of British Columbia. All experiments were performed in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the Universtify of British Columbia Animal Care Committee (**Appendices A-B**). Twenty-eight virgin female Sprague-Dawley rats were paired with age-matched males (250-275g; University of British Columbia Animal Care Services) and individually housed in polycarbonate cages (46x24x20 cm) with Carefresh contact bedding (Absorption Corp., Bellingham, WA) for a one-week adaptation period. The breeding colony room was kept at a constant temperature of 21^0 C and lights turned on from 7:00-19:00 h. After the adaptation period, males and females were paired in suspended wire mesh cages (63x24x18 cm) that were checked twice daily for vaginal plugs. The presence of a vaginal plug was used to indicate gestation day 1 (GD1).

2.1.2 Diet Administration

The *in utero* group assignments and feeding schedules were performed using procedures that are well-established to produce control, dietary restricted and ethanol exposed animal groups (Keiver et al., 1997; Keiver et al., 1996; Keiver and Weinberg, 2003; Keiver and Weinberg, 2004). On GD1, pregnant females were individually housed in polycarbonate cages and assigned to one of three feeding groups: (i) The **ETHANOL** group, in which females were given ad libitum access to a liquid diet containing ethanol (35.5% ethanol-derived calories; 6.61% v/v), (ii) the **PAIR-FED** group consisting of a similar liquid diet as the **ETHANOL** group but with an isocaloric substitution of maltose-dextrin for ethanol; **PAIR-FED** dams were offered a quantity of food that matched the amount of food consumed in g/kg by an **ETHANOL** dam on the corresponding day of gestation; and (iii) **AD LIBITUM** animals that were given ad libitum access to standard rat chow. All groups had ad libitum access to water throughout gestation. The **ETHANOL**, **PAIR-FED** and AD LIBITUM diets were administered from GD1-GD22. The **ETHANOL** dams were slowly introduced to the ethanol during the first three days of gestation by combining 1/3 ethanol diet with 2/3 pair-fed diet on GD1, 2/3 ethanol diet with 1/3 pair-fed diet on GD2 and 3/3 ethanol diet from GD3-21. On GD22, ETHANOL and **PAIR-FED** diets were replaced with ad libitum access to standard rat chow to reduce any further deleterious effects of ethanol exposure on offspring (Weinberg, 1989). The **ETHANOL** and **PAIR-FED** diets contained all of the necessary nutrients to provide adequate nutrition to females in both conditions, despite the decrease in the total amount of diet consumed when compared to the **AD LIBITUM** animals (Weinberg, 1985). The liquid diets were obtained from Dyets Inc. (Bethlehem, PA, USA) and are sold as Weinberg/Keiver High Protein Liquid Diet-

Control (#710109) for the **PAIR-FED** diet and Weinberg/Keiver High Protein Liquid Diet-Experimental (#710324) for the **ETHANOL** diet. To determine consumption, the diet bottles were removed from the **ETHANOL** and **PAIR-FED** cages each day and weighed to determine the amount of diet consumed the previous night. The liquid diet bottles were then replenished with freshly prepared diets and administered in the late afternoon prior to lights out, which helps to prevent a shift in the CORT circadian rhythm that can be observed in animals, fed a restricted diet (Weinberg and Gallo, 1981).

During pregnancy, females were weighed on GD 1, 7, 14 and 21 while their cages were being cleaned so that the animals were minimally disturbed. The day on which females gave birth was indicated as postnatal day 1 (PND1). On PND2, litters were culled to 10 pups (5 males and 5 females) and dams and pups were weighed on PND8, PND 15 and PND22, again during cage cleaning. During this postnatal period, all animals were given the same *ad libitum* rat chow diet so that maternal diet was not different between groups at this point. The pups generated by these procedures were then weaned and group housed according to sex on PND22, and also allowed *ad libitum* access to rat chow. It is important to note that these animals were never exposed to ethanol or dietary restriction after they were born, and it is these animals, and not the dams, that were used for the subsequent electrophysiological studies between PND30-35.

2.1.3 Blood Ethanol Concentration Measurements

To determine the maternal blood ethanol concentrations (BECs) during pregnancy, a tail blood sample was acquired on GD15 two hours after the presentation of the ethanol diet. These samples were obtained from a separate group of **ETHANOL** dams from those whose offspring were included in the study so that the stress of the procedure would not impact

the experimental animals. Blood was collected and allowed to sit at 4°C overnight and centrifuged at 3500rpm for 10-15 minutes and serum was stored at -20°C until assay. BECs were measured using an alcohol reagent kit (no. A7504-150) and alcohol standard (no. A7504-STD) from Pointe Scientific Inc. (Lincoln Park, MI); assay was performed according to manufacturer's instructions.

2.1.4 Stress Protocols

On the day that electrophysiological recordings were being made, the offspring were randomly assigned to NAÏVE or STRESS conditions with the experimenter blind to the animal group designation. The STRESS condition involved placing a single rat on an elevated platform (10x10 cm and 1.6m high) in the middle of a brightly lit room for 30 minutes (Xiong et al., 2004; Xiong et al., 2003; Xu et al., 1998). After this period, the animal was immediately injected with urethane. Once anesthetized (within approximately 2 minutes of urethane adminisatration) and a tail sample of blood was obtained to determine corticosterone levels. To analyze corticosterone levels, blood was collected in Fischer microcentrifuge tubes and centrifuged at 6000rpm for 10-15 minutes. Serum was collected and stored at -20°C until assay. Groups were identified in the following manner: male (n=8) and female (n=13) **AD LIBITUM** NAÏVE animals; male (n=10) and female (n=9) **AD LIBITUM** STRESS animals; male (n=11) and female (n=13) **PAIR-FED** NAÏVE animals; male (n=9) and female (n=9) **PAIR-FED** STRESS; male (n=10) and female (n=10) **ETHANOL** NAÏVE animals; and male (n=9) and female (n=7) **ETHANOL** STRESS animals.

2.1.5 Corticosterone Assay

Serum corticosterone levels were determined from the tail blood samples acquired during the electrophysiological recording period and analyzed using a commercial radioimmunoassay kit (MP Biomedials, Orangeburg, NY; catalog # 07-0120103) according to the manufacturer's instructions.

2.1.6 Electrophysiology

All electrophysiology was performed in the offspring of dams from the **ETHANOL**, **PAIR-FED**, and **AD LIBITUM** groups when they were between PND30-35. It is important to note again here that animals in the **ETHANOL** and **PAIR-FED** groups were only exposed to their respective conditions *in utero*, and that all animals were on equivalent diets from birth until their use in these experiments. All animals were anesthetized with urethane (1.5g/kg) and placed in a stereotaxic apparatus (Kopf Instruments). Rectal temperature was maintained at 37 $\pm$ 1°C with a grounded homeothermic temperature control unit (Harvard Instruments, MA, USA). Two ground screws were inserted in the skull anterior to bregma and at lambda to ground the recording and stimulating electrodes, respectively. A 125-µm stainless steel recording electrode was directed through a trephine hole into the CA1 (3.0mm posterior, 3.0mm lateral to bregma). Additionally, a 125-µm monopolar stimulating electrode was directed through the same trephine hole to stimulate the Schaffer-collateral/commissural pathway. The final depth of the stimulating and recording electrodes was determined by adjusting both electrodes to yield a maximal field excitatory postsynaptic potential (EPSP). The final stimulation intensity was adjusted to elicit an EPSP that was 60% of the maximal EPSP size, within a 1-2 mA range.

Paired-pulse facilitation was assessed by administering two pulses at both 50 and 100ms inter-pulse intervals. Following the presentation of the paired-pulse stimuli, baseline evoked responses were acquired using single pulse stimuli (120 μs) delivered every 15s. After acquiring a stable baseline for at least 15 minutes, low frequency stimulation (LFS: 900 pulses at 3Hz) was administered using the same pulse width. Following LFS, baseline stimulation was again administered for 2h to assess the long-term effects of the LFS (followed by paired-pulse facilitation stimulation). All electrical signals were amplified and filtered (1 Hz and 3 kHz using a differential amplifier (Getting Instruments, San Diego, CA, USA) and then digitized at 5 kHz before being stored on a PC using custom-written software (Lee Campbell; Getting Instruments) and National Instruments data acquisition hardware.

2.1.7 Data and Statistical Analysis

The initial phase of the EPSP slope (10-80%) was assessed at 115-120-min post-LFS to assess LTD expression. All LTD data are presented as the mean percent EPSP change from baseline ± SEM. Either planned comparisons or analysis of variance (ANOVA) was performed on data and where appropriate the ANOVA was followed by Newman-Keuls post hoc analyses. All analyses were performed using Statistica software (Statsoft, Tulsa, OK) with statistical significance set at $p<0.05$.

2.2 Results

2.2.1 Effects of Sex and Diet on the Development of the Offspring

The average ethanol intake for the **ETHANOL** dams throughout gestation was 11.83 ± 0.27 g/kg body wt/day and the average BEC on GD15 was 192 ± 21 mg/dL, a value similar to

that observed in previous studies from our laboratory (Christie et al., 2005; Redila et al., 2006). The developmental data for **ETHANOL**, **PAIR-FED** and **AD LIBITUM** females and offspring is presented in **Table 2.1**. As we have reported previously (Christie et al., 2005), the ethanol diet delays parturition by 1.023 days on average compared to **AD LIBITUM** animals. The pair-fed animals gave birth 0.45 days later than the **AD LIBITUM** animals on average. Prenatal diet did not affect postnatal weight as there was no significant difference in weight between PND2-22 for male **ETHANOL**, **PAIR-FED** and **AD LIBITUM** animals ($F_{(2,25)}$=1.96, p=0.16) or female **ETHANOL**, **PAIR-FED** and **AD LIBITUM** animals ($F_{(2,25)}$=0.61, p=0.54). Furthermore, between PND2-22 there were no weight differences between males and female animals ($F_{(2,50)}$=0.24, p=0.78), regardless of prenatal diet. However, between PND30-35, male offspring did weigh more than female offspring ($F_{(1,111)}$=38.75, p=0.01), although at this age both **ETHANOL** males and females weighed less than their **PAIR-FED** and **AD LIBITUM** counterparts ($F_{(2,11)}$=6.56, p=0.01).

2.2.2 Effects of Acute Stress on Corticosterone Levels

Previous studies have found that ethanol exposed animals are not hyper-responsive to stress at PND39, compared to control animals, although they do still exhibit a stress response (Weinberg and Gallo, 1982). Therefore, it was of interest to compare corticosterone levels between **ETHANOL**, **PAIR-FED** and **AD LIBITUM** animals to determine if **ETHANOL** animals between PND30-35 exhibit a similar stress response as **AD LIBITUM** animals. Basal corticosterone levels were different between males and females ($F_{(1,70)}$=6.99, p=0.01), with higher corticosterone levels in females (62.70 ± 5.35 ng/ml) than in males (59.68 ± 3.79 ng/ml). Exposure to the elevated platform for 30 minutes increased corticosterone levels in both males and females above that observed in naïve

animals ($F_{(1,70)}$=154.54, p=0.01; **Figure 2.1**). There was no effect of prenatal diet on corticosterone levels ($F_{(2,70)}$=0.71, p=0.49), and all groups responded equally to the acute stress paradigm with significantly increased corticosterone levels.

2.2.3 Effects of Ethanol, Diet and Sex on Paired Pulse Facilitation.

Paired-pulse facilitation was examined to determine if stress or prenatal diet altered presynaptic transmitter release in the CA1 region. In male animals, there was a significant main effect of inter-pulse interval ($F_{(1,108)}$= 131.01, p= 0.01) with greater PPF at 50ms than 100ms (p=0.01). Comparisons were then made in female animals revealing a significant main effect of inter-pulse interval ($F_{(1,94)}$=126.41, p=0.01); a 50ms IPI elicited greater PPF than at 100ms (p=0.01). Importantly, there was no effect of prenatal diet on the amount of PPF elicited in males ($F_{(2,108)}$= 1.18, p=0.31) or females ($F_{2,94)}$=3.01, p=0.06) indicating that neither PNEE nor prenatal food deprivation alter presynaptic neurotransmitter release. Paired-pulse facilitation results are summarized in **Figure 2.2**.

2.2.4 Effects of Stress on LTD in Male and Female Animals

To determine if male and female animals differed in their capacity to exhibit LTD in the CA1 region, we examined the effects of LFS in both the NAÏVE animals and in the animals that were exposed to acute stress (platform isolation) for 30 minutes. The change in EPSP slope at 120 minutes post-LFS was different between males and females, as shown in **Figure 2.3A**. Specifically, NAIVE **AD LIBITUM** males did not exhibit LTD (-3.16 ± 3.22%; $t_{(8)}$= -0.98, p=0.36) while significant LTD was observed in STRESS **AD LIBITUM** males (-29.61 ± 8.44%; $t_{(10)}$= -3.51, p=0.01), similar to previous studies (Xiong et al., 2004; Xiong et al., 2003; Xu et al., 1998). On the other hand, significant LTD was

observed in NAIVE **AD LIBITUM** females (-18.33 ± 5.99%; $t_{(12)}$= -3.06, p=0.01) but not in the STRESS **AD LIBITUM** females (2.64 ± 4.43%; $t_{(9)}$= 0.60, p=0.57; **Figure 2.3B**). The amount of LTD in the NAIVE **AD LIBITUM** females was not significantly different from that observed in the STRESS **AD LIBITUM** males ($t_{(20)}$=1.12, p=0.23).

2.2.5 Effects of Prenatal Food Deprivation on LTD in Male and Female Animals

Identical experiments to those conducted in the offspring of the **AD LIBITUM** dams were also conducted in the offspring of the **PAIR-FED** dams. In contrast to the results obtained in the **AD LIBITUM** offspring, the male **PAIR-FED** offspring showed significant LTD in the absence of stress (NAIVE: -23.77 ± 4.44%; $t_{(12)}$= -5.35, p=0.00). In addition, a significant level of LTD was observed in the male STRESS **PAIR-FED** animals (-15.73 ± 5.28%; $t_{(9)}$= -2.98, p=0.02). The amount of LTD observed at 120 minutes post-LFS in the two male **PAIR-FED** offspring conditions was not significantly different ($t_{(19)}$= -1.17, p=0.26; **Figure 2.4A**).

A similar pattern was observed in the female **PAIR-FED** offspring. Significant LTD was observed in female **PAIR-FED** NAIVE offspring (-12.57 ± 5.57%; $t_{(13)}$= -2.25, p=0.04) as well as in the female **PAIR-FED** STRESS animals (-19.48 ± 4.96; $t_{(9)}$= -3.93, p=0.01). Once again, the amount of LTD observed in both female **PAIR-FED** offspring groups was not significantly different ($t_{(20)}$= 0.88, p=0.39; **Figure 2.4B**). In addition, the amount of depression observed in both **PAIR-FED** male and female offspring was not significantly different, regardless of the stress condition ($F_{(1,39)}$= 2.03, p= 0.16).

2.2.6 Effects of Prenatal Ethanol Exposure on LTD in Male and Female Animals

To determine if prenatal exposure to ethanol had deleterious effects on synaptic plasticity in the hippocampus, we examined LTD in male and female offspring from **ETHANOL**

dams. Male **ETHANOL** offspring did not exhibit significant LTD in the absence of stress (-8.01 ± 7.51%; $t_{(10)}$= -1.06, p=0.31) but, similar to **AD LIBITUM** offspring, did exhibit significant LTD in the STRESS condition (-9.64 ± 4.04%; $t_{(9)}$= -2.38, p=0.04), even though the magnitude of the change in EPSP slope in the NAIVE and STRESS **ETHANOL** male offspring was not significantly different ($t_{(17)}$=0.18, p=0.86; **Figure 2.5A**).

Although not significant, there was a trend for NAIVE **ETHANOL** female offspring to exhibit LTD (-15.57 ± 7.10%; $t_{(10)}$= -2.19, p=0.06). However, similar to STRESS **AD LIBITUM** female offspring, significant LTD was not induced following stress (-4.21 ± 5.88%; $t_{(7)}$= -0.72, p=0.50). Again, there was no difference in the magnitude of change in the EPSP slope in the STRESS and NAIVE **ETHANOL** female offspring ($t_{(15)}$= -1.15, p=0.27; **Figure 2.5B**).

2.2.7 Effect of Acute Stress on LTD across Prenatal Diets

The results thus far indicate that acute stress does not uniformly affect CA1 LTD in males and females. As well, the capacity for LTD in CA1 is enhanced following prenatal food deprivation because significant LTD was observed in NAÏVE **PAIR-FED** offspring. Interestingly, prenatal ethanol exposure does not seem to have deleterious effects on CA1 LTD in males because **ETHANOL** male offspring exhibit LTD only following acute stress, similar to control males. On the other hand, **ETHANOL** female offspring do not exhibit LTD in the absence of stress, in contrast to NAÏVE **AD LIBITUM** female offspring. Taken together, these results suggest that the capacity for LTD in males is unaffected by prenatal ethanol exposure but enhanced by prenatal nutrional deprivation. In females, prenatal ethanol exposure reduces LTD in NAÏVE **ETHANOL** female offspring but enhances LTD in **PAIR-FED** female offspring.

To determine whether the magnitude of LTD in **PAIR-FED** and **ETHANOL** offspring was similar to **AD LIBITUM** animal offspring, comparisons were made between male offspring, and between female offspring, from each group. In addition, the animals from each group were further subdivided into either stress or non-stress (NAIVE) conditions (**Figure 2.6**). A one-way ANOVA revealed a main effect of diet in male NAÏVE offspring ($F_{(2,27)} = 3.94$, $p = 0.03$) and post-hoc analyses revealed that male **PAIR-FED** offspring had significantly more LTD (-23.77 $\pm$ 4.44) than male **AD LIBITUM** offspring (-3.16 ± 3.22, $p = 0.03$); there was a trend toward greater LTD in **PAIR-FED** male offspring than in **ETHANOL** male offspring (-8.01 ± 7.50, $p = 0.06$). Surprisingly, there was also a trend toward a significant relationship of prenatal diet and LTD in STRESS male offspring ($F_{(2,25)} = 2.61$, $p = 0.09$), with less depression in **ETHANOL** male offspring than controls ($p = 0.09$).

Female NAÏVE offspring exhibited similar amounts of LTD, regardless of prenatal diet ($F_{(2.32)} = 0.23$, $p = 0.79$) but there was a main effect of prenatal diet in females exposed to acute stress ($F_{(2.22)} = 5.44$, $p = 0.01$). Post hoc analyses did not reveal significant LTD in **AD LIBITUM** and **ETHANOL** female offspring following LFS (2.63 ± 4.43 and -4.20 ± 5.87, respectively; $p = 0.34$). In contrast, female **PAIR-FED** offspring did show significant LTD (-19.48 ± 4.95, $p = 0.04$) indicating that food restriction can enhance LTD in the CA1 region of females, whether or not they are exposed to stress.

2.3 Discussion

There were a number of important and interesting findings in the present study. First, the capacity for LTD expression *in vivo* is different in male and female animals, even when they are tested prior to puberty, as was the case in this study. The LFS produced

significant LTD in NAÏVE **AD LIBITUM** female offspring, while LTD was only observed in STRESS **AD LIBITUM** male offspring. In contrast, food deprivation *in utero* (**PAIR-FED** animals) established a more permissive environment for the induction of LTD in both male and female offspring. Furthermore, LTD could be observed in both sexes following prenatal food deprivation regardless of whether they were exposed to stress or not. Surprisingly, prenatal ethanol exposure did not affect the capacity for LTD in NAÏVE female offspring, but there was a trend toward reduced LTD in STRESS **ETHANOL** offspring compared to STRESS **AD LIBITUM** male offspring. The capacity for LTD across groups was not associated with changes in presynaptic release mechanisms, as paired-pulse facilitation was not significantly different across sex or prenatal diet.

2.3.1 Effects of Stress on LTD in Male and Female Animals

Previous studies have shown that exposure to stress can facilitate the induction of LTD in male animals (Xiong et al., 2004; Xiong et al., 2003; Xu et al., 1998), which was replicated in the current study. In contrast to the **AD LIBITUM** male offspring, female offspring reliably expressed LTD in the absence, but not in the presence of stress. This is the first study to investigate the effects of stress on LTD in young female animals *in vivo,* and as such, it is important to acknowledge that the observed differences in synaptic plasticity between males and females might reflect differences in estradiol levels (Day and Good, 2005; Desmond et al., 2000; Good et al., 1999; Shiroma et al., 2005; Zamani et al., 2000). These previous studies provide evidence that estradiol can enhance LTP and reduce LTD. Since LTD was enhanced in control females with the current study, it does not seem likely that estradiol influenced synaptic plasticity in these prepubescent females. However, we did not directly determine estradiol levels in the animals used in this study,

and future studies will need to investigate whether there is any impact of sex hormones on synaptic plasticity at PND30-35, because this is within the range for the onset of puberty (Gabriel et al., 1992). It is noteworthy that although the stress response appears equivalent in male and female animals at PND39 (Weinberg and Gallo, 1982), our results indicate that sex differences do exist in both the physical response to stress and the effect of stress on synaptic plasticity as early as PND30. Acute stress differentially affects learning and memory in male and female animals (Beiko et al., 2004; Conrad et al., 2004; Hodes and Shors, 2005; Shors, 2004), with impairments more common in males. As glucocorticoid receptors (GRs) are active in response to stress (Reul et al., 1987; Reul and de Kloet, 1985; Spencer et al., 1993), these findings suggest that acute stress may not lead to uniform GR occupancy in males and females. In adult females, GRs have greater B_{max} and higher K_d values than males, which is not affected by prenatal food deprivation or PNEE; GRs in females have similar properties and are likewise unaffected by prenatal diet (Weinberg and Petersen, 1991). It has previously been shown that MR and GR density is not altered by PNEE in the female hippocampus (Kim et al., 1999b) subtle differences in receptor density were apparent when assessed across the estrous cycle. Specifically, reduced mineralocorticoid receptor (MR) mRNA in adult **ETHANOL** females was most apparent during proestrus (Sliwowska et al., 2008). On the other hand, adult **ETHANOL** females had significantly more GR mRNA during proestrus (compared to other phases of the estrous cycle), an effect that was not observed in **AD LIBITUM** of **PAIR-FED** females (Sliwowska et al., 2008). However, changes in MR and/or GR protein levels was not assessed in this study so it remains to be determined if the observed changes in mRNA expression translate to changes in protein levels. Less corticosterone is required

for the activation of MRs than GRs (Reul and de Kloet, 1985) and the receptors have opposing effects on synaptic plasticity such that MR activity enhances LTP (Pavlides et al., 1996) while GR activity enhances LTD (Xu et al., 1998). Therefore, in order to depress synaptic plasticity, optimal levels of corticosterone are required in order to activate GRs. However, if too little corticosterone is produced in response to stress, predominant MR activation might result which would enhance synaptic plasticity. Within the current study, NAÏVE **AD LIBITUM** and **ETHANOL** female offspring exhibited robust LTD, which was blocked by acute stress suggesting that the acute stress may have occupied MRs, effectively shifting the induction threshold for LTP. Although the exact mechanism through which stress affects CA1 LTD in females has yet to be determined, it is clear that there are basic sex differences in CA1 synaptic plasticity that are apparent as early as PND30-35.

2.3.2 Effects of Prenatal Food Deprivation on Synaptic Plasticity

The prenatal diets employed in this study were designed to provide adequate nutrition to dams, regardless of the amount of food diet consumed (Weinberg, 1985), although **PAIR-FED** and **ETHANOL** dams still consume less protein and fewer calories overall compared to **AD LIBITUM** dams (Weinberg, 1985). This prenatal food deprivation did alter CA1 plasticity, as significant LTD was observed in **PAIR-FED** male and female offspring, regardless of the stress condition. Previous studies have found that prenatal stress can enhance the expression of LTD (Yang et al., 2006) and since significant LTD was observed in both NAÏVE and STRESS **PAIR-FED** male and female offspring, it is possible that prenatal stress associated with this restricted feeding regimen may have affected offspring development. Although restricted feeding is not intended as a stressor, several

observations suggest that the **PAIR-FED** dams are stressed. Specifically, they responded to cues associated with feeding (e.g. removing bottles from cages, sounds of the bottles) and sniffed at the lid when the experimenter approached the cage. When fresh food was presented, dams immediately began eating and consumed all available food by morning. These behaviors suggest that the feeding regimen has an unavoidable element of mild stress. The stress associated with the restricted diet can affect the stress response in **PAIR-FED** dams in that corticosterone levels remain elevated following the termination of a stressor (Weinberg and Gallo, 1982). Therefore, restricted feeding may serve as a mild stressor thereby enhancing the induction of LTD in offspring. However, this is a necessary group when assessing the effect of prenatal ethanol exposure on offspring because dams with the ethanol diet do not consume all of the available food, which adds an element of food restriction to this diet as well. The pair-fed diet thus serves as a control for this effect.

2.3.3 Effects of Prenatal Ethanol Exposure on Synaptic Plasticity

The direct effects of ethanol on LTD have yielded mixed results, with one report showing LTD is blocked while the other shows it to be enhanced (Hendricson et al., 2002; Thinschmidt et al., 2003). The current study is the first to investigate the relationship between prenatal ethanol exposure and LTD and found that prenatal exposure to this teratogen does not produce a long-lasting impairment in the ability of animals to express LTD. Our initial comparisons indicated that stress was required for LTD in **ETHANOL** male offspring while there was a trend toward blocked LTD in **ETHANOL** female offspring following acute stress. These findings were similar to the respective **AD LIBITUM** control offspring, so comparisons were made across prenatal diets. These

revealed that LTD was not significantly affected by prenatal ethanol exposure. Specifically, there was no significant difference in the amount of LTD observed between STRESS **AD LIBITUM** and **ETHANOL** male offspring or between NAÏVE **AD LIBITUM** and **ETHANOL** male offspring. Furthermore, there was no significant difference between the amount of LTD in NAÏVE **AD LIBITUM** and **ETHANOL** female offspring, nor between STRESS **AD LIBITUM** and **ETHANOL** male offspring. Therefore, in contrast to the results obtained in **PAIR-FED** offspring, the effect of stress on CA1 LTD in males and females is preserved following prenatal ethanol exposure.

Previous studies have indicated that prenatal ethanol exposure attenuates hippocampal LTP in adult animals (Christie et al., 2005; Sutherland et al., 1997; Swartzwelder et al., 1988) although LTP does appear to be altered in young males (Krahl et al., 1999). A heightened stress response is also not apparent in young animals (Weinberg and Gallo, 1982). Taken together, this suggests that the adolescent brain might not be as susceptible to the deleterious effects of prenatal ethanol exposure, but that these impairments become apparent with age. The results of the current study support this notion since basal and stress CORT levels, as well as the amount of LTD in **ETHANOL** males and female offspring was not significantly different from their **AD LIBITUM** counterparts. The functional integrity of GRs and MRs in the hippocampus are not affected by prenatal ethanol exposure (Weinberg and Petersen, 1991), which may account for the LTD in the male **ETHANOL** STRESS condition, since it presumably relies upon functional GRs in the hippocampus (Xu et al., 1998). Furthermore, the acute stress may have preferentially occupied MRs in **ETHANOL** female offspring, which would account for the reduced LTD observed following acute stress.

It is curious that significant LTD was not observed in NAIVE and STRESS **ETHANOL** male offspring and female offspring, as was found in **PAIR-FED** animals. **ETHANOL** dams have elevated corticosterone levels, when compared to both **PAIR-FED** and **AD LIBITUM** dams, in response to acute stress (Weinberg and Gallo, 1982) suggesting that the ethanol diet can alter HPA activity. Ethanol can also directly stimulate the HPA axis (Ogilvie and Rivier, 1996; Rivier, 1993; Rivier, 1996; Rivier and Lee, 1996) which might cause enhanced HPA activity in response to stress, compounding the elevated corticosterone levels in **ETHANOL** dams following acute stress. However, the actions of ethanol on the HPA axis might not be "stressful" but instead simply activates the HPA axis. Indeed, animals with PNEE have elevated ACTH levels when exposed to a stressor but not following infusion of exogenous CRH (Lee et al., 2000) suggesting that artificial stimulation of the HPA axis by exogenous CRH might not produce the same effect as actually experiencing stress. Therefore, the ethanol-exposed dams might have elevated corticosterone due to the stimulatory effects of ethanol on the HPA axis but the **PAIR-FED** dams *experienced* the stress of food deprivation. Thus LTD was expressed in both NAÏVE and STRESS pair-fed offspring due to the mild stress experienced by the **PAIR-FED** dam throughout gestation. This hypothesis is supported by the finding that prenatal stress can enhance the induction of LTD in offspring (Yang et al., 2006). In contrast to **ETHANOL** offspring, **PAIR-FED** offspring do not always exhibit elevated corticosterone levels in response to stress (Weinberg, 1988; Weinberg, 1992; Weinberg and Gallo, 1982) and LTP in **PAIR-FED** animals is not different from controls (Christie et al., 2005; Savage et al., 2002; Sutherland et al., 1997). This suggests that LTD is more sensitive to prenatal food deprivation and prenatal stress than it is to prenatal ethanol exposure, even though

prenatal ethanol exposure can impact LTP (Christie et al., 2005; Savage et al., 2002; Sutherland et al., 1997).

2.3.4 Summary

Young male and female animals possess different capacities to exhibit LFS induced LTD in vivo, with females being more likely to exhibit LTD than males. Acute stress has opposing effects on synaptic plasticity in each sex, enabling the induction of LTD in males, but impairing it in females. Although normally a control group for prenatal ethanol exposure, the **PAIR-FED** offspring all showed LTD, indicating that this manipulation can affect synaptic plasticity. More importantly, this LTD could be induced in the **PAIR-FED** offspring irrespective of whether they were exposed to acute stress prior to experimentation or not. This would indicate that the prenatal stress associated with restricted food intake *in utero* is a main factor in influencing the capacity for LTD in these animals. Surprisingly, this is not the case for LTP, which does not appear to be inhibited by the pair-fed diet (Christie et al., 2005; Sutherland et al., 1997). In contrast to our expectations, prenatal ethanol exposure did not significantly impair LTD in young males or females, although there was a trend for it to reduce LTD in NAÏVE male offspring, as compared to **AD LIBITUM** male offspring.

2.4 Acknowledgements

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Table 2-1 Developmental Data for Ethanol, Pair-fed and Ad libitum Dams and Offspring from Birth to PND35

	Treatment		
	Ethanol	Pair-fed	Ad libitum
Pregnancy outcome variable			
Number of pregnant dams	8	10	10
Gestation Length (days)	22.25 ± 0.25 [a]	21.8 ± 0.133	21.48 ± 0.15
Number of male pups	6.62 ± 0.53	7.1 ± 1.10	7.40 ± 0.80
Number of female pups	7.37 ± 0.80	6.9 ± 0.87	7.90 ± 0.92
Male pup weight (g)			
PND2	5.03 ± 0.10	6.57 ± 1.39	6.56 ± 1.42
PND8	13.57 ± 0.47	17.66 ± 3.75	17.77 ± 4.70
PND15	27.54 ± 4.03	33.11 ± 6.79	32.52 ± 6.66
PND22	42.48 ± 5.98	55.21 ± 11.35	49.77 ± 7.71
Female pup weight (g)			
PND2	5.92 ± 1.26	5.82 ± 0.80	7.04 ± 0.77
PND8	13.73 ± 3.56	14.80 ± 2.45	16.19 ± 3.35
PND15	26.57 ± 4.52	29.29 ± 4.35	27.15 ± 6.82
PND22	41.15 ± 9.27	46.48 ± 7.87	46.28 ± 2.80
PND 30-35 weight (g)			
Male [b]	103.00 ± 3.08	112.57 ± 3.56	116.26 ± 7.62
Female	78.38 ± 1.78	88.04 ± 3.99	98.72 ± 3.45

[a] Significantly longer than Pairfed and Ad libitum

[b] Significantly larger than females on corresponding PND.

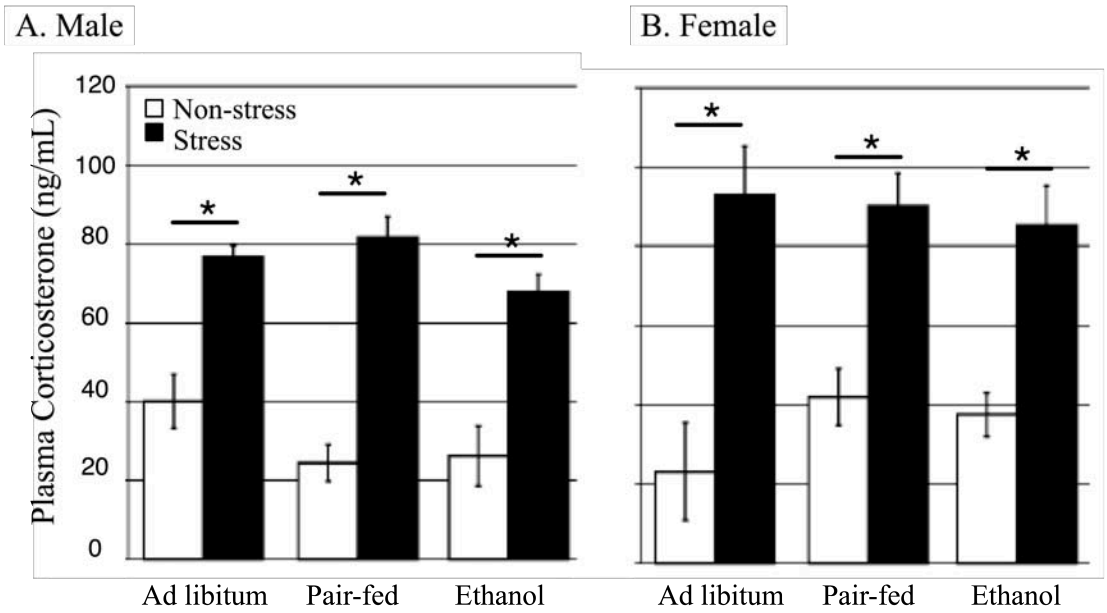

Figure 2.1 CORT levels are increased in males and females following acute stress.

A). Basal CORT levels are not significantly different amongst male AD LIBITUM, PAIR-FED and ETHANOL animals. Exposure to elevated platform stress significantly increased CORT in male offspring. B). Basal CORT levels are not significantly different amongst AD LIBITUM, PAIR-FED and ETHANOL females. Exposure to an elevated platform stress significantly increased CORT levels in female offspring. * $p<0.05$

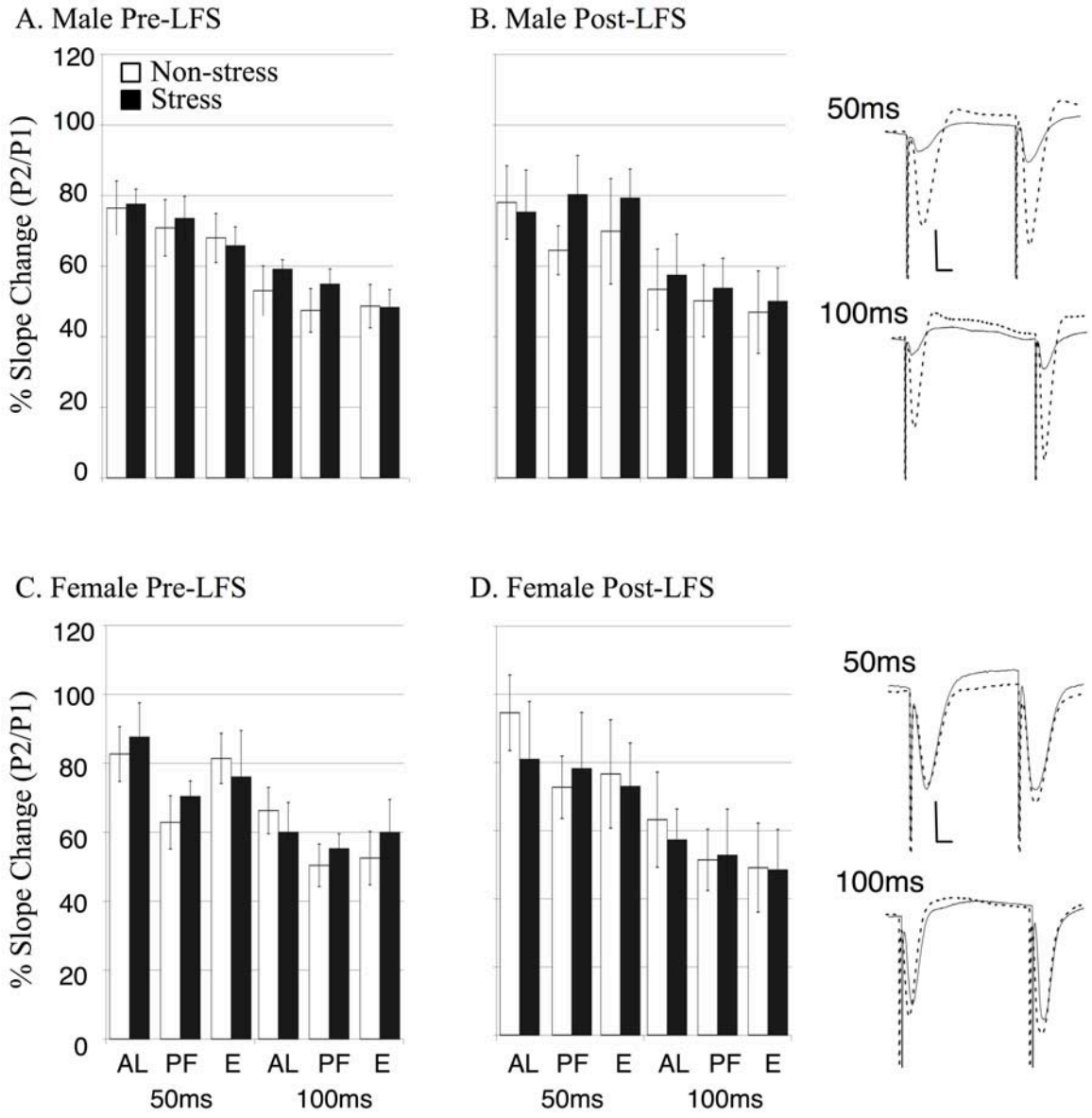

Figure 2.2 Paired-pulse facilitation before and after LFS.

Paired-pulses administered at 50 and 100ms intervals in males (A) before LFS and (B) after LFS. Identical results were obtained in females before (C) and after (D) LFS. There was no significant difference between any of the groups before or after LFS, however, overall less facilitation was observed at 100ms than at 50ms (p=0.00). Representative traces are taken from control animals and dotted lines are traces prior to LFS. Scale bar is 1mV by 5ms. AL: AD LIBITUM, PF: PAIR-FED, E: ETHANOL.

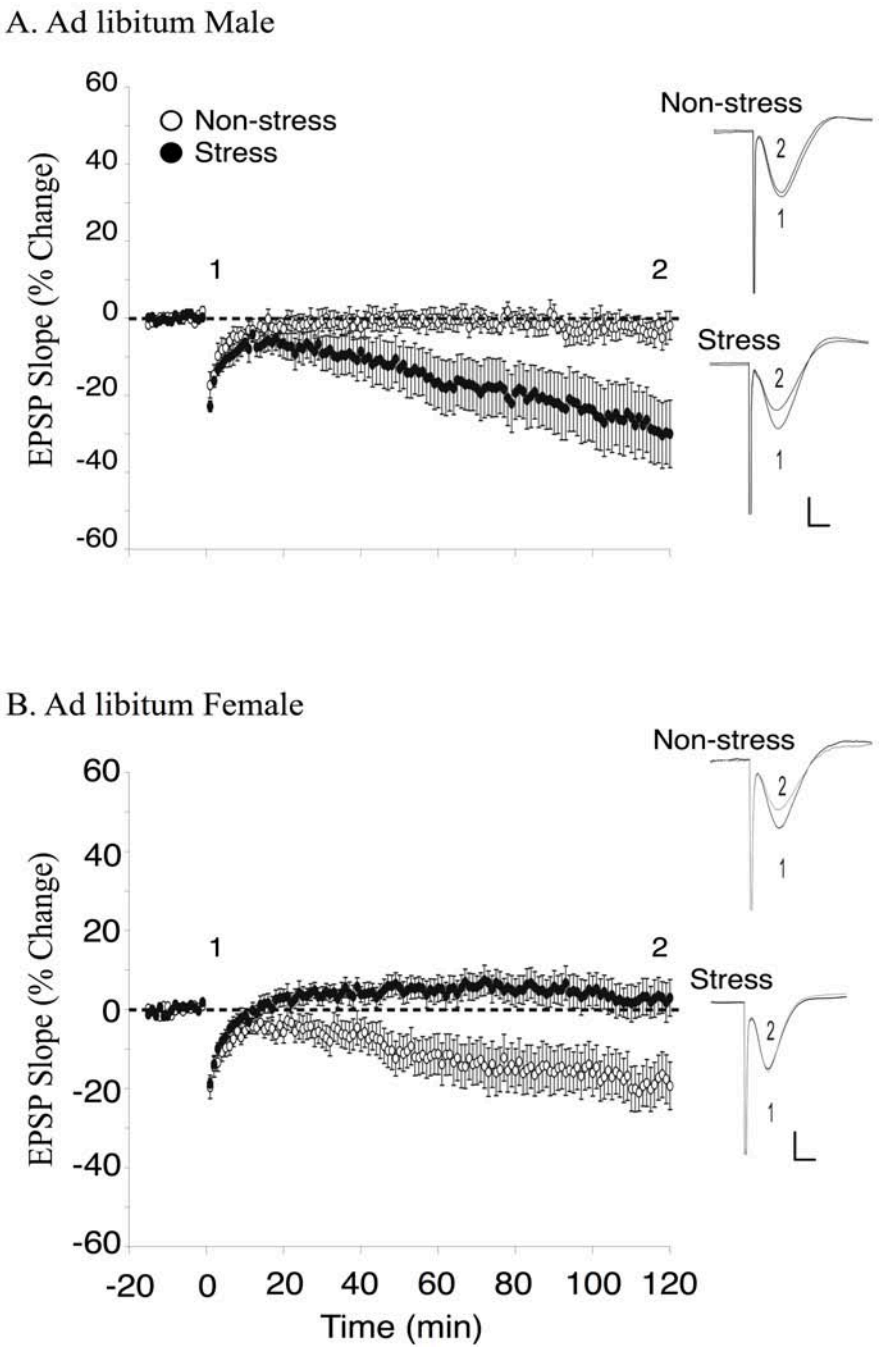

Figure 2.3 Long-term depression in male and female ad libitum animals.

A). 3Hz LFS does not induce LTD in male **AD LIBITUM** NAIVE (n=8) animals but does result in LTD in male **AD LIBITUM** animals exposed to an acute stress (elevated platform) for 30 minutes (n=10). Note that despite the persistent downward trend in the STRESS group, the final 20 minutes is stable. B). Female **AD LIBITUM** NAIVE animals (n=13) exhibits LTD following 3Hz stimulation whereas female **AD LIBITUM** STRESS animals (n=9) do not show LTD. Scale bar is 1mV by 5ms.

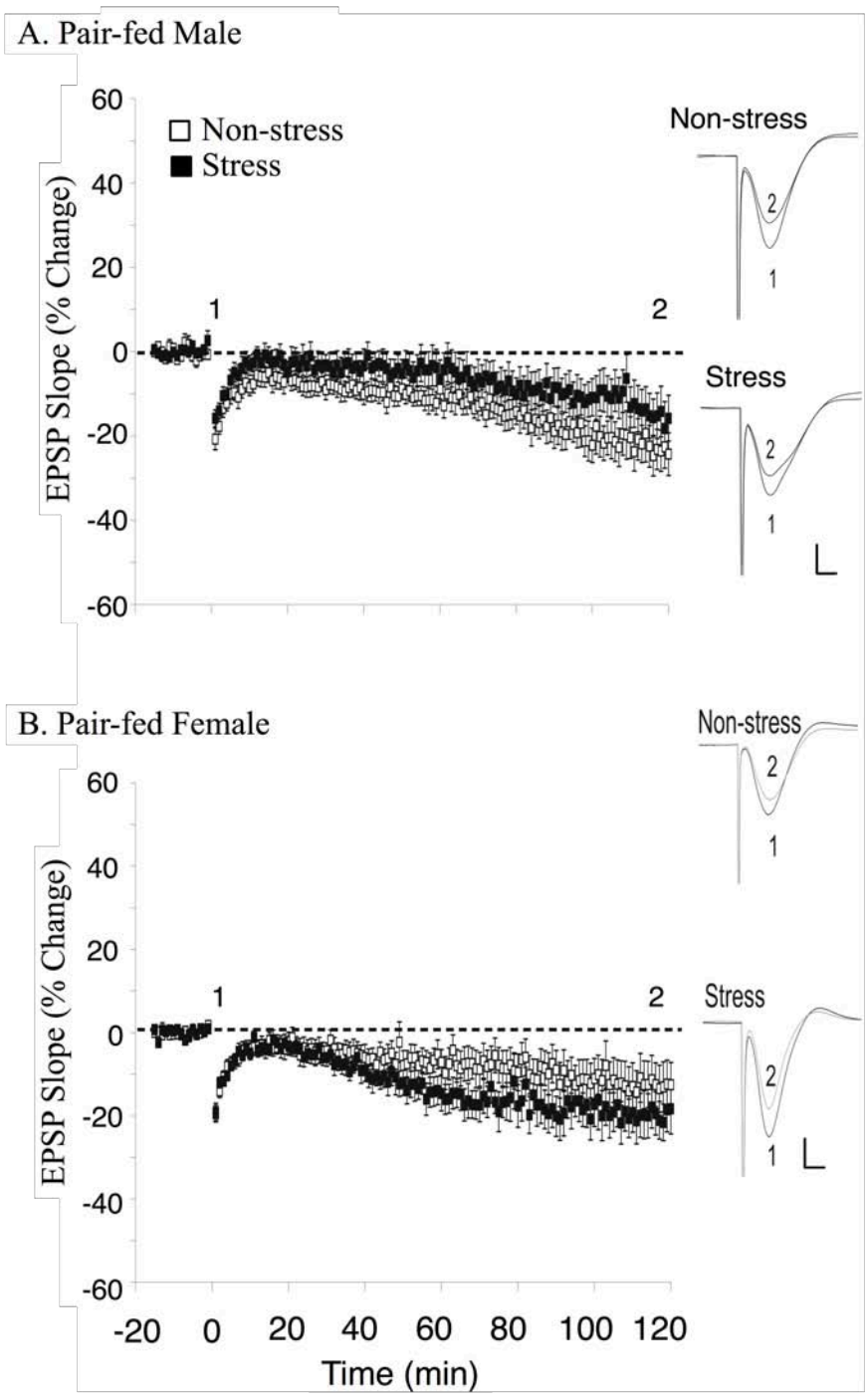

Figure 2.4 Long-term depression in male and female pair-fed animals.

A. LTD can be induced in male PAIR-FED NAIVE (n=11) animals and there is a depression of EPSP slope in male PAIR-FED STRESS (n=9) animals. B. Stress was required for LTD in female PAIR-FED animals (n=9) as there was a slight depression of EPSP slope in female PAIR-FED NAIVE (FPNS, n=13) animals, although this did not reach significance. Traces correspond to immediately prior to LFS (1) and 2h after LFS (2). Scale bar is 1mV by 5ms.

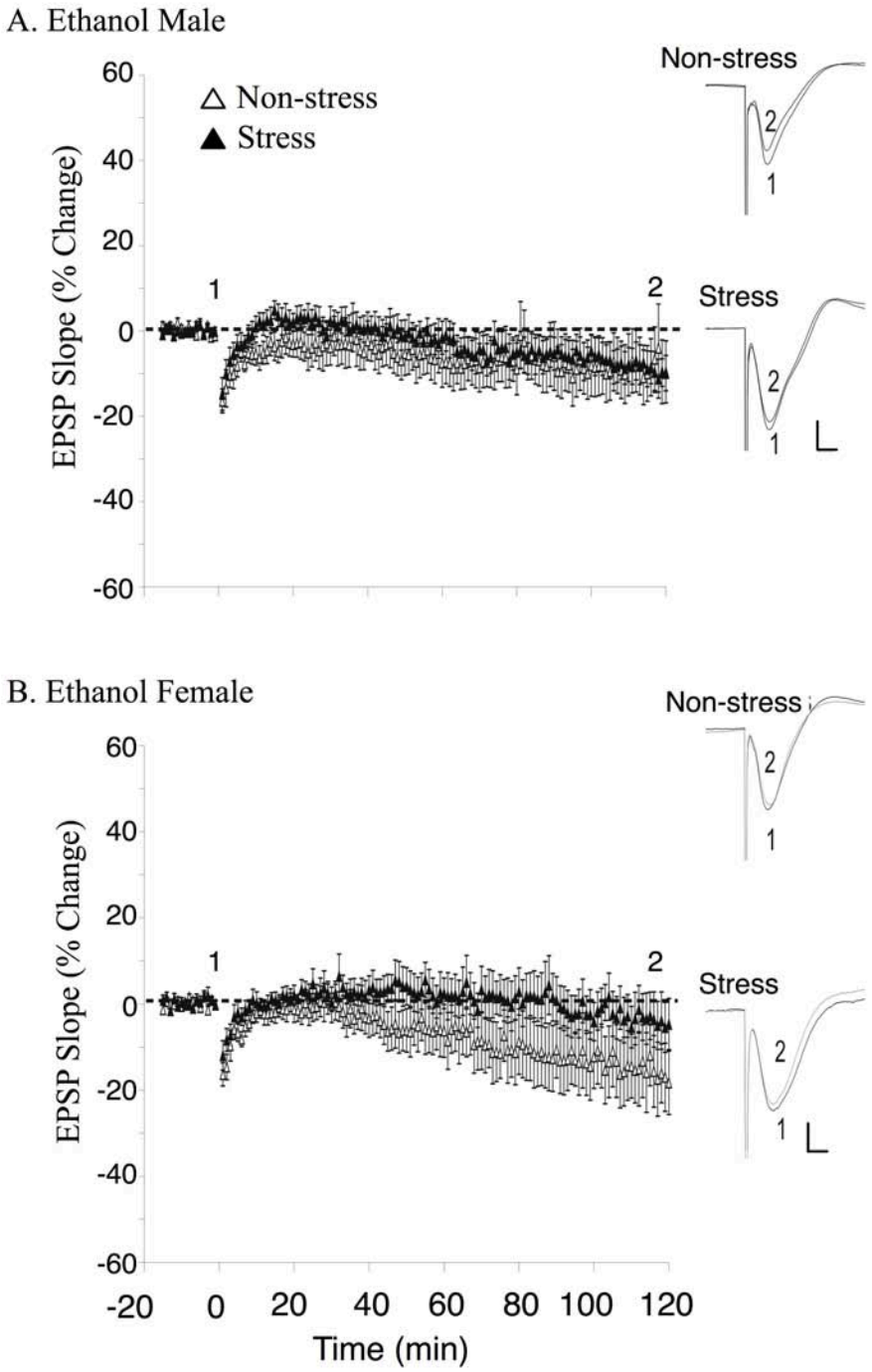

Figure 2.5 Long-term depression in male and female ethanol animals.

A. Neither male **ETHANOL** NAIVE animals (MENS, n=10) nor male **ETHANOL** STRESS animals (n=9) showed LTD following 3Hz conditioning. B. LTD was also not apparent in either female **ETHANOL** NAIVE (n=10) or female **ETHANOL** STRESS animals (FES, n=7). The slight depression in NAÏVE **ETHANOL** females was not significantly different from baseline. Traces correspond to immediately prior to LFS (1) and 2h after LFS (2). Scale bar is 1mV by 5ms.

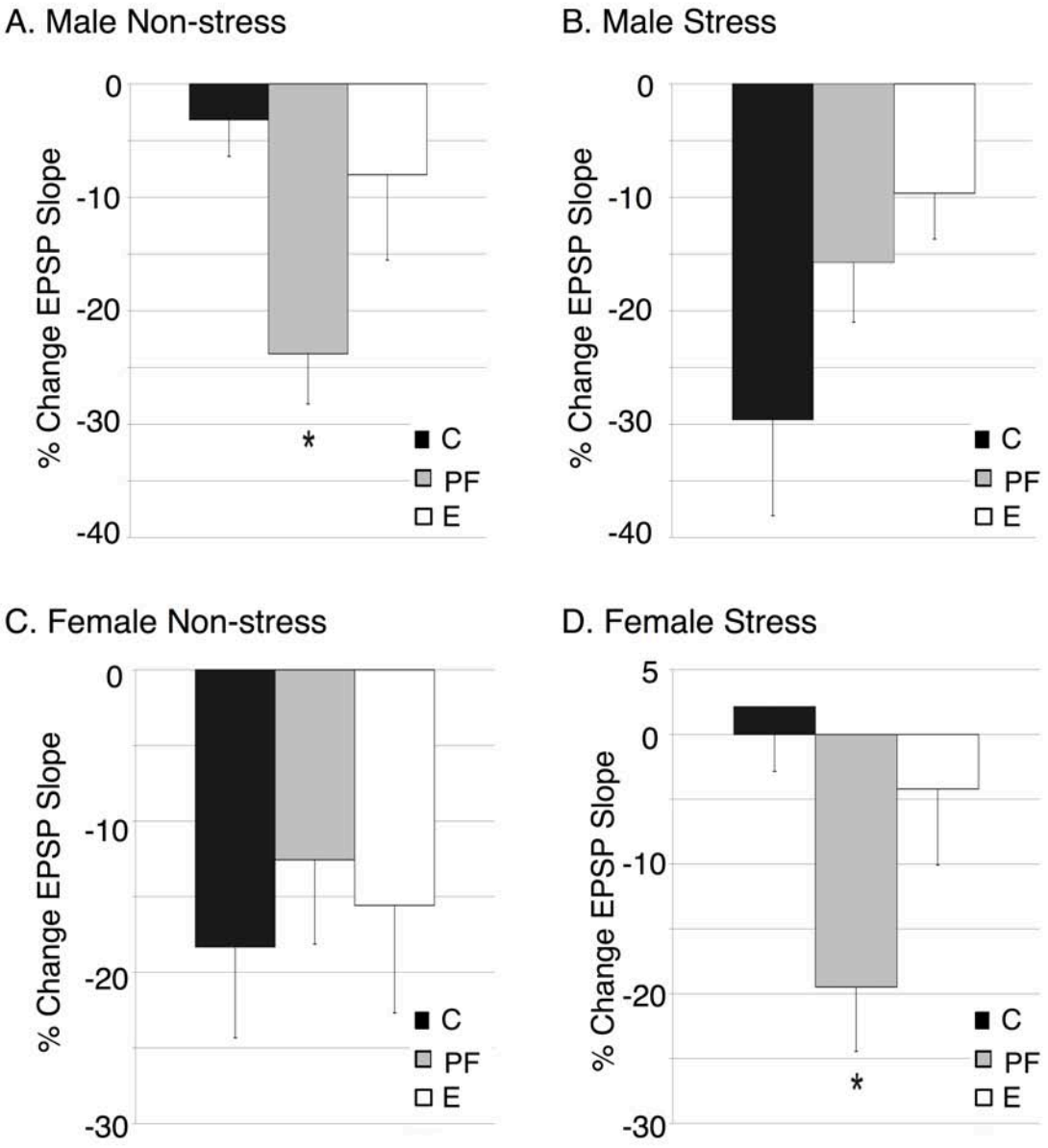

Figure 2.6 Summary of long-term depression across prenatal diets.

A. In the absence of stress, PAIR-FED males exhibited significantly more LTD than AD LIBITUM; E animals were not significantly different from AD LIBITUM or PAIR-FED animals. B. Equivalent LTD was observed when males across all prenatal diets were exposed to stress, although there was a trend toward less LTD in ETHANOL compared to AD LIBITUM. C. NAIVE females across all prenatal diets exhibit comparable LTD. D. Significant LTD was observed in STRESS PAIR-FED females only, compared to controls. Asterisks indicate significantly different from corresponding control animals. AL: AD LIBITUM, PF: PAIR-FED, E: ETHANOL.

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3 Prenatal Ethanol Exposure Enhances NMDAR-dependent LTP in the Adolescent Female Rat Dentate Gyrus [2]

3.1 Introduction

Changes in the *in utero* environment can have a profound impact on the developing fetus. The consumption of alcohol during pregnancy, in particular, can lead to a host of abnormalities that include central nervous system (CNS) dysfunction, impaired cognition, and reduced growth. Collectively these deficits are referred to fetal alcohol spectrum disorder or FASD (Hoyme et al., 2005). High levels of maternal stress can also negatively impact the development and maturation of the CNS. However it is not clear if stress would interact with prenatal ethanol exposure synergistically.

Human and animal studies have shown that hippocampal-dependent spatial learning and memory is impaired in male offspring following exposure to ethanol *in utero* (Reyes et al., 1989; Richardson et al., 2002; Ryan et al., 2008; Uecker and Nadel, 1996; Uecker and Nadel, 1998; Wilcoxon et al., 2005). Consistent with an impairment of hippocampal-dependent learning, long term potentiation **(LTP)** of synaptic efficacy, a putative model of learning and memory (Bliss and Collingridge, 1993), can be reduced in males following prenatal ethanol exposure **(PNEE)** across gestation (Christie et al., 2005; Richardson et al., 2002; Sutherland et al., 1997; Swartzwelder et al., 1988). These studies suggest that the hippocampus is sensitive to insult following PNEE but it is unknown how PNEE affects hippocampal synaptic plasticity in females.

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Prenatal stress can alter hippocampal structure and function similar to that observed following PNEE. Specifically, prenatal stress **(PS)** can impair spatial learning and memory (Hosseini-Sharifabad and Hadinedoushan, 2007; Son et al., 2006; Yaka et al., 2007; Yang et al., 2006; Yang et al., 2007) and LTP in the CA1 region of the hippocampus (Son et al., 2006; Yaka et al., 2007; Yang et al., 2006). Activation of the hypothalamic-pituitary-adrenal **(HPA)** axis can elevate levels of the stress hormone corticosterone (CORT). CORT can readily cross the placenta and may directly contribute to some of the deleterious effects of prenatal stress on hippocampal function (Barbazanges et al., 1996; Zagron and Weinstock, 2006). Interestingly, ethanol consumption can significantly elevate CORT levels above controls following an acute stress (Weinberg and Gallo, 1982) and directly stimulates HPA activity (Nash and Maickel, 1988; Pohorecky, 1990). This suggests that ethanol consumption and stress during gestation might act synergistically to affect hippocampal function. Indeed, combined exposure to both ethanol and stress *in utero* can produce more severe impairments in learning (Schneider et al., 2001), hyperactivity (Schneider et al., 2001), responsivity to stressors experienced later in life (Schneider et al., 2004) and sexual function (Ward et al., 1999; Ward et al., 1994; Ward et al., 2002) than exposure to either ethanol or stress alone.

Despite evidence of sex differences in spatial learning and memory (Driscoll et al., 2005; Isgor and Sengelaub, 1998; Kanit et al., 2000; Mendez-Lopez et al., 2009; Takase et al., 2008), and synaptic plasticity (Maren, 1995; Maren et al., 1994; Titterness and Christie, 2008; Yang et al., 2004), little work has been done to investigate how PNEE affects synaptic plasticity in females. We have recently shown that acute stress

differentially affects long-term depression in control males and females and PNEE does not alter this sexual dimorphism (Titterness and Christie, 2008). However, it is unknown how PNEE affects LTP in females. Therefore, the goals of the current study were to determine 1) how PNEE affects LTP in the dentate gyrus of adolescent females, 2) whether prenatal stress alters DG LTP in males and females and 3) if PNEE and prenatal stress act synergistically to affect DG LTP.

3.2 Methods

3.2.1 Animals

Male (275-300g) and virgin female (250-275) female Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, PQ, Canada). The colony room was maintained on a 12hr light: dark cycle (lights on at 0600 h) with constant humidity temperature (22°C) with *ad libitum* access to standard rat chow (Lab Diets, 5001) and water. All animal procedures were performed in accordance with the University of Victoria, University of British Columbia, and the Canadian Council for Animal Care policies (**Appendix C**).

3.2.2 Breeding and Diets

Following an acclimation period of at least one week, individual females were paired with a single male. A timeline for the experiments is shown in **Figure 3.1**. Vaginal smears were taken daily at 09:00h and checked for the presence of sperm, indicating gestation day 1 (GD1), at which point females were individually housed and randomly assigned to one of three feeding conditions: 1) ethanol (E): *ad libitum* liquid diet containing 35.5 % ethanol derived calories (EDC); 2) pair-fed (PF): liquid diet with

maltose-dextrin isocalorically substituted for EDC. PF dams received the same amount of food in g/kg/day as the matched E dam; and 3) ad libitum (AL): *ad libitum* access to standard rat chow. All dams had unrestricted access to water. E dams were gradually introduced to the liquid diet across the first three days of gestation by combining 1/3 ethanol liquid diet with 2/3 pair-fed diet on GD1, 2/3 ethanol diet with 1/3 pair-fed diet on GD2 and 3/3 ethanol diet on GD3. Freshly prepared liquid diets were given 2 hours prior to lights off to prevent a shift in the CORT circadian rhythm (Weinberg and Gallo, 1982), at which point bottles from the previous day were weighed to determine the amount of food consumed. Liquid diets were obtained from Dyets (Bethlehem, PA) and are sold as Weinberg/Keiver high protein liquid diet-control (no. 710109) for the pair-fed diet and Weinberg/Keiver high protein liquid diet-experimental (no. 710324) for the ethanol diet. Both liquid diets are nutritionally fortified to provide adequate nutrition for pregnant rodents (Weinberg, 1985). Liquid diets were replaced with *ad libitum* access to standard rat chow on GD22, which helps to reduce any further deficits of ethanol exposure on offspring (Weinberg, 1989).

Females were weighed on GD1, 7, 14, and 21 during routine cage changing to minimally disturb the animals. The day on which females gave birth indicated postnatal day 1 (PND1) and litters were culled to 10 pups (5 male/5 female when possible) on PND2. After birth, cages were changed twice weekly and dams and offspring were weighed on PND 2, 8, 15 and 22. Offspring were weaned on PND 22 and group housed (2-3 per cage) by sex and electrophysiological experiments were performed on adolescent offspring between PND30-35. To reduce litter effects (Zorrilla, 1997), only two male and female offspring from each dam were used for electrophysiology recordings.

3.2.3 Prenatal Stress

E, PF and AL dams were randomly assigned to one of two housing conditions: 1) stress (S): three, 45-minute restraint sessions (09:00, 12:00 and 15:00h) during gestation days 12-21 or 2) non-stress (NS): remained undisturbed in their home cage. Restraint stress was performed by placing females individually in a clear plastic tube (diameter=7 cm; length=19cm) under bright light (Zuena et al., 2008). Tubes contained holes at either end for airflow. The last restraint session occurred at 15:00 h to ensure that the timing of liquid diet administration was consistent across stress/non-stress conditions.

3.2.4 Blood Collection

3.2.4.1 Blood Ethanol Concentration

To determine blood ethanol concentrations (BEC), tail vein blood samples were taken on GD15 from ethanol dams approximately 2 hours after lights out. Blood was sampled from three randomly chosen E dams from both stress and non-stress conditions. Blood was collected in a microcentrifuge tube and allowed to clot overnight at 4^0C. The following day, samples were centrifuged at 3000xg and supernatant was collected and stored at -20^0C until assayed. BECs were determined using the Analox Alcohol Analyzer (Model AMI; Analox Instruments, Lunenberg, MA).

3.2.4.2 Corticosterone

To determine the CORT response to the prenatal restraint stress, tail blood samples were collected from restraint dams immediately following cessation of the first restraint (~9:45 am) on GD 12, 17 and 21. Blood samples were also collected from E, PF and AL dams that were not subjected to the restraint stress on GD 12; offspring from these non-stress

dams were not used in the current study. Samples were collected from non-stress dams within 2 minutes of touching the cage in order to reduce the influence of HPA activation on the basal blood sample (Davidson et al., 1968). Blood samples were collected and allowed to clot for approximately 30 minutes at room temperature then centrifuged at 3,000xg for 15 minutes; supernatant was collected and stored at -20^{0}C until assayed. CORT levels were assayed via enzyme-linked immunoassay (ELISA, Assay Designs; Ann Arbor, MI; catalog #900-097) according to manufacturer's instructions. The minimum detection of the kit is 26.99 pg/mL and has the cross reactivity is 100% for corticosterone, 28.6% for deoxycorticosterone, 1.7% for progesterone and less than 0.3% for testosterone, aldosterone and cortisol.

3.2.5 Electrophysiology

Animals were anesthetized with urethane (1.5g/kg, i.p.) and placed on a Kopf stereotaxic apparatus. Rectal temperature was monitored and maintained at 37± 1^{0}C with a grounded homeothermic temperature control unit (Harvard Instruments, MA). One electrode was inserted into the skull anterior to bregma and a second posterior to lambda to serve as a reference and ground for the recording and stimulating electrodes, respectively. A 125 µm diameter stainless steel recording electrode was directed through a trephine hole to the dorsal dentate gyrus (3.5 mm posterior, 2.0 mm lateral to bregma). A monopolar stimulating electrode (125 µm diameter) was directed through a trephine hole to the ipsilateral perforant path (7.4mm posterior, 3 mm lateral to bregma). Once both stimulating and recording electrodes were lowered to elicit a maximal response, the minimal stimulation required to elicit a population spike that was ~1-2 mV was determined. The baseline excitatory postsynaptic potential **(EPSP)** was assessed by

delivering a single pulse (0.12ms in width) at 0.067Hz for a minimum of 15 minutes. Once a stable baseline was obtained, long-term potentiation (LTP) was induced by administering a theta-burst protocol consisting of 4 trains of 10 bursts of 5 pulses at 400Hz with a 200ms interburst interval. The pulse width was changed to 0.24ms during theta-burst stimulation (TBS) and there was a 15s delay between trains. Following TBS, baseline stimulation resumed for 60 minute period, after which the animal was euthanized by an overdose of urethane to the brain. Electrical signals were acquired using custom written software (Lee Campbell; Getting Instruments) and National Instruments data acquisition hardware. Signals were amplified and filtered at 1Hz and 3Hz using a differential amplifier (Getting Instruments, San Diego, CA) and digitized at 5 kHz before being stored on a PC. All electrophysiology data are presented as the percent change from baseline (mean ± SE) of the initial phase of the EPSP slope (10-80%). The following is a summary of the number of offspring in each group: ad libitum, non-stress male (n=10); ad libitum, non-stress female (n=12); ad libitum, stress male (n=11); ad libitum, stress female (n=11); pair-fed, non-stress male (n=10); pair-fed, non-stress female (n=14); pair-fed, stress male (n=11); pair-fed, stress female (n=11); ethanol, non-stress male (n=10); ethanol, non-stress female (n=13); ethanol, stress male (n=8); ethanol, stress female (n=14).

3.2.6 Drug

The competitive antagonist against N-methyl-D-aspartate receptors (NMDARs) (±)-3-(2-Carboxypiperazin-4-yl) propyl-1-phosphonic acid (**CPP**) was obtained from Sigma and dissolved in 0.9% saline. Drug was administered intraperitoneal 90 minutes prior to application of TBS at a dose of 10mg/kg (Farmer et al., 2004).

3.2.7 Data and Statistical Analyses

All data are presented as mean ± standard error of the mean (S.E.M.). A 2-way analysis of variance (ANOVA) for prenatal diet (AL, PF, E) and stress condition (NS, S) was conducted on dam weight gain across gestation. Pup weight was analyzed using a 2-way ANOVA for sex (male, female) x diet (AL, PF, E) on PND2 because PNEE has been shown to reduce birth weight (Christie et al., 2005; Weinberg and Gallo, 1982); offspring weight for PND 8-35 were analyzed repeated measures ANOVA. LTP data were analyzed by assessing the initial phase of the EPSP slope (10-80%) at 55-60 minutes post-TBS. A 3-way ANOVA of prenatal diet (AL, PF, E) X prenatal stress (NS, S) and sex (male, female) was performed on LTP data. For all ANOVAs, Newman-Keuls *post hoc* tests were performed where appropriate. Based on the *a priori* hypothesis that PS would reduce LTP in ad libitum male offspring, we also performed a student's t-test between male ad libitum stress and non-stress offspring and between female ad libitum stress and non-stress offspring. We also hypothesized that application of CPP will block LTP and thus performed t-tests to determine if the percent change in EPSP slope was 1) significantly different from baseline and 2) significantly reduced compared to saline counterparts. To control for multiple comparisons, Bonferroni corrections were applied when analyzing the data and statistical significance was set at $p<0.016$ for CPP data. Statistical analyses were performed using Statistica software (Statsoft, Tulsa, OK) with statistical significance set at a $p < 0.05$ unless otherwise stated.

3.3 Results

3.3.1 Developmental Data

There was not a significant difference in blood ethanol concentration in non-stress ethanol dams (86.91 ± 14.90 mg/dl) compared to stress ethanol dams (114.29 ± 26.50 mg/dl; $t_{(11)}$=0.9161, p<0.3792). Maternal weight gain was affected by prenatal diet ($F_{(2, 74)}$=8.38, p<0.0005) with a significant reduction in weight gain by ethanol dams (26.74 ± 2.02%) compared to ad libitum (38.34 ± 3.15, p<0.0006) and pair-fed (33.04 ± 1.76%, p<0.036) dams. Prenatal stress also attenuated weight gain across gestation ($F_{(1,74)}$=15.40, p<0.0001). The altered weight gain was not due to reduced litter size as neither prenatal stress ($F_{(1,70)}$=0.49, p<0.482) nor prenatal diet ($F_{(2,70)}$=0.656, p<0.522) affected litter size. Although a significant interaction between prenatal diet and stress was observed ($F_{(2,70)}$=3.28, p<0.043) for the ratio of male/female pups born, post hoc analyses failed to reveal any significant differences in the sex ratio amongst treatment groups. Finally, gestation length was not affected by prenatal diet ($F_{(2,47)}$=0.15, p<0.863) or prenatal stress ($F_{(1,47)}$=0.07, p<0.792). Gestation developmental data for dams are summarized in **Table 3.1**.

We next analyzed offspring weight across the postnatal period to determine if prenatal treatments altered weight gain. When assessed on PND 2, a significant main effect of prenatal diet ($F_{(2,140)}$=6.381, p<0.002) indicated that birth weight was significantly reduced in ethanol-exposed animals compared to ad libitum (p<0.002) and pair-fed (p<0.008) offspring. There was a trend toward a main effect of both sex ($F_{(1,140)}$=3.621, p=0.059) and stress ($F_{(1,140)}$=3.510, p=0.063) with males weighing less than females (p<0.034) and stress reducing birth weight (p<0.014). Across PND 8-35, all

offspring gained weight during the postnatal period as indicated by a significant main effect of postnatal day ($F_{(3,321)}$=1,923.321, p<0.0001). A significant main effect of prenatal diet ($F_{(2,107)}$=3.584, p<0.031) revealed that ethanol-exposed offspring weighed significantly less than ad libitum offspring (p<0.015); there was a trend toward reduced weight compared to pair-fed offspring (p=0.058). A significant main effect of sex ($F_{(1,107)}$=24.050, p<0.000) indicated that males weighed significantly more than females (p<0.0001) but this difference in weight was not apparent until PND35 ($F_{(3,321)}$=12.530, p<0.0001).There was a trend for and effect of prenatal stress on offspring weight ($F_{(1,107)}$=2.761, p=0.099) but a significant interaction between postnatal day and prenatal stress ($F_{(3,321)}$=6.139, p<0.0004) revealed that the reduction in weight did not occur until PND30-35 (p<0.0001). The developmental data for all offspring are presented in **Table 3.2**.

3.3.2 Ethanol Consumption does not Exacerbate the CORT Response to Restraint Stress

To determine if the restraint stress increased CORT levels, we compared the CORT levels on GD12 of non-stress and stress dams. As shown in **Figure 3.2A**, CORT was significantly increased in all dams following restraint stress ($F_{(1,37)}$=34.1400, p<0.0000). There was also a main effect of diet ($F_{(2,37)}$=3.7690, p<0.0323) with a trend toward increased CORT in ethanol dams compared to ad libitum dams (p<0.094). CORT levels following restraint stress were then compared across gestation to determine if dams habituated to the stressor. A repeated measures ANOVA revealed no main effect of gestation day ($F_{(2,48)}$=0.1283, p<0.8799) or diet ($F_{(2,48)}$=1.2801, p<0.2963), shown in

Figure 3.2B, indicating that CORT levels following restraint stress did not decrease across gestation.

3.3.3 Prenatal Ethanol Exposure Reduces LTP in Adolescent Males but Enhances LTP in Adolescent Females

We sought to determine how prenatal events (i.e., PNEE and prenatal stress) alter LTP in the dentate gyrus of adolescent male and female rodents. A 3-way factorial ANOVA for sex (male, female) x prenatal diet (ad libitum, pair-fed, ethanol) and prenatal stress (non-stress, stress) revealed a significant main effect of stress ($F_{(1,123)}= 9.05$, $p<0.05$) and sex ($F_{(1, 123)}=12.06$, $p<0.05$), as well as significant interactions between prenatal diet and sex ($F_{(2,123)}=5.45$, $p<0.05$) and prenatal diet x prenatal stress x sex ($F_{(2,123)}=5.91$, $p<0.05$). The post-hoc analyses for this 3-way interaction are presented below.

In the absence of prenatal stress, LTP was significantly reduced in ethanol-exposed males (27 ± 2%) compared to ad libitum males (39 ± 1%, $p< 0.05$; **Fig. 3.3A**). LTP in pair-fed males (32 ± 2%) was not significantly different from either ad libitum ($p> 0.05$) or ethanol ($p> 0.05$) males. In contrast, LTP in ethanol-exposed females (34 ± 3%) was significantly greater than ad libitum females (21 ± 2%, $p< 0.05$; **Fig. 3.3B**). LTP in pair-fed females (30 ± 2%) was not significantly different from ad libitum ($p> 0.05$) or ethanol ($p> 0.05$) females.

As shown in **Figure 3.3C**, ad libitum females had significantly less LTP than ad libitum males ($p<0.05$), supporting previous findings of basic sex differences in LTP capacity during early adolescence (Maren et al., 1994). This basic sex difference in LTP was abolished following either prenatal food deprivation ($p> 0.05$) or prenatal ethanol exposure ($p> 0.05$). Interestingly, LTP in ethanol-exposed females was equivalent to that

observed in ad libitum males ($p > 0.05$), while the LTP in ethanol-exposed males was now equivalent to that observed in ad libitum females ($p > 0.05$). That is, prenatal ethanol exposure increased female LTP levels to a point normally seen in ad libitum males, but reduced LTP in males to a level normally observed in ad libitum females. These findings are graphically depicted in **Figure 3.3D**.

3.3.4 Prenatal Stress Reduced LTP in Ethanol Exposed Adolescent Females but not Males

A test of our *a priori* prediction confirmed that prenatal stress significantly reduced LTP in ad libitum males (31 ± 3%) compared to non-stress ad libitum males (39 ± 1%; $t_{(19)}=2.18$, $p<0.05$; **Fig 3.4A**). Conversely, prenatal stress did not reduce LTP in pair-fed (28 ± 4%, $p>0.05$; **Fig. 3.4B**) or ethanol (28 ± 1%, $p>0.05$; **Fig. 3.4C**) males compared to non-stress pair-fed (32 ± 2%) or ethanol (27 ± 2%) counterparts. Results of prenatal stress on DG LTP in adolescent males are summarized in **Figure 3.4D**.

Post-hoc analyses also revealed that prenatal stress did not reduce LTP in ad libitum females (24 ± 2%; $p > 0.05$) compared to non-stress ad libitum females (21 ± 2%), which was further supported by a student's t-test of our *a priori* prediction ($t_{(23)}=-0.09$, $p > 0.05$; **Fig. 3.5A**). Prenatal stress also did not reduce LTP in pair-fed females (23 ± 3%, $p > 0.05$; **Fig. 3.5B**) compared to non-stress pair-fed females (30 ± 2%). However, combined exposure to stress and ethanol *in utero* significantly reduced LTP (21 ± 3%, $p<0.05$; **Fig. 3.5C**) compared to non-stress ethanol females (34 ± 3%). The effect of prenatal stress on DG LTP in adolescent females are summarized in **Figure 3.5D**.

3.3.5 Prenatal Stress Alters NMDAR Contribution to DG LTP in Adolescent Females

In order to determine if PNEE altered NMDAR contribution to LTP in the DG, CPP, a competitive NMDAR antagonist, was administered 90 minutes prior to the application of theta-patterned stimulation. The change in EPSP slope was significantly different from baseline in ad libitum ($t_{(17)}$=-3.67, p<0.016) and pair-fed males ($t_{(12)}$=-3.33, p<0.016) but not in ethanol males ($t_{(11)}$=-1.84, p> 0.016) following application of CPP as shown in **Fig. 3.6A**. LTP was significantly reduced in all male offspring compared to saline counterparts (all p values < 0.016). In females, the EPSP slope was significantly different from baseline in ad libitum females ($t_{(19)}$=-7.68, p<0.016) but not ethanol ($t_{(6)}$=-1.77, p> 0.016) and pair-fed females ($t_{(6)}$=-0.68, p> 0.016; **Fig. 3.6B**). The EPSP slope was significantly reduced in ethanol ($t_{(15)}$=4.17, p<0.016) and pair-fed females ($t_{(16)}$=4.36, p<0.016) and there was a trend toward reduced LTP in ad libitum females following CPP administration ($t_{(21)}$=2.59, p=0.017).

We next investigated whether prenatal stress altered NMDAR contribution to LTP. LTP was significantly reduced by CPP in ethanol ($t_{(16)}$=4.05, p<0.016) and pair-fed ($t_{(14)}$=4.26, p<0.016) males but not ad libitum males ($t_{(14)}$=2.16, p>0.016; **Fig. 3.7A**). The change in EPSP slope was not significantly different from baseline in ad libitum males ($t_{(8)}$=-2.96, p> 0.016), pair-fed ($t_{(18)}$=-0.82, p> 0.016) or ethanol males ($t_{(14)}$=-1.92, p>0.016) following CPP administration. In females, the percent change in EPSP slope following CPP administration was not significantly different in ad libitum ($t_{(13)}$=0.11 p>0.016) or ethanol females ($t_{(17)}$=2.00, p>0.016) compared to saline counterparts; the EPSP slope was reduced by CPP in pair-fed females ($t_{(28)}$=6.70, p<0.016; **Fig. 3.7B**).

Furthermore, the EPSP slope was not significantly different from baseline in ad libitum ($t_{(6)}$=-3.00, p>0.016), pair-fed ($t_{(14)}$=1.56, p>0.016) or ethanol ($t_{(8)}$=-2.75, p>0.016) females.

3.4 Discussion

The present study revealed that PNEE reduced LTP in the DG of adolescent male offspring but *enhanced* LTP in the DG of adolescent females. These results are consistent with previous research in the adult male hippocampus (Christie et al., 2005; Richardson et al., 2002; Sutherland et al., 1997; Swartzwelder et al., 1988). This is the first study to show that PNEE produces disparate effects on synaptic plasticity in the adolescent male and female hippocampus. In addition, these studies showed that prenatal stress produces sex specific reductions in synaptic plasticity. That is, prenatal stress reduced LTP in the DG of adolescent ad libitum males, but did not produce a similar deficit in adolescent ad libitum females. Surprisingly, prenatal stress reduced the potentiating effect produced by PNEE in adolescent females, but did not further alter synaptic plasticity in adolescent males.

The behavioral ramifications of alterations in the capacity for LTP following PNEE are not fully understood. It might be tempting to extrapolate that reduced hippocampal synaptic plasticity will result in impaired spatial learning. Support for this notion comes from previous studies showing that both hippocampal LTP and spatial learning are impaired in adult males following PNEE (Blanchard et al., 1987; Christie et al., 2005; Cronise et al., 2001; Kim et al., 1997; Matthews and Simson, 1998). Spatial learning and memory can be impaired in adolescent males and females following PNEE (Zimmerberg and Weston, 2002) suggesting that the enhanced LTP in adolescent females

may not be advantageous for hippocampal-dependent learning and memory. Indeed, impaired spatial performance can be accompanied by enhanced LTP (Vaillend et al., 2004) and the magnitude of LTP can be negatively correlated with spatial performance (Jeffery, 1995). It is likely that an optimal amount of hippocampal synaptic plasticity is required for successful spatial learning and deviations from this can likewise impair spatial performance.

It has previously been suggested that the HPA axis contributes to the deleterious effects of PNEE on offspring. Ethanol can stimulate HPA activity (Rivier, 1996; Spencer and McEwen, 1990; Wand and Dobs, 1991; Wilkins and Gorelick, 1986) thereby increasing CORT levels. CORT can readily cross the placenta (Macdonald and Matt, 1984) and maternal adrenalectomy can rescue behavioral deficits imposed by PNEE (Slone and Redei, 2002; Taylor et al., 2002; Wilcoxon et al., 2003). Exposure to stressors can significantly elevate CORT levels in ethanol consuming dams above stressed control dams (Weinberg and Gallo, 1982) and can increase ethanol consumption in non-pregnant rats (Nash and Maickel, 1988; Pohorecky, 1990). Prenatal stress can impair spatial learning (Gal and Marta, 2006; Gi Hoon et al., 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Mohammad and Hossein, 2007; Mueller and Bale, 2007; Rami et al., 2007; Son et al., 2006) and LTP (Yaka et al., 2007; Yang et al., 2006; Yang et al., 2007) indicating that gestational elevations in CORT can have long lasting consequences on hippocampal function. These findings suggest that hippocampal function in offspring that were exposed to ethanol and stress *in utero* might be more impaired than offspring exposed to either ethanol or stress alone. We found that prenatal stress reduced LTP only in adolescent males but not females. This is consistent with impaired spatial performance

only in males following prenatal stress (Zagron and Weinstock, 2006). Surprisingly, prenatal stress did not alter the reduced LTP in ethanol-exposed males but reduced the enhanced LTP in ethanol-exposed adolescent females. This indicates that prenatal stress and prenatal ethanol produce distinct, sexually dimorphic effects on synaptic plasticity in the adolescent hippocampus.

The contribution of NMDARs to synaptic plasticity following prenatal stress and PNEE was investigated and a complex relationship between sex and prenatal condition was found. Within the hippocampus, NMDARs are recruited for the induction of LTP following theta-burst stimulation (Capocchi et al., 1992; Farmer et al., 2004; Larson and Lynch, 1988; Mott and Lewis, 1992). Application of the competitive NMDAR antagonist CPP significantly reduced, but did not block, DG LTP in adolescent ad libitum males and females. Shankar et al (1998) found that hippocampal LTP is more reliant upon NMDARs in the adult vs. aged male rat (Shankar et al., 1998) and NMDAR antagonist blocks LTP in adult but not adolescent rats (de Marchena et al., 2008). We have previously demonstrated that CPP does block DG LTP in adult males (Farmer et al., 2004) indicating that there is a developmental contribution of NMDARs to synaptic plasticity. The EPSP slope of ethanol exposed males and females was not significantly different from baseline following CPP application suggesting that LTP is solely reliant upon NMDARs following PNEE. This would be surprising because PNEE reduces [^{3}H]MK-801 binding, NMDAR-mediated calcium entry, and expression of NR2A and NR2B NMDAR subunits in males (Diaz-Granados et al., 1997; Lee et al., 1994; Spuhler-Phillips et al., 1997) and activation of intracellular pathways that contribute to LTP (Samudio-Ruiz et al., 2009). Prenatal stress can reduce NMDAR subunit expression in

the hippocampus (Son et al., 2006; Yaka et al., 2007) indicating that altered expression and/or function of NMDARs following prenatal ethanol or prenatal stress might affect NMDAR contribution to synaptic plasticity in the adolescent hippocampus.

There is a dearth of evidence as to how PNEE affects hippocampal synaptic plasticity in adolescent female offspring. We have previously shown that PNEE abolished CA1 LTD in ethanol-exposed females (Titterness and Christie, 2008) but the present data indicate that DG LTP is significantly enhanced in adolescent ethanol females compared to ad libitum females. These data suggest that the threshold for bidirectional synaptic plasticity is shifted in favor of LTP in females following PNEE. Alterations in the expression of gonadal hormones might account for this change. In the adult female, LTP is significantly enhanced during the proestrus phase of the estrous cycle (Good et al., 1999; Warren et al., 1995) when estradiol levels are highest (Haim et al., 2003). Surprisingly, estradiol can reduce LTP in adolescent rats (Ito et al., 1999) indicating developmental differences of estradiol on synaptic plasticity. Pubertal estradiol is closely related to vaginal opening (Germain et al., 1978; Ojeda et al., 1976) and since vaginal opening is delayed in females following PNEE (McGivern et al., 1992; McGivern and Yellon, 1992; Sliwowska et al., 2008) then pubertal estradiol levels might be reduced in females following PNEE. This intriguing possibility might account for the enhanced LTP in the adolescent ethanol-exposed female because of the lack of the depressing actions of estradiol on synaptic plasticity and requires further investigation.

It is important to consider the influence of food deprivation on the results observed in the current study. The diets employed within the current study were designed to impart proper nutrition to dams regardless of the amount of diet consumed (Weinberg,

1985) yet ethanol dams tend to consume less of the liquid diet, and therefore, fewer calories than control dams (Weinberg, 1985). As a result, the pair-fed dams receive a limited ration of food. Although not meant as a stressor, the behavior of pair-fed dams suggests that an element of stress results from this feeding regime. Specifically, pair-fed dams are sensitive to cues associated with feeding and rush to the front of the cage when the food is presented. Pair-fed dams also immediately start to consume the liquid diet upon presentation and typically consume all of the diet that is presented. Corticosterone levels in stress pair-fed dams were not significantly different from ethanol or control dams indicating that all of the dams responded equally to the restraint stress. LTP in pair-fed offspring was not significantly different from either control or ethanol offspring suggesting that the reduced food intake of ethanol dams might contribute to LTP changes observed in ethanol-exposed offspring. LTP in ethanol-exposed animals was significantly different from controls, as opposed to pair-fed offspring, so there were specific alterations that resulted from the ethanol diet. Therefore, it is possible that prenatal food restriction may partially mitigate or augment the LTP in ethanol exposed males and females, respectively. However, the LTP in ethanol-exposed offspring was significantly different from controls suggesting distinct effects of the ethanol and pair-fed diets on LTP.

3.4.1 Summary

Prenatal ethanol exposure produced sex-specific alterations to NMDAR-dependent DG LTP in early adolescent offspring. As this is the first study to investigate how PNEE alters LTP in females and it remains to be determined if the enhanced LTP is correlated with improved performance on hippocampal-dependent learning and memory tasks. We also illustrated that LTP in males and females is differentially affected by prenatal stress

and PNEE shifts the sexually dimorphic sensitivity to insult. These data highlight a complex relationship between sex, prenatal stress and prenatal ethanol exposure on hippocampal function and future research should be aimed at elucidating the mechanisms and behavioral implications of these interactions.

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Table 3-1 Gestation Outcome Measures for Ad libitum, Pair-fed and Ethanol Dams

	Ad libitum		**Pairfed**		**Ethanol ***	
Gestation Outcomes Measures	Non-stress	**Stress^**	Non-stress	**Stress^**	Non-stress	**Stress^**
Number of Dams	10	10	18	9	18	15
Gestation Length (days)	22 ± 0.15	21.8 ± 0.20	21.89 ± 0.20	20.78 ± 1.30	21.92 ± 0.14	22 ± 0.00
Dam Weight Gain (% Change from GD1)	42.62 ± 4.97	34.05 ± 3.64	35.29 ± 2.22	28.13 ± 2.14	32.63 ± 2.56	19.69 ± 2.13
Number of Live Pups	15.42 ± 0.47	14.37 ± 0.92	14.46 ± 1.14	13.83 ± 1.76	15.05 ± 0.63	15.23 ± 0.50
Pup Sex Ratio (Male/Female)	1.11 ± 0.18	1.61 ± 0.33	1.51 ± 0.19	1.06 ± 0.13	1.28 ± 0.15	0.95 ± 0.12

*Ethanol diet significantly reduced weight gain across gestation.
^Prenatal stress significantly reduced weight gain across gestation.

Table 3-2 Offspring Developmental Data

	Ad libitum		**Pairfed**		**Ethanol**	
Offspring Weight (g)	Non-stress	Stress	Non-stress	Stress	Non-stress	Stress
Male						
PND 2	7.90 ± 0.39	7.59 ± 0.28	7.37 ± 0.36	7.30 ± 0.33	**7.30 ± 0.20***	**6.69 ± 0.14***
PND 8	19.77 ± 0.95	34.96 ± 16.43	19.77 ± 0.79	18.29 ± 0.93	18.04 ± 0.81	16.97 ± 0.38
PND 15	36.87 ± 1.23	35.66 ± 0.56	36.29 ± 0.91	38.20 ± 1.84	37.08 ± 0.84	35.21 ± 1.32
PND 22	59.13 ± 1.92	59.21 ± 1.67	58.12 ± 1.64	60.07 ± 3.31	58.68 ± 2.26	55.28 ± 1.38
PND 30-35	135.46 ± 6.31	127.14 ± 7.18	134.82 ± 4.83	125.67 ± 9.55	124.36 ± 6.20	121.69 ± 5.86
Female						
PND 2	7.30 ± 0.31	7.21 ± 0.27	7.23 ± 0.39	6.90 ± 0.10	**7.01 ± 0.19***	**6.51 ± 0.18***
PND8	17.88 ± 0.52	18.86 ± 1.04	19.28 ± 0.79	17.40 ± 0.49	18.17 ± 0.38	16.01 ± 0.45
PND 15	34.25 ± 0.93	34.71 ± 0.69	33.55 ± 2.12	36.45 ± 0.89	36.57 ± 0.58	32.70 ± 1.12
PND 22	55.68 ± 1.59	56.88 ± 1.56	53.34 ± 3.31	58.06 ± 1.82	58.49 ± 1.69	50.59 ± 1.67
PND 30-35^	119.00 ± 4.11	112.71 ± 5.36	116.45 ± 6.16	105.17 ± 5.90	116.75 ± 6.38	97.77 ± 2.99

*E < AL, PF
^Female < Male

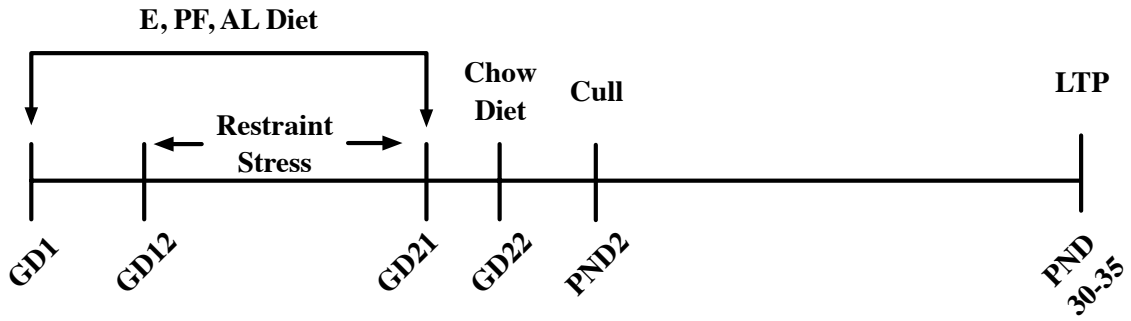


Figure 3.1 Timeline of experiments.

The first day on which sperm was present in the vaginal smear indicated gestation day 1 (GD1). On GD1, females were individually housed and assigned to one of three diets (E, PF, AL). Stress dams were exposed to restraint stress during GD12-21; non-stress dams were left undisturbed in their home cage. Liquid diets were replaced with standard rat chow on GD22, litters were culled to 5 males and 5 females on postnatal day 2 (PND2) and electrophysiology experiments were performed on offspring between PND30-35.

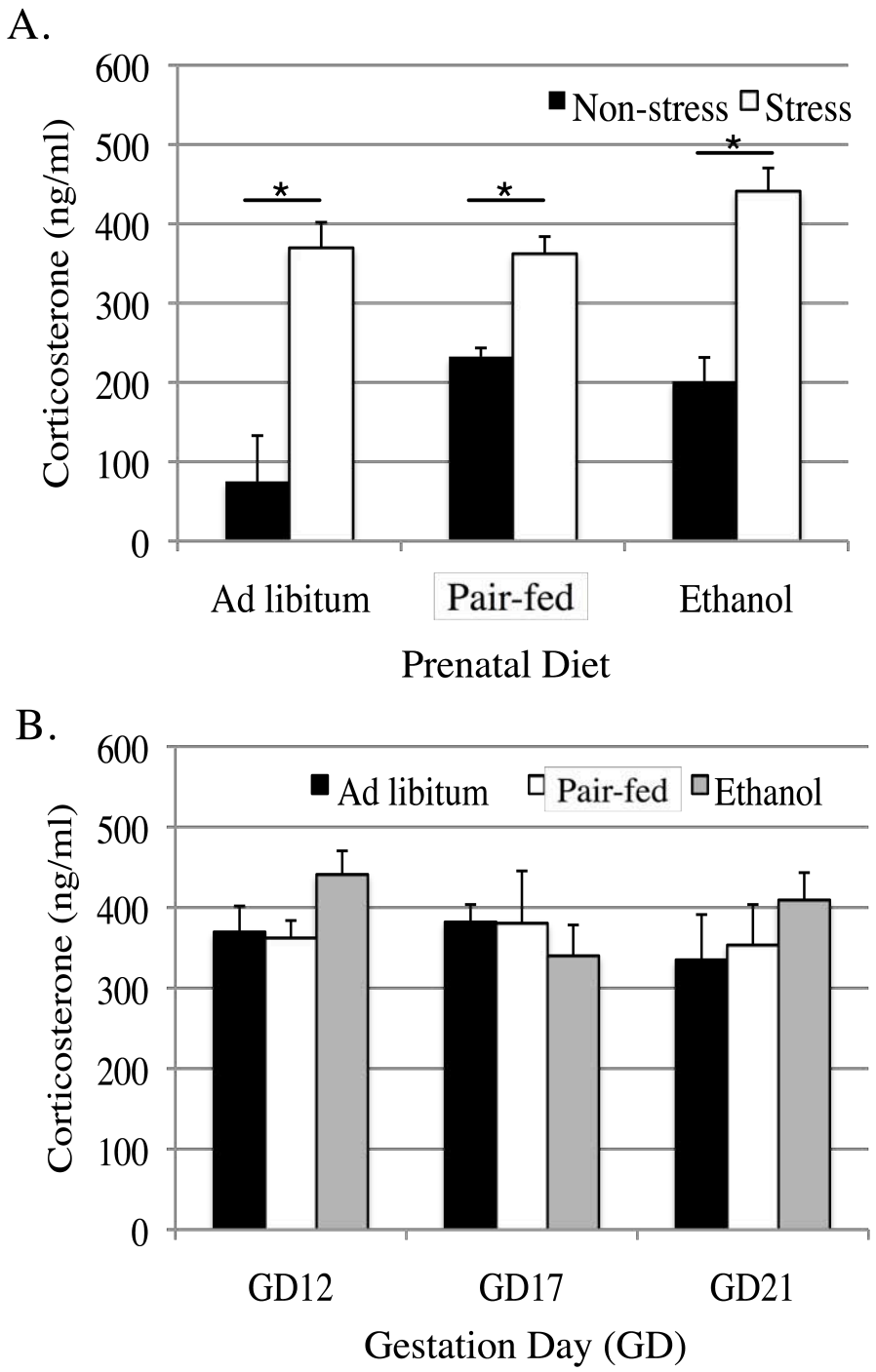


Figure 3.2 Restraint stress increases serum CORT levels equally across prenatal diet.

A. CORT levels were significantly increased following restraint stress on GD12 in ad libitum, pair-fed and ethanol dams. B. When assessed immediately following the first restraint session, CORT levels were not significantly different between ad libitum, pair-fed or ethanol dams on gestation days 12, 17 and 21. There was not a significant difference in CORT levels across gestation days following restraint stress. *$p<0.05$.

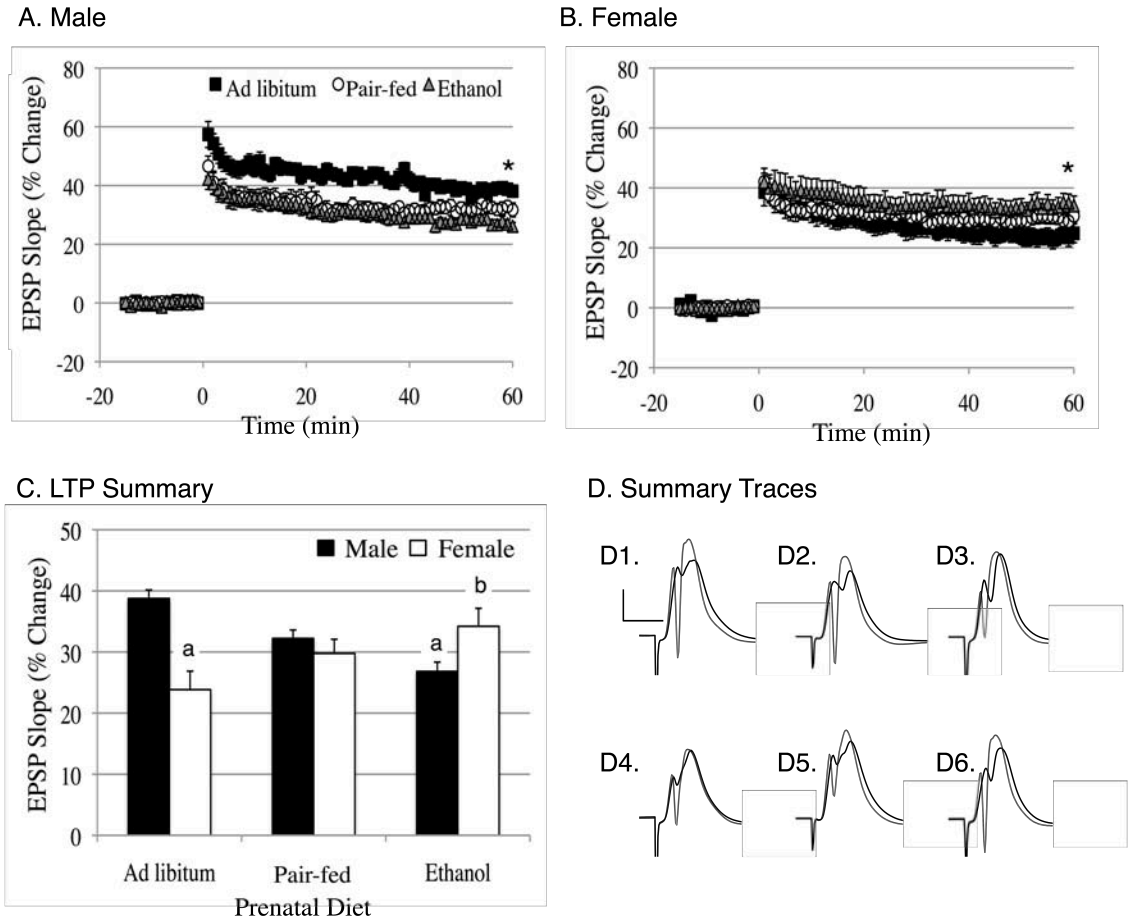


Figure 3.3 Prenatal ethanol exposure produced sex-specific effects on DT LTP.

A. LTP was reduced in ethanol-exposed males compared to ad libitum males. B. LTP was enhanced in ethanol-exposed females compared to ad libitum females. C. Ad libitum females and ethanol males had significantly less LTP compared to ad libitum males. Ethanol females had significantly more LTP than ad libitum females. D. Representative traces from ad libitum, pair-fed and ethanol males (D1-3) and females (D4-6). Darker traces were taken immediately prior to HFS and lighter traces were taken 55-60 minutes post-HFS. *$p<0.05$; a: significantly less than ad libitum male ($p<0.05$); b: significantly greater than ad libitum female ($p<0.05$). Scale bar: vertical: 4mV, horizontal 20ms.

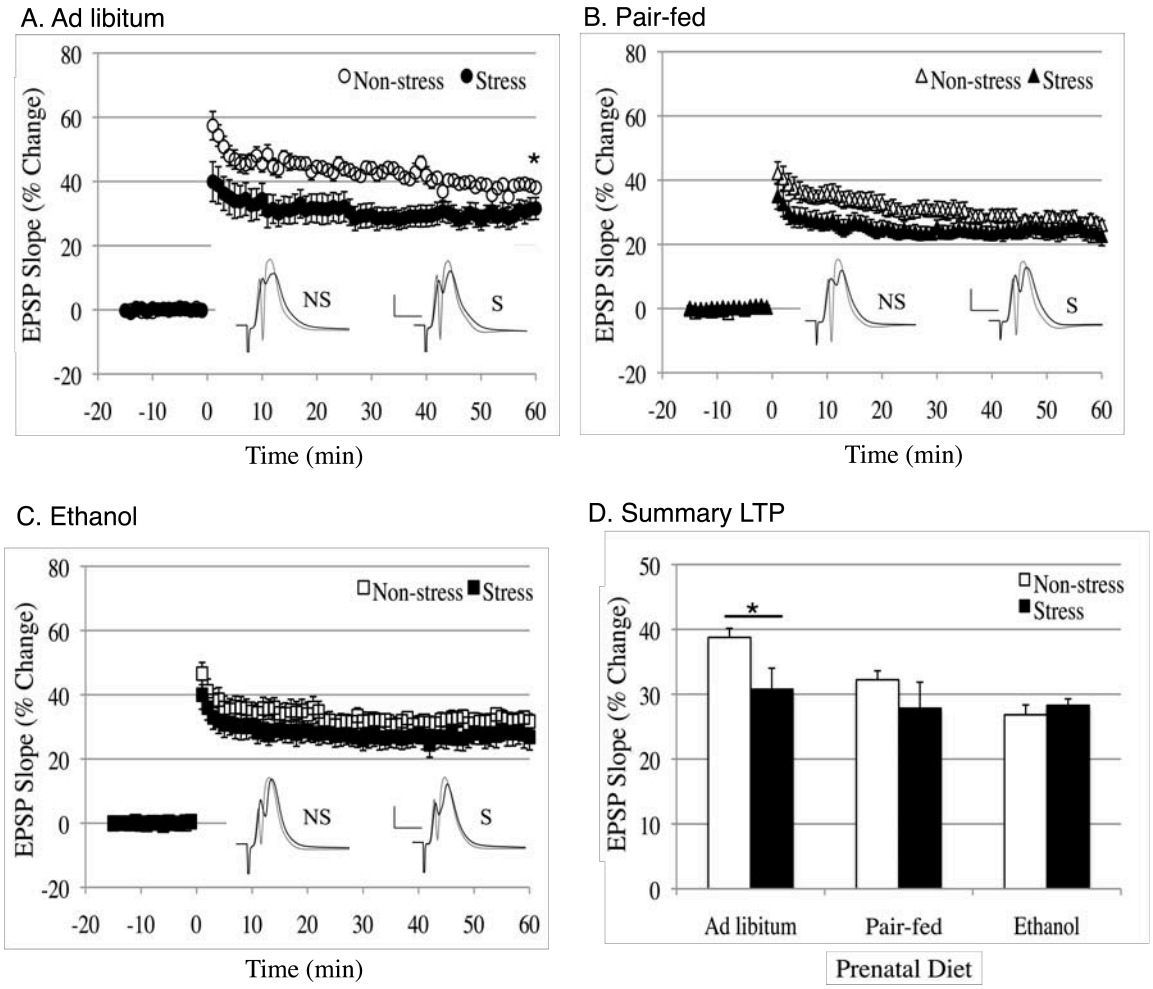


Figure 3.4 Prenatal stress reduced LTP in ad libitum males.

A. Prenatal stress significantly reduced LTP in ad libitum males. B. LTP was not reduced by prenatal stress in pair-fed males. C. LTP in ethanol-exposed males was not further reduced by prenatal stress. D. Summary LTP illustrating that prenatal stress reduced LTP in ad libitum males. Representative traces from prenatal stress offspring. Representative traces from non-stress (NS) and stress (S) offspring are included within ad libitum, pair-fed and ethanol LTP graphs. Dark traces were taken immediately prior to HFS and light lighter traces were taken between 55-60 minutes post-HFS. *$p<0.05$. Scale bar: vertical: 4mV, horizontal 20ms.

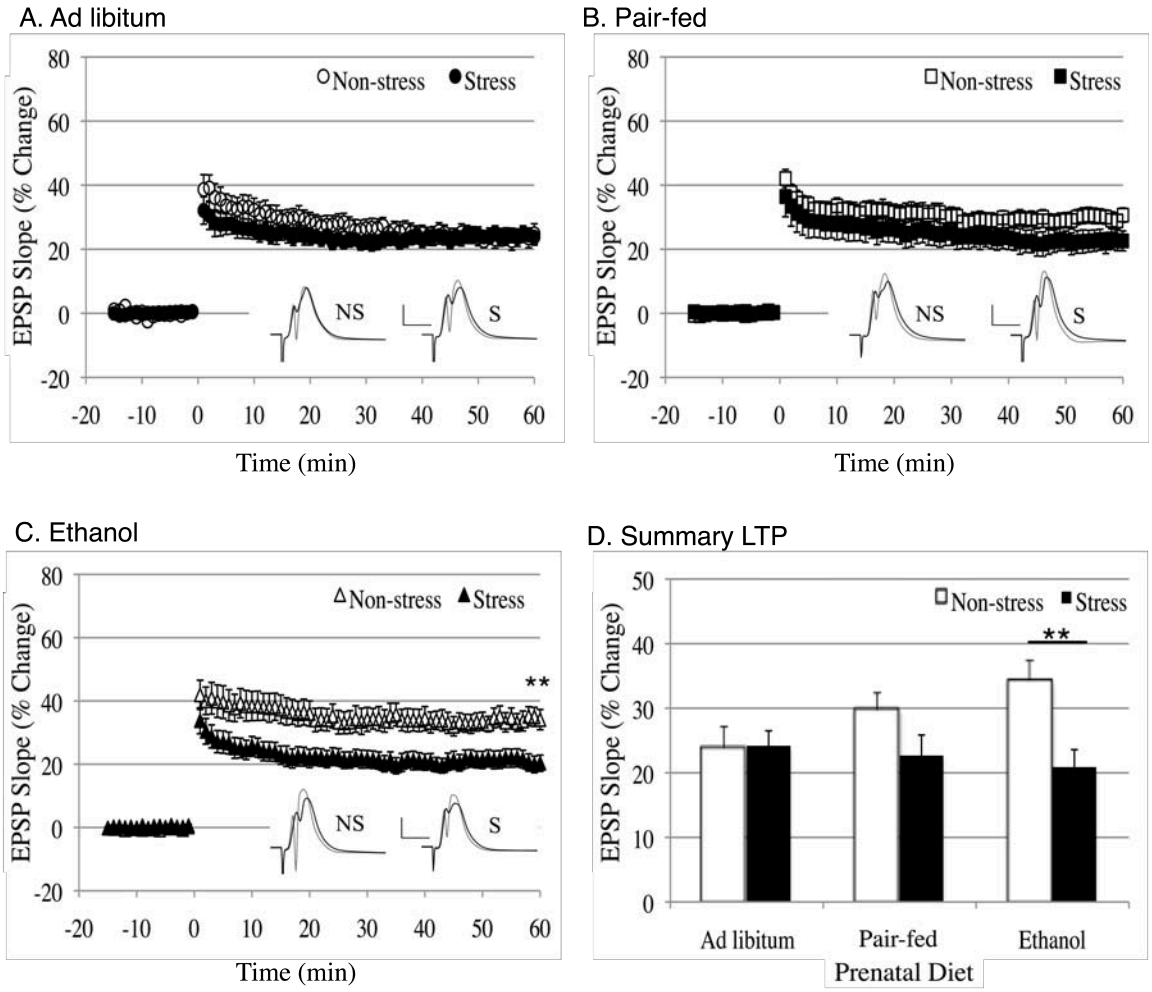
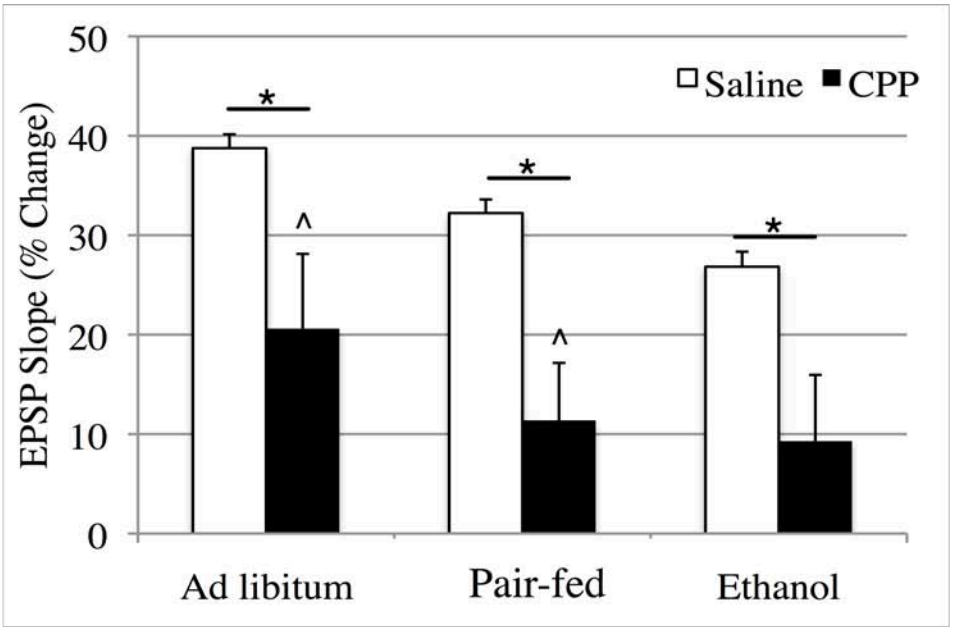


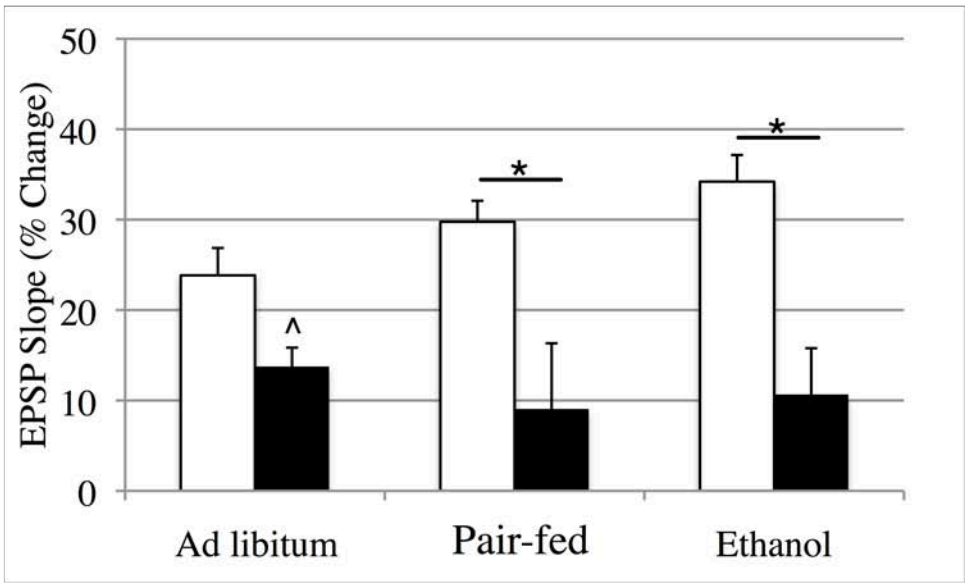
Figure 3.5 Prenatal stress reduced LTP in ethanol females.

A. Prenatal stress did not reduce LTP in ad libitum females. B. LTP was not reduced by prenatal stress in pair-fed offspring. C. Prenatal stress significantly reduced LTP in ethanol-exposed females. D. Summary graph indicating that prenatal stress reduced LTP only in ethanol females. Representative traces from non-stress (NS) and stress (S) offspring are included within ad libitum, pair-fed and ethanol LTP graph. Lighter traces were taken immediately prior to HFS and lighter traces were taken between 55-60 minutes post-HFS. **$p<0.01$. Scale bar: vertical: 4mV, horizontal 10ms.

A. Non-stress Male



B. Non-stress Female



Prenatal Diet

Figure 3.6 CPP blocked LTP in male and female offspring following prenatal ethanol exposure.

A. CPP reduced LTP in ad libitum, pair-fed and ethanol males but blocked LTP in ethanol males. B. Application of CPP reduced in pair-fed and ethanol females but not in ad libitum females. CPP blocked LTP in pair-fed and ethanol females but not ad libitum females by CPP. *significantly different from saline, $p<0.016$; ^significantly different from baseline, $p<0.016$.

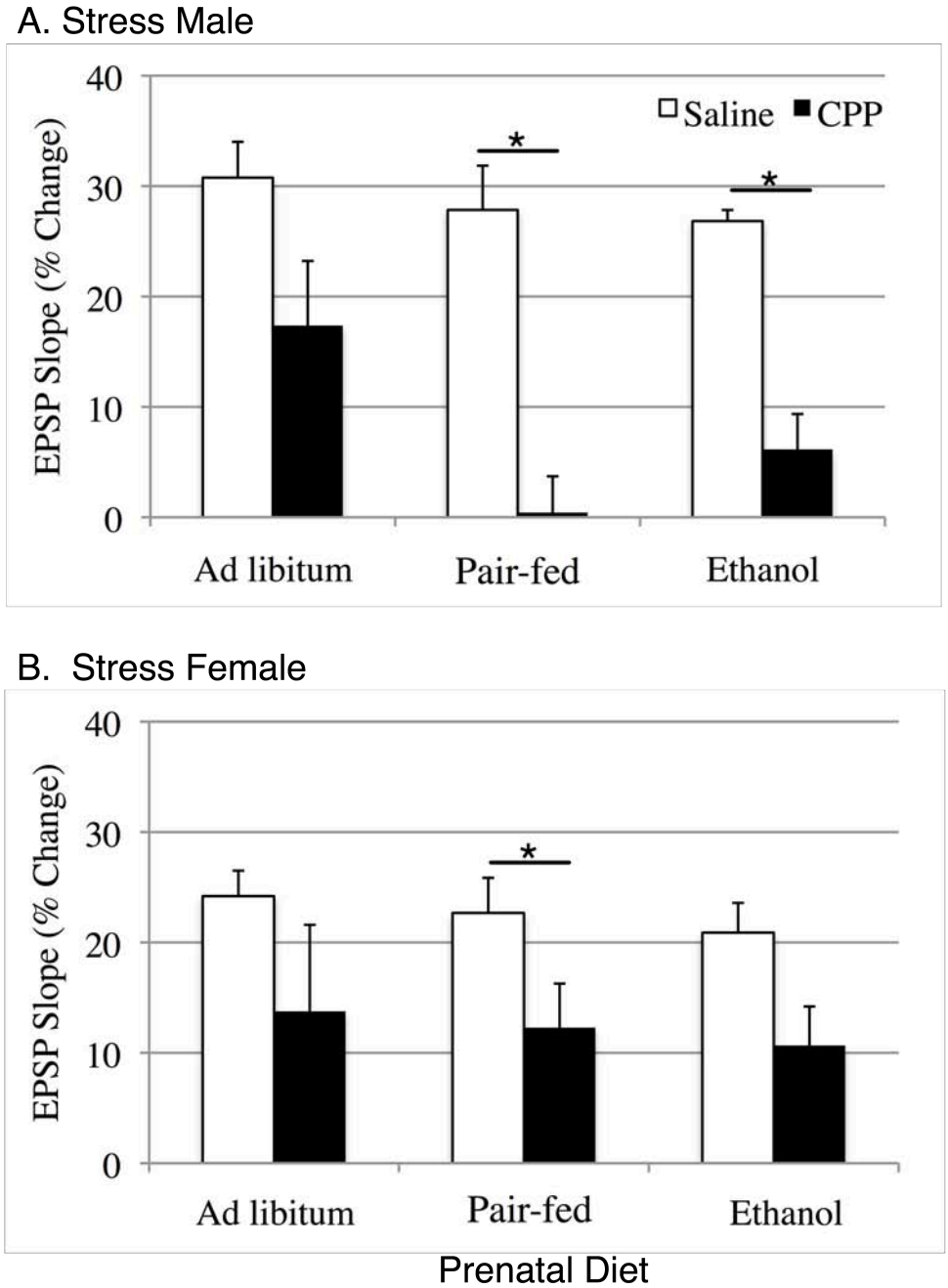


Figure 3.7 Sex-specific effects of CPP on LTP following prenatal stress.

A. LTP was reduced and blocked by CPP in pair-fed and ethanol males exposed prenatal stress. LTP was neither blocked nor reduced in ad libitum males exposed to prenatal stress. B. Application of CPP did not block LTP in ad libitum, pair-fed or ethanol females exposed to prenatal stress. CPP reduced LTP in pair-fed females only. *significantly different from saline ($p<0.016$); ^significantly different from baseline ($p<0.016$).

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4 General Discussion

The studies described in the current thesis were the first to investigate how PNEE alters hippocampal synaptic plasticity in adolescent females. In Chapter 2, we demonstrated that the capacity for LTD in the CA1 of adolescent males and females is dramatically altered by exposure to an acute stress. PNEE reduced CA1 LTD in females but not in males. In Chapter 3, we found that PNEE reduced DG LTP in males but *enhanced* LTP in females. These data suggest that the male and female hippocampus is differentially sensitive to the effects of PNEE. In the following sections, a more systematic review of the experimental findings will be given along with potential pitfalls of the experiments and future directions. The first section will review the basal sex-differences in synaptic plasticity that were independent from PNEE followed by a discussion on the sex-specific effects of PNEE on hippocampal synaptic plasticity in adolescent males and females. The potential influence of maternal food deprivation on the reported results will also be reviewed.

4.1 Basal Sex-differences in Hippocampal Synaptic Plasticity in Adolescent Rats

4.1.1 Effect of Acute Stress on CA1 LTD

Previous studies have shown that CA1 LTD cannot be induced in males by 3 Hz stimulation in the absence of exposure to an acute stressor (Xiong et al., 2004; Xu et al., 1998), a finding that was confirmed in Chapter 2 of this thesis. Significant LTD was

apparent in females, however, in the absence of stress (Figure 2.3). These results indicate that the capacity for CA1 LTD is different in males and females.

The mechanism that contributes to the sexually dimorphic capacity for CA1 LTD is unclear, although gonadal hormones may have played a role. In adult females, removal of the ovaries (ovariectomy-OVX) significantly reduced CA1 LTD but treating females with estradiol restored the LTD (Day and Good, 2005) indicating that estradiol enhances the capacity for LTD in female rats (Day and Good, 2005; Desmond et al., 2000; Mukai et al., 2007; Ogiue-Ikeda et al., 2008; Zamani et al., 2000). ERα mediates the potentiating effect of estradiol on hippocampal LTD (Ogiue-Ikeda et al., 2008) and this is the predominant estradiol receptor in the adolescent hippocampus (Orikasa et al., 2000). Although not systematically studied in the current thesis, vaginal opening in ad libitum adolescent females can occur between PND30-35 (McGivern et al., 1992; McGivern and Yellon, 1992; Sliwowska et al., 2008) and adult levels of unbound estradiol are achieved between PND30-35 (Puig-Duran et al., 1979). It is possible, therefore, that circulating estradiol present in adolescent ad libitum females may have enhanced CA1 LTD via ERα. During adolescence, however, estradiol can produce disparate effects on synaptic plasticity than during adulthood (Ito et al., 1999). Furthermore, LTD can be reduced during the proestrus phase of the estrous cycle (Good et al., 1999) when estradiol levels are highest (Haim et al., 2003) suggesting that estradiol does not always potentiate LTD. Taken together, the influence of estradiol on CA1 in adolescent ad libitum females is unclear and remains to be further investigated.

Consistent with previous studies (Xu et al., 1997), we illustrated that acute stress was required for the expression of CA1 LTD following 3Hz stimulation in male rats. The

stress-induced enhancement of LTD in males has been shown to be dependent upon GRs (Xu et al., 1998) indicating CORT is the mitigating factor in the effect of stress on synaptic plasticity in males. Yet despite a similar stress-induced increase in CORT between males and females (Figure 2.1), acute stress blocked LTD in adolescent ad libitum females. Acute stressors have previously been shown to produce distinct effects on the hippocampus in males and females. Acute stress can increase spine density in adult males but reduced spine density in adult females (Shors et al., 2001) and can impair neurogenesis (the birth of new neurons) in the DG of adult males but not adult females (Falconer and Galea, 2003). Acute stress can facilitate spatial memory in females while impairing memory in males (Conrad et al., 2004). The enhancing effect of stress memory in females was independent of gonadal hormones (Conrad et al., 2004) even though estradiol can protect against stress-induced changes to synaptic plasticity (Foy et al., 2008). Estradiol can potentiate HPA activity (Burgess and Handa; Figueiredo et al.; Vamvakopoulos and Chrousos; van de Stolpe et al.), which may, in part, contribute to greater basal and stress-induced HPA activity in females than males. Sex-differences in HPA activity in response to an acute stress are not always apparent between PND30-35 (Hodes and Shors, 2005; Weinberg and Gallo, 1982), suggesting that stress-induced changes to synaptic plasticity might occur independently from the effects of gonadal hormones. Under certain circumstances, high levels of CORT can enhance LTP (Champagne et al., 2008). Acute stress reduced CA1 LTD in adolescent females and might therefore be seen as “enhancing” synaptic plasticity. Taken together, these studies suggest a complex relationship between acute stress and hippocampal function in adolescent males and females.

4.1.2 Sex-specific Effect of Prenatal Stress on DG LTP

Consistent with previous studies (Maren et al., 1994), we have illustrated that adolescent males exhibit significantly more DG LTP than adolescent females (Figure 3.3). Pubertal testosterone reduces CA1 LTP (Hebbard et al., 2003) but does not affect LTP in the DG (Sakata et al., 2000). Estradiol, on the other hand, can enhance LTP in adult females (Good et al., 1999; Montoya and Carrer, 1997; Warren et al., 1995) but reduces CA1 LTP in adolescent females (Ito et al., 1999); the manner in which estradiol affects DG LTP in adolescent females has yet to be investigated. Therefore, it is possible that gonadal hormones exert little influence over the DG LTP observed in adolescent ad libitum male and female rats. Previous studies have shown that sex-differences in DG LTP are dependent upon the conditioning protocol used (Yang et al., 2004) which may have contributed to the sex-difference observed in the current thesis.

Gestational stress produces distinct effects on DG synaptic plasticity in the adolescent male and female. Previous studies have shown that chronic restraint stress can reduce LTP in the CA1 of males (Gi Hoon et al., 2006; Rami et al., 2007; Yang et al., 2006) and we have extended these findings to the DG. Acute gestational stress can enhance DG LTP (Fujioka et al., 2006) but we have illustrated that chronic gestational stress significantly reduces DG LTP in males (Figure 3.4). In females, however, chronic prenatal stress did not alter DG LTP (Figure 3.5). Although this was the first study to investigate whether prenatal stress differentially affects synaptic plasticity in males and females, previous studies have shown that sex-differences in neurogenesis are abolished by prenatal stress specifically due to reduced neurogenesis in stressed males (Mandyam et al., 2008). We found a basal sex-difference in DG LTP and this effect was abolished

by prenatal stress specifically due to the reduced LTP in males. Interestingly, prenatal stress impairs spatial memory in males but not females (Szuran et al., 2000; Zagron and Weinstock, 2006) suggesting that the male hippocampus is more sensitive to the deleterious effects of prenatal stress than the female hippocampus.

4.1.3 Putative Mechanisms Behind Adolescent Hippocampal Synaptic Plasticity

The exact mechanism through which prenatal stress alters synaptic plasticity is unclear. CORT can rapidly cross the placenta (Arishima et al., 1978; Dupouy et al., 1975; Michaud and Burton, 1977) and maternal stress can cause significant elevations in fetal CORT levels (Ward and Weisz, 1984). The female fetus has higher CORT levels than the male fetus due to greater transplacental passage of CORT for female fetuses than male fetuses (Montano et al., 1993). Prenatal stress, however, can reduce 11β-HSD2 mRNA in the male placenta (Mairesse et al., 2007), which might account for increased placental levels of CORT in male fetuses following prenatal stress (Montano et al., 1991). Maternal adrenalectomy can rescue impaired spatial memory that results from gestational stress (Zagron and Weinstock, 2006). Taken together, the selective prenatal stress-induced reduction of 11β-HSD2 in the male placenta might expose the male fetus to abnormally high levels of CORT and therefore produce long-lasting changes to the male hippocampus.

Another mechanism through which prenatal stress might specifically affect hippocampal function in males is through alterations in perinatal testosterone exposure. There is evidence that perinatal testosterone can masculinize the hippocampus (Roof and Havens, 1992). Fetal testosterone surges that are normally present on gestation days 18 and 19 are absent in male fetuses following the cessation of maternal stress (Ward and

Weisz, 1980; Ward and Weisz, 1984). These gestational surges in testosterone coincide with the critical period for tesosterone-induced masculinization of certain brain regions (Weisz and Ward, 1980). Therefore, stress-induced changes to the prenatal surge of testosterone might alter the masculinization of the hippocampus, thereby affecting the capacity to express LTP.

4.1.4 Potential pitfalls

We did not systematically investigate the age of vaginal opening or the levels of either testosterone or estradiol. As such, we were not able to rule out the influence of gonadal hormones on the hippocampal synaptic plasticity described in this thesis. Although gonadal hormones can produce distinct effects on synaptic plasticity in adolescent males and females compared to adult animals (Ito et al., 1999) it would be advantageous to determine how endogenous hormones expressed during the early adolescent period affect synaptic plasticity.

4.1.5 Future Directions

The effect of prenatal stress on hippocampal synaptic plasticity might vary across development. Chronic prenatal stress can enhance CA1 spine density in adolescent males but reduces spine density in adult males (Martinez-Tellez et al., 2009) indicating that the deleterious effects of prenatal stress might be more apparent during adulthood. Estradiol can protect against stress-induced changes to synaptic plasticity (Foy et al., 2008) suggesting that acute stress might differentially affect synaptic plasticity across the estrous cycle. Taken together, future studies should investigate whether gonadal hormones influence the effect of gestational stress on synaptic plasticity in adult males and females.

In Chapter 3 we illustrated that DG LTP in adolescent males and females is partially mediated by NMDARs. In the CA1, LTP in adolescent males is attenuated by NMDAR antagonists but only blocked with combined exposure to antagonists of NMDARs and voltage-dependent calcium channels (VDCCs) (Shankar et al., 1998). We did not investigate the contribution of different receptor systems to LTP in adolescent animals (i.e., VDCCs) but the role of these different systems in DG LTP should be resolved. MR and GR activation can enhance and depress hippocampal synaptic plasticity (Pavlides et al., 1996; Pavlides et al., 1995), respectively, and it would be interesting to further investigate if these receptors contribute to the basal sex-differences we observed in the current thesis (i.e., CA1 LTD in adolescent females). The downstream cascades that are recruited in adolescent synaptic plasticity should also be further elucidated. Prenatal stress can increase ERK expression in the female hippocampus (Cai et al., 2007) and LTP in adolescent males can recruit different downstream cascades depending on the type of receptor recruited for LTP (Shankar et al., 1998). These studies suggest that multiple receptor systems and intracellular signaling cascades might be recruited for synaptic plasticity during adolescence.

Finally, it is necessary to fully elucidate how hippocampal-dependent learning and memory are affected by acute stress and prenatal stress in early adolescent rats. PS can impair performance on the Morris water maze (MWM) in male offspring (Gal and Marta, 2006; Mohammad and Hossein, 2007; Yaka et al., 2007; Yang et al., 2006) suggesting that the reduced LTP in PS males might underlie the impaired performance. Interestingly, however, PS does not impair spatial learning and memory in female rats (Gal and Marta, 2006; Zuena et al., 2008). As hippocampal subregions differentially

contribute to spatial learning and memory (Goodrich-Hunsaker et al., 2008; Huerta et al., 2000; Hunsaker and Kesner, 2008; McHugh et al., 2007; Rolls and Kesner, 2006), employing regions-specific tasks will provide the most detailed information about the effect of gestational and acute stress on hippocampal function in adolescent male and female rodents.

4.2 Sex-specific Effects of Prenatal Ethanol Exposure on Hippocampal Synaptic Plasticity

4.2.1 Effect of Acute Stress on CA1 LTD

In Chapter 2, we investigated how PNEE altered the effect of acute stress on CA1 LTD. Previous studies have shown that stress-induced CORT levels of ethanol-exposed offspring can be significantly higher than ad libitum offspring (Glavas et al., 2007; Kim et al., 1999a; Lan et al., 2009; Taylor et al., 1982; Weinberg, 1988; Weinberg, 1992; Weinberg et al., 2008; Weinberg et al., 1996). In Chapter 2, however, we illustrated that stress-induced enhancement to CORT levels in ethanol-exposed adolescent males and females was not significantly different than ad libitum offspring (Figure 2.1). Interestingly, the enhanced CORT response to stressors in ethanol-exposed offspring is not always apparent. For example, exaggerated CORT levels were not apparent in ethanol-exposed male and female adolescent offspring (PND39) following following exposure to an acute stressor (Weinberg and Gallo, 1982) suggesting that adolescence is a unique period for HPA activity in ethanol-exposed males and females.

PNEE alters the effect of acute stress on CA1 LTD. Previous studies have shown that acute stress enhances CA1 LTD in males following 3Hz stimulation (Xiong et al.,

2003; Xu et al., 1997), which was confirmed in the current thesis. Although significant LTD was observed in ethanol-exposed males (Figure 2.5) there was a trend toward reduced LTD compared to ad libitum males. In females, on the other hand, LTD was not apparent in ethanol-exposed females, even in the absence of stress. These findings indicate that PNEE differentially affects CA1 LTD in males and females. Previous studies have shown that GRs mediate the effect of acute stress on CA1 LTD (Xu et al., 1998). GR binding capacity, receptor density and mRNA in the hippocampus is not altered by PNEE (Kim et al., 1999b; Sliwowska et al., 2008; Weinberg and Petersen, 1991) suggesting that GR-mediated changes to synaptic plasticity might be intact following PNEE. NMDARs also contribute to the deleterious effects of stress on synaptic plasticity (Kim et al., 1996) and since PNEE impairs the function of NMDARs (Costa et al., 2000; Lee et al., 1994; Morrisett et al., 1989; Puri et al., 2003; Samudio-Ruiz et al., 2009b) it is possible that ethanol-induced changes to NMDAR function might mediate the reduced capacity for stress-induced changes to CA1 synaptic plasticity. This possibility, however, remains to be investigated.

4.2.2 Effect of Prenatal Stress on DG LTP in Ethanol-exposed Offspring

The focus of Chapter 3 was to determine if gestational exposure to ethanol and stress synergistically affect synaptic plasticity in adolescent male and female offspring. Ethanol consumption can increase HPA activity (Ogilvie et al., 1997; Ogilvie and Rivier, 1996; Rivier, 1996; Wilkins and Gorelick, 1986) and CORT levels in ethanol consuming dams can be significantly elevated above stressed *ad libitum* dams following acute stress (Weinberg and Bezio, 1987; Weinberg and Gallo, 1982). Deleterious effects of PNEE can be rescued by maternal adrenalectomy (Slone and Redei, 2002) suggesting that the

negative effects of PNEE can be mediated by maternal CORT (Redei et al., 1993; Wilcoxon et al., 2003). Since prenatal stress can also reduce LTP and impair hippocampal-dependent learning and memory (Byrnes et al., 2004; Gal and Marta, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Mohammad and Hossein, 2007; Son et al., 2006; Yaka et al., 2007; Yang et al., 2006; Zagron and Weinstock, 2006) we hypothesized that prenatal stress will compound the deleterious effects of PNEE on synaptic plasticity. We found that PNEE reduced LTP in males (Figure 3.3), consistent with previous studies (Christie et al., 2005; Richardson et al., 2002; Swartzwelder et al., 1988). This was the first study to investigate the effect of PNEE on LTP in adolescent females and, surprisingly, DG LTP was *enhanced* in ethanol-exposed adolescent females compared to ad libitum females. Ethanol-exposed males and females were also differentially affected by prenatal stress. In contrast to previous studies (Yaka et al., 2007; Yang et al., 2006; Yang et al., 2007) and with the results obtained within this thesis, prenatal stress did not reduce LTP in ethanol-exposed males but significantly reduced LTP in ethanol-exposed females. These findings the capacity for LTP in the DG in males and females is differentially affected by PNEE and prenatal stress.

4.2.3 Putative Mechanisms Behind Hippocampal Synaptic Plasticity in Adolescent Offspring following PNEE

The mechanism through which PNEE affects synaptic plasticity is unclear. We have demonstrated that DG LTP in adolescent ethanol-exposed males and females is NMDAR-dependent (Figure 3.6). This is in contrast to what we, and others (Shankar et al., 1998), have demonstrated in non-ethanol exposed adolescent males. PNEE alters NMDAR subunit expression (Hughes et al., 1998; Nixon et al., 2004; Samudio-Ruiz et

al., 2009a) and reduces MK-801 binding in adult male offspring (Diaz-Granados et al., 1997; Valles et al., 1995). Furthermore, PNEE induces abnormal Mg^{2+} regulation of NMDARs (Morrisett et al., 1989) and decreases [3]glutamate binding site density (Savage et al., 1991) and glutamate binding to NMDARs (Farr et al., 1988) in males. Altered NMDAR expression and/or function, together with reduced glutamate efflux following PNEE (Butters et al., 2000), suggests that PNEE impairs the capacity for excitatory synaptic transmission in male offspring.

The effect of PNEE on NMDAR function in ethanol-exposed females has yet to be investigated. As such, it is difficult to speculate why NMDAR-dependent LTP is enhanced in ethanol females but reduced in ethanol males. One possibility is the delayed onset of puberty in ethanol females. Vaginal opening is delayed in females following PNEE (Creighton-Taylor and Rudeen, 1991; Lan et al., 2009; McGivern et al., 1992; McGivern and Yellon, 1992; Sliwowska et al., 2008) and since pubertal estradiol levels are intimately related to vaginal opening (Germain et al., 1978; Ojeda et al., 1976) then pubertal expression of estradiol might be delayed in females following PNEE. If this were indeed the case, then the depressing effect of estradiol on LTP in early adolescent offspring (Ito et al., 1999) would not be present in ethanol-exposed females. It would therefore be expected that LTP in ethanol-exposed females would be significantly greater than ad libitum females, which is what we found. In the adolescent hippocampus, estradiol blocks the NMDAR-mediated component of the EPSP (Ito et al., 1999) suggesting that estradiol reduces NMDAR contribution to LTP in adolescent females. If pubertal rises in estradiol levels are attenuated by PNEE, then estradiol-induced changes

to NMDAR contribution to LTP would not be apparent and it LTP should be NMDAR-dependent in ethanol-exposed females, which is what we observed.

The mechanism behind LTP in offspring exposed to both prenatal stress and PNEE has yet to be elucidated. It is possible that prenatal stress and PNEE alter synaptic plasticity through similar mechanisms. We found that DG LTP was dependent upon NMDARs in offspring exposed to ethanol and stress *in utero*. Prenatal stress can reduce hippocampal protein levels of NMDAR subunits (Son et al., 2006; Yaka et al., 2007) and NR2B and GluR1 protein in males (Yaka et al., 2007), which is similar to PNEE (Naassila and Daoust, 2002; Puri et al., 2003; Samudio-Ruiz et al., 2009a; Savage et al., 1991). It is therefore possible that prenatal stress and PNEE converge on similar pathways to alter synaptic plasticity. If this were the case, then either manipulation is sufficient to reduce LTP. Indeed, the magnitude of LTP in ethanol-exposed offspring was not significantly different from males with combined exposure to stress and ethanol *in utero*. These findings suggest that, in males, prenatal stress and PNEE might disrupt similar pathways that contribute to hippocampal synaptic plasticity.

In females, however, PNEE enhanced DG LTP but combined exposure to PS and PNEE reduced LTP. The disparate effect of these manipulations on DG LTP in adolescent females does not necessarily indicate that distinct mechanisms underlie these changes. Indeed, CPP significantly reduced LTP in ethanol-exposed females as well as those exposed to stress and ethanol *in utero*. Prenatal treatment of glucocorticoids can lead to a persistent reduction in NR1 mRNA in the adult female hippocampus (Brown et al., 2007) indicating that the expression of NMDARs is disrupted by prenatal stress.

Long-lasting effects of PNEE on NMDAR function and/or expression in the female hippocampus have yet to be investigated.

4.2.4 Potential Pitfalls

We did not systematically assess vaginal opening and/or estradiol levels in the female offspring used in the current studies. Postnatal handling can promote hyperactivation of the HPA axis in adolescent offspring following PNEE (Weinberg and Gallo, 1982) and a systematic investigation into vaginal opening would have required a significant amount of handling. Therefore, we chose not to investigate the onset of vaginal opening in order to avoid alterations to HPA activity, which might subsequently affect synaptic plasticity.

Perhaps the greatest pitfall to the experiments is that we were not able to employ a perfect control for the ethanol liquid diet. Food consumption in ethanol consuming dams is often reduced and the pair-fed group is designed to account for this food restriction. However, we observed specific effects of pair-feeding on synaptic plasticity. In Chapter 2, we demonstrated that significant LTD is apparent in stress and non-stress pair-fed offspring (Figure 2.4) indicating that prenatal food restriction enhances the capacity for LTD in the CA1. In the adolescent DG, LTP in adolescent male and female pair-fed offspring was not significantly different from either ad libitum or ethanol offspring suggesting that the effects of PNEE on LTP might be partially mediated by prenatal food restriction. We cannot rule out these possibilities but cannot ignore that LTP in ethanol-exposed offspring, but not pair-fed offspring, was significantly different from ad libitum animals. Therefore, PNEE, alone or in conjunction with prenatal food deprivation, can produce distinct effects on synaptic plasticity. The following is a brief discussion on the role of maternal nutrition in mediating the effects of ethanol-induced toxicity to the fetus.

4.2.4.1 Maternal Nutrition and Ethanol Metabolism

Pregnancy and maternal nutritional status can alter ethanol metabolism. Ethanol metabolism can increased during pregnancy (Badger et al., 2005; Petersen et al., 1977) an effect that is independent of caloric intake. Within 5 minutes of maternal ethanol consumption, fetal ethanol concentrations are equivalent to maternal blood ethanol concentrations (Zorzano and Herrera, 1989). Fetal ethanol metabolism is low during gestation (Zorzano and Herrera, 1989) suggesting that maternal metabolism of ethanol largely dictates the levels of fetal ethanol exposure. Maternal ethanol metabolism is reduced by undernutrition (Shankar et al., 2006) thus exposing the fetus to ethanol for longer periods of time. Indeed, maternal nutritional status may influence the level of fetal ethanol exposure during gestation (Shankar et al., 2007).

Ethanol can directly alter maternal nutrition (Lieber, 1984) by impairing nutrient and vitamin absorption (Gloria et al., 1997; Green, 1983) or by altering the amount of food consumed (Wiener et al., 1981). The ethanol liquid diets used within the current thesis have been nutritionally fortified to provide adequate nutrition to ethanol-consuming pregnant rats (Weinberg, 1985). The pair-feeding group was designed to control for the altered nutritional state induced by consumption of the ethanol liquid diet. Indeed, caloric and protein intake of pair-fed dams is significantly less than ad libitum dams but similar to ethanol dams (Weinberg, 1985) controlling for reduce caloric and protein intake imposed by the ethanol liquid diet (Weinberg, 1985). Prenatal protein deprivation can reduce DG LTP (Austin et al., 1986), indicating that reduced maternal protein intake might mediate some of the deleterious effects of PNEE on hippocampal function. Maternal blood ethanol concentration, however, does not vary as a function of

caloric or protein intake (Weinberg, 1985) indicating that these components of the diet do not influence maternal ethanol metabolism. Offspring of ethanol consuming dams are not only exposed to reduced protein levels throughout gestation but to ethanol as well which might account for the specific effects of PNEE on synaptic plasticity.

4.2.4.2 Reduced Food Intake

Previous studies have suggested that the deleterious effects of stress are mediated by reductions in food intake (Lesage et al., 2001; Lingas and Matthews, 2001). In Chapter 3, we found that the percentage of weight gain across gestation was significantly affected by the ethanol liquid diet and prenatal stress (Table 3.1). Changes in maternal weight gain across gestation cannot be attributed to litter size, male/female ratio or gestation length as none of these variables were affected by either prenatal stress or prenatal ethanol exposure (Table 3.2). Non-stress and stress dams consumed equivalent amounts of ethanol liquid diet (data not shown) and we made sure that stress- pair-fed dams were paired to stress-ethanol dams to control for any changes in diet consumption due to restraint stress. The significant main effect of diet and stress found in Chapter 3 suggests that ethanol-consuming dams exposed to stress were at greatest risk for altered weight gain across gestation. We cannot rule out the influence of reduced food consumption and/or weight gain on the measures taken, but these factors might be more robust following ethanol consumption.

4.2.4.3 Pairfeeding as a Stressor

A component of the prenatal food restriction (i.e., pairfeeding), might be due to stress. We, and others, have suggested that the pairfeeding regime is stressful to the rats since basal CORT levels in pair-fed dams can be elevated above ad libitum controls (Weinberg,

1985). Maternal undernutrition can increase fetal exposure to CORT possibly by reducing placental 11β-HSD2 levels (Lesage et al., 2001) similar to what is observed following prenatal restraint stress (Pankevich et al., 2009). Prenatal stress can enhance the capacity for LTD in CA1 (Yang et al., 2006) and we illustrated that CA1 LTD was enhanced in pair-fed offspring (Figure 2.4) further suggesting that some of the deletrious effects of pairfeeding might be due to altered maternal HPA activity. In Chapter 3, we found a trend toward increased basal CORT levels in non-stress ethanol and pair-fed dams on GD12 (Figure 3.2) but there was a trend toward increased CORT following restraint stress on GD12 only in ethanol dams. Taken together, these findings suggest that even though pairfeeding might act as a stressor, ethanol exposure had specific effects on measures taken within the current thesis.

4.2.5 Future Directions

4.2.5.1 Mechanisms that Contribute to Hippocampal Synaptic Plasticity Following PNEE

4.2.5.1.1 NMDA Receptor

The manner in which PNEE alters hippocampal synaptic plasticity has yet to be fully elucidated. PNEE reduces NMDAR expression (Naassila and Daoust, 2002; Puri et al., 2003; Samudio-Ruiz et al., 2009a; Savage et al., 1991), glutamate binding to NMDARs in the male hippocampus (Abdollah and Brien, 1995; Farr et al., 1988; Savage et al., 1991) and alters NMDAR-mediated activation of intracellular pathways that contribute to LTP. For example, PNEE can reduce PKC expression (Perrone-Bizzozero et al., 1998)

and activation of ERK signaling induced by NMDAR activation (Samudio-Ruiz et al., 2009b). Additionally, PNEE reduces glutamate release in the hippocampus (Butters et al., 2000; Butters et al., 2003). These findings indicate that ethanol-induced changes to NMDAR function might underlie the impaired synaptic plasticity in males following PNEE. Ethanol exposure, however, enhanced NMDAR-dependent DG LTP in the female hippocampus and the exact mechanism behind this enhancement remains to be determined.

4.2.5.1.2 Estradiol

LTP is significantly enhanced during the proestrus phase of the estrous cycle (Good et al., 1999; Warren et al., 1995) when estradiol levels are the highest (Haim et al., 2003). A putative mechanism through estradiol-induced enhancement of LTP is through regulation of NMDAR expression and function. Specifically, estradiol promotes glutamate binding to NMDARs (Romeo et al., 2005) and can increase mRNA for the NR1 subunit of the NMDAR (Adams et al., 2001). Estradiol increases Ca^{2+}-mediated current through NMDARs (Pozzo-Miller et al., 1999) particularly through NR2B-containing NMDARs (Snyder et al., 2010) implicating NR2B subunits in the potentiating effect of estradiol on LTP. Indeed, blockade of NR2B-contatining NMDARs prevents estradiol-induced increases to LTP (Smith and McMahon, 2006). PNEE increases estradiol levels in ethanol-exposed adult females compared to ad libitum females (Lan et al., 2009) suggesting that PNEE might alter estradiol-induced changes to synaptic plasticity. Estradiol can also regulate GR and MR expression in the hippocampus (Ferrini and De Nicola, 1991; Ferrini et al., 1995; Handa et al., 1994) and protects against stress-induced

reductions to LTP (Foy et al., 2008). PNEE, however, increases GR mRNA in the hippocamus during proestrus (Sliwowska et al., 2008) suggesting a complex relationship between estradiol and GR-induced changes to hippocampal synaptic plasticity following acute stress. Therefore, the effect of acute stress on synaptic plasticity in ethanol-exposed adult females remains to be determined.

4.2.5.2 Ramifications of PNEE on Hippocampal-dependent Learning and Memory

The behavioral effects of PNEE on hippocampal-dependent learning and memory have yet to be fully elucidated. PNEE impairs spatial learning and memory in adult and adolescent male offspring (Blanchard et al., 1987; Iqbal et al., 2004; Matthews and Simson, 1998; Reyes et al., 1989; Richardson et al., 2002; Wilcoxon et al., 2005; Zimmerberg and Weston, 2002) indicating that PNEE depresses hippocampal-dependent learning and memory across development. The effect of PNEE on spatial learning and memory has only recently been investigated and ethanol-exposed adult females are impaired on spatial learning and memory tasks (Zimmerberg et al., 1991). These behavioral deficits should be interpreted with caution, however, because ethanol can change aspects that contribute to spatial learning and memory. For example, PNEE can alter spatial processing to a greater extent in males than females (Blanchard et al., 1987; McAdam et al., 2008) and the acquisition rate of learning is slowed by PNEE (Iqbal et al., 2006) but can be partially overcome with pre-training (Iqbal et al., 2006). These findings suggest that altered spatial processing induced by PNEE might contribute to impaired performance on hippocampal-dependent learning and memory and this remains to be further investigated.

PNEE might induce alterations in the contribution of hippocampal subregions to hippocampal-dependent learning and memory. For example, the DG contributes to metric processing of a spatial environment (Goodrich-Hunsaker et al., 2008) while the CA1 is involved with temporal processing (Hunsaker and Kesner, 2008) and functional deficits in either region might contribute to impaired hippocampal-dependent learning and memory following PNEE. However, studies have yet to investigate how the function of specific hippocampal subregions is affected by PNEE.

4.2.5.3 Role of Corticosterone on Ethanol-induced Changes to Synaptic Plasticity

Previous studies have shown that CORT can mediate some of the deleterious effects of PNEE. For example, ethanol-induced changes to immune function in male and female offspring are differentially mediated by CORT (Redei et al., 1993). We have shown that the ethanol-induced enhancement of LTP in the DG of adolescent females is abolished by prenatal stress. In males, however, the magnitude of LTP in ethanol-exposed males was not significantly different from males exposed to both ethanol and stress *in utero*. These findings indicate that prenatal stress and PNEE produce sex-specific alterations to hippocampal synaptic plasticity. Prenatal stress counteracts the potentiating effect of PNEE on LTP in the DG of adolescent females suggesting distinct effects of gestational stress or ethanol exposure on LTP in females. In males, placental levels of 11β-HSD2 levels are reduced by prenatal stress (Pankevich et al., 2009) but not prenatal ethanol (Wilcoxon et al., 2003). In contrast, placental 11β-HSD2 levels of the female fetus were reduced by PNEE (Wilcoxon et al., 2005) but not by prenatal stress (Pankevich et al.,

2009). It is possible that sex-specific alterations to 11β-HSD2 following gestational stress and ethanol might contribute to sexually dimorphic changes to synaptic plasticity.

4.3 Conclusions

The experiments within the current thesis highlight that basal sex differences in synaptic plasticity that are present during adolescence are sensitive to insult following prenatal ethanol exposure. It remains to be fully elucidated how the altered synaptic plasticity following PNEE alters learning and memory in adolescent offspring. Up until this point, the effect of PNEE on hippocampal function has predominantly been investigated in males, but these studies highlight the need to also focus attention on females.

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Appendices

Appendix A

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0183

Investigator or Course Director: Brian R. Christie

Department: Psychology, Department of

Animals: Rats Sprague-Dawley 300

Start Date: **May 1, 2006** **Approval Date:** **June 9, 2006**

Funding Sources:

Funding Agency: Alcoholic Beverage Medical Research Foundation (US)
Funding Title: Effects of prenatal ethanol administration of hippocampal neurogenesis and synaptic plasticity: Role of NMDA receptor subunit topology

Funding Agency: BC Ministry of Children and Family Development
Funding Title: HELP: Neurogenesis and functional plasticity

Funding Agency: Canada Foundation for Innovation
Funding Title: Fetal Alcohol Syndrome

Funding Agency: BC Ministry of Children and Family Development
Funding Title: Fetal Alcohol Syndrome

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

Appendix B

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A06-0042

Investigator or Course Director: Brian R. Christie

Department: Psychology, Department of

Animals: Rats 40

Approval Date: March 6, 2006

Funding Sources:

Funding Agency:	Alcoholic Beverage Medical Research Foundation (US)
Funding Title:	Effects of prenatal ethanol administration of hippocampal neurogenesis and synaptic plasticity: Role of NMDA receptor subunit topology
Funding Agency:	UBC Operating Budget
Funding Title:	BREEDING: Fetal Alcohol Syndrome
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

Appendix C

RECEIVED
AUG 17 2009

University of Victoria — Office of Research Services, Animal Care Services, PO Box 1700, STN CSC, Victoria BC V8W 2Y2

For Administrative Use Only
Protocol Number: 2009-027(2)
Expiry Date: 23 SEP 2010

Application to Use Animals for Research

Please note, you must use Adobe Acrobat 7 or higher (not Reader) in order to save and submit this form.
Click on blue text to display additional information.

1. Contact Information

PRINCIPAL INVESTIGATOR

Surname	First Name	Initial
Christie	Brian	R

Rank / Position (please indicate)	Department
Associate Professor	Division of Medical Sciences

Business Telephone	Laboratory Telephone	Residence Telephone
250-472-4244	250-721-8798	250-477-4973

Emergency Telephone	Pager / Cell Telephone	E-mail Address
	250-508-8780	brain64@uvic.ca

Laboratory Address
Medical Science Building, Room 250, 3800 Finnerty Road, UVic, Victoria BC V8P 5C2

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES

A. (mandatory)

Surname	First Name	Emergency Telephone
Webb	Evelyn	250-544-0569

B. (optional)

Surname	First Name	Emergency Telephone

2. Project

STATUS

☐ New Application ☐ Renewal of Protocol #:
☐ Pilot Project ☒ Amendment of Protocol #: 2008-027

Title (including major species, e.g. rat, mouse)
Effects of prenatal stress on synaptic plasticity in rats.

3. Declaration

The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and polices of the Canadian Council on Animal Care and those of University of Victoria. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I **understand that this approval is valid for one year and must be approved on an annual basis.**

Principal Investigator — Signature [redacted] — Date
Brian Christie

UVic Department Chair (Required for all submissions) — Signature — Date

(When the Department Chair is the Principal Investigator, the signature of the Dean is required)

4. Approvals

University Veterinarian [redacted] [redacted]

Chairman, University Animal Care Committee [redacted] [redacted]

Protocol Start Date: 23 SEP 2009

Last revised: 08/09

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