PRENATAL ETHANOL EXPOSURE: GONADAL AXIS INFLUENCES ON HYPOTHALAMIC-PITUITARY-ADRENAL RESPONSIVENESS IN PREPUBERTAL RATS

by

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ABSTRACT

The mechanisms underlying altered hypothalamic-pituitary-adrenal (HPA) activity following prenatal ethanol exposure are unknown. Previous findings from our laboratory have revealed that rats prenatally exposed to ethanol (E) differ from their control counterparts in HPA responding both neonatally and in adulthood. Furthermore prenatal ethanol differentially alters HPA activity in males and females, suggesting a possible role for altered HPG function in HPA axis dysregulation. Prenatal ethanol exposure alters both activational and organizational effects of HPG hormones. This thesis was designed to determine if maturation of the HPA stress response is delayed in prepubertal E rats and to investigate the possibility that ethanol-induced changes to HPG organizational effects, as perhaps indicated by altered basal and/or stress hormone regulation, may underlie some of the differential HPA responsivity observed between E males and females compared to their control counterparts.

Prepubertal (PN 25-26) male and female rats from ethanol, pair-fed (PF), and *ad libitum*-fed control (C) dams were examined at 0, 30 or 60 min following the onset of a 30 min restraint stress. Overall, prepubertal E pups were hyporesponsive to stressors compared to controls, demonstrated by blunted CORT responses following restraint. Both MR and GR mRNA levels were differentially altered by stress in E compared to C pups, suggesting prenatal ethanol delayed and/or altered the development of CORT feedback mechanisms. HPG axis development was delayed and/or altered in E rats, as LH and E2 levels were lower and the LH/E2 correlation was absent in E compared to C female pups, and the HPG hormones responsivity to stressors was altered in both E males and females compared to controls. Importantly, prenatal ethanol both differentially altered the pattern

of CORT responding, and interfered with the normal HPA/HPG hormone interactions in prepubertal males and females compared to control counterparts.

These data indicate that prenatal ethanol delayed and/or altered HPA and HPG development during the prepubertal period. Furthermore HPA responding to stressors was differentially altered in prepubertal E males and females compared to their control counterparts, suggesting that altered HPA activity may be mediated, in part, by changes to the HPG organizational effects.

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ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AFP	α-fetoprotein
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ARBD	Alcohol related birth defects
ARND	Alcohol related neurodevelopmental disorder
AVP	Arginine vasopressin
B-EP	β-endorphin
BAL	Blood alcohol level
BNST	Bed nucleus of the stria terminalis
C	Control
CBG	Corticosterone binding globulin
CNS	Central nervous system
CORT	Corticosterone
CRF	Corticotropin releasing factor
DEX	Dexamethasone
dl	Decilitre(s)
E	Prenatally exposed to ethanol
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
FAS	Fetal Alcohol Syndrome

FSH	Follicle stimulating hormone
G	Gram(s)
GD	Gestation day
GDX	Gonadectomy
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
icv	Intracerebroventricula
LH	Leutinizing hormone
LSD	Least significant difference
mg	Milligrams
ME	Median eminence
min	Minute
ml	Millilitre(s)
MR	Mineralcorticoid receptor
mRNA	Messenger ribonucleic acid
n	Sample size
NE	Norepinephrine
ng	Nanogram(s)
OVX	Ovariectomy
PBS	Phosphate buffer solution
PF	Pair-fed

pg	Picogram(s)
PN	Postnatal day
POA	Preoptic area
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
r^2	Pearson's product-moment correlation
RIA	Radioimmunoassay
sc	Subcutaneous
SDN-POA	Sexually dimorphic nucleus of the preoptic area
SEM	Standard error of the mean
SHRP	Stress hyporesponsive period
SSC	Standard saline citrate
TEA	Triethanolamine

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CHAPTER 1: INTRODUCTION

A. Fetal Alcohol Syndrome

1. Introduction

The association between birth defects and maternal alcohol consumption has been recognized for hundreds of years. In the Bible, Judges 13:7, it is written: "You will conceive and give birth to a son. Now then, drink no wine or other fermented drink." A report to the British House of Commons in 1834 described a starved, shriveled and imperfect look to infants of alcoholic mothers (as cited in Streissguth, Landesman-Dwyer et al. 1980). It wasn't, however, until the late 1960's and early 1970's when two teams of scientists independently published their observations of altered growth, central nervous system (CNS) development and specific facial features amongst children born to alcoholic mothers (Jones and Smith, 1973; Jones et al., 1973; Lemoine, 1968), that the term Fetal Alcohol Syndrome (FAS) was attached to this set of birth defects (Jones and Smith, 1973) and that FAS received widespread attention.

Since the identification of FAS over three decades ago, several diagnostic categories have been developed (Stratton 1996). FAS is characterized by: (1) pre- and postnatal growth retardation, (2) facial anomalies such as short palpebral fissures, low nasal bridge, indistinct philtrum and flat midface, and (3) central nervous system (CNS) neurodevelopmental deficits, including brain structure abnormalities, impaired fine motor skills and poor eye-hand coordination. FAS may be diagnosed with or without a history of maternal alcohol consumption. If an individual fits some but not all the criteria for FAS, that individual may be diagnosed with partial FAS, alcohol related birth defects (ARBD) or alcohol related neurodevelopmental disorder (ARND). Partial FAS is defined

by the presence of at least some of the facial anomalies, and alterations in at least one of: growth retardation, CNS neurodevelopmental deficits, or behavioural impairment such as poor impulse control, hyperactivity or attention problems. Diagnosis of partial FAS requires confirmation of maternal alcohol consumption. ARBD and ARND are diagnostic categories reserved for individuals with confirmed fetal alcohol exposure but who display only physical or neurodevelopmental deficits, respectively (Stratton, 1996).

FAS impacts our communities and the economy. Some experts estimate that 1% of the North American population suffers from FAS or partial FAS. Of these individuals, 95% will have mental health problems, 82% will not be able to live independently, 68% will have trouble with the law and 70% of females and 50% of males will have alcohol and drug problems. An individual with FAS or partial FAS may cost taxpayers up to $2 million in his or her lifetime for health problems, special education, psychotherapy and counselling, welfare, crime and the criminal justice system (Buxton and Philcox, 2000). Given these statistics, research into the symptomatology and etiology of FAS is essential.

2. FAS Research in Humans

Studies on FAS in humans have assessed physical and behavioural abnormalities at all stages of development to understand how fetal alcohol exposure is manifested throughout the lifespan. Infants with FAS fail to thrive (Jones and Smith, 1975). At birth, weight and head circumference are negatively correlated with maternal alcohol intake. Neonates of heavy drinkers show poor habituation and arousal responses and deficits in operant behaviour tasks (Streissguth et al., 1980). Children with FAS have below average intelligence, are hyperactive and show perceptual-motor disturbances (Streissguth et al., 1980). These cognitive and behavioural problems persist. In adolescence and adulthood

individuals with FAS often have below average intelligence and display maladaptive behaviours such as poor concentration, dependency, social withdrawl and impulsivity. Decreased height and head circumference, as well as facial and joint anomalies, are evident throughout development (Streissguth et al., 1991).

While human studies are fundamental for elucidating symptomatology of FAS, this research has intrinsic confounds. First, polydrug use may be a risk factor in alcohol-induced birth defects. For example, smoking is positively correlated with alcohol consumption (Streissguth et al., 1980) and maternal smoking during pregnancy in itself leads to behavioural abnormalities in the offspring (Naeye and Peters, 1984). Second, alcohol consumption can change the nutritional status of the drinker. For example, calories of alcohol replace calories of more nutritious foods. As well, alcohol disrupts ingestion, absorption and utilization of nutrients in the mother (Thomson and Pratt, 1992), placental function, and transfer of nutrients to the fetus (Fisher et al., 1982). Nutrient deficiency is associated with fetal growth deficiency (Brasel and Winick, 1972). Finally, the accuracy of alcohol intake reporting is difficult to determine. Factors such as type of drink, and rate and volume of alcohol consumption may be inconsistently recorded on the self-report scales used in many clinical experiments (Streissguth et al., 1980). Thus while clinical studies of FAS are essential for determining the impact of *in utero* alcohol exposure on human development and behaviour, the role that polydrug use, nutritional deficiencies and blood alcohol levels of the mother have in clinical observations of the offspring is difficult to evaluate. To control for these issues and to address specific questions regarding mechanisms and timing of alcohol insult scientists have turned to animal research.

3. FAS Research in Animals

Animal models of prenatal ethanol exposure are advantageous because they eliminate the influence of polydrug use and allow control over nutrient consumption and alcohol dosage in pregnant females. Furthermore, scientists can easily observe changes throughout a laboratory animal's lifespan and assess the biological constructs that may be underlying alcohol-induced effects. Rodents are the most common laboratory animals used in FAS research. Similar to humans with FAS, rodents exposed to ethanol *in utero* show pre- and postnatal growth deficiencies, CNS and craniofacial abnormalities, decreased organ weights, physiological alterations and numerous behavioural problems including poor response inhibition, hyperactivity and learning deficits (Abel and Dintcheff, 1978; Bond and Di Giusto, 1977; Gallo and Weinberg, 1982; Gallo and Weinberg, 1986; Riley et al., 1979; Streissguth et al., 1980; Sulik et al., 1981; Weinberg and Gallo, 1982).

Issues that influence the interpretation of animal-based studies are the dose of ethanol, timing of ethanol alcohol exposure, and route of administration. Doses of ethanol may be manipulated to evaluate teratogenic levels of ethanol exposure. For example offspring of pregnant rats given *ad libitum* access to a liquid ethanol diet throughout gestation show deficits in response inhibition when 35 %, but not 17 % of the calories are ethanol derived (Riley et al., 1979). These results are analogous to findings from human research in that greater alcohol consumption during pregnancy is associated with greater behavioural impairments (Streissguth et al., 1980). The period of ethanol exposure may be varied to determine the critical periods during development when the fetus is susceptible to alcohol-induced damage. For example, acute ethanol administration to

mice on gestation day 7 increases incidences of eye malformations and abnormalities of nasal and upper lip regions. Ethanol administration at this time may be interfering with formation of the neuroepithelium, resulting in the facial dysmorphology characteristic of FAS (Sulik et al., 1981). At later times in gestation, ethanol may disrupt behavioural and physiological function without causing gross malformations. For example, during the second week of gestation, when the fetal pituitary-adrenal system undergoes considerable development (Eguchi, 1969), exposure to ethanol vapors enhances corticotrophin-releasing factor (CRF) biosynthesis and adrenocorticotropin (ACTH) signaling later in life (Lee, Imaki et al. 1990). During the last week of gestation ethanol interferes with the prenatal testosterone surge (McGivern, Raum et al. 1988), which may underlie some of the long term reproductive deficits observed in ethanol-exposed offspring. Finally, administration of ethanol during the early postnatal period, equivalent to the third trimester of a human fetus and the time of most rapid brain growth, alters neuronal circuitry (West and Hamre, 1985) and causes severe neuronal loss (West et al., 1986). By controlling levels and timing of ethanol exposure in animal models of FAS, researchers can elucidate critical periods during fetal development when ethanol exposure has specific adverse effects.

Another experimental consideration in animal research of FAS is the method used to administer ethanol. Several techniques have been developed for administering ethanol to pregnant dams, including intubation, placing ethanol in drinking water and feeding ethanol as part of a liquid diet. With intubation, doses of ethanol can be closely controlled and high blood alcohol levels are achieved. The disadvantage is that this procedure involves a great deal of handling and thus may be quite stressful (Riley and Meyer,

1984). Prenatal stress can alter endocrine function later in life (Walker et al., 2002), thereby potentially confounding experimental results. Placing ethanol in drinking water is the method with the most ecological validity. However, most animals find the taste of ethanol aversive and will reduce their fluid intake. This technique therefore yields low blood alcohol levels. Furthermore, upon reducing water intake animals also reduce their food intake, resulting in nutritional deficiencies (Riley and Meyer, 1984).

Placing ethanol in a liquid diet is the technique used in our laboratory. Dams on a liquid ethanol diet achieve high blood alcohol levels without being exposed to excessive handling or stressful procedures (Weinberg, 1985). Ethanol is blended with a diet mix that is specifically formulated to provide adequate nutrition for pregnant females (Lieber and DeCarli, 1982). However, dams on a liquid ethanol diet still have reduced diet intake because of ethanol's anorexigenic effect and high caloric content. To account for the reduction in nutrient intake a pair-fed (PF) group is used as an experimental control. Dams in the pair-fed group are given a liquid diet in which maltose-dextrin, a non-nutritive carbohydrate, is isocalorically substituted for ethanol. The pair-fed dams are fed an amount equivalent to the amount consumed by an ethanol-consuming partner on the same day of gestation (g/kg body weight/day of gestation). It should be noted that the pair-fed group is not a perfect control, as pair-feeding cannot control for the secondary effects of ethanol on absorption and utilization of nutrients. As well, pair-fed females receive less food than they would consume *ad libitum*, which creates a state of mild food deprivation and results in the consumption of the entire food ration within a short time after food presentation (Gallo and Weinberg 1981). Both food deprivation and a meal feeding regime alter HPA responsiveness of pair-fed rats (Weinberg and Gallo 1982) and

thus pair-feeding may serve as a mild prenatal stress in the offspring (Weinberg, 1984; Weinberg, 1985). Therefore a second control group, in which dams are given *ad libitum* access to standard laboratory rat chow, is included in our studies to tease out ethanol-induced effects from effects arising because of nutritional deficiencies and/or prenatal stress associated with the pair-feeding procedure. When experimental data from rats born to both ethanol (E) and pair-fed (PF) dams differ from what we observe in rats born to control (C) dams, the effects may be due primarily to nutritional variables alone or to a combination of nutritional variables and ethanol in E rats and nutritional variables and stress or other experimental factors in PF rats. In these circumstances further testing is required to elucidate the mechanisms underlying the observations. When E rats differ from PF and C, the effects can be attributed specifically to the exposure to ethanol *in utero*.

We and others have shown that offspring of dams exposed to a liquid ethanol diet (36% ethanol-derived calories) during pregnancy have reduced postnatal weight gain (Osborn et al., 1996; Weinberg, 1988; Weinberg, 1992; Weinberg and Vogl, 1988), altered stress responsiveness in the neonatal period (Taylor et al., 1986a; Weinberg, 1989; Weinberg and Gallo, 1982) and adulthood (Lee et al., 2000; Nelson et al., 1986; Taylor et al., 1982; Weinberg, 1988; Weinberg, 1992), deficits in immune function (Giberson and Weinberg, 1995; Redei et al., 1993; Taylor et al., 1999; Weinberg and Petersen, 1991) and long term effects on neurotransmitter systems, such as the serotonin (Hofmann et al., 2002; Kim and Druse, 1996), catecholamine (Detering, Collins et al. 1980; Cooper and Rudeen 1988) and gamma-aminobutyric acid (GABA) (Osborn et al., 1998) systems. Hyperactivity (Blanchard and Hannigan 1994), altered maternal

behaviour (Barron and Riley 1985) and deficits in learning and memory (Nagahara and Handa, 1999; Wilcoxon et al., 2005) have also been observed in offspring exposed prenatally to a liquid ethanol diet. These rats have neurodevelopmental deficits in the absence of structural abnormalities, and thus represent a model of ARND rather than full FAS.

B. Hypothalamic-Pituitary-Adrenal (HPA) Axis

Living organisms maintain a complex dynamic equilibrium, called homeostasis, for their internal environment. When adverse forces, called stressors, challenge this equilibrium the result is a state of threatened homeostasis, or stress (Johnson et al., 1992; Tsigos and Chrousos, 2002). Stressors can be disruptions within the internal environment, such as low blood glucose or oxygen levels, perturbations to external environments, such as extreme heat or cold, or stimuli that provoke emotion, with fear-, anxiety- or frustration-inducing stressors amongst the most potent. In response to stressors, organisms activate adaptive responses. Behavioural adaptations include increased alertness, enhanced memory, and altered cognitive and sensory thresholds. Peripheral adaptations involve the release of energy substrates, stimulation of cardiac and pulmonary functions, suppression of anabolic processes such as digestion, growth, reproduction and immunity, and containment of the stress response (Johnson et al., 1992).

The body's response to stressors is controlled by two interacting brain centers: the paraventricular nucleus (PVN) of the hypothalamus and locus coeruleus-norepinephrine (LC-NE) autonomic system in the brain stem. Stimulation of the PVN by higher brain regions initiates a cascade of hormonal responses in the hypothalamic-pituitary-adrenal (HPA) axis, which ultimately increases glucocorticoid secretion from the adrenal cortex.

Plasma glucocorticoids are the final effectors of the HPA axis and participate in the maintenance of homeostasis under basal conditions as well as the body's behavioural and physical or physiological adaptive responses following stress. Activation of the LC and other catecholaminergic cell groups in the medulla and pons of the brain stem stimulates the autonomic nervous system (ANS). Centrally, NE serves as a global alarm to decrease neurovegetative functions such as eating and sleeping, and in the periphery postganglionic sympathetic fibers release NE to coordinate rapid responses to stressors by innervating smooth muscle, fat and many organs. The HPA axis and SNS interact in a bidirectional manner, such that CRF and arginine vasopressin (AVP) neurons in the PVN and catecholaminergic neurons in the brain stem reciprocally innervate each other, and glucocorticoid feedback inhibits both systems. Behavioural and physical responses to stressors are therefore coordinated by co-activation of the HPA axis and the ANS (Tsigos and Chrousos, 2002).

One focus of this thesis is on the effects of prenatal ethanol exposure on development of the HPA response to stressors and the role of the hypothalamic-pituitary-gonadal (HPG) hormones in modulating this response. The PVN represents the final common pathway for the integration of stress responding in the brain (Vazquez, 1998). They receive multiple inputs, including cholinergic and serotonergic stimulatory signals and GABA and opioid inhibitory signals. When activated, neurons in the medial parvocellular region of the PVN release CRF and AVP into the hypophyseal portal system. CRF is the principal hypothalamic stimulus to the anteriour pituitary gland. AVP is co-localized and co-secreted with CRH in 20-50% of nerve terminals in the median eminence and synergizes with it to potentiate its effect in the anteriour pituitary. CRF and

AVP stimulate the synthesis and secretion of the pro-opiomelanocortin (POMC)-derived peptide ACTH from pituitary corticotroph cells. ACTH is released into the general circulation and where it signals the adrenal cortex to produce and secrete glucocorticoids: cortisol in humans and corticosterone (CORT) in rats. In the adult animal approximately 95% of circulating CORT is bound to an alpha globulin called corticosterone binding globulin (CBG) and is physiologically inactive (Johnson et al., 1992; Tsigos and Chrousos, 2002).

Prolonged or chronic stress can have detrimental effects on growth, reproduction, immune function and psychological well-being (Johnson et al., 1992). Therefore one critical role for CORT is to provide negative feedback to the HPA axis by acting at receptors in the pituitary and hypothalamus, as well as in higher brain centers that project either directly or indirectly to the hypothalamus, such as the bed nucleus of the stria terminalis (BNST), the preoptic area (POA) and the hippocampus. Two types of CORT receptors regulate HPA activity. Mineralocorticoid receptors (MR), or Type I receptors, are located primarily in the lateral septum and hippocampus. They have a high affinity for CORT and are up to 90% occupied by both basal and stress CORT levels. Glucocorticoid receptors (GR), or Type II receptors, are more widely distributed in the brain and are found in regions including the anteriour pituitary, lateral septum, hippocampus, PVN, and PVN-projecting cell groups such as the arcuate nucleus, BNST, and POA. GR have a lower affinity for CORT and only become occupied when plasma CORT levels rise in response to stressors or during the peak of the circadian rhythm. Because MR are nearly saturated under basal conditions this receptor is implicated in regulating basal activity and controlling the sensitivity of the HPA axis to stress. As

CORT levels increase, the coactivation of MR and GR functions to mediate the termination of and recovery from stress (De Kloet et al., 1998; Herman and Cullinan, 1997; Jacobson and Sapolsky, 1991; Reul and de Kloet, 1985).

CORT negative feedback occurs in three time domains and the mechanisms underlying each domain is unique. Fast, rate-sensitive feedback (within seconds to minutes of stressor onset) occurs when CORT levels are rising, and is sensitive to the rate of CORT increase. During this time domain, CORT acts via nongenomic mechanisms within the brain to limit the immediate ACTH and CRF response to a stressor. Intermediate feedback (between 1-10 hrs after stressor onset) depends on the dose and duration of CORT exposure. During the intermediate time domain, CORT feedback promotes termination of the stress response by inhibiting ACTH and CRF release, and limits the responses to subsequent stressors by suppressing ACTH and CRF biosynthesis. Slow feedback (beginning 12 hrs after stressor onset) inhibits both hormone release and synthesis in order to protect the body against the deleterious effects of prolonged CORT exposure (Keller-Wood and Dallman 1986). Negative feedback in all three time domains is achieved by direct actions of CORT on receptors in the pituitary and hypothalamus, as well as by indirect actions at higher brain centers. In particular, the hippocampus has received considerable attention for its role as a regulator of the HPA axis, as it is densely populated with CORT receptors, its activation is essential for basal and stressor-induced HPA regulation, and it is one of the most susceptible brain regions to damage by environmental insults (Sapolsky, Krey et al. 1986; Jacobson and Sapolsky 1991).

1. Postnatal Development of the HPA Axis

Despite having detectable levels of HPA hormones during gestation (Almazan et al., 1989; Boudouresque et al., 1988; Grino et al., 1989), rats do not show significant HPA axis activity in response to stress until the third week of life. This neonatal period of reduced HPA responsiveness, that lasts from approximately postnatal day (PN) 3 or 4 to PN 14 is called the Stress Hyporesponsive Period (SHRP), and is characterized by blunted ACTH (Dent et al., 2000a; Dent et al., 2000b; Vazquez et al., 1996; Walker et al., 1986a; Walker et al., 1991) and CORT (Levine et al., 1967; Schoenfeld et al., 1980; Walker et al., 1986b; Walker et al., 1991) responding to a variety of stressors. The SHRP is likely the result of a number of developmental changes occurring simultaneously within the HPA axis of neonatal rats. First, during the first two weeks of life the adrenal cortex displays limited capacity to respond to ACTH (Levine et al., 1967). For example, stressors that elicit significantly different ACTH responses do not result in significantly different CORT responses (Walker et al., 1991). Second, there is a higher ratio of free versus bound CORT levels in neonatal rats, likely due to low CBG levels (Henning, 1978; Walker et al., 1990). Thus at this stage of development there is a greater availability of bioactive CORT to target tissues, including brain regions sensitive to CORT negative feedback (Walker et al., 1986b). Third, anteriour pituitary corticotrophs are more sensitive to CORT negative feedback in neonates than adults (Sakly and Koch, 1983). For example CORT is more effective at inhibiting CRF-induced ACTH secretion in neonatal compared to adult pituitary extracts. As well, less CORT is required to inhibit urethane-induced ACTH secretion in neonates compared to adults (Walker et al., 1986b). Fourth, neural inputs to the PVN, such as the stimulatory serotonergic (Roth et al., 1991) and cholinergic (Coyle and Yamamura, 1976) systems, display considerable development

postnatally. Ontogenetic development of differential HPA responding to specific stimuli may reflect the concurrent development of PVN projecting neurotransmitter systems (Rosenfeld et al., 1992; Walker et al., 2002).

The SHRP has an adaptive function. While low levels of glucocorticoids are required for normal development, for example determination of neurotransmitter phenotype and induction of glial enzyme activity, excessive glucocorticoid secretion can be detrimental, as high CORT levels inhibit neurogenesis, gliogenesis, cell division and myelination (Rosenfeld et al., 1992; Sapolsky and Meaney, 1986; Walker et al., 2002). Therefore the ability for an organism to maintain stable CORT levels during the neonatal period is essential for proper CNS development.

As rat pups emerge from the SHRP they show adult-like responses to stress but are slow to terminate their stress response. For example, Goldman et al. (1973) demonstrated that PN 25 pups had a peak CORT response to ether and shock stress 30 min after stress onset and that this increase over basal was still present 60 min after stress onset. Adults, on the other hand, had peak CORT levels at 15 min and returned to baseline by 60 min. In the same experiment, pretreatment with dexamethasone, a synthetic GR agonist, suppressed stress-induced CORT in adults but not in weanlings, suggesting that glucocorticoid receptor-regulated feedback was less effective in weanlings. Vazquez and Akil (1993) replicated and extended these findings. Not only did PND 25 pups have a later and more sustained CORT response to ether stress than adults, their ACTH response was also prolonged.

Delayed and prolonged stress responding in weanling rats may be due to a number of factors. First, the adrenal cortex does not fully mature until after puberty (Van

Dorp and Deane, 1950) and may be slow to release CORT upon ACTH stimulation. Second, a delayed release of CORT from immature adrenal glands may result in less effective negative feedback, because rapid feedback inhibition of the HPA axis is rate sensitive (Keller-Wood and Dallman, 1984). Third, glucocorticoid receptor concentrations in the hippocampus are still developing during the post-weaning period (Olpe and McEwen, 1976; Rosenfeld et al., 1988; Sapolsky et al., 1985). These developmental changes may be responsible for the decreased responsiveness to negative feedback even when CORT levels are high (Vazquez et al., 1993). Finally, ACTH metabolic clearance takes longer in weanlings compared to adults (Vazquez et al., 1997) thus prolonging the ACTH effect on CORT release from the adrenal gland. In summary, HPA activity and inhibition show different patterns of development, and the result is a phase of negligible CORT responding to stress, the SHRP, in neonates, followed by a period of delayed and prolonged CORT responding in weanlings.

2. Prenatal Ethanol Exposure and HPA Axis Activity

Ethanol interferes with fetal brain development both directly and indirectly. Ethanol easily passes across the placental barrier and can directly damage the fetal brain. For example, high levels of alcohol intake can induce hypoxia, increase free radical generation, and interfere with neuronal migration, differentiation and survival (Guerri, 1998). Ethanol also alters the maternal hormone balance and disrupts the hormonal interactions between the mother and fetus. Hormonal dysregulation can disrupt normal fetal development and may underlie some of the changes to endocrine function observed later in life (Gabriel et al., 1998; Weinberg and Gallo, 1982).

Considerable development of the HPA axis occurs prenatally, rendering this system a viable target for damage by prenatal ethanol exposure. Indeed, on gestational day 21 E fetuses have blunted CORT levels (Weinberg, 1989) and after birth postnatal day (PN) 1 E pups have higher CORT (Angelogianni and Gianoulakis, 1989; Kakihana et al., 1980; Taylor et al., 1982; Weinberg, 1989) and β-endorphin (Angelogianni and Gianoulakis, 1989) levels as well as lower CBG binding capacity (Weinberg, 1989) compared to same age controls. During the SHRP, when the HPA response to stressors is blunted, E pups are even more hyporesponsive than controls. For example, data indicate that CORT responses to a variety of stressors including ether, novelty, saline, morphine, ethanol and cold are lower in E compared to control pups (Angelogianni and Gianoulakis, 1989; Taylor et al., 1986a; Weinberg, 1989). Prior to weaning (PN 18) the HPA response to saline, morphine and ethanol challenge does not differ from controls (Taylor et al., 1986a) while responses to other stressors, such as novelty, ether and ACTH are still blunted (Weinberg, 1989).

Following puberty a new pattern of HPA dysregulation develops, as adult E rats display increased and prolonged HPA responses to a variety of stressors. Increased HPA activity may be at least partially due to increased drive to the system. For example, within 30 min of the onset of cardiac puncture (Taylor et al., 1982), noise and shake (Taylor et al., 1982) or footshock (Lee et al., 2000; Nelson et al., 1986) stress, plasma levels of CORT and/or ACTH are elevated in E compared to control rats. Central mediators of HPA drive are also enhanced in E animals. Under basal conditions CRF mRNA levels are increased in adrenalectomized (ADX) E males compared to controls (Glavas et al., in preparation). When endogenous HPA activity is blocked by dexamethasone (DEX), a

synthetic CORT that mimics negative feedback signals, infusion with CRF induces ACTH hypersecretion from the pituitary of E versus C rats (Osborn et al., 2000). Finally, during acute footshock stress E rats have elevated c-fos mRNA and CRF hnRNA in the PVN compared to controls (Lee et al., 2000).

Previous studies suggest there may also be deficits in negative feedback regulation of the HPA axis following prenatal ethanol exposure. Alterations to fast feedback regulation are controversial. While footshock stress elicits ACTH hypersecretion during the fast feedback time domain in E compared to C rats (Taylor et al., 1986b), injection of CORT immediately prior to swim and ether stress attenuates ACTH responding equally in E and C rats (Hofmann et al., 1999). Deficits in intermediate feedback are more consistent. For example, compared to controls, E rats show elevated basal CORT levels 4 hrs following CORT administration (Nelson et al., 1986; Nelson et al., 1985), and elevated CORT responses to stress 3 and 6 hrs following treatment with DEX (Osborn et al., 1996). In chronic stress regimes (i.e. slow feedback time domains) E animals show poor habituation of HPA axis activity compared to controls (Kim et al., 1999b; Weinberg, 1996; Weinberg et al., 1996). The mechanisms underlying these deficits may involve changes in the regulation of MR and GR gene expression. For example, although basal and chronic-stress induced MR and GR protein concentrations are normal in E adult rats (Kim et al., 1999b; Weinberg and Petersen, 1991), regulation of MR and GR gene transcription by exogenous CORT treatment following ADX is altered by prenatal ethanol exposure (Glavas et al., in preparation).

While both E males and females demonstrate HPA axis dysregulation, their stress responses appear to be differentially altered by prenatal ethanol exposure, with the effects

appearing to depend on the nature, intensity and time course of the stressor. For example, both E males and females demonstrate deficient HPA axis recovery following stress, but under different circumstances. E females have elevated CORT compared to C females after 60 min of restraint stress (Weinberg, 1988), while in a slightly different restraining paradigm E males have higher CORT than C males after 180 min (Weinberg, 1992). During repeated restraint E males and females show differential deficits in stress response habituation. Immediately after 10 daily exposures to stress E females have elevated ACTH, while E males have lower ACTH but elevated β-endorphin levels compared to controls (Weinberg et al., 1996). In a similar repeated restraint paradigm E females were found to have lower pituitary POMC mRNA levels compared to controls throughout testing, while after restraint on days 1 and 10 E but not C males show acute increases in POMC mRNA (Leo et al., 2002). There is some evidence that deficits in feedback regulation may also be differentially altered between E males and females. For example, following ADX and CORT replacement, both E males and females show deficits in utilizing exogenous CORT to normalize MR mRNA, while only E males showed significant deficiencies in regulating GR mRNA (Glavas et al., in preparation).

The HPA axis does not function in isolation and interacts in a bidirectional manner with another important neuroendocrine system: the HPG axis, which controls the reproductive system. There is a normal sexual dimorphism in stress responding, such that females generally have greater basal and stress-induced HPA activity than males, and this is due to activational and organizational effects of sex hormones. One possible mechanism of the differential HPA dysregulation in E males and females may be the effect of prenatal ethanol exposure on HPG axis function.

C. Hypothalamic-Pituitary-Gonadal Axis

Reproductive capacity is under the control of another neuroendocrine system: the HPG axis. Activity of this axis is initiated with the synthesis and secretion of gonadotropin releasing hormone (GnRH). While GnRH-producing neurons are few in number (about 1200 neurons in the rat brain) and widely dispersed throughout the forebrain, clusters of neurons are found in the periventricular region of the medial preoptic area of the hypothalamus, diagonal band of Broca and septal nuclei. Fibers from GnRH neurons project caudally to other CNS regions such as the olfactory and limbic systems, where GnRH plays a role in modifying reproductive behaviour, and rostrally to the median eminence where GnRH is released into the hypophyseal portal system and controls the synthesis and secretion of gonadotropins from anteriour pituitary gonadotroph cells (Witkin et al., 1982). The gonadotropins, leutinizing hormone (LH) and follicle stimulating hormone (FSH), enter the systemic circulation and act on the gonads. In adult males LH promotes the synthesis of androgens by testis Leydig cells and FSH stimulates seminiferous tubule maturation and spermatogenesis. Androgens are steroid hormones that promote the growth of male tissue and include testosterone (T), the main secretagogue, and dihydroxytestosterone (DHT). Both T and DHT are potent stimulators of their target tissues, and T, but not DHT, can be aromatized to estradiol (E2) and activate E2-sensitive systems as well. In adult females LH stimulates the production of ovarian steroids, E2 and progesterone (P), as well as ovulation and FSH stimulates E2 and P production, ovarian follicle development and ovulation (Yen and Jaffe, 1986).

1. Postnatal Development of the HPG Axis

Ontogentic studies have revealed that adult-like distribution of GnRH neurons and fibers is attained by gestation day 19 in the rat (Jennes, 1989). However, the GnRH system continues to develop postnatally, as connectivity (Wray and Hoffman, 1986), biosynthetic capacity (Jakubowski et al., 1991), and levels of GnRH mRNA (Jakubowski et al., 1991) and GnRH protein (Araki et al., 1975) increase through the neonatal and prepubertal period. The synthetic capacity of anterior pituitary gonadotrophs is fully established shortly after birth (Harris and Jacobsohn, 1952) but the secretory patterns vary throughout the neonatal and prepubertal periods. In males, LH and FSH show adult-like levels during the first 4 weeks of life and are elevated slightly but significantly at puberty onset (PN 35-45). Females have variable LH plasma levels during the first three weeks of life but consistently show a surge akin to the preovulatory LH surge at or just before puberty onset (PN 30-35). FSH levels are elevated early in life, drop abruptly around PN 17, and rise again at puberty (Dohler and Wuttke, 1975). Since synthetic capacity of gonadotrophs is established soon after birth it is believed that prepubertal elevations of plasma gonadotropin levels is mediated by stimulation by central and peripheral branches of the HPG axis. Specifically, an increase in excitatory tone in conjunction with a decrease in inhibitory tone to GnRH neurons leads to an increased hypothalamic drive to pituitary gonadotrophs, while subtle increases in ovarian secretions in conjunction with increased sensitivity to E2 positive feedback in females (Ojeda and Tesawa, 2002; Urbanski and Ojeda, 1986) and decreased sensitivity to T negative feedback in males (Negro-Vilar et al., 1973) account for peripheral stimulation to gonadotrophs.

2. Sexual Dimorphism of Reproductive Function and Behaviour

Mature males and females have sexually distinct reproductive capabilities and behaviours. This sexual dimorphism is the result of several interacting forces including genetic influences, which control gonad determination during fetal development, organizational effects, which are the permanent structural changes arising from the actions of perinatal sex hormones, and activational effects, which are the direct and temporary influences of circulating gonadal hormones on function and behaviour following puberty and throughout adulthood (Kelly et al., 1999).

Gonad formation is genetically determined. Genetic males possess a testis-determining factor, the SRY gene, on their Y chromosome that initiates testis development. Subsequently the developing testes secrete two hormones, T and Mullerian Inhibiting Hormone (MIH), which control organizational effects of HPG development in males. By a process called masculinization, T promotes the maturation of the Wolffian ducts into male sex accessory structures, including the epididymis, vas deferens, seminal vesicles and ejaculatory duct. By a process called defeminization, MIH causes the regression of the Mullerian ducts, the precursor for the female sex accessory structures. In the absence of these hormones, such as in the case of a genetic female, the Wolffian ducts regress and the Mullerian ducts develop into the oviducts, uterus, cervix and vagina.

Gonadal hormone secretion controls sexual differentiation of the brain and behaviour. For permanent masculinization of behaviour both aromatizable (i.e. T and its aromatized form E2) and non-aromatizable (i.e. DHT) androgens are required. For example, when males are castrated neonatally they are capable of normal ejaculatory responses in adulthood only if they are treated with both T and DHT immediately after

castration (Hart, 1979). During the critical period of pre- and postnatal development (gestation day 18 through to the second week of life in the rat) aromatizable androgens (i.e. T and E2) are important for defeminization of behaviour. For example, when neonatal males are treated with aromatase inhibitors or E2 receptor antagonists, these males are capable of displaying female-typical sexual receptivity and mating behaviour in adulthood (McEwen et al., 1977). There is also a role for ovarian steroids in the organization of sexual differentiation (McEwen, 1983). For example, the sexual dimorphism of cortical thickness is altered by OVX of neonatal females (Pappas et al., 1978). Both testicular and ovarian hormonal secretions also promote the organization of CNS systems involved in non-reproductive behaviours. For example, social play in male rats depends on neonatal androgen status (Meaney and Stewart, 1981) and the normally high levels of open field activity in adult females are attenuated by neonatal, but not prepubertal, OVX (Steward and Cygan, 1980). Thus organizational effects on sexual differentiation of the brain and behaviour rely on perinatal levels of testicular and ovarian steroids.

Activational effects of sex steroids arise at puberty. Elevated gonadal hormone levels promote sex-specific anatomical function. In males, T supports sex organs including the penis, prostate and seminal vesicles. In females E2 and P are released according to a 4-5 day estrous cycle that consists of 4 phases: diestrus 1, diestrus 2, proestrus and estrus. Within the cycle, P is elevated in diestrus 1, decreases throughout diestrus 2 and early proestrus, rises to a peak during late proestrus and early estrus and then falls to a minimum in mid- and late estrus. E2 levels rise slowly during diestrus 1 and 2, peak late in proestrus and then fall to a minimum in estrus. The fluctuating levels

of ovarian steroids during the estrous cycle are necessary for maintaining reproductive function and directing sexual receptivity.

Post-pubertal circulating sex hormones interact with sexually differentiated brain structures to promote sex differences in behaviour. Behaviours whose form and frequency differ between males and females are called sexually dimorphic behaviours (Kelley, 1988), and can include reproductive, i.e. courtship, mating reflexes and parental care (Kelley, 1988), and non-reproductive, i.e. activity, learning and memory and intraspecies aggression (Beatty, 1979) behaviours. Mating behaviour depends on both circulating hormones and sexually differentiated brain structures. For example, in females, sexual receptivity is abolished by ovariectomy (OVX) and reinstated with estrogen replacement (Lisk, 1962), and in males the typical pattern of mounts, intromissions and ejaculations in response to a sexually receptive female is eliminated by gonadectomy (GDX) and reinstated with subsequent testosterone replacement (Davidson, 1966). However, no amount of estrogen in males or testosterone in females can elicit a full mating pattern typical of the opposite sex, indicating that the activational effects of circulating hormones are only effective if the appropriate structural components, derived from the effects of perinatal sex hormones, are in place (Connor and Levine, 1969; Levine, 1971). Aggression, a sexually dimorphic non-reproductive behaviour, is also influenced by both activational and organizational effects of the sex steroids. For example, fighting behaviour is decreased in GDX males and rises to normal levels when testosterone is replaced. In females, however, no amount of testosterone treatment can elicit the level of aggression seen in males, indicating that aggression depends not only on circulating levels of testosterone but also on differential CNS responsiveness to

testosterone between males and females (Connor and Levine, 1969). Sexual dimorphism of reproductive and non-reproductive behaviours in adulthood depends on both the organizational effects of neonatal sex steroids, as they promote the development of permanently sexually differentiated brain structures, and the activational effects of circulating sex steroids, as they directly and temporarily activate the sexually differentiated systems.

3. Prenatal Ethanol Exposure and the HPG Axis

Exposure to ethanol *in utero* causes permanent changes to reproductive function and behaviour. For example, during mating E males show increases in mounting latency and decreases in intromission frequency (Udani et al., 1985), and E females have lower quality lordosis responses (McGivern et al., 1995). E females also have a shorter reproductive lifespan (McGivern et al., 1995) and display decreased maternal behaviour (Barron and Riley, 1985) compared to control females. Non-reproductive behaviours are also altered, as the normal sex differences observed in behaviours such as saccharin preference and maze learning are absent in E animals (McGivern et al., 1984). Associated with these behavioural changes are long-term changes in the HPG axis. In adulthood, E males have a significantly reduced volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Barron et al., 1988) as well as lower testis weight and plasma levels of LH and T (Handa et al., 1985; Udani et al., 1985). E females have a smaller SDN-POA volume prior to puberty (Ahmed et al., 1991) but this difference is no longer significant in adulthood (Barron et al., 1988). E females show reduced LH secretion following OVX and E2 (Creighton-Taylor and Rudeen, 1991; Handa et al., 1985; Wilson et al., 1995) or GnRH (Creighton-Taylor and Rudeen, 1991) treatment in adulthood, and

reduced basal plasma LH levels both pre- and postpubertally (Esquifino et al., 1986; Handa et al., 1985; Morris et al., 1989). Pubertal E females have lower hypothalamic GnRH protein content (Morris et al., 1989) and altered GnRH neuron morphology (McGivern and Yellon, 1992) which may account for the delay in puberty onset, determined by the delay in vaginal opening (Boggan et al., 1979; Esquifino et al., 1986; McGivern and Yellon, 1992) and behavioural estrous (Hard et al., 1984), observed in E females.

Importantly, ethanol interferes with the perinatal gonadal hormone milieu. In males, ethanol administration late in gestation decreases Leydig cell number, testis response to LH, testicular enzymatic activity, basal T levels and the prenatal T surge (Kelce et al., 1989; McGivern et al., 1998b; McGivern et al., 1988). Since perinatal androgen status is critical for neurobehavioural differentiation of the brain, ethanol may have long-term effects on the HPG axis by altering testicular hormone output (McGivern et al., 1984). The affect of ethanol in HPG organizational effects in females is less obvious. E females have masculinized reproductive behaviours and while this may suggest greater exposure to androgens and/or estrogens during the critical period of HPG development, these hormones are not elevated in perinatal E females (Ahmed et al., 1991; Kakihana et al., 1980). Another possibility lies in altered availability of these hormones to their target receptors. Under normal conditions there are protective mechanisms which buffer against inappropriate developmental effects of androgens. These include α-fetoprotein (AFP), which is found in fetal serum and brains and binds E2 to prevent it from activating E2 receptors (Savu et al., 1974; Vannier and Raynaud, 1975), and P, which comes from the placenta and via progestin receptor activation has antiandrogenic

and antiestrogenic properties (Kincl and Maqueo, 1965). Both AFP (Halmesmaki et al., 1987b) and P (Halmesmaki et al., 1987a) have been found to be low in pregnant women who abuse alcohol. Thus ethanol may disrupt HPG organizational effects in female offspring indirectly by interfering with the activity of AFP and/or P.

As previously mentioned not only is the development of the reproductive system dependent on HPG organizational effects, a number of non-reproductive systems are as well (McEwen, 1983). It has been speculated that one mechanism underlying some adverse effects of *in utero* exposure to ethanol is ethanol's ability to interfere with HPG organizational effects. By disrupting HPG organizational effects, alcohol may thus alter the development of systems that rely on these organizational effects (Blanchard and Hannigan, 1994; Chen and Smith, 1979; McGivern et al., 1998a). One such system is the HPA axis.

D. Bidirectional Interactions Between the HPA and HPG Axes

1. Sex Steroids Influence HPA Activity

As previously noted, there is a normal sexual dimorphism of the HPA axis both in terms of basal activity and in response to stressors. Females display higher plasma CORT and/or ACTH levels than males under basal conditions (Atkinson and Waddell, 1997; Patchev and Almeida, 1995) and in response to a variety of stressors (Figueiredo et al., 2002; Kitay, 1961; Lesniewska et al., 1990; Ogilvie and Rivier, 1996; Rivier, 1999). This sexual dimorphism of the HPA axis is due to both activational and organizational effects of sex steroids.

There is a wide body of evidence to suggest that circulating sex hormones modulate HPA axis activity. In general, E2 has stimulatory effects and T has inhibitory

effects on HPA activity. For example, intact females have higher stress-induced CORT and ACTH levels in proestrous, when E2 levels are maximal, compared to other phases of the estrous cycle (Carey et al., 1995; Viau and Meaney, 1991) and compared to males (Kitay, 1961; Lesniewska et al., 1990). OVX lowers both basal and stress-induced CORT, and subsequent treatment with E2 restores CORT responding to control values (Burgess and Handa, 1992; Carey et al., 1995; Lesniewska et al., 1990; Seale et al., 2004b; Seale et al., 2004a). GDX increases basal and stress CORT and ACTH in males, and T replacement restores CORT responding to normal (Lesniewska et al., 1990; Seale et al., 2004b; Seale et al., 2004a; Viau and Meaney, 1996). Furthermore, GDX males treated with E2 have increased HPA activity and OVX females treated with T have decreased HPA activity compared to their GDX and OVX vehicle-treated counterparts (Lund et al., 2004; McCormick et al., 2002).

Sex steroids exert their effects at various levels of the HPA axis. For example, independent of sex, E2 enhances basal CORT and T inhibits ACTH-stimulated CORT secretion from adrenocortical cells *in vitro* (Nowak et al., 1995). *In vivo* there are estrous cycle-related changes to basal adrenal secretions, with CORT release being highest at proestrous (Atkinson and Waddell, 1997). At the level of the pituitary, corticotrophs are more responsive to CRF in OVX + E2 treated females compared to OVX + vehicle treated females (Coyne and Kitay, 1969). Central structures of the HPA axis are also influenced by the activational effects of sex steroids. E2 enhances and T inhibits stress-induced CRF and AVP hnRNA (Lund et al., 2004) and mRNA (Seale et al., 2004b) levels in the PVN. E2 receptors have been localized on CRF neurons in the PVN (Simerly et al., 1990) and may exert their effects directly. T receptors are absent from

median eminence-projecting PVN neurons and likely exert their effects via trans-synaptic pathways (Viau and Meaney, 1996). Finally, sex steroids modulate the recovery of CORT and ACTH levels following stress activation. That is, there is evidence that E2 impairs GR- (Burgess and Handa, 1992) and MR- (Carey et al., 1995) mediated negative feedback, and that T enhances GR binding (Viau and Meaney, 1996). Thus circulating sex steroids act at various levels of the HPA axis to modulate responding to stressors.

Sexual dimorphism of the HPA axis relies on more than just activation by circulating gonadal hormones. For example, before puberty females already display greater HPA activity in response to ether (Hary et al., 1984; Tang and Phillips, 1977) and immune challenge (Rivier, 1994; Shanks et al., 1994) than males. In adulthood GDX and OVX often decrease, but do not eliminate, sex differences in HPA responding (McCormick et al., 2002; Nock et al., 1998; Rivier, 1994; Sibilia et al., 2000; Skelton and Bernardis, 1966; Wilson and Biscardi, 1994). These observations suggest that organizational effects of sex hormones may underlie some of the observed sex differences in HPA axis responding. To address this hypothesis experiments have been performed to evaluate the effects of neonatal gonadectomy and/or hormone treatment on HPA activity. For example, intact PN 3 females show higher CORT and ACTH in response to immune challenge compared to males, and this difference in eliminated by GDX and OVX at birth (Shanks et al., 1994). When males are gonadectomized at birth and treated with T in adulthood, their HPA response to stress is elevated compared to that of intact controls: the neonatally gonadectomized males are not able to utilize T to suppress stress-induced HPA activity. However, when males are neonatally gonadectomized and treated with T neonatally and in adulthood, their HPA responding is

indistinguishable from that of controls (McCormick et al., 1998). When females are exposed to high levels of E2 at birth, to mimic the levels observed in neonatal males, there is masculinization of their HPA axis such that steady state CRH mRNA levels in the PVN are increased and GR mRNA levels in the hippocampus are decreased towards those mRNA levels observed in males (Patchev and Almeida, 1995). Thus the sex difference in stress responding is due to interacting influences of activational and organizational effects of sex steroids.

2. Stress Hormones Influence HPG Activity

Just as gonadal steroids can modulate stress responding, stress hormones can alter reproductive function. The nature of these effects depend on the length of exposure, intensity of stressor and hormonal milieu. Chronic stress is generally inhibitory. For example, prolonged stress has been shown to delay puberty (Almeida et al., 2000; Ramaley, 1974), suppress LH and T levels in males (Almeida et al., 1998; Charpenet et al., 1981) and disrupt ovulation and pregnancy outcome in females (Brann and Mahesh, 1991; Yang et al., 1969). Glucocorticoids suppress anabolic processes and therefore CORT has been implicated as a principal mediator for stress-induced inhibition of reproductive function. Indeed exogenous CORT treatment delays development (Ramaley and Schwartz, 1980; Smith et al., 1971), lowers basal and GnRH-stimulated LH levels (Baldwin, 1979; Rosen et al., 1988), and disrupts ovulation (Baldwin, 1979; Smith et al., 1971). CORT receptors have been localized in the medial basal forebrain (Reul and de Kloet, 1985), anteriour pituitary gonadotrophs (Kononen et al., 1993), and gonads (Evain et al., 1976; Schreiber et al., 1982) and are thus positioned to modulate reproductive function at every level of the axis. Centrally, GnRH activity is influenced by CORT such

that chronic stress (Lopez-Calderon et al., 1990) or CORT administration (Rosen et al., 1988) inhibits GnRH secretion. In the pituitary, LH responsivity to GnRH is significantly reduced both *in vitro* (Kamel and Kubajak, 1987; Rosen et al., 1988) and *in vivo* (Breen and Karsch, 2004) by prolonged CORT exposure. Finally, glucocorticoids act directly on the gonads and sustained CORT elevations directly inhibit gonadotropin-induced testicular (Charpenet et al., 1981; Evain et al., 1976) and ovarian (Hsueh and Erickson, 1978) steroid production.

Inhibition of the HPG axis may also be mediated by CRF activity. *In vitro*, application of CRF to medial basal hypothalamus and median eminence tissue inhibits GnRH release, and this effect is reversed by treatment with CRF receptor antagonists (Gambacciani et al., 1986). *In vivo*, central CRF administration causes a rapid and prolonged dose-related suppression of LH secretion and peripheral treatment inhibits the LH surge and disrupts pregnancy (Rivier and Vale, 1984). Thus stress suppresses reproductive function through the actions of CORT at every level of the HPG axis and CRF at the hypothalamic-pituitary levels of the axis.

Acute stress has variable effects on pituitary and gonadal secretory patterns. Plasma LH levels have been shown to increase (Briski, 1996; Briski and Sylvester, 1988; Lopez-Calderon et al., 1990; Mann et al., 1986), decrease (Briski, 1996; Briski and Sylvester, 1988; Kam et al., 2002) or not change (Briski and Sylvester, 1988) following a variety of acute stressors. The intensity of the stressor and the magnitude of the stress-induced CORT response may play a role in determining the gonadotropin response to stress. For example, differential CORT sensitivity to mild versus severe stressors is related to the LH response pattern: stressors that elicit relatively moderate CORT

responses result in transient LH increases while stressors that elicit greater CORT responses suppress LH (Briski, 1996). HPA hormones may be acting centrally to modulate GnRH release (Du Ruisseau et al., 1979; Rivier and Vale, 1984), or peripherally to alter basal and GnRH- and/or E2-stimulated LH release (Baldwin, 1979; Breen and Karsch, 2004; Kamel and Kubajak, 1987; Suter and Schwartz, 1985). Interestingly, independent of LH, acute stress may result in rapid elevations of sex steroids. Both T (Almeida et al., 2000; Mann and Orr, 1990; Orr and Mann, 1992) and E2 have been shown to increase immediately following stress and this effect may be due to direct stimulatory actions of CORT on the gonads (Orr and Mann, 1992; Shors et al., 1999).

E. Thesis Objective

Alcohol is damaging to a developing fetus, and rats prenatally exposed to ethanol show altered HPA responsiveness throughout life. For example, during the SHRP, when normal pups show blunted responses to stressors, E pups are even more hyporesponsive than controls. Under normal conditions when pups emerge from the SHRP they encounter another stage of HPA development, in which inhibitory regulation of HPA activity is just beginning to develop and as a result, their responses to stressors are delayed and prolonged compared to adults. Little is known about the HPA responding of E rats during this period. However, some transition in HPA activity and regulation in E rats clearly occurs during the period between weaning and puberty because following puberty and throughout adulthood E rats are hyperresponsive to stressors compared to controls. Interestingly, data from our lab consistently show that adult E males and females have differentially altered HPA responsiveness to stressors compared to their

control counterparts. As noted above, the HPA axis is modulated by hormones of the HPG axis, and specifically by organizational effects of perinatal sex hormones and activational effects of circulating sex hormones in adulthood. There is evidence that prenatal ethanol exposure alters both organizational and activational effects of sex hormones. Thus, one possible mechanism underlying the differentially altered stress responsiveness in E males and females may be the ability for ethanol to alter HPG organizational effects, thus interfering with the late fetal and/or early postnatal sexual differentiation of the HPA axis. To investigate this possibility, this thesis was designed to determine if (1) development of the HPA axis during the prepubertal period has been altered by prenatal ethanol exposure, and (2) sexually dimorphic effects of ethanol on E males and females may be due, in part, to ethanol's ability to interfere with HPG organizational effects. We hypothesized that: (1) prepubertal E pups would show a delay in the maturation of HPA responding to stressors, as seen by an extension of the SHRP and/or a delay in the maturation of HPA feedback mechanisms, (2) there would be evidence for delayed HPG development in E rats, evidenced by blunted basal hormone levels and altered hormone responses to stressors, and (3) E males and females would show differentially altered HPA responding to stressors as well as altered HPA/HPG interactions compared to control counterparts.

To test these hypotheses, prepubertal (PN 25-26) male and female rats from ethanol, pair-fed, and *ad libitum*-fed dams were examined under basal, stress and recovery conditions. This age range was selected because it allowed pups a few days to adjust to weaning (PN22) but was still several days before the beginning of puberty (PN 30-35 in the female and PN 35-45 in the male). The 30 min stress time point was selected

on the basis of previous studies that showed that the peak CORT response to stress was at 30 min in PN 25 pups (Goldman et al., 1973; Vazquez and Akil, 1993). A 60 min recovery time point was selected because previous work in our lab has shown that E males and females show differential stress responding at this time (Weinberg 1988; Weinberg, Taylor et al. 1996).

HPA drive was assessed by measuring plasma levels of CORT and ACTH. As an indicator of feedback, MR and GR mRNA were measured in the hippocampus. HPG function was assessed by measuring plasma E2, T and LH, and GnRH mRNA in the basal forebrain. Interactions between the HPA and HPG axes were assessed by correlating HPA and HPG plasma hormone levels with each other and with central measures of HPA and HPG function. If prenatal ethanol exposure alters HPA and/or HPG development, then basal levels of HPA or HPG hormones and/or gene expression may be altered. Exposure to a stressor may further unmask changes in either of these axes as the hormone and gene expression levels and response patterns following stress may differ between E and control pups.

If prenatal ethanol has interfered with fetal and/or neonatal sexual differentiation of the HPA axis, then E males and females should show differential CORT, ACTH, MR and/or GR mRNA regulation prior to puberty as well as altered correlations between HPA and HPG measurements. If results from the current study indicate that prepubertal E males and females have sexually differentiated HPA activity as well as altered HPG function, then HPA dysregulation may be due, in part, to altered HPG organizational effects.

CHAPTER 2: METHODS

A. Animals and Breeding

Sprague-Dawley rats (Charles River Laboratories, Montreal, PQ, Canada) were bred and their offspring were tested. Prior to breeding, males (250-275 g, n=12) and females (225-250 g, n=24) were group-housed by sex for a one to two week adaptation period, during which time they were maintained on standard laboratory chow (Jamison's Pet Food Distributors Ltd., Delta, BC, Canada) and water *ad libitum*. Following this period males were placed in stainless steel hanging breeding cages (25 x 18 x 18 cm) with mesh floors and fronts and maintained on *ad libitum* lab chow and water. Three to four days later females were randomly paired with a male, with the heavier females being placed on the breeding rack first. Wax paper located under the hanging cages was checked every morning for the presence of vaginal plugs. Discovery of a vaginal plug indicated day 1 of gestation (G1). Colony rooms were temperature controlled (21°C) and maintained on a 12 hr light-dark cycle, with lights on from 0600 hr to 1800 hr. All animal use procedures were in accordance with the National Institutes of Health guidelines and were approved by the University of British Columbia Animal Care Committee.

B. Diets and Feeding

On G1, females were singly housed in polycarbonate cages (24 x 16 x 46 cm) and randomly assigned to one of three treatment groups: 1) Ethanol (E) females (n = 7) had *ad libitum* access to a liquid ethanol diet in which 36 % of the calories were ethanol-derived, 2) Pair-fed (PF) females (n = 7) were given a liquid control diet, in which maltose-dextrin was isocalorically substituted for ethanol, in an amount that matched the amount consumed by her E partner (g/kg body weight/day of gestation), and 3) Control

(C) females (n = 10) had *ad libitum* access to standard laboratory chow. All females had *ad libitum* access to water. E females were introduced to the ethanol diet gradually by increasing the ratio of ethanol:control liquid diet, with 1/3, 2/3, 100% ethanol diet on G1, G2 and G3, respectively. The liquid diets, provided by Dyets Inc., Bethlehem, P.A., USA, were formulated to provide adequate nutrition for pregnant dams regardless of ethanol content (Lieber and DeCarli, 1982). Fresh diet was administered within 1.5 hr of lights off to minimize shifts in the circadian rhythms of plasma CORT (Weinberg and Gallo, 1982). The diet consumed by each E and PF dam was determined daily by subtracting the weighback mass of the bottle and diet from the mass of bottle and diet provided the night before. Liquid diets were administered from G1 to G21, and on G22, the night before parturition, E and PF dams resumed the *ad libitum* standard laboratory chow diet. Pregnant females were handled only on G1, G7 and G14 for weighing and cage changing and on G21 for weighing only. Litters were checked at 0800 hr and 1600 hr for births. On the day of birth (postnatal day [PN] 1) the entire litter was weighed. Litters were then culled to 10 pups (5 females and 5 males), weights were recorded for the 5 males and 5 females separately, and pups and dam were placed in clean cages. On one occasion, 3 pups were cross fostered between control litters, following measurement of entire litter weights, to maintain litter size. Dams and pups were weighed and cages were changed again on PN8, PN15 and PN22, but litters were otherwise undisturbed. Males and females from each litter were weighed separately and mean values were used to analyze weight gain over days.

C. Blood Alcohol Level Measurements

Three pregnant E females was randomly selected for blood alcohol level (BAL) testing between G14 to G16. Sampling occurred 2 to 3 hrs after lights off, which is immediately following the rats' major diet consumption. Pregnant dams were removed to a room adjacent to the colony room and, under red light, samples were collected by tail bleeding. The blood was stored at room temperature for 2 hours and then centrifuged at 3200 rpm for 10 min at 4°C. Serum was removed to 0.6 ml polypropylene micro centrifuge tubes and stored at -20°C until the time of assay. BALs were determined using a Pointe Scientific (Lincoln Park, MI) (kit #A7504-150) Ethanol Assay kit. The reaction is based on the conversion of ethanol to acetaldehyde by alcohol dehydrogenase (ethanol + NAD -> acetylaldehyde + NADH). The conversion of NAD to NADH results in a change in UV absorbance that is detected by a spectrometer. The minimum amount of detectable ethanol is 10 mg/dl and the curve is linear to 150 mg/dl.

D. Testing

Animals were tested during the circadian trough of stress hormone levels, between 0930 hr and 1100 hr, when the HPA hormones are at a minimum, allowing clearer differences to be seen between experimental groups. On PN22, pups were individually weighed and randomly assigned to a testing condition: 1) basal, nonstressed (n=10), 2) stress, a 30 min restraint stress (n=10), and 3) recovery, a 30 min restraint stress followed by a 30 min homecage recovery period (n=10). Animals were marked at the base of their tail with a felt marker to designate their assigned condition. The pups were then housed in groups of twos and threes, according to litter, sex and condition, such that pups of the same litter and sex but different experimental conditions were housed together. Testing took place on PN 25 or 26.

On the day of testing, basal animals were terminated immediately upon removal from the homecage. Stress animals were restrained in a clear ventilated 100 ml specimen cup (UBC Biosciences Store) and then sacrificed. Recovery animals were restrained and then returned to their homecage in a room adjacent the testing room. Restraint is primarily a psychological stressor and caused no pain or injury.

Animals were sacrificed by rapid decapitation. Trunk blood was collected in ice-chilled 1.5 ml epindorf tubes containing 50 µl of a 3.75 % ethylenediamenetetraacetic acid (EDTA) disodium salt/aprotinin (1000 KIU) solution to prevent blood coagulation and ACTH denaturation. After testing each day blood was centrifuged at 3500 rpm at 4°C for 10 min. The plasma was transferred to 0.6 ml micro centrifuge epindorf tubes and frozen at -70°C until assayed. Brains were quickly removed from the skulls, snap frozen on powdered dry ice, and then stored at -70°C until slicing.

E. Radioimmunoassays

1. Plasma Corticosterone (CORT) Levels

Total CORT (bound and free) was measured via radioimmunoassay (RIA) in plasma, extracted with 95 % ethanol to precipitate out the proteins and corticosterone binding globulin (CBG), using our adaptation (Weinberg and Bezio, 1987) to the method of (Kaneko et al., 1981). CORT antiserum was obtained from Immunocorp (Montreal, P. Q., Canada) and tritiated CORT from Mandel Scientific (Guelph, ON, Canada). Dextran-treated charcoal (Fisher Scientific Ltd., Nepean, ON, Canada) was used to absorb unbound steroids, and Scintisafe Econo 2 scintillation fluid (Fisher Scientific Ltd., Nepean, ON, Canada) was added to the supernatant for counting radioactivity. CORT antiserum cross-reacts 100% with CORT, 2.3% with desoxycorticosterone, 0.47% with

testosterone, and 0.17% with progesterone. The minimum detectable amount of CORT was 0.25 μg/dl. Mid-range intra- and interassay coefficients of variation were 1.55 % and 4.26 % respectively.

2. Plasma Adrenocorticotrophin (ACTH) Levels

Plasma ACTH was measured with a competitive binding ACTH radioimmunoassay (RIA) kit (Diasorin Inc., Stillwater, MN, USA, Cat # 24130). This assay was modified such that all reagents were halved and only 50 μl, instead of 100 μl, of plasma was tested. ACTH antibody cross-reacts 100% with ACTH, and less than 0.01% with a-melanocyte-stimulating hormone or β-endorphin. The minimum detectable ACTH concentration was 20 pg/ml, and the mid-range intra- and interassay coefficients of variation were 3.9 % and 6.5 % respectively.

3. Plasma 17β-Estradiol (E2) Levels

Plasma 17β-estradiol was measured in female pups using a competitive binding 17β-estradiol RIA kit (MP Biomedicals, Inc., Costa Mesa CA). All reagents and samples were halved for this assay. 17β-estradiol antibody cross-reacts 100% with 17β-estradiol and 0.68% with 17α-estradiol. There is no cross reactivity with progesterone or testosterone (<0.01%). The minimum detectable 17β-estradiol concentration was 10 pg/ml, and the mid-range intra- and interassay coefficients of variation were 4.7 % and 9.1 % respectively.

4. Plasma Testosterone (T) Levels

Plasma T was measured in male pups. A T competitive binding assay kit was obtained from MP Biomedicals, Inc. (Costa Mesa CA). An initial pilot determined that plasma T levels of the pups were near or below the lowest standard. Thus to achieve

detectable T levels with the experimental plasma, each plasma sample was "spiked" with an alloquot of diluted standard. Specifically, the highest standard (10 ng/ml) was diluted by a factor of 10 with steroid diluent (to yield a concentration of 1.0 ng/ml) and included as a portion of the plasma sample: instead of 50 µl of pup plasma, 25 µl of pup plasma and 25 µl of diluted standard was pipetted into every tube. Testosterone antibody cross-reacts 100% with testosterone, 3.4% with 5a-dihydroxytestosterone, 2.2 % with 5a-androstane-3b and 17b-diol (2.2%) and 2% with 11-oxotestosterone. There is no cross reactivity with progesterone, estrogen or glucocorticoids (<0.01%). The minimum detectable testosterone concentration was 0.1 ng/ml, and the mid-range intra- and interassay coefficients of variation were 4.6 % and 7.5 % respectively.

5. Plasma Leutinizing Hormone (LH) Levels

Plasma LH levels were measured by RIA in the laboratory of National Hormone and Peptide Program, A. F. Parlow (Harbor-UCLA Medical Center, CA, USA). The minimum detectable LH concentration of the assay was 0.2 ng/ml.

F. In Situ Hybridizations

1. Brain Preperation

Frozen brains were sectioned in the coronal plane (thicknesses of 16 µm at the dorsal hippocampus and 20 µm at the POA). Slices were mounted on poly-L-lysine (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and gelatin (Fisher Scientific Ltd., Nepean, ON, Canada) coated slides and stored at -70 °C until processing.

2. Probes

MR riboprobe (fragment subcloned into Bluescript Skt, courtesy of J. P. Herman,) and GR riboprobe (fragment subcloned into pGem4, courtesy of J. P. Herman) were used

to detect MR and GR mRNA in the dorsal hippocampus. MR and GR mRNA sense riboprobes were used as controls. Riboprobes were transcribed and labeled by incubating linearized MR and GR DNA with transcription buffer, DTT, Rnasin, ATP, CTP, GTP, 35S-UTP and polymerase. A purified antisense oligonucleotide probe (synthesized at the Nucleid Acids Protein Services Unit, University of British Columbia, BC, Canada) was used to detect GnRH mRNA in the POA. The sense oligonucleotide probe was used as a control. Antisense and sense probes were labeled on the 3' end with 35-S ATP (Amersham Biosciences Inc., Piscataway, NJ, USA) using terminal dioxy-transferase (New England Biolabs Ltd., Mississauga, ON, Canada). Labeled riboprobes and oligonucleotide probes were purified using G-25 and G-50 sephadex Roche Quick Spin columns, respectively (Roche, Indianapolis, IN, USA, Cat # 1-274-015).

3. Hybridization

Sections were thawed to room temperature and then fixed in 4 % formaldehyde in phosphate buffer. Fixed POA slices were then washed in phosphate buffer saline, followed by triethanolamine containing 0.25% acetic anhydride, then sodium saline citrate solution before being defatted in chloroform and dehydrated with graded concentrations of ethanol. Fixed hippocampus slices were given the same treatment with an additional Proteinase K wash before the acetic anhydride. After drying completely MR and GR mRNA probes contained in 75 % hybridization buffers and GnRH mRNA probe contained in a 50 % hybridization buffer were applied to their respective sections. Slides were coverslipped and incubated overnight at 55 °C for MR and GR mRNA and 37 °C for GnRH mRNA.

The next day, coverslips were removed. Slides labeled for MR and GR mRNA were placed in a warm RNase A solution. Slides labeled both for MR and GR mRNA, and GnRH mRNA underwent a series of stringent saline solutions washed and then were dehydrated in ethanol. After drying completely, MR and GR mRNA slides were exposed to Kodak Biomax MR Film (Eastman Kodak Co., Rochester, NY, USA). C-14 standards were also placed under each of the autoradiographic films to establish the film's saturation threshold. Exposure times were 3 days for MR mRNA and 6 days for GR mRNA. GnRH mRNA slides were coated with Kodak NTB2 liquid autoradiographic emulsion of a 1:1 (with $RO{\cdot}H_2O$) dilution, dried and then stored with dessicant in light tight boxes at 4 °C. Emulsion coated slides were developed after 24 days, stained with 1 % toluene blue and coverslipped.

4. Autoradiographic Analyses

Semiquantitative densitometric analysis was performed using Image J 1.31v (National Institutes of Health, Bethesda, MD, USA) for PC imaging software. Images of X-ray autoradiographs for MR and GR mRNA were digitized and grey level measurements were taken over the regions of interest. Four subfields of the dorsal hippocampus were analyzed for MR and GR mRNA: CA1, CA2, CA3 and the dentate gyrus (DG) (Figure 6). The hippocampal subfields were divided according to both the specifications of Paxinos & Watson (2005), using the visible pattern of signal intensity in MR mRNA slides. Signal intensity was greatest in CA2 and was weaker in the surrounding CA1 and CA3 regions. Since the subfields were less distinct for GR mRNA, the boundaries determined in the MR slides were applied to the GR slides. Measurements were taken bilaterally and one slice was analyzed per animal. All grey level

measurements were compared to C-14 standards exposed with the slides to ensure levels were below saturation point. Background measurements, where no specific MR or GR mRNA hybridization was detected, were taken from a region between the dentate gyrus and CA1 subfields. Background was subtracted from mean grey level measurements obtained for each subfield to determine the mean grey pixel value associated with the MR and GR signals.

Autoradiographic emulsions were analyzed for GnRH mRNA. Emulsion dipped slides were digitized under darkfield (31.5X magnification) using a Q-Imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope and images were captured using Northern Eclipse 6.0v (Empix Imaging Inc., Mississauga, ON, Canada). Two anatomically matched sections were evaluated from each brain, corresponding to the region of the rostral POA that surrounds the rostral tip of the 3rd ventricle. Using Image J, neurons labeled for GnRH mRNA were outlined and each cluster was analyzed for mean grey value (grey value per pixel divided by the number of pixels). Total grey value per 40 μm selection of the POA was calculated by multiplying the mean grey value of each cluster by its area (in pixels) and then summing the measured grey values for each brain.

G. Statistics

Developmental data were analyzed using 1-way ANOVAs with treatment as a between factor variable for gestation length, number of liveborn pups and pup weight at birth. 2-way repeated measures ANOVAs, with treatment as between factor variables and PN day as within factor variables, for dam weight gain during gestation and lactation. Experimental data were analyzed first with 3-way prenatal treatment by time by sex

ANOVAs. Data for males and females were then analyzed separately with 2-way treatment by time ANOVAs, with time as the repeated measures variable where applicable. Newman-Keuls post hocs were used to further analyze significant main effects and interactions found in the ANOVAs. Fisher LSD tests were used to address our a priori hypotheses. Specifically, to determine whether the SHRP was prolonged or development of feedback was delayed in E compared to control pups, HPA hormone levels were compared among groups at 30 and 60 min following stressor onset. To assess whether prenatal ethanol delayed HPG development, basal hormone levels were compared among groups and the pattern of HPG hormone responding to stress was assessed within each group. Finally, to determine if prenatal ethanol differentially altered the pattern of stress responding between males and females, the pattern of HPA hormone responding across time was assessed for each prenatal treatment group. The Fisher LSD tests were run on the interaction results from the 2-way treatment by time ANOVAs, with error terms adjusted by replacement with the error term from the 3-way ANOVAs. Pearson r correlation coefficients were used to examine the relationships between hormones. Specifically, correlating measures within the same axis provides information about drive or feedback regulation within that axis. Correlating HPA and HPG measures with each other provides information about the interactions between the axes. These tests were run separately for males and females at specific time points.

CHAPTER 3: RESULTS

A. Developmental Data

1. Ethanol Intake and Blood Alcohol Levels

Ethanol intake by pregnant dams averaged 9.42 ± 2.85, 12.98 ± 2.18, and 12.81 ± 2.25 g/kg body weight/day of gestation, for weeks 1, 2 and 3 respectively. Blood alcohol levels were 130 - 156 mg/dl.

2. Maternal Body Weights and Gestation Lengths

Repeated measures ANOVAs were performed on maternal weight during gestation (G) (Table 1) and lactation (L) (Table 2). Analysis of gestational weight revealed main effects of treatment [$F(2, 18) = 16.46$, $p<0.01$] and day [$F(3, 54) = 449.80$, $p<0.01$], and a treatment by day interaction [$F(6, 54) = 9.33$, $p<0.01$]. Only C dams gained weight during the first week of gestation ($p<0.01$), and all dams gained weight during the 2nd and 3rd weeks of gestation ($ps<0.01$). C dams were significantly heavier than E and PF dams on G14 ($ps<0.05$) and G21 ($ps<0.01$). Analysis of lactational weight gain revealed a main effect of day [$F(3, 60) = 47.11$, $p<0.01$] and a treatment by day interaction [$F(6, 60) = 3.27$, $p<0.01$]. All dams gained weight during the first 2 weeks of lactation whereas on L22 dam weights were significantly lower than on L15 ($p<0.05$) but equal to weights on L8. E dams weighed significantly less than C on L1 ($p<0.05$) but were no longer different from C by L8.

A 1-way ANOVA was used to compare gestation lengths among E, PF and C dams. No significant differences were found (Table 3).

3. Pup Data

There was no difference in the number of live born pups among E, PF and C dams (Table 3), nor were there any stillborns in any litter. Birth weights (utilizing initial weight o fthe entire litter) did not significantly differ among the groups.

A prenatal treatment by postnatal day by sex repeated measures ANOVA on average weight per pup throughout the preweaning period revealed a trend for a sex effect ($p=0.06$) and a significant sex by day [$F(3, 114) = 2.910$, $p<0.05$] interaction (Figure 1). By PN 22 females weighed less than males ($p<0.05$). 2-way ANOVAs were then used to analyze data from females and males separately. There was a main effect of day [$F(3, 54) = 1231.995$, $p<0.01$] for females and an effect of day [$F(3, 60) = 1711.058$, $p<0.01$], a trend for a treatment effect ($p=0.07$), and a treatment by day interaction [$F(6, 60) = 2.255$, $p<0.05$] for males. Both females and males gained weight through the postnatal period. On PN 22 male E ($p=0.08$) and PF ($p=0.10$) pups were marginally lighter than male C pups.

B. Plasma Hormone Data

1. Plasma CORT

A 3-way factorial ANOVA revealed significant main effects of time [$F(2, 159) = 484.301$, $p<0.01$], sex [$F(1, 159) = 6.915$, $p<0.01$], and treatment [$F(2, 159) = 4.810$, $p<0.01$] for CORT levels (Figure 1). Overall, CORT was increased at 30 and 60 min compared to basal ($ps<0.01$), females had higher CORT levels than males ($p<0.01$) and E and PF had lower CORT than C ($ps<0.05$).

Separate analyses for data from females and males revealed that the overall treatment effect was driven mainly by reduced CORT levels in E compared to C females at 60 min ($p<0.01$), and in E and PF compared to C males at 30 min ($ps<0.05$).

Furthermore, prenatal treatment altered the pattern of CORT responding over time among males: E males appeared to be delayed in reaching peak CORT levels following stress, and showed a significant CORT increase between 30 and 60 min ($p<0.05$), whereas there were no significant differences between 30 and 60 min CORT levels for PF or C males.

2. Plasma ACTH

A 3-way ANOVA on ACTH levels revealed a main effect of time [$F(2, 159) = 218.828$, $p<0.01$] (Figure 2). ACTH levels were highest at 30 min ($ps<0.01$) and decreased but remained elevated over basal levels at 60 min ($p<0.01$). Separate analyses for females and males revealed a trend for a treatment by time interaction for females ($p=0.055$): ACTH levels were higher in PF compared to E and C females at 60 min ($ps<0.01$).

3. Plasma E2

A treatment by time ANOVA revealed a marginal treatment effect for E2 ($p=0.08$), as E females had marginally lower E2 levels compared to C females (Figure 3). In addition a significant effect of time [$F(2, 70) = 7.624$, $p<0.01$] indicated that E2 levels were higher at 30 and 60 compared to 0 min ($ps<0.01$). However, inspection of Fig 3 suggests that this effect was driven primarily by increases in E2 levels at 30 and 60 compared to 0 min in E ($ps<0.05$) and at 60 compared to 0 min in PF females ($p<0.05$). C females showed a small but not significant increase in E2 with stress.

4. Plasma T

There was a significant effect of time of T levels [$F(2, 72) = 4.055$, $p<0.01$] (Figure 3), such that levels at 30 and 60 min were higher than basal levels ($ps<0.05$). Further analyses revealed that the increase over basal was significant in C pups at 30 and

60 min (ps<0.05) and in E pups at 60 min (p<0.05). PF pups showed no significant increase with stress.

5. Plasma LH

The 3-way ANOVA on LH levels revealed a main effect of sex [$F(1, 144) = 2.426$, $p<0.05$], as well as significant time by sex [$F(2, 144) = 7.723$, $p<0.01$] and treatment by time by sex [$F(4, 144) = 1.7438$, $p<0.05$] interactions (Figure 4). Separate ANOVAs were run for female and male data.

Among females there was a trend for a treatment effect ($p=0.051$), a main effect of time [$F(2, 72) = 5.867$, $p<0.01$] and a treatment by time interaction [$F(4, 72) = 4.278$, $p<0.01$]. C females had significantly higher basal LH levels than E and PF females (ps<0.01). Following stress LH levels were suppressed in C (ps<0.01) but not E or PF females. Among males, there was a main effect of time [$F(2, 72) = 5.598$, $p<0.01$], such that LH levels were lower at 30 min compared to 0 ($p=0.07$) and 60 ($p<0.01$) min. Further analyses revealed that LH decreased in C pups at 30 min ($p<0.05$) and rebounded to above basal values at 60 min ($p<0.05$). In contrast, E and PF pups showed no significant changes in LH over time.

C. Brain Measures

1. MR and GR mRNA

Figure 5 shows the MR and GR mRNA pattern in the dorsal hippocampus and indicates the subdivisions for densometric analyses, i.e. CA1, CA2, CA3 and DG. Semiquantitative analysis of MR and GR mRNA in the different subfields of the hippocampus revealed that mRNA levels vary across subfields. For MR mRNA, levels in CA1>CA2>DG>CA3, whereas for GR mRNA, levels in CA1>DG>CA3>CA2 in both

males and females. Separate 3-way ANOVAs on MR and GR mRNA levels for each hippocampal subfield revealed a trend for a time by sex interaction in the DG ($p=0.07$) for MR mRNA, and a trend for a treatment by sex interaction in DG ($p=0.06$) for GR mRNA. Data for females and males were then analyzed separately.

For MR mRNA among females, there was a marginal treatment effect in CA3 ($p=0.08$) and marginal treatment by time interactions in CA1 ($p=0.06$) and DG ($p=0.052$) (Figure 6). These effects were due to lower MR mRNA levels in C compared to E females at 30 min (CA1: $p<0.01$; CA3 and DG: $ps<0.05$).

For MR mRNA among males, no significant effects were found in the 2-way ANOVAs. However, when each group was separately analyzed for the pattern of MR mRNA responding across time it was found that at 30 min MR mRNA decreased below basal levels in C, but not E or PF, pups in CA1 ($p<0.01$), CA3 and DG ($ps<0.05$). This decrease resulted in significantly lower MR mRNA levels in C compared to E males in all three subfields (CA1 and CA3: $ps<0.05$) (Figure 7).

For GR mRNA, there was no significant effect of prenatal treatment among females (Figure 8). Among males, GR mRNA levels were lower in E (CA1 and DG: $ps>0.05$) and PF (DG: $p<0.05$) compared to C pups at 60 min (Figure 9).

2. GnRH mRNA

Figure 10 is a diagrammatic representation of the POA and a representative section showing GnRH mRNA expression. A 3-way ANOVA for GnRH mRNA levels revealed a significant time by sex interaction [$F(2, 84) = 5.125$, $p<0.01$] (Figure 12). Separate analyses were then run for data from females and males.

There was a significant treatment by time interaction for females [$F(4, 42) = 2.938$, $p<0.05$]. GnRH mRNA levels were decreased 30 and 60 min following stress onset in E ($ps<0.01$) but not PF or C females. In contrast, among males there was a main effect of time [$F(2, 42) = 5.069$, $p<0.05$], which is mainly due to an increase in GnRH mRNA levels at 60 compared to 0 min in E males ($p=0.057$) and compared to 0 and 30 min in PF males ($ps<0.01$).

D. Correlations

1. Correlations Between HPG Measures

1.1 Basal (0 min) Sex Steroids and LH

Under basal conditions, there was a significant positive correlation between E2 and LH levels in C ($r=0.6811$, $p<0.05$) but not E or PF females (Figure 12A). LH did not correlate with testosterone in males for any group (Figure 12B).

2. Interactions Between HPA and HPG Axes

2.1 CORT and Sex Steroids Following Stress (30 and 60 min collapsed)

CORT and E2 negatively correlated in E, but not PF or C females following stress ($r=-0.6815$, $p<0.01$) (Figure 13A). CORT and T did not correlate for any group following stress (Figure 13B).

2.2 Basal (0 min) MR mRNA and Sex Steroids

Among males, MR mRNA and T showed a borderline positive correlation in C ($p<0.10$) and a borderline negative correlation in E ($p<0.10$) pups under basal conditions (Figure 14A). Among females basal MR mRNA and E2 did not correlate in any group (Figure 14B).

Table 1 Maternal body weights (g) (mean ± SEM) during gestation

Maternal body weights (g) of E (n = 7), PF (n = 7) and C (n = 10) dams during gestation (G). Only C dams gained weight during the first week of gestation ($^{@}p<0.01$) and all dams gained weight during the second (+ps<0.01) and third ($^{\&}ps<0.01$) weeks of gestation. By G14 ($^{*}p<0.05$) and 21 ($^{\#}p<0.01$), C dams were significantly heavier than E and PF dams.

Diet	G1	G7	G14	G21
Ethanol	271.1±5.1	272.1±5.6	$308.0\pm6.6^{+}$	$374.7\pm9.1^{\&}$
Pair-fed	275.1±5.1	269.0±6.1	$295.9\pm8.0^{+}$	$365.9\pm9.8^{\&}$
Control	282.6±3.7	$306.4\pm4.0^{@}$	$339.8\pm4.2^{+*}$	$431.8\pm6.6^{\&\#}$

Table 2 Maternal body weights (g) (mean ± SEM) during lactation

Maternal body weights (g) of E (n = 7), PF (n = 7) and C (n = 10) dams during lactation (L). Weights for all dams were increased over L1 on L8, 15, 22, and while weights on L22 were lower than on L15, they were equal to weights on L8. C dams were heavier than E dams on L1 only ($^{\#}p<0.01$).

Diet	L1	L8	L15	L22
Ethanol	294.3±12.4	341.6±5.9	359.3±6.0	335.0±4.2
Pair-fed	309.9±9.2	334.3±7.2	355.1±8.4	334.3±6.9
Control	328.2±5.0$^{\#}$	349.3±4.4	359.1±3.5	338.8±4.3

Table 3 Gestation data of E, PF and C dams

Gestation length in days (mean ± SEM), number of liveborn pups (mean ± SEM), and birth weight (initial weight of entire litter [mean±SEM]) for E (n = 7), PF (n = 7) and C (n = 10) litters. There were no effects of prenatal treatment on gestation length, the number of liveborn pups, or birthweight.

Prenatal Treatment	Gestation Length (days)	Liveborn Pups (# pups)	Birthweight (g)
Ethanol	23.1±0.1	14.4±0.6	6.1±0.2
Pair-fed	22.9±0.3	15.9±1.0	5.9±0.1
Control	22.9±0.1	15.3±1.0	6.4±0.3

Table 4 Postnatal pup body weights (g) (mean ± SEM)

Postnatal (PN) body weights (g) (mean ± SEM) of E (n = 7 litters per group), PF (n = 7 litters per group) and C pups (n = 10 litters per group). All pups gained weight during the postnatal period. On PN 22 females weighed significantly less than males ($p<0.05$) and among males there was a trend for E and PF to weigh less than C pups (*p=0.08 and p=0.10 respectively).

Prenatal Treatment	PN1 Females	PN22 Females	PN 1 Males	PN22 Males
Ethanol	5.96±.021	45.38±1.83	6.22±0.24	49.62±2.06
Pair-fed	5.66±0.16	46.74±2.21	6.12±0.13	48.72±1.59
Control	6.144±0.18	50.97±1.75	6.62±0.22	54.21±1.73*

Figure 1 Plasma CORT levels (ng/ml) (mean ± SEM)

Plasma CORT (ng/ml) levels of E, PF and C females (A) and males (B) at 0, 30 or 60 min after stress onset (n = 9-10 per condition). Overall, CORT levels were higher in females compared to males and at 30 and 60 min compared to 0 min (*ps<0.01) and in C compared to E and PF pups (ps<0.05). E females had lower CORT compared to C females ($^{@}$p<0.01) at 60 min and E and PF males have lower CORT than C males ($^{\&}$ps<0.05) at 30 min. As well, there was a significant CORT increase between 30 and 60 min in E males ($^{\#}$p<0.05).

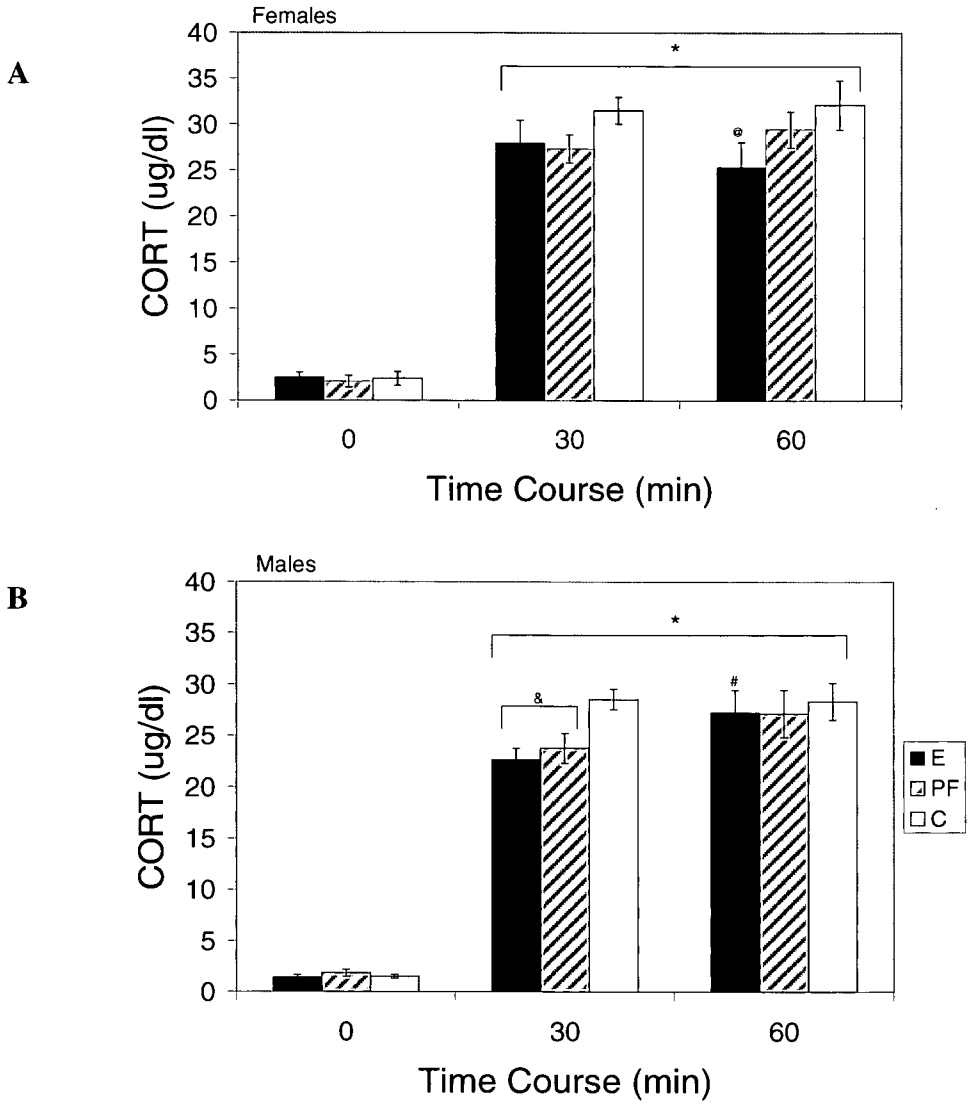


Figure 2 Plasma ACTH levels (pg/ml) (mean ± SEM)

Plasma ACTH levels (pg/ml) of E, PF and C females (A) and males (B) at 0, 30 or 60 min after stress onset (n = 9-10 per condition). ACTH levels were highest at 30 min (*ps<0.01) and remained elevated over basal at 60 min ($^{\#}$p<0.01). At 60 min ACTH levels were elevated in PF compared to E and C females ($^{@}$ps<0.01).

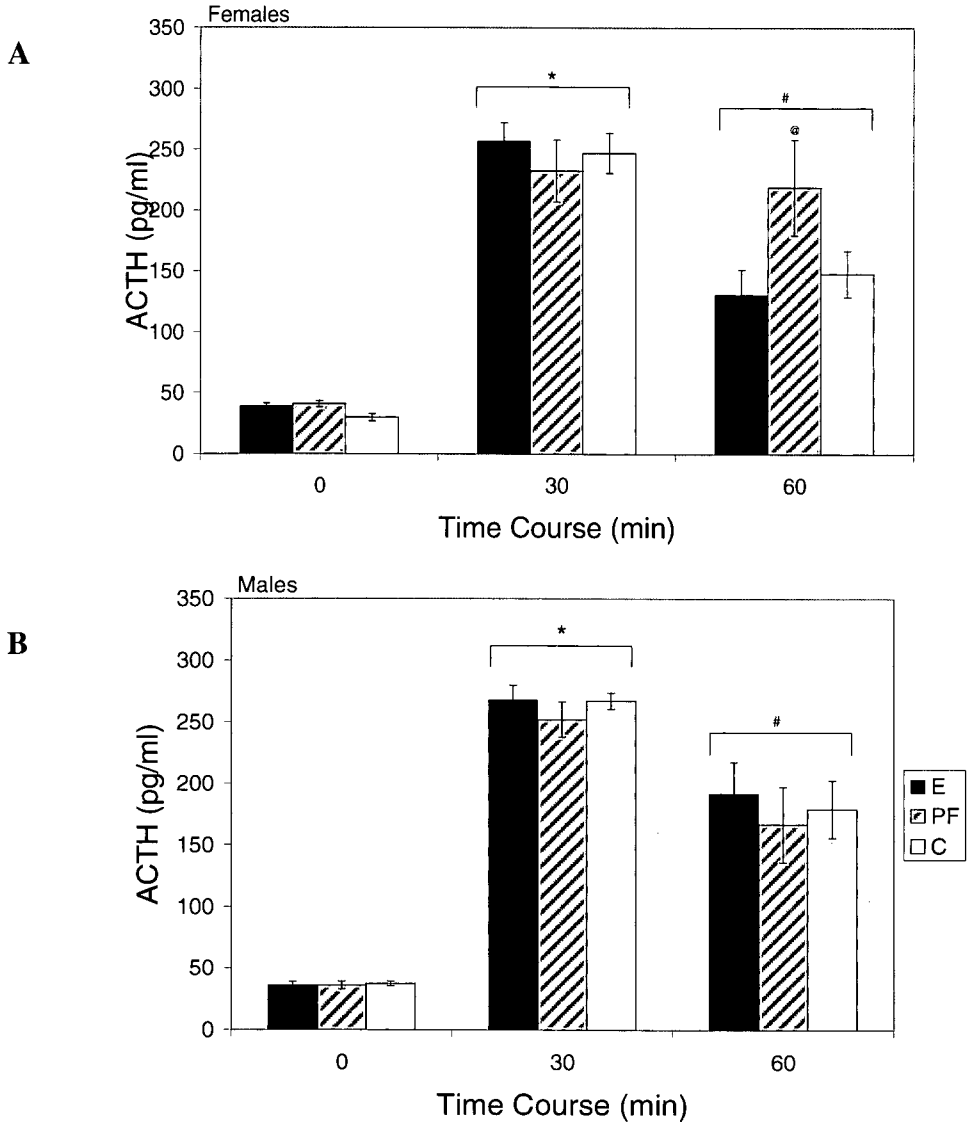


Figure 3 Plasma E2 (pg/ml) and plasma T levels (ng/ml) (mean ± SEM)

Plasma E2 (pg/ml) in females (A) and T (ng/ml) in males (B) of E, PF and C pups at 0, 30 or 60 min after stress onset (n = 7-10 per condition). E2 levels were lower overall in E compared to C females (p=0.08). While E2 increased with stress in all females (*p<0.01), the effect was significant at 30 and 60 min in E females ($^{\%}$ps<0.05) and at 60 min in PF ($^{++}$p<0.05) pups. While there was an overall increase in T after stress ($^{@}$p<0.05) the effect was significant at 30 and 60 min in C pups ($^{\#}$ps<0.05) and at 60 min in E pups ($^{\$}$p<0.05).

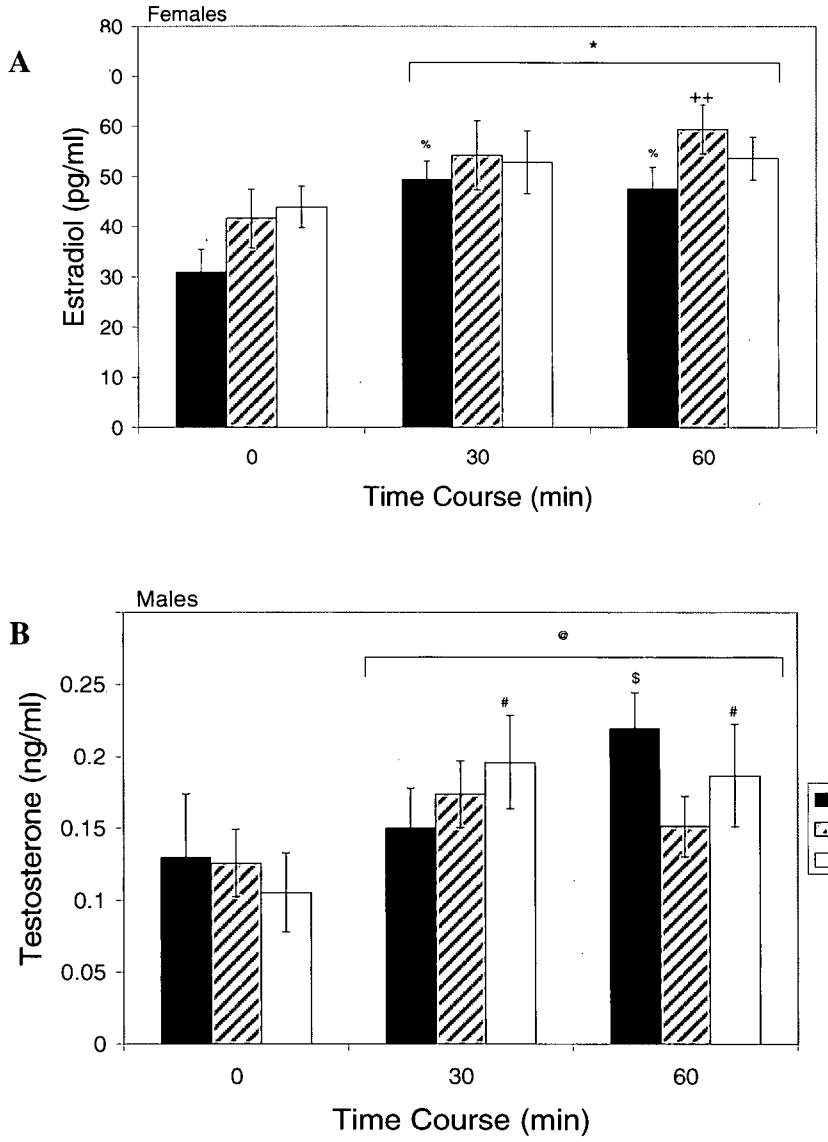


Figure 4 Plasma LH levels (ng/ml) (mean ± SEM)

Plasma LH levels of E, PF and C females (A) and males (B) at 0, 30 or 60 min after stress onset (n = 7-10 per condition). Among females, LH levels were higher in C compared to E and PF females under basal conditions ($^{\#}$ps<0.01). After stress LH was suppressed in C females only ($^{+}$ps<0.01). Among males, there was an overall LH decrease at 30 compared to 0 ($^{@}$p=0.07) and 60 ($^{@}$p<0.01) min. This was driven mainly by changes in C pups, as LH decreased at 30 min ($^{\&}$p<0.05) and rebounded to above basal values at 60 min (*p<0.05).

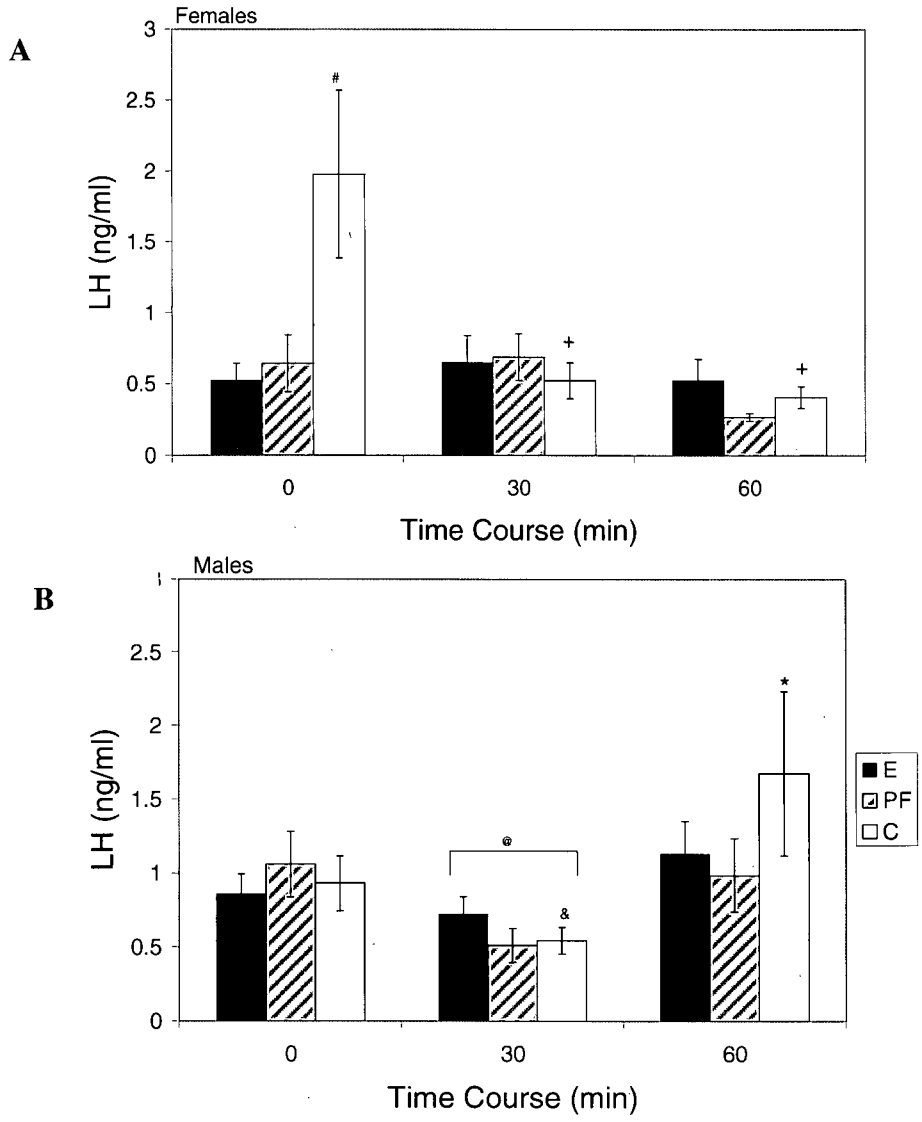


Figure 5 Representative autoradiographs of the dorsal hippocampus

A representative autoradiograph of the dorsal hippocampus (A) indicating the divisions utilized for analysis (CA1, CA2, CA3 and DG) and representative autoradiograph sections(B) labeled for MR mRNA and GR mRNA from E, PF and C females at 30 min. The CA1, CA2, CA3 and dentate gyrus (DG) subfields were analyzed for both MR and GR mRNA.

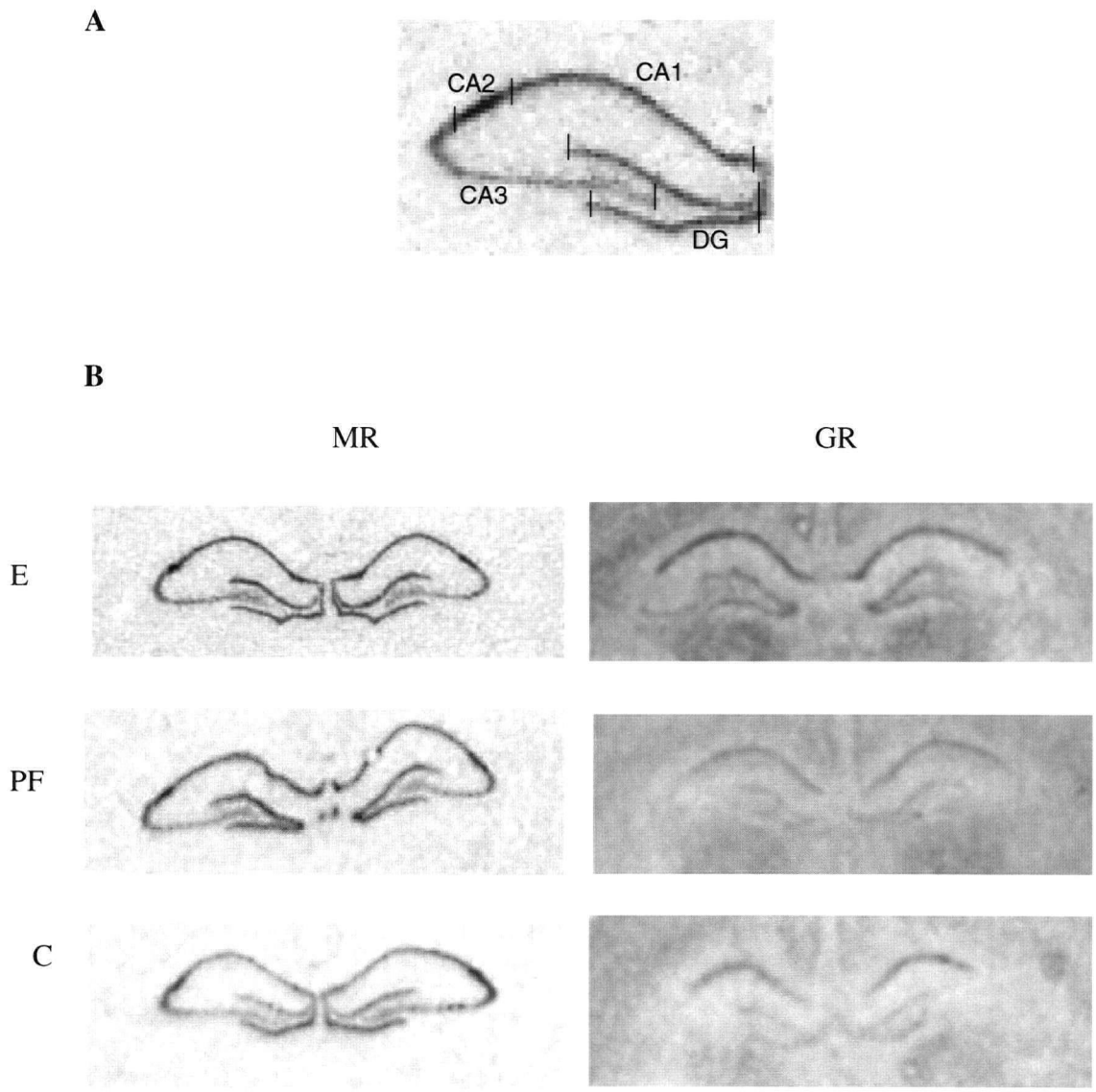


Figure 6 MR mRNA levels (mean grey value ± SEM) in females

MR mRNA levels (mean grey value) in the CA1, CA2, CA3 and DG subfields of the hippocampus in E, PF and C females at 0, 30 of 60 min following stressor onset (n = 5-7). At 30 min MR mRNA was lower in C compared to E females (CA1: *p<0.01, @CA3 and DG: ps<0.05).

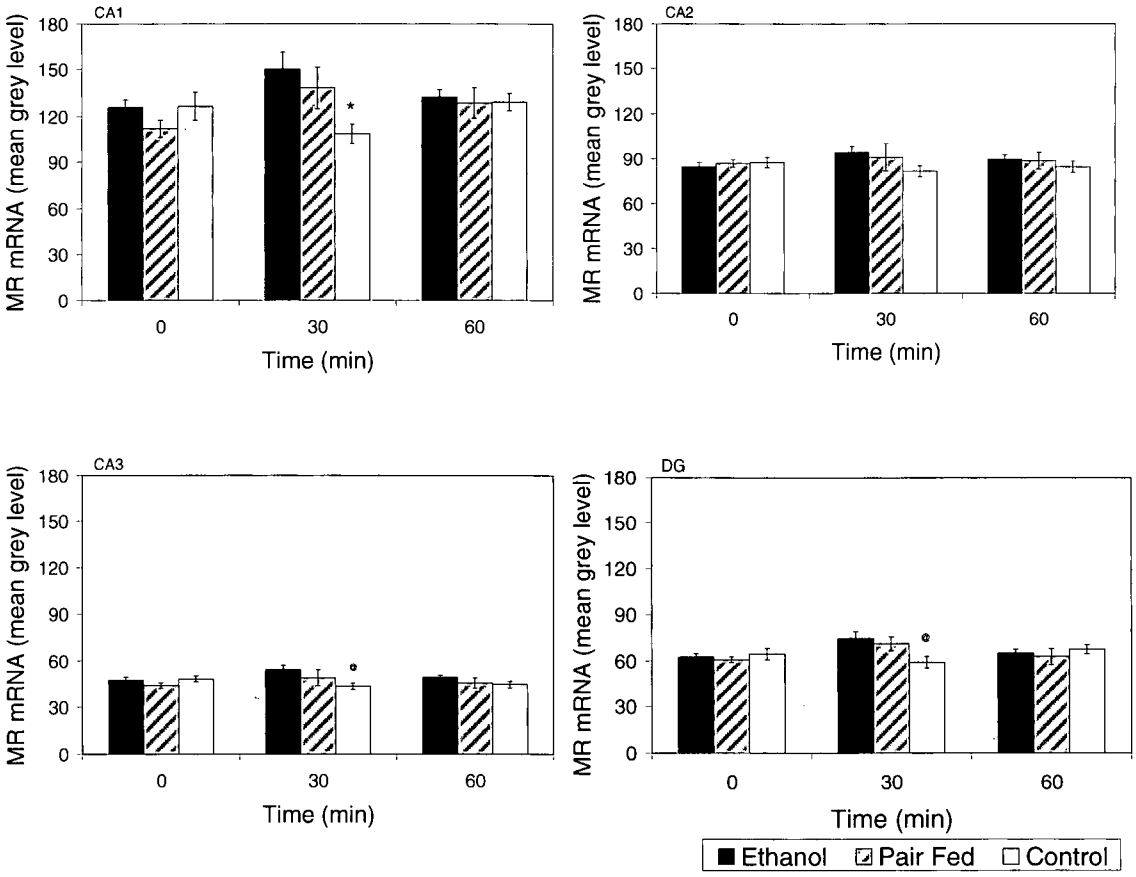


Figure 7 MR mRNA levels (mean grey value ± SEM) in males

MR mRNA levels (mean grey value) in the CA1, CA2, CA3 and DG subfields of the hippocampus in E, PF and C males at 0, 30 of 60 min following stress onset (n = 6). MR mRNA levels were decreased at 30 min compared to basal in C males in CA1 (*p<0.01), CA3 and DG ($^{@}$ps<0.05). Additionally, in CA1, CA3 and DG MR mRNA levels were lower in C compared to E pups at 30 min (CA1 and CA3: $^{+}$ps<0.05).

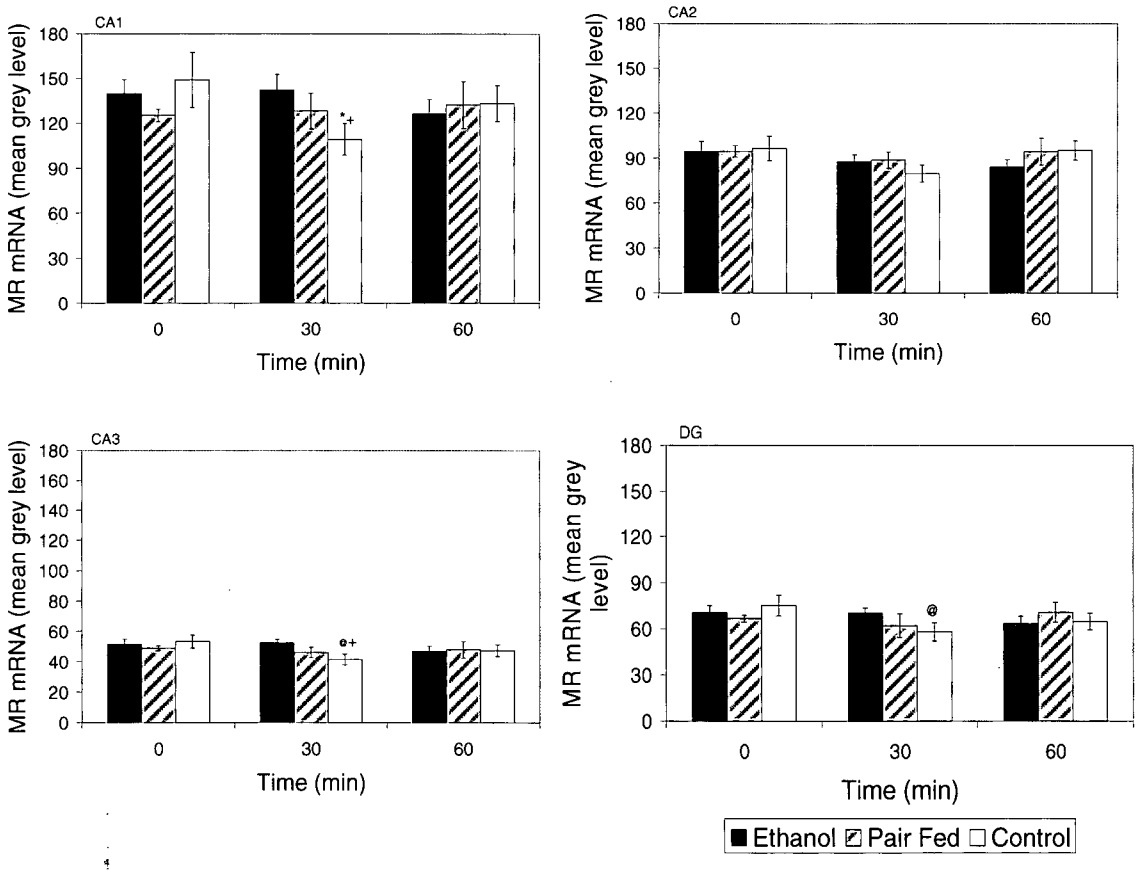


Figure 8 GR mRNA levels (mean grey value ± SEM) in females

GR mRNA levels (mean grey value) in the CA1, CA2, CA3 and DG subfields of the hippocampus in E, PF and C females at 0, 30 of 60 min following stress onset (n = 5-7). Among females, there were no effects of prenatal treatment or time on GR mRNA.

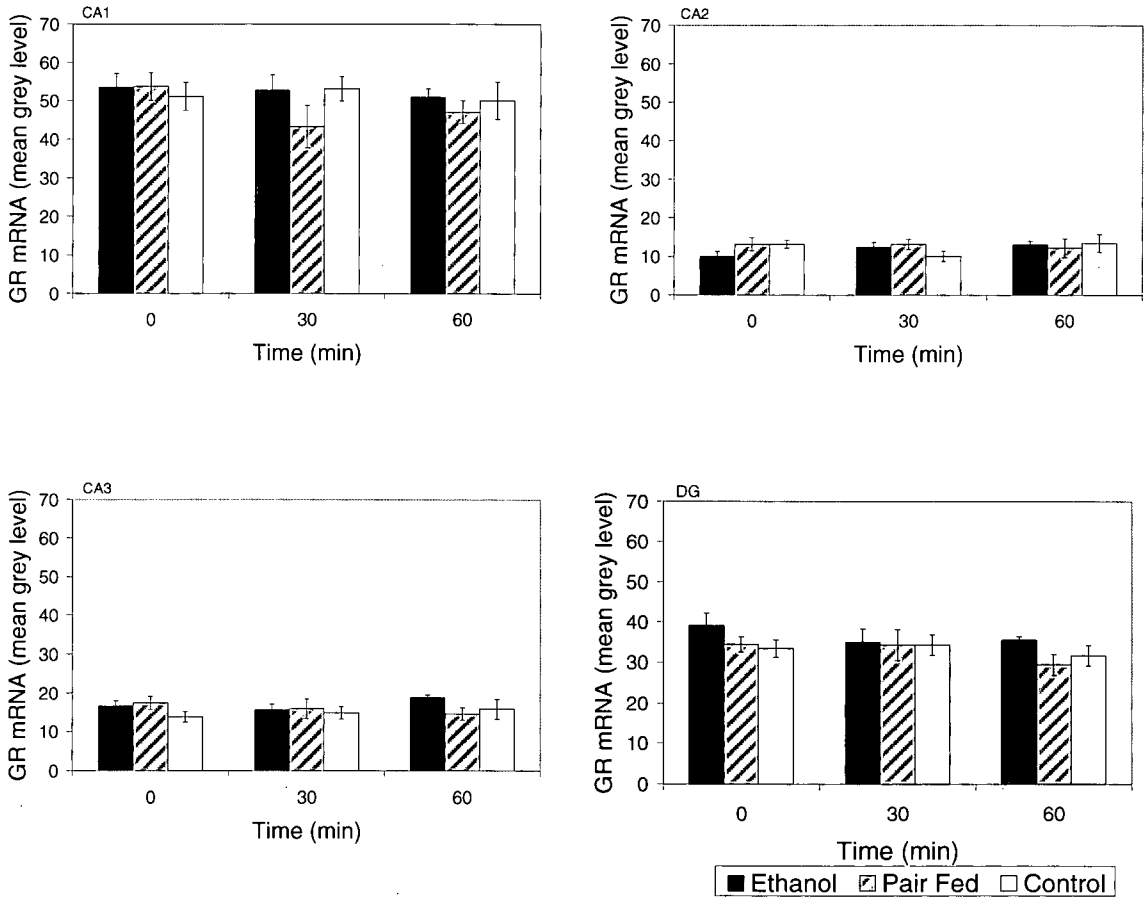


Figure 9 GR mRNA levels (mean grey value ± SEM) in males

GR mRNA levels (mean grey value) in the CA1, CA2, CA3 and DG subfields of the hippocampus in E, PF and C males at 0, 30 of 60 min following stress onset (n = 5-7). E males had lower GR mRNA in CA1 and DG (*ps<0.05), and PF males had lower GR mRNA in DG ($^{@}$p<0.05) compared to controls at 60 min.

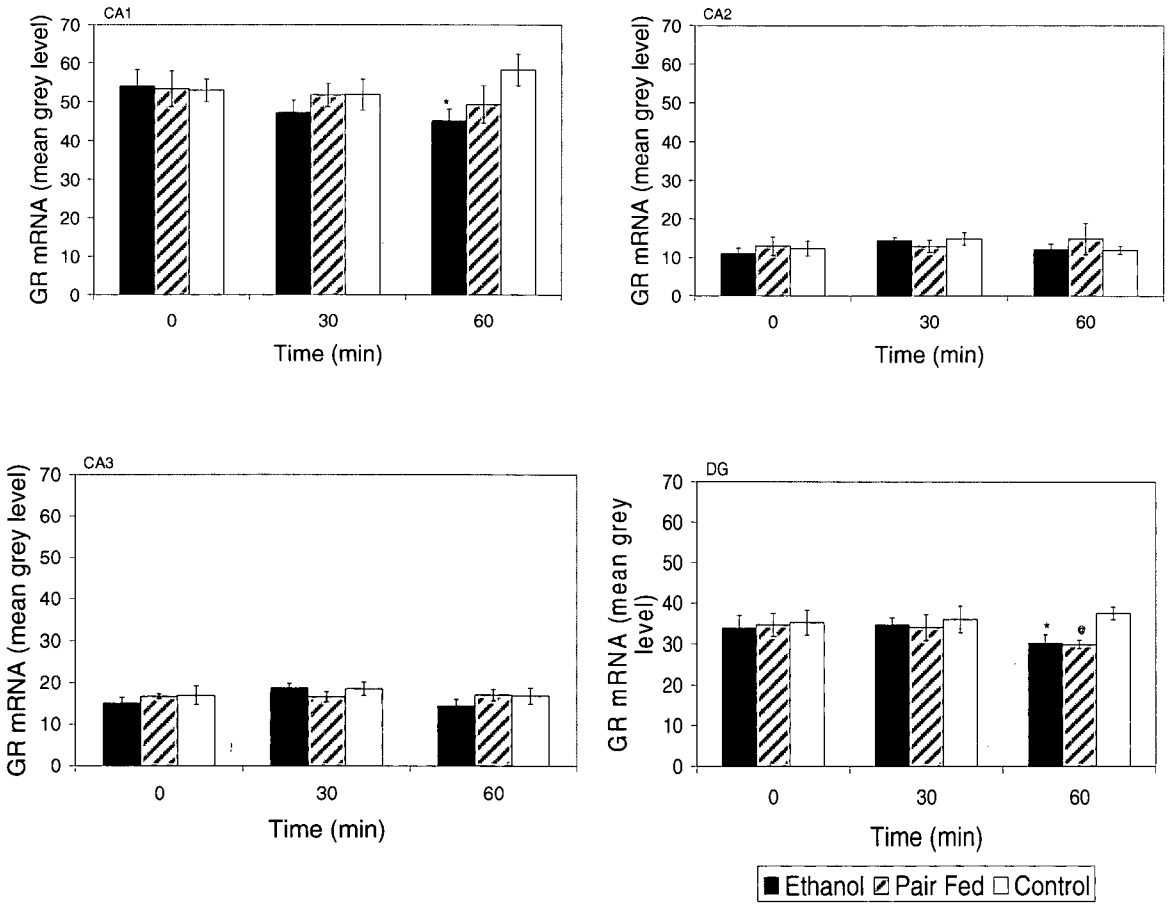


Figure 10 Schematic representation of the preoptic area

A schematic representation of the preoptic area (A), including the medial preoptic area (MPA) and median preoptic nucleus (MnPO), and representative nuclear emulsion-dipped slides of GnRH mRNA expression (B) in E, PF and C females and males.

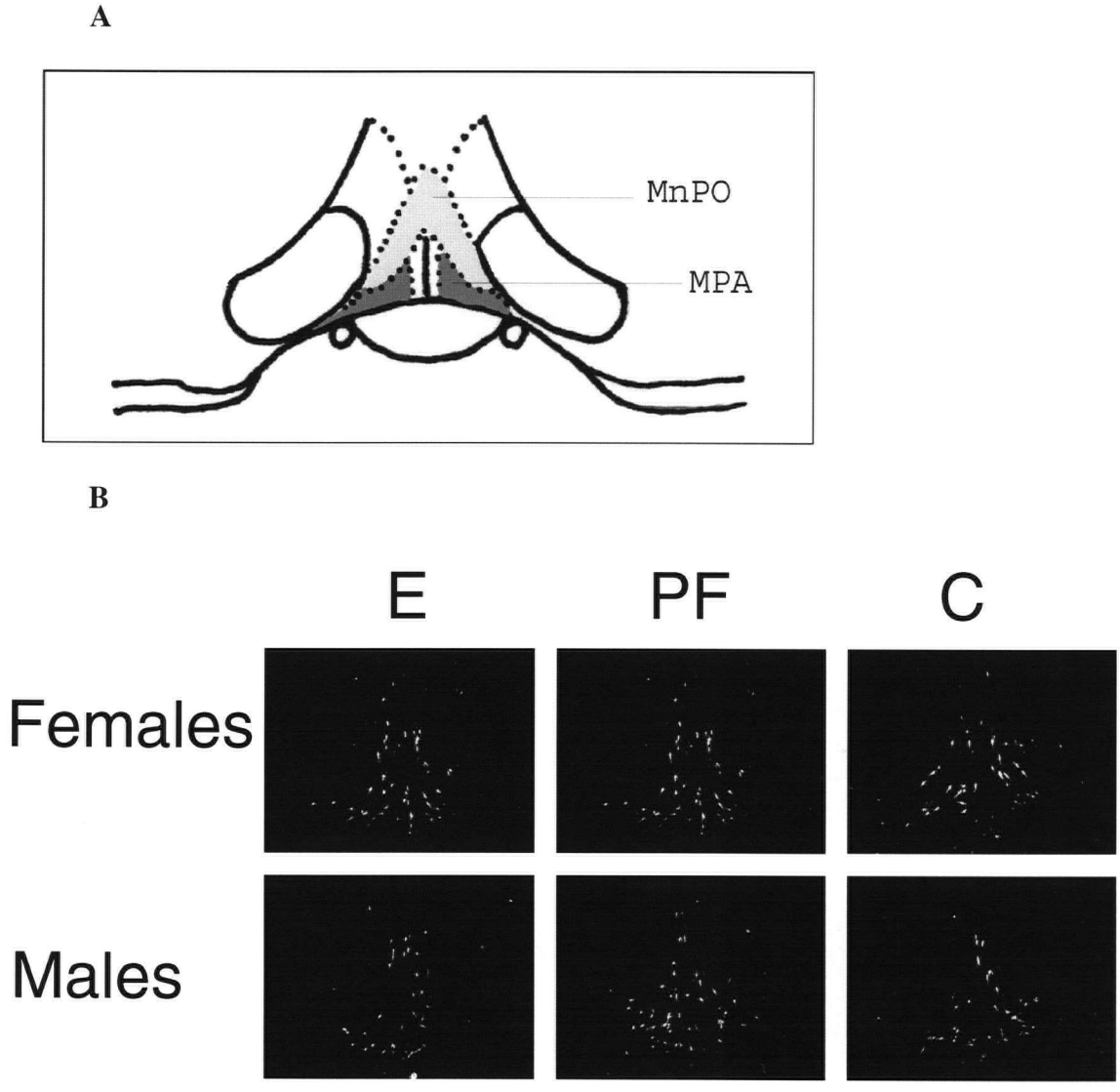


Figure 11 GnRH mRNA levels (total of (pixel area/cluster) X (mean grey value/cluster)) (mean ± SEM)

GnRH mRNA expression in the rostral POA in E, PF and C females (A) and males (B) at 0, 30 or 60 min after stress onset (n = 4-6 per condition). Among females GnRH mRNA levels decreased at 30 and 60 compared to 0 min in E pups only (@ps<0.01). Among males, GnRH mRNA increased at 60 compared to 0 min in E pups (^p=0.057) and at 60 min compared to 0 and 30 min in PF pups (*ps<0.01).

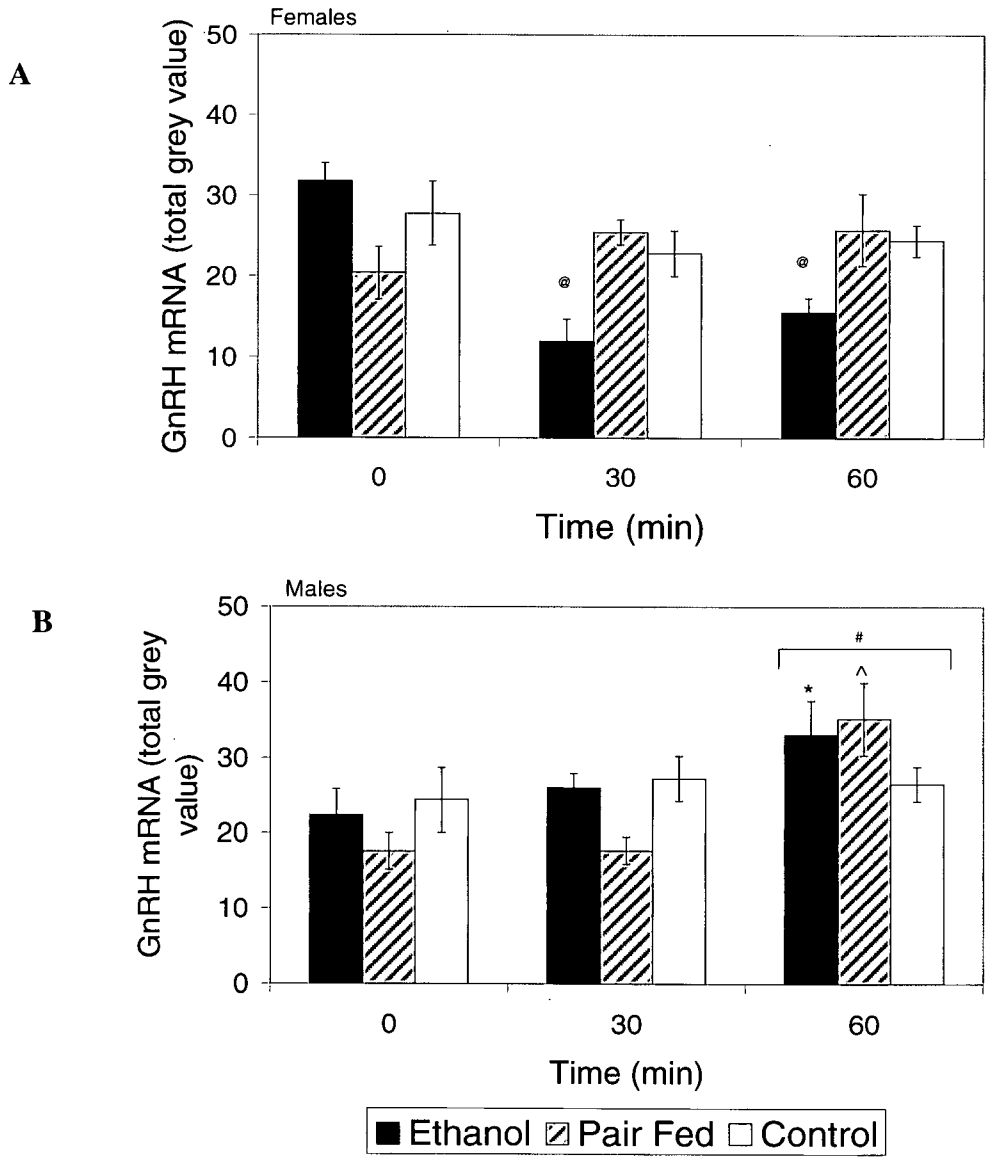
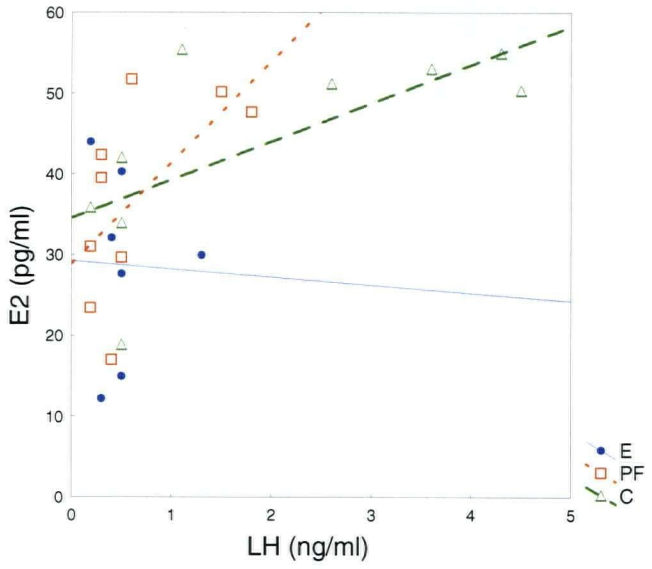


Figure 12 Correlations between basal sex steroids and LH (ng/ml)

Correlations between E2 (pg/ml) and LH (ng/ml) in E, PF and C females (A; n = 8-9) and between T (ng/ml) and LH in E, PF and C males (B; n = 7-10) at 0 min. E2 and LH positively correlated in C ($r=0.6811$, $p<0.05$) but not E or PF females at 0 min. LH did not correlate with testosterone for any group.

A



B

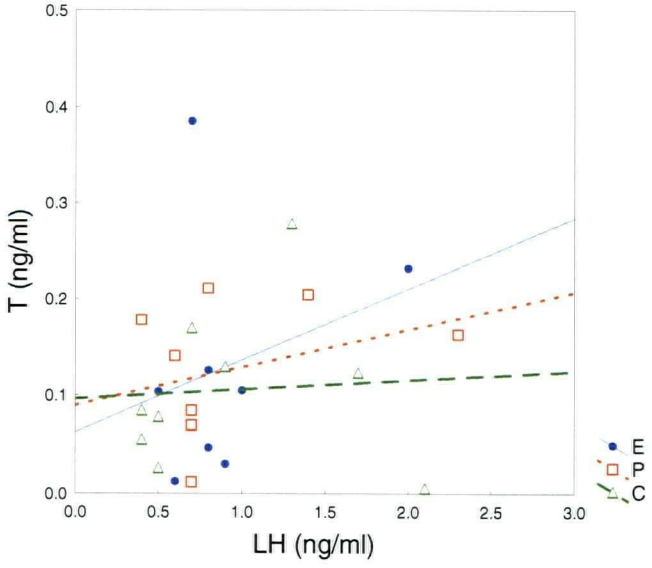


Figure 13 Correlations between CORT (ng/ml) and sex steroids following stress

Correlations between CORT (ng/ml) and E2 (pg/ml) in E, PF and C females (A; n = 8-10) and between CORT and T (ng/ml) in E, PF and C males (B; n = 7-10) following stress (30 and 60 min collapsed). CORT and E2 negatively correlate in E females (r=-0.6815, p<0.01), while CORT and T do not correlate in any group.

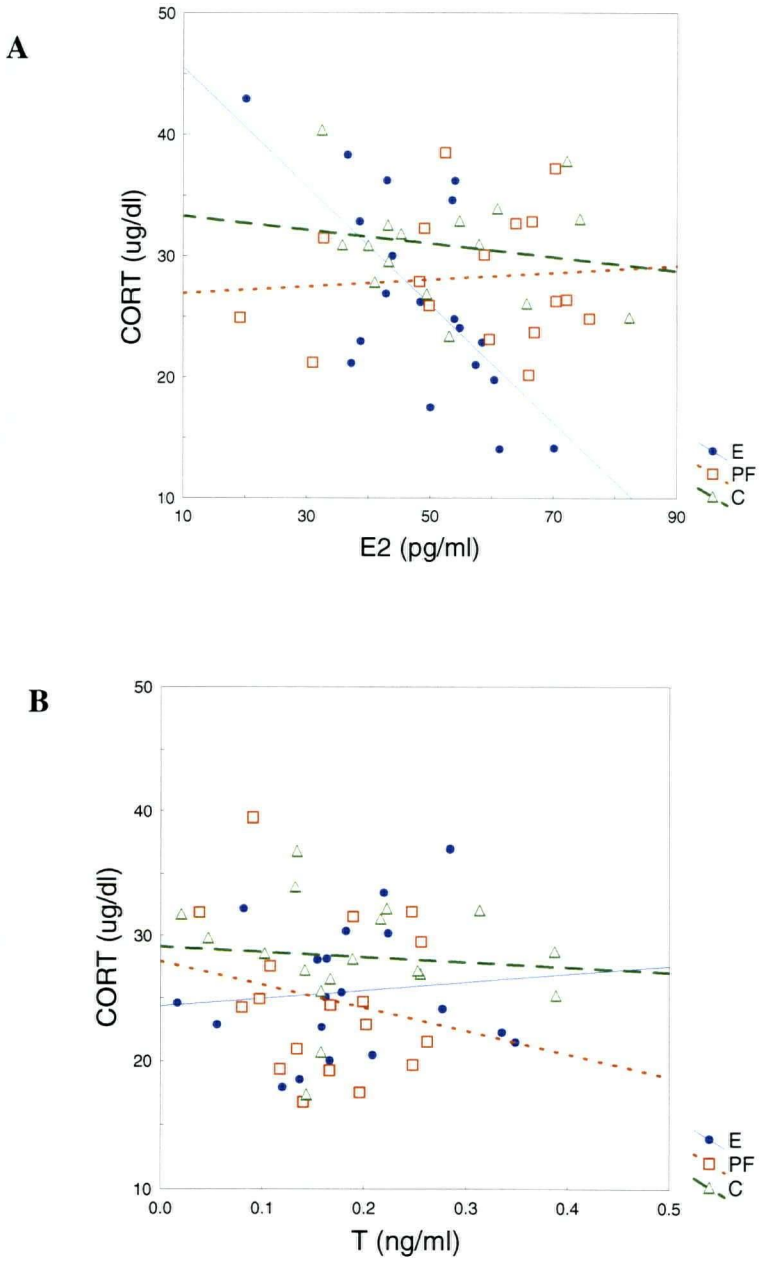
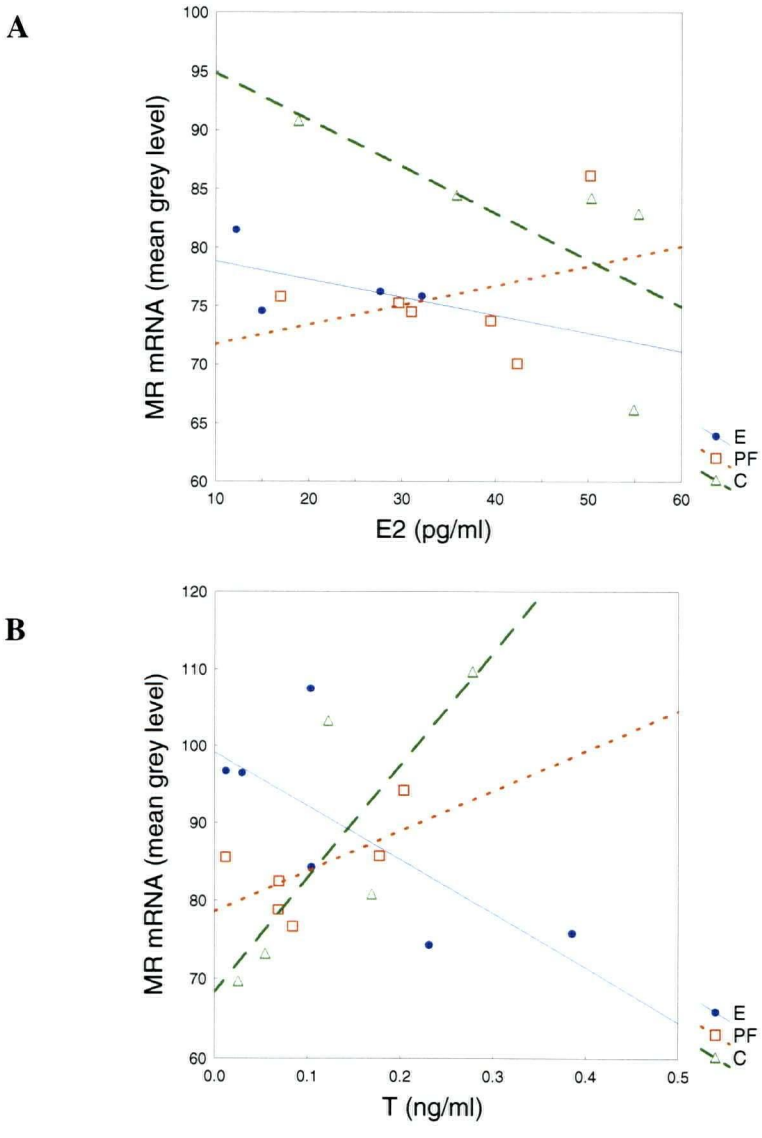


Figure 14 Correlations between basal MR mRNA (mean grey level) and sex steroids

Correlations between MR mRNA (averaged across subfields, mean grey level) and E2 (pg/ml) in E, PF and C females (A; n = 4-6) and between MR mRNA and T (ng/ml) in E, PF and C males (B; n = 6). Among females MR mRNA did not correlate with E2 for any group. Among males MR mRNA and T showed a borderline positive correlation in C ($p<0.10$) and a borderline negative correlation in E ($p<0.10$) pups.



CHAPTER 4: DISCUSSION

A. Summary

The mechanisms underlying altered HPA activity following prenatal ethanol exposure are unknown. Previous findings from our laboratory have revealed that E rats differ from their control counterparts in HPA responding both neonatally and in adulthood, indicating the HPA activity is altered throughout the lifespan. Furthermore prenatal ethanol differentially alters HPA activity in males and females, suggesting a possible role for altered HPG function in HPA axis dysregulation. Prenatal ethanol exposure is known to alter both activational and organizational effects of HPG hormones. This thesis was designed to determine if maturation of the HPA stress response is delayed in prepubertal E rats and to investigate the possibility that ethanol-induced changes to HPG organizational effects, as perhaps indicated by altered basal and/or stress hormone regulation, may underlie some of the differential HPA responsivity observed between E males and females.

Overall, prepubertal E pups were hyporesponsive to stressors compared to C pups, demonstrated by blunted CORT responses following restraint. The data also suggest changes in CORT feedback regulation, as both MR and GR mRNA levels were differentially altered in E compared to C pups following stress. HPG development appeared to be delayed, i.e. LH and E2 levels were lower and the LH/E2 correlation was absent in E compared to C female pups, and the LH response to stress was blunted and the gonadal hormone response was altered in both E males and females compared to controls. These results suggest that during the prepubertal period HPA and HPG development are delayed and/or altered by prenatal ethanol exposure.

Importantly, this study indicates that prenatal ethanol (1) differentially altered HPA responding in males and females compared to control counterparts, and (2) altered HPA/HPG hormone interactions in both males and females. There is evidence in this study to suggest that HPG organizational effects may have been altered, and this alteration may be one mechanism by which prenatal ethanol exposure interfered with HPA function.

B. Effect of Ethanol on Pregnancy Outcome

Ethanol consumption was consistently high throughout gestation, averaging 9.42 +/- 2.85, 12.98 +/- 2.18, and 12.81 +/- 2.25 g/kg body weight/day of gestation, for weeks 1, 2 and 3 respectively. This resulted in a peak blood ethanol level of between 130 - 156 mg/dl. Consistent with previous studies in our lab (Gabriel et al., 2001; Weinberg, 1988; Weinberg, 1992), E and PF dams failed to gain weight during the first week of gestation, and on gestation days 14 and 21 weighed significantly less than C dams. There was postpartum catch up, as the weight difference among dams was eliminated by PN 8. It should be noted that while there were no weight differences between E and PF dams during gestation, ethanol disrupts the digestion, absorption and utilization of nutrients (Weinberg, 1984) and thus may cause dietary deficiencies that cannot be controlled for with a pair-feeding regimen.

Neither gestation length nor the number of live born pups was affected by prenatal treatment in the present experiment. Previous reports found some variability in the effects of ethanol on both gestation length (e.g. increases [Chen and Smith 1979; Hard, Dahlgren et al. 1984; Weinberg 1988, 1992] or no change [Blanchard and Hannigan 1994; Glavas et al. unpublished data]) and number of live born pups (e.g. decreases [Weinberg 1989;

Becker, Hale et al. 1993] or no change [Taylor, Branch et al. 1982; McGivern 1984; Weinberg 1988, 1992]). In contrast, there was an effect of prenatal treatment on postnatal pup weight, as E and PF males weighed less than C males by PN 22. This data is consistent with previous work showing delays or deficits in postnatal development (Taylor, Branch et al. 1986; Cooper and Rudeen 1988; Becker, Hale et al. 1993; Weinberg, Taylor et al. 1996). These data are also consistent with previous work showing that in some breedings males may be more affected than females (Becker et al., 1993). Finally, we found that by weaning females weighed less than males and this is also consistent with the literature (Becker et al., 1993; Gabriel et al., 2001; Weinberg, 1992).

C. Prenatal Ethanol Exposure Alters HPA Axis Activity During Development

HPA activation following exposure to stressors is typically delayed and prolonged in prepubertal rats compared to adults. The characteristics of HPA activity observed in this stage of development have been attributed to slow adrenal activation following ACTH stimulation, a longer metabolic half life of ACTH, and immature negative feedback regulation (Vazquez, 1998). Evidence from the current study indicates that prenatal ethanol exposure disrupts the normal progression or maturation of HPA development during this time, and that the effects may be mediated by changes in adrenal activation as well as deficits in feedback regulation as reflected in indices from central HPA measures.

The present study found that following restraint stress CORT secretions were blunted in prepubertal E, and to some extent PF, pups. Previous studies have shown that during the SHRP, when normal HPA responses to stressors are blunted, E rats are even more hyporesponsive (Angelogianni and Gianoulakis, 1989; Taylor et al., 1986a; Weinberg, 1989). By PN 18 the HPA responsivity to some stressors, such as saline injection,

morphine or ethanol challenge, begins to normalize (Taylor et al., 1986a) but following a number of other stressors, such as novelty, ether or ACTH administration, plasma CORT responses are still blunted (Weinberg, 1989). Data from the present study demonstrate that blunted HPA responsiveness to restraint may extend past weaning age. The reduced stress CORT levels of E pups may be mediated in part at the level of the adrenal. For example, Weinberg (1989) found that PN 18 E pups with blunted stress CORT levels had concomitantly elevated adrenal CORT levels, suggesting that CORT was not being released upon ACTH stimulation to the same degree in E as in C pups. Further evidence that reduced CORT responsiveness may be mediated peripherally comes from the work of Lee, Imaki et al. (1990), who showed that PN 21 pups had enhanced drive at the hypothalamus and pituitary levels, demonstrated by increased basal CRF expression and stress-induced ACTH levels, but that this enhanced drive was not reflected in enhanced CORT secretions. These investigators suggested the possibility that the absence of CORT hypersecretion, despite elevated ACTH levels, following stress in E pups may be due to a disruption in adrenal gland development and that prenatal ethanol exposure may result in a degree of adrenal insensitivity to ACTH stimulation during development (Lee et al., 1990). The mechanism of impaired adrenal responding in E pups is unknown, but given that prenatal ethanol exposure can disrupt or delay postnatal organ growth (Gallo and Weinberg, 1986; Murillo-Fuentes et al., 2001), and that adrenal gland structure and function continues to develop through puberty (Sencar-Cupovic and Milkovic, 1976; Vazquez and Akil, 1993), CORT hyposecretion of immature E rats may be due, in part, to an alteration or delay in adrenal development. PF pups also had lower CORT than C,

indicating that in the present study the ethanol effect on adrenal development is at least partially nutritionally mediated.

Although we observed a blunted CORT response to restraint in prepubertal rats, we (Weinberg 1988; Weinberg 1992; Gabriel, Ellis et al. 2002) and others (Taylor, Branch et al. 1982; Nelson, Taylor et al. 1986; Lee, Schmidt et al. 2000) have shown that in adulthood restraint-induced CORT levels are elevated in E compared to C rats. Collectively the data indicate that the transition from hypo- to hyperresponsiveness, at least in response to some stressors, occurs after PN 26, perhaps later in the prepubertal period or during puberty, which coincides with completion of adrenal gland development (Sencar-Cupovic and Milkovic, 1976; Van Dorp and Deane, 1950; Vazquez and Akil, 1993). It may be, therefore, that completion of adrenal development is important for this transition in HPA responding, and that after PN 26 the adrenal develops the capacity to transduce enhanced central drive into CORT hypersecretion.

We did not observe a difference in ACTH levels between E and C pups. However this does not necessarily indicate normal pituitary function in E rats. First, during the prepubertal period the mechanisms involved in ACTH metabolic clearance are undergoing developmental changes, evidenced by prolonged ACTH clearance in prepubertal rats compared to adult rats (Vazquez et al., 1997). The development of ACTH clearance in E rats in unknown, and a more thorough examination ACTH kinetics would be needed to confirm whether and to what extent prenatal ethanol affects ACTH metabolic clearance. The second caveat to drawing conclusions from the ACTH observations is that, similar to adults, the ACTH response to stress in PN 25-26 pups typically peaks 5 to 15 min after stress onset (Vazquez and Akil, 1993). Thus with the

initial time point of 30 min measured in the present study we may have missed the ACTH peak, and possibly differences among prenatal treatment groups. Since ACTH measurements taken at 30 min may not accurately reflect the pituitary drive evoked by restraint in PN 25-26 pups, we cannot at this time rule out the possibility that prenatal ethanol has altered pituitary ACTH secretion.

In contrast to the data for E pups, PF females had significantly elevated ACTH at 60 min compared to E and C females. These data point out a methodological issue, in that pair-feeding is itself a treatment condition. PF dams receive less food than they would eat if allowed *ad libitum* access to control diet, and are therefore hungry. Thus pair-feeding may be a mild prenatal stressor (Weinberg and Gallo, 1982). Prenatal stress disrupts endocrine regulation later in life: for example both stress responsiveness (McCormick et al., 1995) and reproductive function (Collu et al., 1984) are altered in adult prenatally stressed rats. In the present study, ACTH remained elevated at 60 min following stressor onset in PF females, suggesting that nutritional and/or prenatal stress effects of maternal pair-feeding has disrupted offspring HPA development, resulting in delayed HPA recovery following stressor onset. Furthermore, the disruption was sex-specific, which is consistent with previous research showing that the HPA axis activity of males and females are differentially altered by prenatal stress (Matthews, 2002; McCormick et al., 1995).

Hippocampal MR and GR systems continue to develop until PN 35. For example, while MR protein concentrations reach adult levels by PN 10 (Rosenfeld et al., 1988; Sarrieau et al., 1988), GR protein concentration increases steadily from birth until the 5th week of life (Meaney et al., 1985; Olpe and McEwen, 1976; Rosenfeld et al., 1988;

Sarrieau et al., 1988). MR mRNA has been shown to increase steadily from birth to adulthood (Vazquez et al., 1996), whereas data on GR mRNA have been inconsistent, such that the gene transcript has been shown to either increase (Bohn et al., 1994; Van Eekelen et al., 1991) or decrease (Vazquez, Morano et al. 1993; Vazquez, Van Dours et al. 1996) throughout the neonatal and prepubertal period. Since in adults CORT downregulates its own receptors (Herman, Patel et al. 1989; Herman and Watson 1995; Herman and Spencer 1998; Paskitti, McCreary et al. 2000; Hugin-Flores, Steimer et al. 2004) the findings that GR protein and MR (and perhaps GR) mRNA increase throughout the prepubertal period, during which time CORT levels are also increasing, suggest that the mechanisms underlying CORT receptor regulation are not fully developed until adulthood (Meaney, Sapolsky et al., 1985; Bohn, Dean et al. 1994). Finally, there is considerable postnatal structural development of the hippocampus, as neurogenesis, neuronal migration, and the formation synaptic connections continue postnatally (Gould et al., 1991; Seress et al., 1989). As mentioned above, an immature negative feedback system may play a role in the prolonged HPA activity following stress in pups emerging from the SHRP, and it has been hypothesized that a combination of immature MR and GR regulation as well as underdeveloped hippocampal connectivity underlie the prolonged stress responding of post-weanling pups (Vazquez, 1998).

In the present study MR and GR mRNA intensities varied across subfields. For MR mRNA, levels in CA1>CA2>DG>CA3, whereas for GR mRNA, levels in CA1>DG>CA3>CA2 in both males and females. The intensity pattern for MR mRNA appears more similar to what we have observed previously in adult rats (Glavas, 2003) than to previous observations in PN 28 rats (DG>CA2>CA1>CA3; Vazquez, Morano et

al. 1993). This suggests that the distribution of MR mRNA expression is approaching maturity, and between PN 25-26 and PN 35, when MR mRNA regional distribution fully develops (Vazquez, Morano et al. 1993) there will be an increase in signal intensity in CA2 relative to CA1. The regional organization of GR mRNA resembles adult distribution (CA1>DG>CA3>CA2) by PN 12 (van Eekelen, Bohn et al. 1991; Vazquez, Morano et al. 1993) and our findings in PN 25-26 pups are consistent with this.

There was no effect of prenatal treatment on basal MR or GR mRNA levels in prepubertal rats. This extends upon previous work in our lab showing that basal levels of MR and GR gene expression and protein concentrations in adult E rats does not differ from controls (Glavas et al., in preparation; Kim et al., 1999a; Weinberg and Petersen, 1991). Thus results from this study show that under basal conditions the ontogenetic progressions of MR and GR mRNA are unaltered by prenatal ethanol exposure. Prenatal treatment effects were observed, however, following acute restraint. For example, MR mRNA levels were lower in C compared to E pups in CA1, CA3 and DG 30 min after stressor onset. These effects may be partially nutritionally mediated as PF pups showed the same directional trends as their E counterparts, although they did not significantly differ from either E or C pups. Furthermore, among males GR mRNA levels were lower in E and PF compared to C pups 60 min after stressor onset.

The effects of prenatal ethanol on MR mRNA levels following stress were seen in both males and females. MR mRNA was lower in C compared to E pups at 30 min, and among males this was due to stress-induced decreases of MR mRNA that were significant in C pups. Among females, the differences at this time point were due to different patterns of response, with non-significant MR mRNA decreases in C pups in

combination with non-significant increases in E pups. Hippocampal MR levels are sensitive to available levels of steroid ligand, and in adult rats stress-induced glucocorticoid secretion downregulates CORT receptors, typically beginning 1 to 4 hours following stressor onset (Herman and Watson, 1995; Karandrea et al., 2000; Karandrea et al., 2002; Paskitti et al., 2000). While the decreases in MR mRNA found here are early compared to the temporal lag following stressor onset typically reported in adults, it is possible that transcription rates may be different in immature compared to adult brains. Indeed, recent experiments examining the effects of stress on CRF and AVP mRNA in the PVN of immature rats have shown that in developing brains stress-induced changes in gene expression can be seen within 15 min of stressor onset (Dent et al., 2000a; Dent et al., 2000b). If our data indicate changes in gene transcription, they would extend on previous findings and show that in PN 25-26 control pups, MR mRNA is rapidly downregulated in the dorsal hippocampus by restraint. Alternatively, changes in MR mRNA levels may also reflect changes to post-transcriptional processes, such as transcript stability or degradation. Either way, the downregulation is transient, and by 60 min MR mRNA levels have returned to basal levels. Whether the rapid changes in MR mRNA are translated into changes in MR protein has yet to be elucidated.

In contrast to controls, E and PF pups did not show MR mRNA decreases following stressor exposure. This failure to downregulate MR mRNA at 30 min may be due to the changes in CORT levels observed in E and PF pups. As E and PF pups had blunted CORT levels at 30 min compared to controls, and as MR mRNA downregulation is dose-dependent (Hugin-Flores et al., 2004), it stands to reason that less steroid ligand feeding back to the hippocampus would result in less effective signaling to MR receptors and the

consequent changes in MR downregulation. Another possibility is that the absence of MR mRNA response to stress in E and PF rats may reflect changes in post-transcriptional processes, as the regulation of MR gene expression via post transcriptional mechanisms, such as mRNA stability and degradation, may have been altered by prenatal treatment. It should be noted that while neither E nor PF pups showed the changes in MR mRNA levels that C pups demonstrated, statistically significant differences were seen only between E and C pups, such that MR mRNA levels were significantly lower in C compared to E pups at 30 min. We have previously shown a specific effect of ethanol on CORT regulation of MR transcription. Specifically, E pups were unable to utilize exogenous CORT to normalize ADX-induced MR mRNA upregulation (Glavas et al., in preparation). Thus the inability of E, and to some extent PF, pups to downregulate MR mRNA in the present study may be due to a combination of reduced receptor activation by lower CORT levels, alterations to post-transcriptional processing mechanisms, and/or a deficiency in the molecular mechanisms involved in transducing CORT signaling into changes in receptor gene expression.

Although MR mRNA was not affected by stress in E and PF males, downregulation of GR mRNA was observed in E, and some extent PF, males compared to C males at 60 min. Like MR, GR mRNA is mediated by CORT, and transcript downregulation in the hippocampus can be observed as early as 1 hr following the onset of acute stress in both adults (Karandrea et al., 2000; Paskitti et al., 2000; Tritos et al., 1999) and neonates (Vazquez et al., 1996). In this study we show that in prepubertal pups exposed to ethanol *in utero*, GR mRNA is more sensitive to downregulation by circulating CORT levels, and that this effect is partially nutritionally mediated. The sensitivity of GR transcript levels

to stressor exposure is in contrast to what we observed in MR mRNA and suggests that regulation of the CORT receptor balance is altered in E, and to a lesser extent PF, males. The dual action of MR and GR is essential to maintain basal HPA activity and ensure the return of homeostasis following stress (De Kloet et al., 1998; Vazquez, 1998), and disruption to the balance, as we see in E males, may contribute to the deficits in recovery following stressor exposure seen in adulthood.

The MR and GR mRNA results suggest that changes in transcript levels occur rapidly in prepubertal rats. Further analyses of the time course for changes in MR and GR hnRNA, mRNA, and protein levels following acute stress are required to identify the mechanisms underlying these observations and determine the physiological relevance of the findings i.e. whether the changes in transcript levels are translated into changes in receptor concentration. Importantly, the altered regulation of MR and GR mRNA in E rats following acute stressor exposure is a novel finding and suggests a differential sensitivity of MR and GR gene expression to stressor-induced plasma CORT levels in E compared to C pups. Prenatal nutritional effects may be partially mediating these changes, as MR and GR mRNA levels in PF pups showed intermediate levels of responding. The findings in this study may begin to provide insight into the mechanisms underlying altered stress responding observed later in life. In adulthood E rats show prolonged HPA responding to acute stress (Taylor et al., 1988; Weinberg, 1988; Weinberg, 1992), failure to habituate to chronic stressors (Weinberg et al., 1996) and sensitization to acute stressors superimposed in a chronic stress regime (Kim et al., 1999a). The interactive effects of MR and GR are important for regulating feedback inhibition to the HPA axis, and results from the current study suggest that deficits in MR

and GR autoregulation following acute stressor exposure may participate in mediating the deficits in stress response inhibition that is consistently observed in E rats.

D. Prenatal Ethanol Exposure Alters HPG Development

Normal pubertal development depends on the intricate endocrine interactions between the hypothalamus, the pituitary and the gonads. Females reach puberty before males (PN 30-35 for females and PN 35-45 for males) and indeed in this study there is evidence that C females are approaching puberty. In females prenatal ethanol exposure altered and/or delayed the normal pubertal progression, and effects were observed at all levels of the HPG axis. Although these data were collected at only one age within the complex process of sexual maturation, in combination with the literature they help provide insight about the effects of prenatal alcohol on pubertal development in the female rat.

Throughout the prepubertal period the female reproductive system undergoes a series of changes that function to prepare the organism for the transition into adulthood. Central to this process is the development of the pituitary's ability to integrate and respond to hypothalamic and ovarian signals with episodic LH releases of increasing amplitude. During the days leading up to puberty these episodic LH secretions develop into minisurges, which represent the initial attempts of the HPG axis at mounting the preovulatory surge of gonadotropins seen during the adult estrous cycle (Ojeda and Tesawa, 2002). In the present study C females had higher LH levels compared to E and PF females, and higher E2 levels compared to E females. Furthermore, LH and E2 positively correlated in C females only. These data suggest that C females are more advanced in their pubertal development, such that the gonadotrophs in C females are eliciting greater LH pulses, perhaps as a result of increasing sensitivity to positive E2

feedback. There is a great deal of variability in the basal LH levels of C females, and this may reflect the variability in the timing of the LH pulses or individual differences in the timing of the progression towards puberty onset.

In E females the delay of puberty onset reported in the literature (Boggan et al., 1979; Esquifino et al., 1986; McGivern and Yellon, 1992) may arise due to reduced gonadotropin secretion, as both pubertal (Esquifino et al., 1986; Morris et al., 1989) and adult (Handa et al., 1985; Wilson et al., 1995) E females show reduced mean LH levels and blunted LH surges compared to controls. Previous experiments have indicated that these observations may be due to alcohol-induced changes at the pituitary and/or the hypothalamus. For example, decreased pituitary responsiveness to stimulation by E2 and GnRH have been observed in adult E females *in vivo* (Handa et al., 1985; Wilson et al., 1995) and *in vitro* (Creighton-Taylor and Rudeen, 1991). At the level of the hypothalamus, while steady state GnRH mRNA levels of E females are similar to controls (Wilson et al., 1995) there are suggestions that post-translational events may be altered, as decreases in GnRH protein have been observed in pubertal E females (Morris et al., 1989).

E and PF females in this study had lower basal LH levels than C females (Esquifino et al., 1986; Morris et al., 1989). On the one hand, this may be due, in part, to an absence of a positive feedback effects from E2, as E females had lower E2 levels and E and PF females did not have an E2/LH correlation. This is consistent with the literature showing that prenatal ethanol interferes with pituitary sensitivity to E2 (Creighton-Taylor and Rudeen, 1991; Handa et al., 1985; Wilson et al., 1995). In E females, this finding may also be due to a specific effect of ethanol on GnRH mRNA levels. While there were no

prenatal treatment effects on basal GnRH expression, following stress GnRH mRNA was significantly decreased in E but not PF or C females. These data are consistent with the reduced GnRH transcript levels observed previously in adult E females following acute stress (Yamashita, 2004). One possible explanation for these data is that GnRH gene expression of E females may be more sensitive to downregulation by rising CORT levels than controls. Enhanced responsivity of the GnRH transcript to changes in the cellular endocrine environment may be one mechanism underlying reduced GnRH protein content previously observed in prepubertal E females (Morris et al., 1989). It is also possible that this decrease may be suggestive of inhibited GnRH release, as decreases in GnRH mRNA following stress have been associated with increases in GnRH content in the median eminence (Kam et al., 2000). If GnRH release was inhibited in E females, then the finding that LH levels did not also decrease with stress suggests that the pituitary of E females is insensitive to changes in GnRH levels, which has been reported in adult E females (Creighton-Taylor and Rudeen, 1991). Overall, the LH data in this study support and extend previous reports (Esquifino, Sanchis et al. 1986; Morris, Harms et al. 1989) showing that LH secretion is blunted in prepubertal E females. Furthermore, our results provide insight into possible mechanisms underlying reduced LH levels, as we present data suggesting that prenatal ethanol exposure may have altered the positive feedback effect from ovarian steroids and/or caused deficits in hypothalamic drive to pituitary gonadotrophs.

It has been suggested that decreased stimulation to the ovaries by blunted gonadotropin secretion in E females may result in inadequate or delayed rises in prepubertal E2 levels, causing the E2-dependent event of vaginal opening to be delayed

(McGivern et al., 1992). Results from this study provide novel findings that lend support to this possibility, as we demonstrate for the first time that prepubertal E females have lower E2 levels compared to controls. There are several possible explanations for these observations. First, blunted E2 may be partially due to decreased LH secretion. However, as both E and PF females had low LH levels but only E females had low E2 levels, there may be a specific effect of prenatal ethanol-induced on ovarian function. It is unlikely that prenatal ethanol exposure resulted in an inability of the ovary to produce or secrete E2, as E females were capable of mounting an E2 response to stress. Instead, prenatal ethanol exposure may have decreased ovarian responsivity to stimulation by gonadotropins. Indeed, studies in both pubertal (Rudeen and Hagaman, 1988) and adult (McGivern et al., 1992; McGivern and Yellon, 1992) E females have shown that prenatal ethanol exposure alters ovarian sensitivity to gonadotropins. Another possibility is that low E2 levels may be mediated by disruptions to the FSH secretory pattern. Regulation of E2 requires the coordinated stimulation of the ovaries by both LH and FSH. In the second week of life prenatal ethanol alters FSH secretion such that the normal developmental peak of FSH is delayed (Wilson and Handa, 1997), and in adulthood intact E females have reduced FSH pulse amplitudes and OVX E females have decreased pituitary FSH mRNA compared to controls (Wilson et al., 1995). FSH was not measured in this study and cannot be ruled out as a possible mediator of altered E2 secretion. In summary, the data to date suggest that reduced basal E2 levels may be due to altered gonadotropin secretions and ovarian responsitivity to gonadotropins. It is possible that this E2 decrease underlies the delayed vaginal opening reported in E females by other laboratories (Boggan et al., 1979; Esquifino et al., 1986; McGivern and Yellon, 1992).

The reduced LH and E2 levels seen in prepubertal E females in the present study are in many ways akin to the HPG changes observed in neonatally androgenized females (Becu-Villalobos et al., 1997; Handa et al., 1985; Lookingland and Barraclough, 1982). Boggan et al. (1979) reported that the changes in differentiation vaginal epithelium of ethanol-exposed mice were similar to those seen in mice treated neonatally with E2. Handa et al. (1985) related the decrease in the phasic pattern of LH secretion observed in adult E females with the change in LH secretion described in lightly androgenized female rats. It has been speculated that alcohol may be altering the central mechanisms controlling LH secretion by inducing a perinatal androgenization effect (Handa et al., 1985; McGivern et al., 1984). Thus one possible mechanism for the alcohol-induced HPG deficits observed in the E females in this study may be alcohol's effects on the organizational effects of the HPG hormones.

Puberty onset in the male occurs later than in the female and involves a more gradual change in the endocrine environment. Both LH and T levels are low until the fourth week of life, and thereafter gradually increase until puberty (Dohler and Wuttke, 1975). In the male, puberty is characterized by the descent of the testes and the ability to make mature sperm, and is initiated by a combination of increasing androgen levels and decreasing pituitary sensitivity to T negative feedback (Dutlow et al., 1992; Negro-Vilar et al., 1973).

In contrast to females where group differences in E2 and LH were seen, no group differences were observed in basal HPG parameters among males. Previous studies have shown that prenatal ethanol exposure alters HPG hormonal regulation at various stages throughout the lifespan. Both pre- and postnatal T surges are blunted in E males

(McGivern et al., 1998b; McGivern et al., 1993; McGivern et al., 1988) and this effect may be due to deficits in steriodogenic enzyme activity (Kelce et al., 1989). During the preweaning period the trough of LH secretion observed in control males is blunted following prenatal ethanol exposure (Wilson and Handa, 1997). While little is known about the HPG development of E males following weaning, during puberty and in adulthood E rats show altered testicular morphology (Fakoya and Caxton-Martins, 2004), testicular weight (Udani et al., 1985), and lowered serum levels of both T (Udani et al., 1985) and LH (Handa et al., 1985; Udani et al., 1985). The current data extend on previous findings of HPG development in E males by showing that at PN 25-26 basal T and LH levels do not differ from controls.

While all HPG parameters were responsive to stress, prenatal treatment altered the pattern of HPG hormone responding to stress, as LH levels showed a significant decrease in C but not E females and males 30 min after stressor onset. Studies in adult rats have shown that acute stressors or glucocorticoid treatments reduce LH levels (Baldwin, 1979; Breen and Karsch, 2004; Briski and Sylvester, 1988; Rivier and Vale, 1984), and there is evidence to suggest that this is mediated both peripherally and centrally. At the level of the pituitary, glucocorticoids inhibit basal and E2- (in females) and GnRH- (females and males) stimulated LH release (Baldwin, 1979; Breen and Karsch, 2004; Kamel and Kubajak, 1987; Suter and Schwartz, 1985). Centrally, both feedback from glucocorticoids and direct actions of CRF (Du Ruisseau et al., 1979; Rivier and Vale, 1984) are involved in inhibiting GnRH secretion. Finally, stress reduces LH levels by activating other neural pathways that inhibit gonadotrophs, such as the opioid system (Petraglia et al., 1986).

In the present study there is no evidence to indicate that the LH response to stress in C pups is due to altered hypothalamic input, as GnRH mRNA levels did not show the same directional changes as LH following stress, in either females or males. Whether stress altered the responsiveness of LH to GnRH is difficult to determine, as these measures did not correlate under basal or stress conditions. In C females, stress may have disrupted the positive feedback effect of E2 on LH secretions. LH did not decrease after stress in E and PF females. This may be because under basal conditions LH secretion was unaffected by stimulatory effects of E2, and therefore LH levels were already minimal, resulting in a 'floor effect' such that no further decreased could occur. Furthermore, in both C females and males, decreased LH levels following stress may be due to the modulatory effects of alternate neural pathways, such as the opioid system. Under nonstressed conditions opioid administration decreases LH levels (Cicero et al., 1976; Pang et al., 1977), and treatment with an opioid antagonist prior to stress blocks the stressor-induced LH decrease (Briski et al., 1984; Briski and Sylvester, 1988). The notion that stressor-induced opioid activity may be mediating decreases in LH levels in C but not E pups is consistent with previous studies showing that prenatal ethanol exposure blunts beta-endorphin levels in immature rats (Angelogianni and Gianoulakis, 1989). Since PF pups were similar to E pups in showing no LH decrease with stress, this blunting of the LH response in E pups may be at least partially nutritionally mediated. Finally, we cannot rule out the possibility that the blunted LH levels and responsiveness in E and PF pups may be due simply to delayed maturation of the HPG axis and/or other stress responsive systems that modulate HPG activity.

At 60 min following stressor onset, C males showed elevated LH levels. This elevation may be due to input from catecholamines that are released during stress and are known to stimulate LH. For example, norepinephrine (NE), a main secretagogue of the SNS response to stress, has a modulatory role in GnRH release from the median eminence (Negro-Vilar et al., 1979). Furthermore, NE antagonists inhibit both basal and GnRH-stimulated LH release (Rettori et al., 1981), and as well as acute-stressor induced increases in LH (Martin et al., 1995). LH did not increase in E or PF males, and this may be due to deficits in NE responding. Indeed, the NE system is susceptible to the effects of prenatal treatment effects, as E, and to some extent PF, rats exhibit deficient catecholamine activity under basal conditions at weaning (Cooper and Rudeen, 1988; Detering et al., 1980) and show normal basal catecholamine levels but altered catecholamine responses to stress in adulthood (Rudeen and Weinberg, 1993). Interestingly, the LH response to stress is not altered by prenatal ethanol exposure in adulthood (Lan et al., 2004a; Yamashita, 2004), suggesting that the blunted LH response to stress observed in prepubertal pups is a transient phenomenon.

Despite reduced or unchanged LH levels, gonadal hormones increased after stress. In females, acute stressor-induced increases in E2 level have been reported (Cardenas, 1992; Shors et al., 1999; Yamashita et al., 2003) and this effect may be due to stimulation by CORT acting on MR and GR in corpus luteum and granulosa cells of the ovary (Shors et al., 1999; Tetsuka et al., 1999). While the trend for E2 to increase was observed in all groups, the effect was driven mainly by increases in E, and to a lesser extent PF females. On the one hand, this may suggest that the ovaries of these pups were more responsive to CORT. However, a comparison of the stress-induced E2 increases among adult females

found that E2 was responsive to stress in C but not E or PF females (Yamashita et al., 2004). Thus if the ovaries of E and PF females are more responsive to CORT during prepuberty, this effect does not persist into adulthood. Another possibility is that the relative E2 increases following stress may be related to the amount of stimulatory input present under basal conditions. Specifically, LH levels were low in E and PF females, indicating that the stimulatory input from higher levels of the HPG axis was minimal in these pups. Thus CORT induced a relatively large increase in E2 in E and PF pups. Basal LH levels were high in C females and thus additional CORT stimulation following stress elicited relatively small and nonsignificant E2 increases. Therefore the magnitude of the E2 response to stress may be indicative of the basal stimulation by LH present in the system before stressor onset, and these data may provide further evidence suggesting that HPG development is delayed in E, and to some extent PF, females.

T has been shown to increase (Frankel and Ryan 1981; Orr and Mann 1992) or decrease (Charpenet et al., 1981; Evain et al., 1976) following stress depending on the timing and the nature of the stressor. In the present study T levels in males increased overall with stress, and the pattern of responding differed among the prenatal treatment groups. In C males, T increased immediately after stressor onset and remained elevated at 60 min. Stressor-induced T elevations independent of LH elevations have been previously reported (Orr and Mann, 1992; Sapolsky, 1986; Siegel et al., 1981) and these effects may be mediated by the direct actions of CORT on gonadal receptors (Orr and Mann, 1992), and/or by sympathetic innervation to the testes (Frankel and Ryan, 1981; Sapolsky, 1986; Sapolsky et al., 1986). T level increases were delayed in E males and absent in PF males. These observations may be due to blunted CORT levels at 30 min.

This possibility is supported by the finding that the rise in T levels reached significance in E males following a significant rise in CORT between 30 and 60 min. It is also possible that the delayed (E males) or blunted (PF males) T response to stress may be due to prenatal ethanol and/or nutritional-induced deficiencies in central catecholamine systems, which is consistent with previous reports showing disruptions in NE regulation in E and PF rats (Cooper and Rudeen, 1988; Detering et al., 1980; Rudeen and Weinberg, 1993). Importantly, this notion is in line with the data from this study showing that E and PF males were not able to mount an LH increase in response to stress, which we might also speculate is mediated by NE deficiencies. Finally, the delayed T response to stress may reflect the effects of prenatal ethanol on testes function. Prenatal ethanol exposure has been shown to alter testicular weight, morphology and function under basal conditions (Fakoya and Caxton-Martins, 2004; Handa et al., 1985), and while we did not see changes in testicular function under basal conditions our data may indicate that the ability of the testes to mount a T response to stress is altered in E males.

Finally, GnRH mRNA levels did not show the same directional changes following stress that LH showed in any prenatal treatment group. In females GnRH mRNA was suppressed in E pups at 30 and 60 min and, as mentioned previously, this may indicate either rapid inhibition of GnRH release or alterations to GnRH transcript stability. In males, GnRH mRNA levels in C pups were constant across time. As indicated above, we believe this suggests that GnRH release is not altered by stress, and thus stress-induced changes in the LH secretory patterns may be mediated at the level of the pituitary. Following prenatal treatment, on the other hand, GnRH mRNA levels increased significantly between 30 and 60 min and showed a marginal increase between 0 and 60

min in PF and E pups respectively. Increases in GnRH mRNA levels may be indicative of increased GnRH release. If this is true then the data indicate that there may be a degree of gonadotroph insensitivity to GnRH in E and PF males, as LH levels did not show corresponding changes. This possibility is consistent with the finding that in E females changes in GnRH mRNA were observed without concomitant changes in LH. It is also possible that stress interfered with the translational processing of GnRH mRNA, resulting in a greater pool of GnRH transcript. To determine the mechanisms underlying these changes in GnRH mRNA levels observed in both males and females, a time-course analysis evaluating GnRH hnRNA and mRNA in the POA and GnRH protein content in the median eminence before and after stress would be needed. Either way, results indicate that regulation of the GnRH transcript has been altered in E male and female pups, and, among males, that this alteration is mediated at least in part by prenatal nutritional effects.

E. Organizational Effects of HPG Hormones May Be Involved in Altered HPA Responding

In this study we predicted that prenatal ethanol exposure would differentially alter HPA responding to stressors in male and female prepubertal rats compared to their control counterparts. This is indeed what we see in the CORT data: C males and females showed no change in CORT during the recovery period. In contrast, the CORT response in E males was blunted at 30 min and showed a significant increase between 30 and 60 min, whereas CORT in E females peaked at 30 min, and was lower than levels in C females at 60 min following stressor onset. We also predicted that prenatal ethanol exposure would disrupt the interactions between the HPA and HPG hormones. Indeed,

we found that the HPA/HPG correlations were differentially altered in E males and females compared to controls, and these altered interactions may, at least in part, underlie the altered CORT responding observed between E males and females.

In E males, the finding that CORT levels were significantly higher at 60 compared to 30 min may be due to reduced drive in combination with deficits in feedback. Reduced drive is suggested by blunted CORT levels in E and PF compared to C males at 30 min and, as described above, this may be due to immature adrenal gland function. Deficits in feedback may be, in part, peripherally mediated, as the adrenal response of E males was either delayed or reduced, thus compromising the efficacy of fast (rate-sensitive) and intermediate (dose-dependent) feedback mechanisms. Importantly, deficits in feedback may also be due to brain factors, and specifically altered sensitivity to T. The relationship between T and MR mRNA was positive in control males and negative in E males, and while these correlations did not reach significance, perhaps due to the small sample size, these relationships may provide some indication about how prenatal ethanol has altered the interactions between the HPA and HPG axes. Since MR is the primary regulator of basal or tonic HPA activity and acts together with GR to regulate stressor-induced HPA activity (De Kloet et al., 1998; Herman and Cullinan, 1997; Jacobson and Sapolsky, 1991), the positive relationship seen in control pups fits with data demonstrating that T typically suppresses HPA activity (Lesniewska et al., 1990; Seale et al., 2004b; Seale et al., 2004a; Viau and Meaney, 1996). Indeed, as the hippocampus contains both androgen receptors (Kerr et al., 1995; Kerr et al., 1996) and CORT receptors (Herman et al., 1989; Reul and de Kloet, 1985), this structure may be one interface between the HPA and HPG axes (Kerr et al., 1996). Interestingly, the negative T/MR relationship in prepubertal E

males is consistent with the finding of reduced sensitivity to androgens seen in adult males (Lan et al., 2004b), and collectively these data suggest that prenatal ethanol exposure may alter HPA activity in males, at least in part, by compromising HPA responsitivity to androgens. While the data reported above suggest that immature drive, in conjunction with altered feedback mechanisms are contributing to the delayed rise to peak CORT values observed in E males, the 60 min time point utilized in this study represents the beginning of the intermediate feedback domain and thus further studies evaluating longer time courses are necessary to determine the pattern of CORT recovery during the intermediate time domain in E males.

Altered HPA/HPG correlations may be at least partially responsible for changes in HPA regulation in E females as well. In E but not PF and C females, CORT began to decline between 30 and 60 min, and this observation may be due, in part, to the significant negative E2/CORT correlation observed in E but not PF or C females. This negative correlation may be partially mediated by altered PVN, and specifically CRF mRNA, to E2. For example, while under normal conditions, E2 increases CRF mRNA in the PVN of adult females (Patchev and Almeida, 1995), previous work in our lab has shown that CRH expressing neurons in the E females may be less responsive to E2 (Yamashita, 2004). CRF responsivity to E2 is organizationally mediated, as neonatal androgenation eliminates the E2-induced changes in CRF mRNA levels (Patchev and Almeida, 1995). Also, altered E2/CORT correlations may be mediated at the level of the adrenal gland. Under normal conditions adrenal steroidogenesis is enhanced by E2 (Lesniewska et al., 1990; Nowak et al., 1995). However, we have shown that prenatal ethanol exposure not only alters adrenal gland development in E rats (present data;

Weinberg 1989) but it also decreases adrenal sensitivity to E2 in adulthood (Yamashita et al., 2004). Adrenal sensitivity to E2 may also be mediated by the organizational effects of the HPG hormones, as gonadectomy and ovariectomy in adulthood reduce but do not eliminate the sex difference in adrenal responding to sex hormones (McCormick et al., 2002). Thus the altered CORT responding of E females may be due, in part, to prenatal ethanol exposure-induced altered sensitivity to E2 at both central and peripheral branches of the HPA axis.

We can utilize these correlations to begin to speculate on the mechanisms underlying ethanol-induced alterations in HPA development. The finding that prenatal ethanol exposure may have altered HPA sensitivity to T and E2 in E males and females respectively suggests that deficient androgen and E2 receptor numbers and/or function may underlie altered CORT responding. Neither androgen nor E2 receptors have been studied before in E rats. Given that both receptor types show rapid development during the critical period for HPG organizational effects (MacLusky et al., 1979; McAbee and Doncarlos, 1999; O'Keefe et al., 1995), and that prenatal ethanol exposure disrupts HPG organizational effects (Blanchard and Hannigan, 1994; Chen and Smith, 1979; McGivern et al., 1984; McGivern et al., 1998a), it may be that prenatal ethanol exposure differentially alters CORT responding in male and female prepubertal rats, in part, by altering the HPG organizational effects on T and E2 receptor system development.

F. Conclusions

The results presented in this thesis suggest that development of both the HPA and HPG axes is delayed and/or altered during the prepubertal period in E rats. E males and females were hyporesponsive to restraint, suggesting that the SHRP extends beyond the

age of weaning (PN 22) in these animals. There was evidence for alterations to hippocampal-mediated negative feedback, suggesting that the mechanisms involved in CORT feedback inhibition may also altered and/or developmentally delayed during the prepubertal period in E rats. Prenatal ethanol exposure appears to delay the progression to puberty onset in E females, as LH and E2 levels were blunted and the E2/LH correlation was absent in E compared to C females. The stress response of HPG hormones was altered in both E males and females, and this may provide further support for the notion that HPG axis development was delayed and/or altered by prenatal ethanol. Importantly, prenatal ethanol both differentially altered the pattern of CORT responding and interfered with the normal HPA/HPG interactions in male and female pups compared to their control counterparts. Although not specifically proven in this study, our results suggest that altered HPA activity may be mediated, in part, by changes to the HPG organizational effects. Alterations in HPA sensitivity to HPG hormones may be due, in part, to prenatal ethanol-induced changes to HPG organizational effects, and possibly the development of E2 and T receptor systems. Given the data presented in this study, further experiments investigating the link between disruptions to HPG organizational effects and altered HPA/HPG hormone interactions and/or altered HPA activity in E rats are warranted.

CHAPTER 5: FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

A. Future Directions

Prenatal ethanol exposure alters stress responding throughout the lifespan. While the teratogenic effects of ethanol may be mediated by direct actions on the developing HPA axis, evidence from this study suggests that ethanol-induced changes to HPG organizational effects may also underlie HPA axis dysfunction. Specifically, prenatal ethanol may be altering the perinatal determination of T and E2 receptor number and/or function at various levels of the HPA axis, or in HPA afferents.

Evidence from this study as well as previous research fom our lab (Lan et al., 2005; Sliwowska et al., 2005) suggests that the sex steroid regulation of hippocampal MR and GR and hypothalamic CRF and AVP is altered by prenatal ethanol exposure. T and E2 receptors are located in the hippocampus and may influence MR and GR activity through direct interactions (Burgess and Handa, 1993; Kerr et al., 1996; Shughrue and Merchenthaler, 2000; Weiland et al., 1997). T receptor distribution in the PVN is almost to the exclusion of ACTH regulating neurons (Bingham et al., 2004), and therefore mediates CRF and AVP transsynaptically, via activation of the POA, central and medial nuclei of the amygdala, and BNST (Herman et al., 2003; Simerly et al., 1990; Viau, 2002; Zhou et al., 1994). There is some evidence to suggest that E2 acts directly on CRF and AVP containing neurons (Lund et al., 2004; Miller et al., 2004), although transsynaptic mechanisms may also be playing an important role in integrating the CRF and AVP response to E2 (Axelsen and Van Leeuwen, 1990; Herman et al., 2003; Simerly et al., 1990; Viau et al., 2005).

In the first follow up study, T (males) and E2 (males and females) receptor mRNA and protein levels as well as binding affinity in the hippocampus, the PVN (E2 receptors only) and in PVN projecting neurons (T and E2 receptors) will be compared between E, PF and C rats. HPA responsivity to gonadal hormones matures over the course of puberty (Gomez et al., 2004; Romeo et al., 2004; Viau et al., 2005), so to ensure that differences in sex steroid receptor systems are due to prenatal ethanol-induced changes to the pre- and perinatal hormone environments, rather than to changes in pubertal development, prepubertal rats will again be tested.

If we find that sex steroid receptors are altered in E rats, we will next determine whether changes are due to prenatal ethanol-induced alterations to HPG organizational effects. Specifically, at parturition male and female rat pups will either be left intact or treated with an androgen antagonist and/or aromatase inhibitor (males), or with E2 (females) during the neonatal period. This brief drug exposure has been shown to demasculinize and defeminize HPA activity in male and female rats, respectively (Orikasa and Sakuma, 2004; Patchev and Almeida, 1995; Pinilla et al., 1993; Seale et al., 2005). Pups will again be tested during the prepubertal period. If manipulation of sex steroid milieu over the critical period for HPG organizational effects eliminates group differences in sex steroid receptor systems as well as the altered sexual dimorphism of HPA responding, then we can conclude that prenatal ethanol exposure is mediating it's effects on differential HPA responding between E males and females, in part, by altering HPG organizational effects.

B. Clinical Implications

Pre- and postnatal environmental manipulations may interfere with development and result in structural and functional changes that persist throughout life. Fetal programming refers to the actions of environmental/non-genetic factors during a sensitive period of *in utero* development to permanently alter the development and organization of vulnerable systems (Seckl, 2004). One such environmental insult is alcohol, as it readily crosses the placental barrier and reprograms the fetal HPA axis, such that stress responding is altered in the offspring (Zhang et al., 2005). Using animal models, scientists have shown that prenatal ethanol exposure affects the HPA axis at multiple levels (Lee et al., 1990; Osborn et al., 2000; Weinberg, 1989), resulting in an inability to appropriately respond to and recover from stressor exposure. Furthermore, the effects are sexually dimorphic as E males and females have differentially altered HPA responsiveness to stressors (i.e. Weinberg 1988, 1992; Weinberg, Taylor et al. 1996). In the present study we show that prenatal ethanol exposure disrupts HPA/HPG interactions and differentially alters HPA responsiveness to stressors in E males and females in the prepubertal period, suggesting that disruptions to HPG organizational effects may be one mechanism underlying altered HPA activity.

Dysregulated HPA activity is associated with a number of mental health problems, such as depression, anxiety, and drug abuse (Charmandari et al., 2004; De Kloet et al., 1998; Herman et al., 2003). Consistent with the notion that prenatal alcohol exposure permanently alters the HPA axis, individuals with alcohol-related birth defects are at a higher risk for developing psychiatric-related conditions than the average population (Famy et al., 1998). Interestingly, prenatal alcohol exposure appears to alter the normal sexual dimorphism in the incidence of certain mental illnesses. For example,

while women are usually more prone to depression (Young and Altemus, 2004), clinical investigations in adults with FAS reveal that men and women are equally likely to develop depression (Famy et al., 1998). This finding suggests that in humans prenatal alcohol exposure may interfere with the normal HPA/HPG interactions, and it highlights the importance of understanding not only how the HPA system is altered following prenatal alcohol exposure but also how prenatal alcohol may interfere with the interactions between the HPA axis and other essential neuroendocrine systems, such as the HPG axis.

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