THE ROLE OF ESTRADIOL IN MEDIATING HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVITY IN FEMALE RATS PRENATALLY EXPOSED TO ETHANOL

by

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The University of British Columbia
Vancouver, BC Canada
ABSTRACT:

Prenatal ethanol exposure results in a broad range of physical, physiological and behavioral abnormalities. Experiments in this thesis focused on ethanol-induced alterations in physiological function. Two important systems that are altered by prenatal ethanol exposure are the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes, which are involved in the stress response and reproductive function. Previous studies have shown that rats prenatally exposed to ethanol (E) are typically hyperresponsive to stressors and have altered reproductive development and function. Furthermore, the normal sexual dimorphism of the HPA axis is differentially altered in E males and females, suggesting altered HPA-HPG interactions in E compared to control animals. Since the HPA and HPG axes develop in parallel and interact in a bi-directional manner, the aim of the present study was to determine the possible role of estradiol (E2) in the altered HPA response to stress in E females. We hypothesized that prenatal ethanol exposure would alter HPG development and activity and that altered HPA activity in E females may be due, at least in part, to altered HPA axis sensitivity to E2.

Female offspring from E, pair-fed (PF) and ad lib-fed (C) dams were tested in adulthood. Animals were randomly assigned to one of four surgical groups: Sham, Ovariectomized (OVX), OVX + low (OVX-L) or high (OVX-H) E2 replacement. At testing animals were terminated directly from the home cage or following 30 min restraint stress and plasma, organs and brains were collected for analysis.

E females had decreased uterine sensitivity to E2 compared to C females and lower GnRH mRNA expression/neuron in the MPOA following stress compared to PF females. Furthermore, acute stress failed to increase plasma E2 in E and PF females, as it
did in controls. Following OVX, E and PF females had lower basal plasma CORT levels compared to C females. In addition, under both basal and stress conditions, E females had lower plasma ACTH levels compared to C females overall. Moreover, under stress conditions, plasma ACTH levels in E females did not change across surgical treatments, while PF and C females had increased ACTH levels following OVX, which returned to Sham levels with E2 replacement. Also, under resting conditions, AVP mRNA expression was higher in E compared to C females and both CRH and AVP mRNA expression were increased in PF compared to C females.

The present data suggest that E females have altered HPG activity and HPA responsiveness to stressors as well as altered bidirectional interactions between the HPA and HPG axes. Overall, E females appear to be less responsive than controls to the effects of E2 on both reproductive and non-reproductive measures. Furthermore, E females appear to have decreased tissue responsiveness to E2 and altered HPG sensitivity to acute stress at various levels of the axis.
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LIST OF ABBREVIATIONS:

ACTH  adrenocorticotropic hormone
ADX  adrenalectomy
ANOVA  analysis of variance
ARBD  alcohol-related birth defects
ARND  alcohol-related neurodevelopmental disorder
AVP  arginine vasopressin
β-EP  β-endorphin
BAL  blood alcohol level
BW  body weight
C  control
CBG  corticosteroid-binding globulin
c  cubic centimetre
cm  centimetre
CNS  central nervous system
CORT  corticosterone
CRH  corticotropin-releasing hormone
DEX  dexamethasone
dl  decilitre
DTT  dithiotreitol
E  prenatally exposed to ethanol
E2  estradiol
EDTA  ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER(α/β)</td>
<td>estradiol receptor (α/β)</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FAS</td>
<td>fetal alcohol syndrome</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>G</td>
<td>gestation day</td>
</tr>
<tr>
<td>GAP</td>
<td>GnRH-associated peptide</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>hnRNA</td>
<td>heterogeneous nuclear ribonucleic acid</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>Kg</td>
<td>kilogram(s)</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>mg</td>
<td>milligram(s)</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre(s)</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
</tbody>
</table>
n sample size
NAD/NADH nicotinamide adenine dinucleotide (reduced form)
ng nanogram(s)
OVX ovariectomy
OVX-L ovariectomy with low estradiol replacement
OVX-H ovariectomy with high estradiol replacement
PBS phosphate buffer solution
PF pair-fed
pg picogram(s)
PN postnatal
POMC proopiomelanocortin
PR progesterone receptor
PVN paraventricular nucleus
r\(^2\) Pearson’s product-moment correlation
RIA radioimmunoassay
sc subcutaneous
SEM standard error of the mean
Sham sham ovariectomized
SSC standard saline citrate
TEA triethanolamine
ug microgram(s)
ACKNOWLEDGEMENTS:

“In the name of the best within you, do not sacrifice this world to those who are its worst. In the name of the values that keep you alive, do not let your vision of man be distorted by the ugly, the cowardly, the mindless in those who have never achieved his title. Do not lose your knowledge that man’s proper estate is an upright posture, an intransigent mind and a step that travels unlimited roads. Do not let your fire go out, spark by irreplaceable spark, in the hopeless swamps of the approximate, the not-quite, the not-yet, the not-at-all. Do not let the hero in your soul perish, in lonely frustration for the life you deserved, but have never been able to reach. Check your road and the nature of your battle. The world you desired can be won, it exists, it is real, it is possible, it’s yours”

- Ayn Rand

*Atlas Shrugged*

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CHAPTER 1: General Introduction

1.1 FETAL ALCOHOL SYNDROME:

Alcohol is a known teratogen which, when consumed during pregnancy, can lead to a spectrum of long-lasting deleterious effects on the developing fetus (Sampson et al., 1997). The effects of prenatal alcohol exposure run along a continuum ranging from subtle behavioral problems to perinatal death. The extent and severity of the child’s condition depends on several factors, such as peak blood alcohol level (BAL) achieved by the pregnant mother, her pattern and timing of alcohol consumption, polydrug use and genetic susceptibility (Phillips & Cragg, 1982; Bonthius et al., 1988a, 1988b, 1989, 1990; Goodlett et al., 1989; Schenker et al., 1990; Coles, 1994; Abel & Hannigan, 1995; Maier et al., 1997; Chen et al., 1998). Fetal Alcohol Syndrome (FAS) is the most severe outcome of prenatal alcohol exposure and was first reported by Lemoine et al. in 1968 and later by Jones and Smith in 1973. FAS is characterized by (a) prenatal and/or postnatal growth deficits, (b) craniofacial abnormalities, and (c) central nervous system (CNS) dysfunction (Jones & Smith, 1973). Individuals who do not meet the formal criteria for FAS but exhibit significant cognitive and behavioral deficits are identified as having partial FAS, alcohol-related birth defects (ARBD) or alcohol-related neurodevelopmental disorder (ARND) (Stratton et al., 1996; Mattson et al., 1998b; Warren & Foudin, 2001). The incidence of FAS in the United States is estimated to range from 0.5 – 2.0 per 1,000 births (May & Gossage, 2001), thus it is considered the most common non-hereditary cause of mental retardation today (Abel & Sokol, 1987).
The growth deficiency typical of children with FAS starts prenatally and continues into adulthood. Height, weight and head circumference are affected, although short stature and microcephaly appear to be the more long-lasting growth deficiencies (Streissguth et al., 1991). The characteristic facial features of a child with FAS include short palpebral fissures, long smooth philtrum, thin vermillion border, broad nasal bridge, and epicanthal folds (Jones & Smith, 1973; Mattson et al., 1998a). The CNS dysfunctions among individuals with FAS are diverse and can include mild to severe mental retardation (Streissguth et al., 1978, 1990, 1991; Mattson et al., 1997), hyperactivity (Hanson et al., 1976), attention deficits (Nanson & Hiscock, 1990), learning disabilities (Don et al., 1993), maladaptive behaviors (Streissguth et al., 1991) and psychotic and other mental illnesses (Streissguth et al., 1980; Mattson et al., 1998b; Famy et al., 1998). Physiological and neuroendocrine systems may also be altered by prenatal ethanol exposure. Studies in both humans and rodents have found alterations to the secretory function of gonads, adrenals and thyroid, as well as altered growth hormone (GH), prolactin and arginine vasopressin (AVP) secretion (Weinberg et al., 1986). The focus of this thesis will be on alterations to the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes induced by prenatal ethanol exposure.

1.2 ANIMAL MODELS OF FETAL ALCOHOL SYNDROME:

Animal models have been an invaluable tool in the study of prenatal alcohol exposure because they allow for control over factors that are uncontrolled for or are unethical to manipulate in human studies. For example, the dose, timing and duration of alcohol exposure, nutritional effects, social and environmental variables, genetic
background, multi-drug use and sample size can all be controlled in animal models. Also, with animal models, the mechanisms for an induced abnormality can be investigated (Riley & Meyer, 1984). Most studies have been conducted in rats because of their ease of handling, large litters, short gestation, low cost and the availability of extensive scientific data. They also have low variability in developmental landmarks and a relatively low rate of spontaneous malformations (Palmer, 1971).

Many of the effects of prenatal alcohol exposure found in humans have been replicated in animals (Norton & Kotkoskie, 1991). Retarded pre- and postnatal growth and development, including microcephaly, have been reported in a wide variety of species (Tze & Lee, 1975; Becker et al., 1994). Organ anomalies, such as craniofacial defects, limb malformations, cardiovascular malformations and urogenital anomalies, as well as immune system deficiencies, endocrine and reproductive dysfunction and CNS and behavioral alterations have also been observed (Randall & Taylor, 1979; Beauchemin et al., 1984; Becker et al., 1988, 1994; Norton & Kotkoskie, 1991).

Though rodents provide an excellent model of prenatal alcohol exposure, several issues should be considered. Firstly, rodents are born at a stage of brain development equivalent to the beginning of the third trimester of pregnancy in primates (Dobbing & Sands, 1979). Therefore, in order to study the effects of alcohol on brain development during the third trimester equivalent in humans, alcohol would have to be administered postnatally in the rat. Secondly, there are differences in metabolism among species. For example, rats metabolize ethanol at a higher rate than humans, so a higher dose must be used for results to be comparable to human studies. Thus, blood alcohol levels attained by
pregnant dams should be used for cross species comparisons rather than amount of alcohol consumed (Zajac & Abel, 1992).

There are a number of methods of alcohol administration. For acute administration, alcohol can be injected intraperitoneally or via a feeding tube directly into the stomach (intragastrically). Both methods give rise to high BALs and the dose of ethanol administered can be controlled. However, these methods require extensive handling of the pregnant females and may be stressful, which may itself affect the offspring. More chronic alcohol administration can occur either through a liquid diet (orally), through the drinking water, intragastrically or by inhalation (Ponnappa & Rubin, 2000). The simplest method for administering alcohol chronically is in the drinking water. However, most rodents will not consume alcohol voluntarily, and if placed in drinking water, will cut down their fluid intake. This not only results in low BALs but inadequate nutrition as well, since food intake decreases along with fluid intake. Thus this method may be unsuitable for most studies. Another method involves a liquid diet containing alcohol, which allows for better control over nutritional intake and yields higher BALs. The downside to a liquid diet is that animals consume greater amounts of water than they would with a pelleted diet. Increased fluid intake could affect water balance and/or kidney function, which could contribute to fetal distress (Weinberg, 1984). The liquid ethanol diet can also be administered intragastrically, which allows for more specific control of dose and timing of alcohol exposure. However, as mentioned before, intubation is probably stressful and involves extensive handling of the dam, which could confound the results. Lastly, alcohol can be administered through inhalation using a vapor chamber. While this method yields high BALs and requires minimal handling of
the animal, this route of alcohol administration does not relate to human situations, since human inhalation of alcohol vapor for several hours per day would occur only in limited circumstances (Maciejewski-Lenoir, 1993).

To control for the nutritional effects of prenatal alcohol exposure, control animals, matched by strain, age and body weight to the experimental animals, are included in animal models of prenatal alcohol exposure. A pair-fed (PF) group is always employed to account for the undernutrition that can occur with chronic alcohol consumption. Alcohol contains calories that are not associated with vitamins, minerals, proteins or other essential nutrients (empty calories), yet it has a high energy value (7.1 kcal/g) and may displace other food in the diet. Additionally, alcohol has anorexigenic effects, which may be further compounded in animal models of prenatal alcohol exposure since most experimental animals find the taste of alcohol aversive (Dexter et al., 1979; Sorette et al., 1980). In pair-feeding, the amount of diet and alcohol consumed are determined and PF animals are given identical amounts of diet with a caloric substitute, such as maltose-dextrin, isocalorically substituted for ethanol. Although PF animals are matched by body weight, there may still be differences in metabolic rate. Therefore, PF animals may be underfed or overfed compared to animals in the alcohol group. Furthermore, because PF animals are restricted in their diet intake, they are in a state of constant hunger. This has been shown to alter the pattern of the HPA response to stress in PF dams (Weinberg & Gallo, 1992). Thus, an ad libitum fed control (C) group should also be included in animal models of prenatal ethanol exposure since pair-feeding, in addition to serving as a nutritional control group, is itself a treatment condition. In the C group, animals are given ad libitum access to the liquid control or a pelleted diet with nutrient density adjusted so
that the daily intake of nutrients and calories is equivalent to animals receiving the liquid diets (Weinberg, 1984).

1.3 HYPOTHALAMIC-PITUITARY-ADRENAL AXIS:

The ability of an organism to maintain a relatively steady internal environment, or homeostasis, is essential for life. Stress is defined as a state of threatened homeostasis, by intrinsic or extrinsic variables, which is counteracted by a multitude of physiological and behavioral responses to ensure survival (Tsigos & Chrousos, 2002). The intrinsic or extrinsic variables that disrupt homeostasis are known as stressors. The stress response requires the integrated activation of neuroendocrine, immune and sympathoadrenomedullary systems.

The HPA axis is the key neuroendocrine system involved in the stress response. It consists of a cascade of hormones that are activated in response to stress. The cascade begins at higher brain centers with perception of the stressor. These higher centers activate the paraventricular nucleus (PVN) of the hypothalamus, which represents the final common pathway for the integration of the stress response in the brain (Vazquez, 1998). There are many neuronal inputs to the PVN that modulate the stress response, including input from catecholaminergic, serotonergic and cholinergic brain nuclei (Johnson et al., 1992; Vazquez, 1998). Two of the most important brain regions that send efferents to the PVN are the hippocampus and the amygdala. The hippocampus sends projections to the arcuate nucleus and ventromedial hypothalamic nucleus which exert negative feedback on PVN activity. The amygdala contains corticotropin releasing hormone (CRH) neurons with efferent pathways to the hypothalamus via the bed nucleus
of the stria terminalis (Dobson et al., 2003). These neurons have shown stress-induced increases in CRH release and lesions to the central nucleus of the amygdala block the adrenocorticotropic hormone (ACTH) and corticosterone (CORT) elevations following physical and psychological stressors (Chrousos et al., 1995). Factors such as emotion, pain, changes in blood pressure and cytokines also influence PVN activity (Johnson et al., 1992). Neurons of the PVN synthesize and secrete CRH and AVP (Johnson et al., 1992).

CRH is a 41-amino acid peptide that is secreted from the parvocellular division of the PVN (Vale et al., 1981; Antoni, 1986). CRH neurons project to the median eminence (ME) and terminate on the capillaries of the hypothalamo-hypophyseal portal vessels where CRH is released and transported to the anterior pituitary. In the anterior pituitary, CRH regulates the expression of proopiomelanocortin (POMC) and stimulates the release of POMC-derived peptides, such as ACTH and β-endorphin (β-EP). CRH neurons are also found in extrahypothalamic areas such as the midbrain, striatum, hippocampus, cerebral cortex, brainstem, spinal cord sympathetic ganglia and adrenal gland (Johnson et al., 1992). Also, CRH is produced in limbic areas where it controls behavioral, autonomic and visceral responses to stress (Koob et al., 1993).

AVP is expressed in two populations of neurons. It is coexpressed with CRH in the parvocellular neurons, where it is thought to stimulate ACTH release synergistically with CRH, and it is found in high concentrations in the neighboring magnocellular neurons of the PVN, where it plays a role in hypovolaemia or changes in blood osmolarity (Herbert et al., 1992; Grino & Burgunder, 1992; Vazquez, 1998). There appears to be a reciprocal positive interaction between CRH and AVP, with each neuropeptide stimulating the secretion of the other (Tsigos & Chrousos, 2002). The
CRH:AVP ratio is thought to be governed by the intensity and duration of the stressor, with CRH predominating in response to acute, mild stressors whereas AVP levels increase after chronic, repeated or severe stress (Caraty et al., 1990; Ma et al., 1997, 1998). Thus increased AVP synthesis and release potentiates the secretion of CRH and may sustain ACTH release in response to chronic or repeated stress, which is otherwise inhibited by glucocorticoid negative feedback (Viau et al., 2001). Though AVP is a potent synergistic factor with CRH in stimulating ACTH secretion, AVP has little ACTH secretagogue activity alone (Lamberts et al., 1984).

POMC, the prohormone for ACTH, is synthesized in many brain areas, including the arcuate nucleus, zona incerta, lateral septum, nucleus accumbens, periventricular thalamus, periaqueductal gray, locus coeruleus, nucleus tractus solitarius, reticular formation, stria terminalis, medial amygdala and pituitary, as well as in the gastrointestinal tract and reproductive organs (Johnson et al., 1992). In the anterior pituitary, POMC is cleaved into ACTH and β-lipotropin, which is further broken down into smaller active fragments such as β-EP. The end product of POMC cleavage is thought to be dictated by the enzymes and pH of a given tissue or cell type (Johnson et al., 1992).

ACTH is a 39-residue peptide that is released into the systemic circulation where it stimulates glucocorticoid, aldosterone and adrenal androgen production and secretion by the zonae fasciculate, zona glomerulosa and zona reticularis, respectively, of the adrenal gland (Tellam et al., 2000). ACTH has a sensitizing effect on the adrenal cortex, such that prolonged exposure to ACTH will cause adrenal hypertrophy while a decrease
in ACTH secretion will cause adrenal atrophy. ACTH may also enhance attention, motivation, learning and memory retention (Johnson et al., 1992).

The release of glucocorticoids from the adrenal cortex into the general circulation is the final step in the HPA stress response and results in a multitude of behavioral and physiological effects. The primary glucocorticoid is cortisol in humans and CORT in rats and mice. Almost 95% of circulating CORT is bound to an alpha globulin called corticosteroid-binding globulin (CBG). The bound fraction is considered physiologically inactive while the smaller free-fraction is active. Glucocorticoids exert their effects through their ubiquitously distributed intracellular receptors of which there are two: type I or mineralocorticoid receptors (MR) and type II or glucocorticoid receptors (GR) (Munck et al., 1984). MRs are found mainly in neurons of limbic brain regions, such as the hippocampus, and thus play a role in modulating the response to environmental and emotional stimuli. They have a high affinity and specificity for CORT as an agonist, whereas aldosterone appears to be a competitive antagonist (Johnson et al., 1992). GRs are found in high concentrations in the hypothalamus, particularly in CRH neurons, although they are also found in other brain areas that contain POMC, including the hippocampus, lateral septum, amygdala and nucleus tractus solitarius (McEwen et al., 1968). These receptors bind CORT with a lower affinity than MRs. The main function of these receptors is negative feedback of CORT on ACTH release in response to stress. Under conditions of low CORT concentrations, MR is the primary receptor activated and thus is thought to play a key role in regulation of basal HPA activity, while at higher concentrations, MRs saturate and GRs take over and thus play a major role in feedback during stress to return the system to homeostasis (Vazquez, 1998). However, MRs and
GRs appear to complement each other in their modulation of both basal and stress responsiveness of the HPA axis.

The release of glucocorticoids in response to stress results in several adaptive responses: 1) Mobilization of energy. Along with catecholamines and glucagons, glucocorticoids promote glycogenolysis, gluconeogenesis, lipolysis and proteolysis while inhibiting glycogen, protein and fatty acid synthesis and glucose uptake by tissues. 2) Suppressed reproduction. Glucocorticoids inhibit the responsiveness and decrease the sensitivity of the HPG axis at various levels, resulting in decreased sex steroid secretion in both males and females. 3) Suppressed growth. Glucocorticoids have a suppressive effect on osteoblastogenesis and promote the apoptosis of osteoblasts and osteocytes, leading to decreased bone formation. Glucocorticoids may also increase bone resorption by increasing the lifespan of existing osteoclasts and promoting calcium loss through the kidneys and gut (Mushtaq & Ahmed, 2002). 4) Suppressed immunity. Glucocorticoids inhibit the release of most cytokines and decrease the sensitivity and activity of target cells. They also alter trafficking and function of immune cells, block maturation of developing lymphocytes and cause lysis of immune cells (Sapolsky, 1993; McEwen, 2000; Tsigos & Chrousos, 2002). Non-glucocorticoid mediated adaptive responses to stress include increased cardiovascular and cardiopulmonary tone via the sympathetic nervous system, suppressed digestion mediated by the autonomic nervous system and analgesia induced by opioids, such as β-EP which is produced in the anterior pituitary (Sapolsky, 1993).

Although transient, or time-limited, increases in plasma CORT are beneficial to an organism during times of stress, prolonged periods of stress and thus prolonged CORT
elevations can lead to negative outcomes. Repeated stress results in hypersecretion of glucocorticoids, as well as other hormones in the HPA axis cascade. Long-term elevations in plasma glucocorticoid levels lead to a constellation of pathological consequences. Fatigue, myopathy (wasting away of muscles) and steroid diabetes can arise from constant catabolism of proteins, fats and glycogen and the development of insulin resistance (Sapolsky, 1993; Tsigos & Chrousos, 2002). Anovulation, impotency and loss of libido occur from chronic inhibition of the reproductive system. Psychogenic dwarfism and bone decalcification arise from attenuated GH secretion, decreased responsivity of target tissues to GH and altered bone formation. Impaired disease resistance occurs due to the sustained immunosuppressive effects of glucocorticoids. Glucocorticoids also have numerous effects on the brain, thus hypersecretion could alter learning, memory, perception, vulnerability to depression, feeding and sleeping behavior and aggression (Sapolsky, 1993). Therefore, the hormones involved in the HPA axis require tight regulation in order to maintain the health and survival of an organism.

1.4 HYPOTHALAMIC-PITUITARY-GONADAL AXIS:

Reproduction is controlled by a network of neurons and a cascade of neuroendocrine hormones that together form the HPG axis. The pathway begins with a small population of 800-2000 gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus (Gore, 2002). These neurons are arranged in loose networks and are distributed rostrocaudally from the medial septum and diagonal band of Broca to the ventral anterior hypothalamus and are concentrated around the preoptic area and medial forebrain bundle (Freeman, 1994; Smith & Jennes, 2001). GnRH neurons project axons
to various sites within the brain, such as the organum vasculosum of the lamina terminalis, subfornical region, medial amygdala and periaqueductal central gray, although the majority extend to the ME and terminate on the capillaries of the hypothalamo-hypophyseal portal vessels (Smith & Jennes, 2001). Though GnRH neurons have a scattered distribution, they communicate with one another, resulting in the synchronous release of GnRH into the hypophyseal portal circulation (Merchenthaler et al., 1989; Rivest & Rivier, 1995; Lopez et al., 1998).

GnRH is a decapeptide that binds to receptors on gonadotrophs, stimulating the release of gonadotropins from the anterior pituitary (Freeman, 1994; Lopez, 1998). It is synthesized as a prohormone that consists of a 23 amino acid signal peptide, GnRH and a 56 amino acid C-terminal extension called GnRH-associated peptide (GAP) (Clarke, 1996). GAP is thought to be co-secreted with GnRH and has been shown to have potent prolactin-inhibiting activity in vitro (Nikolics et al., 1985). The release of GnRH into the hypophyseal portal circulation is pulsatile. The frequency and amplitude of GnRH release from the hypothalamus regulates the release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Clarke, 1996). The “GnRH pulse generator” is hypothesized to be a set of GnRH neurons with an intrinsic pulse that periodically fire a high frequency volley of action potentials leading to the secretion of a GnRH pulse into the hypophyseal portal vessels (Levine, 1997). Synchronization of these neurons likely involves complex interactions among neurotransmitters, neuropeptides, nitric oxide and GnRH itself, which are then under the control of sex steroids (Levine, 1997). Control of the pulsatile release of GnRH by sex

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steroids most likely involves interneurons, as GnRH neurons have very few receptors for steroid hormones (Shivers et al., 1983; Laflamme et al., 1998; Butler et al., 1999).

LH and FSH are glycoproteins composed of heterodimeric polypeptides with a common α-subunit and a unique β-subunit that confers biological specificity. They are also released in a pulsatile manner as a consequence of the episodic release of GnRH into the portal circulation. In males, LH promotes the synthesis of testosterone by Leydig cells in the testicular interstitium, while FSH acts on Sertoli and germ cells in the seminiferous tubules to stimulate spermatogenesis (Amory & Bremner, 2003). In females, gonadotropins have a more complex role due to the estrous or menstrual cycle. In general, LH is required for ovarian steroidogenesis and ovulation while FSH induces the growth of follicles in the ovary by acting on granulosa cells. FSH also plays a role in estrogen and progesterone synthesis and secretion.

The cascade of hormones in the HPG axis ends with the release of sex steroids from the gonadal organs into the general circulation. Estrogen is produced by the ovary and has a multitude of effects both on reproductive and non-reproductive function. The systemic effects of estrogen include the development and maintenance of secondary sex characteristics, bone maturation and structure, stimulation of hepatic synthesis of proteins and a role in mineral metabolism (Yen & Jaffe, 1986). The role of estrogen in reproduction is evident through its effects on the growth, development and function of the uterus, vagina, cervix and fallopian tubes, sexual development, growth of breast tissue and its role in ovarian growth, which will be discussed in greater detail below (Yen & Jaffe, 1986). Estrogen also affects both organizational and activational events in the brain. Organizational events, which refer to permanent changes in morphogenesis and
differentiation of organ systems, are dependent on steroid milieu and in general, androgens aromatized into estrogen initiate masculinization of the rodent brain (Cooke et al., 1998). In females, the lack of either testosterone or estrogen in the fetus, results in feminization of the brain (Silbergeld et al., 2000). Activational events, which refer to the responses of cells and organ systems to hormones after differentiation and organization, include the estrous cycle in females, where estrogen and progesterone fluctuate across the estrus cycle, thereby maintaining reproductive function. These effects of estrogen may be mediated through specific neurotransmitter pathways, such as gamma-amino butyric acid, and neuropeptides whose expression are sexually dimorphic and responsive to estrogen (Silbergeld et al., 2002).

Progesterone is secreted by the developing follicle and corpus luteum of the ovary and by the adrenal cortex. Progesterone secreted by the developing follicle stimulates the release of proteolytic enzymes from thecal cells, induces migration of blood vessels into the follicle wall and stimulates prostaglandin secretion in follicular tissues in preparation for ovulation. Once ovulation has occurred, large amounts of progesterone are secreted by the corpus luteum to prepare the endometrium for implantation. If pregnancy occurs, chorionic gonadotropin secreted by fetal trophoblasts prolongs the secretion of corpus luteum progesterone, which is essential to maintain early pregnancy (Yen & Jaffe, 1986). Adrenal progesterone constitutes about 50% of plasma progesterone during follicular development and thus also plays a role in inducing ovulation.

Leydig cells are the primary sites of testosterone synthesis and the testes secrete 95% of circulating testosterone in males (Yen & Jaffe, 1986). Testosterone, like estrogen, has a plethora of effects on various tissues: 1) differentiation of Wolffian ducts, external
genitalia and the brain in male fetuses; 2) stimulation of linear body growth and muscular development; 3) stimulation of adult maturation of external genitalia and accessory sexual organs; 4) induction of the enlargement of the larynx and thickening of vocal cords; 5) stimulation of facial and pubic hair growth; 6) facilitation of libido; 7) production of aggressive behavior. Testosterone, as well as estrogen and progesterone, provide negative feedback to the HPG axis, such that increased gonadal steroid secretion leads to inhibition of LH release from the anterior pituitary. Regulation of LH release takes place through alterations to the amount of GnRH secreted by the hypothalamus and/or modulation of the sensitivity of pituitary gonadotrophs to GnRH.

In female rodents, the level of neuroendocrine hormones, and therefore circulating sex steroids, fluctuates across the estrous cycle (Figure 1). The estrous cycle is 4 to 5 days in the rat and consists of four phases – proestrus, estrus, metestrus and diestrus - as exemplified by vaginal cytology. During proestrus, smears consist mainly of nucleated epithelial cells, while estrus is characterized by an abundance of cornified cells with some nucleated epithelial cells or leukocytes. In metestrus, smears typically have an equal number of nucleated epithelial cells, cornified cells and leukocytes, while diestrus is characterized primarily by an abundance of leukocytes (Montes & Luque, 1988; Marcondes et al., 2002). Mean durations of the stages in a 4-day estrous cycle are 12 hrs proestrus, 30 hrs estrus, 6 hrs metestrus and 48 hrs diestrus (Montes & Luque, 1988).

Females are endowed with a surplus of oocytes, which remain in meiotic prophase surrounded by a single layer of granulosa cells until puberty and the onset of the estrous cycle. During proestrus, oocytes in ovarian follicles are selected for maturation and undergo their first meiotic division, resulting in the reduction of the chromosome
number from diploid to haploid. The recruitment, selection and maturation of follicles are triggered by a rise in FSH levels. Elevated levels of FSH induce an increase in the numbers of FSH receptors, which reflects an increase in the number of granulosa cells rather than an increase in the number of receptors per cell. FSH also induces granulosa cells to produce an enzyme responsible for the aromatization of estradiol (E2), an estrogen, from androgens. The resulting increase in E2 leads to an increase in E2 receptors, independent of FSH, and stimulates the rapid division of granulosa cells, promoting follicular growth. E2 also enhances the ability of FSH to recruit LH receptors on granulosa cells, which then leads to the production of progesterone. The preovulatory secretion of progesterone may serve to induce the progressive increase in basal LH levels despite increasing plasma E2, which should have a negative feedback effect on the HPG axis. During this time, theca interna cells develop LH receptors, leading to the synthesis of androgens that are converted to E2 by granulosa cells.

Ultimately, in each cycle, a dominant follicle is selected through its high capacity for E2 production. The dominant follicle inhibits FSH secretion via E2 and inhibin feedback, thereby impeding the maturation of other follicles, while maintaining its dominance through a local positive feedback of E2. E2 increases until the level exceeds a threshold at which point the negative feedback effect of E2 is converted into a positive feedback loop, leading to an LH surge. The positive-feedback effect is exerted at the level of the hypothalamus to increase GnRH secretion and at the level of the pituitary to increase pituitary gonadotrope responsiveness to GnRH. The LH surge leads to various changes within the preovulatory follicle. There is a shift from E2 to progesterone.
production in granulosa cells, a rapid increase in LH receptors on granulosa cells, the
resumption of meiosis and an increase in prostaglandins in preparation for ovulation.

Ovulation of the haploid egg occurs in early estrus at which point the secretion of
LH and FSH, and thus E2 and progesterone, falls rapidly to basal levels. Metestrus is
characterized by a second peak in progesterone, which is produced by the newly formed
corpus luteum. Luteal cells require an adequate number of LH receptors and pulsatile LH
secretion for the maintenance of corpus luteum function. In the absence of fertilization,
progesterone secretion declines through diestrus. If, however, fertilization occurs the
anterior pituitary secretes sufficient amounts of LH to rescue the corpus luteum until
chorionic gonadotropin produced by the placenta takes over and maintains elevated
progesterone levels throughout pregnancy. E2 levels begin to rise in late metestrus
through diestrus to again reach peak levels by proestrus.

1.5 BIDIRECTIONAL INTERACTIONS BETWEEN THE HPA & HPG AXES:

It has been well established that sex differences exist in HPA axis activity under
both basal and stress conditions and conversely, stress activation of the HPA axis
modulates reproductive function. Thus, the HPG axis has the ability to modulate HPA
function at various levels of the axis and the HPA axis has the ability to modulate HPG
function at various levels of the axis. In general, females produce a quantitatively greater
endocrine response to psychological and physical stressors than males and this is
probably due to the stimulatory effect of estrogen and the inhibitory effect of testosterone
on the HPA axis (Kitay, 1961a, 1961b; Lund et al., 2004). This sexual dimorphism is
found at various levels of the HPA axis. Females have a higher concentration of CORT in
the hippocampus, hypothalamus, amygdala and cortex, and cytosolic CORT receptors in
the hippocampus have a higher level of expression and binding capacity for CORT than
in males (Turner & Weaver, 1985; Patchev et al., 1995). At the level of the pituitary,
numerous groups have reported that female rats release more ACTH in response to a
variety of stressors than males (Weinberg, 1992a; Handa et al., 1994a; Rivier, 1999).
Also, female rats release more ACTH in response to CRH and AVP than males (Spinedi
et al., 1994; Rivier, 1999). At the level of the adrenals, female rats have been shown to
secrete greater levels of CORT than males under both basal and stress conditions (Kitay,
1961a). Female rats have also been shown to have a greater diurnal variation in plasma
CORT concentrations than males (Critchlow et al., 1963).

Many studies have investigated the activational effects of estrogen on the HPA
axis. Estrogen administration increases basal CORT secretion as well as the ACTH and
CORT response to physical and psychological stressors in both males and females
(Burgess & Handa, 1992; Handa et al., 1994b). Studies have shown that ovariectomy
(OVX) reduces basal CORT levels and estrogen replacement increases them (Kitay,
1963; Ramaley, 1976). In addition, OVX rats with estrogen replacement have higher
poststress CORT levels than controls (Viau & Meaney, 1991). This is in keeping with
studies showing that peak estrogen levels during the proestrus phase of the estrous cycle
produce elevated CORT levels under basal and stress conditions (Raps et al., 1971;
Buckingham et al., 1978; Viau & Meaney, 1992; Carey et al., 1995; Atkinson & Waddell,
1997). Plasma AVP also parallels estrogen levels during the estrous cycle, as does AVP
protein and CRH mRNA content in the PVN, which are both increased at proestrus
(Skowsky et al., 1979; Greer et al., 1986; Bohler et al., 1990). Estrogen prolongs ACTH
secretion, suggesting that estrogen-enhancement of stress responsiveness may be through impairment of CORT receptor-mediated negative feedback (Burgess & Handa, 1992). Indeed steady-state levels of MR and GR mRNA as well as their binding capacity were found to be decreased in neuroendocrine tissues regulating CORT and ACTH secretion with estrogen treatment (Burgess & Handa, 1993; Carey et al., 1995). Estrogen has also been found to increase CORT production by the adrenal cortex as well as increase the duration of the CORT response to stress (Kitay, 1963; Burgess & Handa, 1992). Anterior pituitary sensitivity to CRH containing hypothalamic extracts was also increased in the presence of estrogen (Coyne & Kitay, 1971). Furthermore, parvocellular neurons of the hypothalamus increase CRH mRNA transcription in response to estrogen (Lightman & Young, 1989). In humans, the CRH promoter region contains five half-palindromic estrogen-responsive element motifs, thus there is estrogenic transcriptional regulation of the CRH gene (Beato, 1989; Vamvakopoulos & Chrousos, 1993). This suggests that the CRH gene may be a potentially important target of ovarian steroids and a mediator of the sexual dimorphism of the HPA axis response to stress.

Although estrogen generally appears to enhance the stress response there is also evidence in the literature suggesting an inhibitory role for estrogen. Chronic estrogen treatment was shown to decrease hypothalamic CRH immunoreactivity in OVX rats (Haas & George, 1988) as well as plasma ACTH levels in response to immune challenge and noise stress (Dayas et al., 2000). Additionally, estrogen treatment of OVX rats induced a decrease in CRH mRNA levels, which was eliminated with adrenalectomy (ADX) (Paulmyer-Lacroix et al., 1996). This suggests that the inhibitory effect of estrogen on CRH mRNA expression is mediated by the adrenal gland such that estrogen
potentiates CORT inhibition of CRH mRNA expression. Furthermore, Young et al. (2001) reported suppression of the ACTH, but not CORT, increase in response to stress following short-term exposure to low doses of estrogen. Thus estrogen appears to have both stimulatory and inhibitory potential on HPA axis activity. The discrepancies in the role of estrogen may due to the experimental paradigm used and the dose and length of estrogen exposure. In general, acute estrogen replacement appears to be stimulatory (Burgess & Handa, 1992; Carey et al., 1995), while chronic estrogen replacement appears to be inhibitory to HPA axis activity (Haas & George, 1988; Dayas et al., 2000). Also, high doses of estrogen replacement appears to be stimulatory (Viau & Meaney, 1991; Burgess & Handa, 1992; Carey et al., 1995) while low doses appear to be inhibitory to HPA axis activity (Young et al., 2001).

The role of progesterone in the modulation of the HPA axis is unclear in the literature. The treatment of estrogen-primed OVX rats with progesterone resulted in basal HPA activity comparable to that of animals treated with estrogen alone and did not result in the attenuation of the magnitude of the stress response (Carey et al., 1995). Also, OVX animals treated with progesterone alone were comparable in basal ACTH responsiveness to OVX controls suggesting that progesterone does not play a role in mediating HPA activity (Carey et al., 1995). However, progesterone has been reported to decrease the binding affinity of hippocampal MR and to reverse the estrogen-induced decrease in MR binding capacity (Carey et al., 1995). Progesterone may be exerting its effects through two different mechanisms, ie. an antagonistic effect on estrogen, and binding with high affinity to MR in a competitive manner. In vitro studies also suggest an inhibitory role for progesterone on HPA function. Progesterone has been shown to inhibit hypothalamic
CRH and pituitary ACTH release in vitro and to inhibit the CRH-induced release of ACTH from cultured pituitaries (Jones & Hillhouse, 1976; Buckingham, 1982). Progesterone has also been shown to have a faster binding time than glucocorticoids to GR, albeit at a different binding site, and progesterone can increase the rate of dissociation of glucocorticoids from GR (Rousseau et al., 1972).

Testosterone appears to have primarily an inhibitory effect on the HPA axis. Castration of prepubescent male rats increased the CORT response to physical stressors and injection of testosterone was effective in returning CORT levels to that of intact males (Gaskin & Kitay, 1971). Castration of adult male rats increased both the ACTH and CORT response to physical and psychological stressors (Handa et al., 1994a). This effect is most likely mediated via changes in CRH containing neurons of the PVN. Testosterone has also been shown to inhibit the ACTH response to stress in a dose-dependent manner, which was strongly correlated with resting-state AVP content, but not CRH content in the ME, suggesting that gonadal influences on HPA activity may be particular to AVP-expressing parvocellular neurons of the PVN (Viau & Meaney, 1996). The actions of testosterone on AVP expression appear to be mediated indirectly via neurons upstream from the PVN, since androgen receptors are not expressed by medial parvocellular neurons (Zhou et al., 1994).

As noted above, there is bi-directional interaction between the HPA and HPG axes. While the HPG axis modulates HPA activity at various levels of the axis, the HPA axis also has the ability to modulate HPG axis activity. It is important to note that while a variety of stressors can alter HPA activity, usually only intense and/or chronic stressors have an effect on reproduction (Rivest & Rivier, 1995). Also, the nature of the HPG axis
response to stress depends on several factors such as the species, age, sex of the animals, the type and duration of the stressors, and the gonadal steroid milieu (Buckingham et al., 1997).

CRH has been shown to decrease GnRH secretion and therefore LH release as well. Intracerebroventricular (icv) administration of CRH attenuated GnRH release into portal circulation in female rats (Petraglia et al., 1987). Additionally, icv, but not peripheral injection of CRH caused a rapid and dose-related inhibition of LH secretion in OVX rats and inhibited ovulation in intact rats (Rivier & Vale, 1984). Furthermore, Roozendaal et al. (1995) reported a complete inhibition of the proestrus LH surge and ovulation in intact cycling rats following a 5 hr restraint stress. Other neurogenic and metabolic challenges have also been shown to decrease plasma LH, and these effects were reversed by administration of CRH antagonists in gonadectomized animals (Rivier et al., 1986; Maeda et al., 1994; Nagatani et al., 1996). The influence of CRH on GnRH neurons may be mediated through synaptic contacts between CRH axon terminals and dendrites of GnRH-secreting neurons in the medial preoptic area (MPOA) (MacLusky et al., 1988; Rivest et al., 1993). The origins of the CRH neurons that interfere with GnRH neuronal activity remain unknown. The CRH-GnRH neuronal interactions could be either local connections from CRH- and GnRH-containing cells located within the MPOA or CRH neural projections from other hypothalamic and extrahypothalamic areas of the brain, such as the amygdala, lateral hypothalamic area or the bed nucleus of the stria terminalis (Swanson et al. 1983; Simerly & Swanson, 1988). CRH may also be exerting its effects through an indirect mechanism. For example, CRH may act as a secretagogue
for β-EP secretion from the arcuate POMC neurons, which then exerts a tonic inhibition on GnRH secretion (Ferin et al., 1984; Nikolarakis et al., 1986).

AVP has been shown to attenuate the preovulatory LH surge and suppress LH secretion by decreasing LH pulse amplitude in female rats (Cates et al., 1999). Estrogen causes a greater suppression of pulsatile LH secretion in response to various stressors. The mechanism by which estrogen sensitizes the HPG axis to the inhibitory influences of stressors is either directly, via GnRH neural systems, and/or indirectly, via changes in neurotransmitter systems implicated in stress-induced suppression of GnRH and LH secretion (Cates et al., 1999). AVP-containing neurons have been shown to have direct synaptic connections with GnRH neurons as well as estrogen receptors and AVP has been shown to inhibit GnRH release from GT1 cells, a GnRH secreting hypothalamic cell line (Weiner, 1992; Hrabovszky et al., 1998). AVP has also been shown to induce β-EP release from rat hypothalamic tissue in vitro and opioid neurons have been reported to have direct synaptic contact with GnRH neurons and express estrogen receptors (Bronstein & Akil, 1990; Simerly et al., 1996). Therefore, both direct and indirect mechanisms for the estrogen-sensitization of the inhibitory effects of AVP on the HPG axis are plausible. Estrogen also has a sensitizing effect on CRH. Estrogen treatment has been reported to increase CRH mRNA expression in the PVN of ovariectomized rats (Patchev & Almeida, 1996; Li et al., 2003). And as mentioned above, CRH mRNA levels were shown to be greatest during proestrus when estrogen levels are at their peak (Nappi et al., 1997). In males, AVP transiently inhibits basal LH release (Rivier & Vale, 1985; Roozendaal et al., 1996).
CORT and ACTH appear to have both stimulatory and inhibitory effects on reproduction in females depending on the length of exposure and the presence or absence of estrogen priming (Brann, 1991a). Acute stress, ACTH or CORT administration usually results in facilitation of reproductive function. Acute stress has been shown to increase fertility in aged, non-cycling mice and to increase LH secretion in estrogen primed female rats (Paris et al., 1973; Briski & Sylvester, 1988). Both acute ACTH and CORT treatment have been reported to facilitate LH and FSH secretion in estrogen primed female rats and acute CORT has also been shown to facilitate ovulation and sexual maturation in estrogen primed rats (Brann et al., 1990, 1991b). Chronic stress, ACTH or CORT administration is usually inhibitory to reproduction. All three have been reported to suppress LH secretion, inhibit sexual maturation and impair ovulation, fertility and pregnancy (Ramaley, 1974; Brann, 1991a; Rivier & Rivest, 1991; Dobson & Smith, 2000). Modulation of the HPG axis by stress, ACTH and CORT appear to be through alterations to GnRH secretion and/or alterations to pituitary responsiveness to GnRH (Brann, 1991a, 1991c; Breen, 2004). CORT may also exert direct effects at the level of the ovary. CORT has been shown to inhibit aromatase activity, which would thereby reduce circulating estrogen levels, and stimulate tissue plasminogen activator activity in granulosa cells, which is thought to play an important role in ovulation (Hsueh & Erickson, 1978; Wang & Leung, 1989).

Generally, chronic activation of the HPA axis has negative consequences for reproductive function, whether it is at the level of the hypothalamus, pituitary or gonads. In terms of the estrous cycle, this may be a mechanism whereby unfavorable environmental conditions for reproduction would signal an inhibition of the HPG axis.
Gonadal steroids can have both protective and antagonistic effects on the development of HPA axis dysregulation. For example, progesterone binding to glucocorticoid receptors and functioning as an antagonist may prevent receptor down-regulation and therefore the negative effects of CORT hypersecretion. Estrogen, on the other hand, increases HPA axis sensitivity to stressors and a number of diseases associated with stress are more common in females, including depression and panic disorder (Young et al., 1995). Thus, the bidirectional interaction between the HPA and HPG axes is an integral part of homeostasis.

1.6 EFFECT OF PRENATAL ETHANOL EXPOSURE ON THE HPA & HPG AXES:

Ethanol consumption by pregnant dams alters maternal HPA axis activity, such that maternal adrenal weights and both basal and stress levels of CORT are increased above those in controls (Weinberg & Bezio, 1987, 1989). Furthermore, CBG binding capacity was found to be similar between ethanol-consuming females and controls (Weinberg & Bezio, 1987), suggesting that increased CORT levels are due to hyperresponsiveness of the maternal HPA axis and are functionally important. These changes occur early in pregnancy, continue throughout gestation and are independent of maternal nutritional status (Weinberg, 1982; Weinberg & Bezio, 1987). Because CORT can cross the placenta in both directions, ethanol-induced increases to maternal HPA axis activity can have deleterious consequences on the developing fetal HPA axis, which is functional before birth (Zarrow et al., 1970; Dupouy et al., 1975; Milkovic et al., 1973, 1976; Chatelain et al., 1980; Tulchinsky & Ryan, 1980). Ethanol can also cross the
placenta and may itself directly stimulate the fetal HPA axis (Weinberg, 1989). At birth, E neonates have elevated plasma, brain and adrenal levels of CORT and decreased CBG binding capacity compared to control animals (Kakihana et al., 1980; Taylor et al., 1982a, 1983; Weinberg, 1989). E neonates also have elevated plasma but reduced pituitary levels of β-EP (Angelogianni & Gianoulakis, 1989). At 3-5 days of age, basal CORT levels are normalized in E animals but E neonates are typically hyporesponsive to stressors, and this blunted stress responsiveness continues throughout the preweaning period (Taylor et al., 1982a, 1986; Weinberg, 1989; Angelogianni & Gianoulakis, 1989). Following weaning (approximately 22 days of age) and continuing into adulthood, E animals are typically hyperresponsive to stressors although the response to stress may vary depending on the type of stressor, time course, hormonal endpoint measured, species and gender (Weinberg, 1982, 1988, 1989; Nelson et al., 1986). Basal plasma CORT and ACTH levels are typically normal in adult E animals (Weinberg, 1982; Taylor et al., 1983). However, in response to stressors such as restraint (Weinberg, 1988), cardiac puncture (Taylor et al., 1982b), noise and shaking (Taylor et al., 1982b), footshock (Nelson et al., 1986), novel environments (Weinberg, 1988), cold (Angelogianni & Gianoulakis, 1989) and ether (Weinberg, 1982; Angelogianni & Gianoulakis, 1989), E animals show increased elevations of CORT and/or ACTH, and/or a delayed return to basal levels.

Though both E males and females exhibit altered HPA axis responses to stressors, prenatal ethanol exposure differentially alters the normal sexual dimorphism of the HPA axis in males and females. To cite just a few examples, following a 60 min restraint stress, E females showed more prolonged CORT elevations compared to control females whereas E males did not (Weinberg, 1988). Similarly, ethanol exposure from postnatal
days 4 to 9 resulted in an increased CORT stress response to swim stress in female but not male rats (Kelly et al., 1991). In contrast, following a 4 hr restraint stress, E males, but not E females, showed more prolonged CORT elevations compared to control males (Weinberg, 1992a). Also, E males showed a reduced CORT response to 5 days of unpredictable novelty stress, whereas E females showed a trend toward an increased CORT response (Weinberg, 1992b).

Multiple mechanisms could mediate the HPA hyperresponsiveness in E animals. These include, increased drive to the PVN, increased sensitivity of the pituitary to secretagogues and delayed/deficient negative feedback regulation. Data from our lab and others, discussed below, suggest that all three mechanisms may play a role in the HPA hyperresponsiveness seen in E animals.

Increased drive is suggested by several experimental findings. E males exhibited increased basal CRH mRNA in the PVN and POMC mRNA in the pituitary compared to controls (Redei et al., 1993). Also, PVN mRNA levels of the immediate early genes c-fos and NGFI-B, and CRH hnRNA were greater in E males and females compared to controls following footshock and endotoxemia, suggesting enhanced stimulatory inputs to the PVN (Lee et al. 2000). Furthermore, under basal conditions, removal of the CORT feedback signal by ADX resulted in significantly greater plasma ACTH levels in E than in controls, again suggesting either enhanced stimulatory inputs to the hypothalamus, and/or increased pituitary sensitivity to secretagogues (Glavas et al., 2001). In support of this latter possibility, CRH infusion following HPA suppression by dexamethasone (DEX) resulted in increased plasma ACTH in E males and increased plasma ACTH and CORT levels in E females compared to controls (Osborn et al., 2000). Thus, increased
HPA axis activation may be due to increased tone or increased stimulatory inputs to the PVN in response to stress or due to increased pituitary sensitivity to secretagogues.

Deficits in CORT negative feedback mechanisms may also contribute to HPA axis hyperresponsiveness. Following DEX administration, E females had increased stress levels of CORT compared to controls at the trough of the CORT circadian rhythm and both E males and females had increased stress levels of CORT and/or ACTH compared to controls at the circadian peak (Osborn et al., 1996). These data suggest deficits in pituitary-adrenal response inhibition in E compared to control animals. However, alterations in hippocampal glucocorticoid receptor binding do not appear to underlie the possible feedback deficits seen in E animals (Weinberg & Petersen, 1991; Kim et al., 1999).

Development and maturation of the HPG axis is also disrupted by prenatal ethanol exposure in both male and female offspring. Since this project focused on female rats prenatally exposed to ethanol, only the literature on females will be discussed here. E females have been shown to be delayed in their sexual maturation and have an earlier incidence of acylicity, a marker of reproductive aging (Esquifino et al., 1986; McGivern et al., 1992a, 1992b, 1995). Delayed sexual maturation is evidenced by delayed onset of puberty, as measured by age of vaginal opening, delayed onset of behavioural estrus and delayed onset of FSH secretion, which is thought to play a role in initiating ovarian activity (Hard et al., 1984; Esquifino et al., 1986; McGivern et al., 1992a; Wilson et al., 1997). Delays in puberty in E animals may be due to increased inhibition of GnRH neurons and functional impairment of LH secretion. Creighton-Taylor and Rudeen (1991b) suggested increased opiate inhibition of GnRH neurons as one possible
mechanism. Alterations in the number or morphology of GnRH neurons have also been implicated in delayed puberty (McGivern et al., 1992b). Decreased stimulation of the ovaries by gonadotropins may then result in insufficient or delayed increases in peripubertal estrogen levels, delaying vaginal opening.

Studies have demonstrated decreased LH levels in E fetuses at 18 days of gestation, in prepubertal and peripubertal E females and in ovariectomized adult E females, thus indicating altered developmental patterns of LH secretion in these animals (Guerri et al., 1984; Handa et al., 1985; Udani et al., 1985; Esquifino et al., 1986; Morris et al., 1989). There is also a decrease in the pulse amplitude of LH and FSH secretion in OVX E rats, and a decreased and delayed preovulatory-like surge of LH in response to estrogen treatment in adult ovariectomized E females (Handa et al., 1985; Wilson et al., 1995). Furthermore, hypothalamic GnRH content is reported to be decreased in E females compared to controls, as was anterior pituitary LH release in response to GnRH and estrogen stimulation in vitro (Morris et al., 1989; Creighton-Taylor & Rudeen, 1991a, 1990). A single acute prenatal exposure to ethanol significantly decreased GnRH neurons in the perinatal period, which is a critical period for sexual differentiation of brain and behaviour (Gavin et al., 1994). However, postnatal catch-up growth likely occurs, as there are no changes in number of GnRH neurons in adult E females, although some studies have reported a trend toward altered distribution of GnRH neurons, with E animals having more neurons in the caudal than rostral areas (McGivern & Yellon, 1992b; Gavin et al., 1994; Wilson et al., 1995). Ethanol-induced disturbances in perinatal GnRH neuronal development may account for altered reproductive and non-reproductive sexually dimorphic behaviours. Masculanization of saccharin consumption patterns, maze
learning performance and play behavior have been observed in E females (McGivern et al., 1984, 1987; Meyer & Riley, 1986). Also, there appears to be a marked decrease in maternal behavior by E females (Barron & Riley, 1985). Ovarian weights of adult E females were shown to be greater than controls, suggesting increased sensitivity to gonadotropin stimulation, possibly as a compensatory mechanism for decreased gonadotropin synthesis and release (McGivern et al., 1992a). Thus there are alterations at all levels of the HPG axis as a result of prenatal ethanol exposure.

1.7 Thesis Objective:

As the HPA and HPG axes develop in parallel and interact in a bi-directional manner, and as prenatal ethanol exposure is disruptive to both axes, the altered response of the HPA axis to stress in E animals may be due, in part, to altered HPG development or activity. The aim of the present study was to determine the possible role of E2 on the altered HPA response to stress in female rats prenatally exposed to ethanol. We tested the following hypotheses: 1) prenatal ethanol exposure alters HPG development and activity; and 2) altered HPA activity in E females may be due, at least in part, to altered HPA axis sensitivity to gonadal steroids, specifically E2. These hypotheses were tested by manipulating plasma E2 levels, through the use of four surgical treatment groups, and testing animals under both basal and stress conditions. OVX was used to assess HPA axis activity without the effects of E2. If the HPA axis of E animals was differentially sensitive to E2, then removal of the main source of E2 would either eliminate or reveal any differences between Sham E and control animals. OVX plus E2 replacement using either, low or high doses of E2, were used to study the effects of physiological levels of
E2 similar to those found at diestrus and proestrus, respectively, on HPA activity. If OVX eliminated HPA activity differences between E and control animals, then these differences should be reinstated with E2 replacement. Contrasting responses in OVX vs OVX with E2 replacement, as well as OVX-L versus OVX-H, may also reveal differences in the sensitivity of the HPG axis to estradiol at the level of the hypothalamus and pituitary in E compared to control animals. CRH and AVP mRNA in the PVN of the hypothalamus, plasma ACTH and CORT, and thymus and adrenal weights were measured as an index of HPA activity. HPG development was measured by age of vaginal opening, which is a marker for the onset of puberty, and GnRH mRNA in the MPOA, plasma LH and uterine weight were measured as an index of HPG function.
Figure 1: Plasma Estrogen and Progesterone Levels Across the Estrous Cycle

Change in plasma estrogen and progesterone across the 4-day estrous cycle of the rat (Chateau & Boehm, 1996).

NOTE: Diestrus 1 is the same as Metestrus
CHAPTER 2: Materials and Methods

2.1 ANIMALS AND BREEDING:

Male (275-300g, n=18) and female (230-275g, n=50) Sprague-Dawley rats (Charles River, Montreal, PQ, Canada) were group-housed by sex for a 1-2 week adaptation period, during which they were maintained on standard laboratory chow and water, ad libitum. The animals were maintained under controlled temperature (21-22°C) and lighting, with lights on from 0600-1800 hr. Following the adaptation period, each male was placed into a stainless steel suspended cage (25 X 18 X 18 cm) with mesh front and floor and provided with ad libitum access to standard laboratory chow and water. Three days later females were randomly paired with males, with the heavier females being placed into the hanging cages first. Wax paper was placed under the hanging cages and was checked every morning for the appearance of vaginal plugs, indicating the first day of gestation. All animal use and care was in accordance with the National Institute of Health and Canadian Council on Animal Care guidelines and approved by the University of British Columbia Animal Care Committee.

2.2 DIETS AND FEEDING:

On day 1 of gestation (G1), females were re-housed into polycarbonate cages (24 X 16 X 46 cm) and males were given one day of rest before the next female was introduced into the hanging cage. Pregnant females were randomly assigned to one of three prenatal treatment groups starting on G1: 1) Ethanol (E), liquid ethanol diet (36% ethanol-derived calories) and water, ad libitum (n=17); 2) Pair-fed (PF), liquid control
diet (maltose-dextrin isocalorically substituted for ethanol), with each animal pair-fed the amount consumed by a female in the ethanol group (g/kg body weight) on the same day of gestation and water, ad libitum (n=14); or 3) Control (C), standard laboratory chow and water, ad libitum (n=18). E animals were introduced to the ethanol diet by gradually increasing the ratio of ethanol:control diet over three days. The liquid diet was formulated to provide adequate nutrition to pregnant females, regardless of ethanol intake, and manufactured by Dyets, Inc., Bethlehem, PA, USA. Fresh diet was offered daily, within 1½ hrs of lights out to maintain regular CORT circadian rhythms which typically reentrain to the feeding time when animals (ie. PF group) are “meal-fed” a restricted ration of diet (Gallo & Weinberg, 1981). At that time, bottles from the previous day were removed and weighed to determine the amount of diet consumed. On day 22 of gestation, experimental animals were placed on standard laboratory chow and water, which they received throughout the lactation period.

Females were weighed on days 1, 7, 14 and 21 of gestation. After birth, on postnatal day 1 (PN1), dam and litter weights were obtained and all litters were culled to 10 (5 males and 5 females). If necessary, pups born on the same day and within the same prenatal treatment group were cross-fostered to maintain a litter size of 10. Pups and dams remained undisturbed, other than weekly weighing and cage changing, until weaning on postnatal day 22 (PN22). On PN22, animals were ear-marked to indicate litter number and littermates were group-housed by sex for approximately 2 months, and then pair-housed by sex until the time of testing. Starting on PN25, females were checked daily for vaginal opening, a marker for the onset of puberty.
2.3 SURGERY:

All surgery was carried out between 0900-1200 hr, under halothane anesthesia, at random stages of the estrous cycle. Females of approximately 3 months of age were randomly selected from E, PF and C prenatal treatment groups and assigned to one of four post-natal surgical treatment groups: 1) Sham ovariectomized (Sham). Bilateral incisions were made in the dorsolateral flank but the ovaries were not removed; 2) Ovariectomized (OVX). Bilateral incisions were made in the dorsolateral flank, ovaries were removed and estradiol placebo pellets, containing everything but estradiol, were subcutaneously (sc) implanted between the shoulder blades; 3) Low estradiol replaced (OVX-L). Animals were OVX, then immediately implanted with low dose estradiol pellets (0.01 mg/pellet), sc; or 4) High estradiol replaced (OVX-H). Animals were OVX, then immediately implanted with high dose estradiol pellets (0.05 mg/pellet), sc. All pellets were 21-day release pellets containing 17-β estradiol, at doses chosen to mimic physiological levels of estradiol at diestru (low) and proestrus (high) and were obtained from Innovative Research of America, Sarasota, FL, USA. All animals received a 0.1cc intramuscular injection of the antibiotic Duplocillin (Intervet Canada Inc., Whitby, ON, Canada, DIN 01983377) and were weighed post-surgery as well as one day prior to testing.

2.4 TESTING AND SAMPLING:

Two weeks after surgery, E, PF and C females from each post-natal surgical treatment group were assigned to one of two post-natal testing conditions: 1) Basal - animals were decapitated directly from the home cage; or 2) Stress – animals were
decapitated following 30 min restraint in a polyvinyl chloride restraint tube. The restraint tubes were 5.5 X 20 cm and had plastic caps at both ends secured with tape. The front cap had four holes 1 cm apart to allow for ventilation and the end cap had an opening for the tail. All testing/sampling was done between 0930-1200 hr, as HPA hormone levels should be at their nadir, allowing for clearer differences to be seen among the three prenatal treatment groups. Trunk blood was collected on ice in 12 X 75 ml polystyrene tubes coated with ethylenediaminetetraacetic acid (EDTA), to prevent blood from coagulating, and aprotinin, a protease inhibitor to protect ACTH from denaturation. Blood samples were centrifuged at 3200 rpm for 10 min at 0°C, and plasma was transferred into 600ul eppendorf tubes and frozen at -70°C until assays could be run.

Brains were quickly removed from decapitated rats, rapidly frozen on powdered dry ice, individually wrapped in parafilm and aluminum foil and stored at -70°C until brains could be sectioned. Pituitaries were also collected and rapidly frozen in RNAse free 1.5 ml microtubes (VWR International, Edmonton, AB, Canada) on dry ice for later determination of pituitary ACTH content. The uterus, thymus and left adrenal from each animal were dissected free of fat and wet weights were measured.

Post-mortem vaginal smears were done to verify the post-natal surgical treatment. The smears were stained with 1% toluene blue and rinsed with tap water prior to analysis. The smears were classified according to the following criteria (Montes & Luque, 1988; Marcondes et al., 2002):

1. Metestrus - thin smear consisting of an equal number of nucleated epithelial cells, cornified cells and leukocytes
2. Diestrus – an abundance of leukocytes with some nucleated epithelial cells and cornified cells

3. Proestrus - an abundance of nucleated epithelial cells with some leukocytes or cornified cells

4. Estrus – thick smear consisting of an abundance of cornified cells with some nucleated epithelial cells or leukocytes

2.5 BLOOD ALCOHOL LEVEL (BAL):

To determine the peak blood alcohol levels achieved by pregnant females on the ethanol diet, blood samples were taken at around 15 days of gestation, two hours after lights off when major eating bouts occur. Blood from three randomly selected E females was sampled by cutting the tip of the tail with a razor blade and collecting tail vein blood into 600ul eppendorf tubes. The blood was allowed to coagulate for two hours at room temperature and then centrifuged at 3000 rpm for 20 min at 4°C and serum was removed and stored in 600ul eppendorf tubes at -20°C until time of assay. BAL was determined using Pointe Scientific Inc. Alcohol Reagent Set (Lincoln Park, MI, USA). The assay is based on the change in absorbance at a wavelength of 340nm which occurs when alcohol dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) to NADH. The change in absorbance is detected by a spectrophotometer and is directly proportional to the alcohol concentration in the sample. The minimum detectable concentration of ethanol is 2 mg/dl with a correlation coefficient of 0.999 and a regression equation of \( y = 1.01x - 1.01 \).
2.6 RADIOIMMUNOASSAYS:

a. **Plasma Corticosterone (CORT)**

Radioimmunoassay (RIA), adapted from Kaneko et al. (1981), was used to determine total corticosterone (bound and free) concentration. Antiserum was obtained from MP Biomedicals (Orangeburg, NY, USA), tritiated CORT tracer from Mandel Scientific (Guelph, ON, Canada) and corticosterone for standards from Sigma Chemical Co (St. Louise, MO, USA). A charcoal suspension (Fisher Scientific Ltd., Nepean, ON, Canada) with dextran was used to absorb free CORT after incubation. The minimum detectable concentration of CORT was 2.5 ng/ml and the intra- and interassay coefficients of variation were 1.55% and 4.26% respectively.

b. **Plasma Adrenocorticotropic hormone (ACTH)**

Plasma ACTH concentration was determined using an adaptation of the Diasorin ACTH RIA kit (Stillwater, MS, USA); all reagents and samples were halved. The minimum detectable amount of ACTH was 20 pg/ml and the intra- and interassay coefficients of variation were 3.9% and 6.5% respectively.

c. **Plasma Estradiol (E2)**

Plasma E2 concentration was determined using an adaptation of the Diagnostic Products Corporation Double Antibody E2 RIA kit (Los Angeles, CA, USA); all reagents and samples were halved. The minimum detectable amount of E2 was 5 pg/ml.
d. **Plasma Luteinizing hormone (LH)**

Samples were assayed by RIA in the laboratory of Dr. A. F. Parlow, National Hormone and Peptide Program (Harbor-UCLA Medical Center, California, USA).

2.7 **IN SITU HYBRIDIZATION:**

a. **Brain Preparation**

Using a cryostat, coronal sections of 14 um were taken from frozen brains through the MPOA (Bregma - 0.23 mm; coordinates from Paxinos & Watson, 1997) (Figure 2) and PVN (Bregma - 1.80 mm) of the hypothalamus (Figure 3). Four different areas of the MPOA were sampled by taking four consecutive slices, then discarding ten, taking four consecutive slices, etc.. Frozen sections were thaw-mounted onto slides coated with gelatin (Fisher Scientific Ltd., Nepean, ON, Canada) and poly-L-lysine (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and stored at -70°C until analysis.

b. **Oligonucleotide Probes**

Purified antisense oligonucleotide probes tail-labeled with $[^{35}\text{S}]$-deoxyadenosine 5'-triphosphate (Amersham Biosciences Inc., Piscataway, NJ, USA) were used to measure CRH and AVP mRNA in the PVN and GnRH mRNA in the MPOA. Probes were synthesized at the Oligonucleotide Synthesis Laboratory, University of British Columbia, Vancouver, BC, Canada: CRH antisense (5'-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3') (Jingami, 1985; Young et al., 1986a), AVP antisense (5'-GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG-3') (Ivell & Richter, 1984; Young et al., 1986b) and
GnRH antisense (5'-TTC AGT ATT TCT CTT CCC CCC AGG GCG CAA CCC ATA GGA CCA GTG CTG-3') (Scott et al., 1995). Sense oligonucleotide probes for CRH, AVP and GnRH mRNA were used as controls. Radioactive antisense and sense oligonucleotide probes were synthesized as follows: Probes were incubated with terminal deoxynucleotidyl transferase (New England Biolabs Ltd., Mississauga, ON, Canada), CoCl$_2$ (2.5mM) (New England Biolabs Ltd., Mississauga, ON, Canada), 10 X NE Buffer 4 (New England Biolabs Ltd., Mississauga, ON, Canada) and NF water at 37°C for 45 min. Following $^{35}$S-ATP tail-labeling, probes were placed on ice and 0.2M EDTA, pH8 (Fisher Scientific Ltd., Nepean, ON, Canada) was added. Probes were purified using G-25 Sephadex Columns for Radiolabeled DNA Purification (Boehringer-Mannheim Corp., Indianapolis, IN, USA), with 1M dithiotreitol (DTT) added to prevent oxidation.

c. **In Situ Hybridization**

Slides were removed from the -70°C freezer and thawed for 20 min. On a shaker with gentle agitation, slides were fixed with formalin for 30 min, followed by two washes of 1 X PBS for 10 min. Slides were then washed with 0.1M TEA containing 0.25% acetic anhydride for 10 min, 2 X SSC for 5 min, then dehydrated through 50% EtOH (1 min), 75% EtOH (1 min), 95% EtOH (2 min), 100% EtOH (1 min), chloroform (5 min) and 100% EtOH (1 min), then air dried for 30 min. 100ul of hybridization buffer (50% formamide, 3 X SSC, 1 X Denhardt’s solution, 100 ug/ml yeast tRNA, 25 mM NaPO$_4$ buffer (pH 7.4), 55 mM DTT, 30% deionized water) was applied per slide and covered with hybrislips (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Slides were incubated overnight at 40°C in 50% formamide humidified containers. Coverslips were removed and slides were washed in 2 X SSC for 20, 2 X SSC for 15 min, 2 X SSC with
DTT at 45°C for 20 min, 1 X SSC at 45°C for 20 min, 1 X SSC with 50% formamide 45°C for 30 min, 1 X SSC for 10 min and 0.5 X SSC for 10 min. Following the washes, the slides had five quick dips in distilled water and were plunged immediately into 70% EtOH for 5 min, then air dried for 30 min. Slides for measurement of CRH and AVP mRNA were exposed to Kodak BioMax MR X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 6d and 1hr, respectively. Then these slides as well as slides for the measurement of GnRH mRNA were dipped in Kodak NTB2 autoradiography emulsion of a 1:1 (with RO·H2O) dilution and exposed for 19d (CRH mRNA), overnight (AVP mRNA) or 24d (GnRH mRNA) in light tight boxes, with dessicant, at 4°C. Emulsion coated slides were then developed with Kodak D-19 developer at 14°C and fixed with Kodak Polymax T fixer at 14°C, then counterstained with Cresyl Violet and coverslips were mounted with permamount.

d. Densitometric Analysis

CRH, AVP and GnRH mRNA positive cells were visualized by using a Q-Imaging monochrome 12-bit camera attached to a Zeiss Axioskop 2 motorized plus microscope. Images were captured using Northern Elite 6.0v (Empix Imaging Inc., Mississauga, ON, Canada) and semiquantitative densitometric analyses were performed using Image J 1.31v (National Institutes of Health, Bethesda, MD, USA) for PC imaging software. Magnification of 63X (6.3X camera, 10X optical) was used for imaging CRH mRNA and 31.5X (6.3X camera, 5X optical) was used for AVP and GnRH mRNA. Image J digitized the continuous range of image gray shades into 255 discrete gray levels. The threshold for CRH and AVP mRNA was set to include gray levels of 70 to 255, and the threshold for GnRH mRNA was set to include gray levels of 60 to 255. The length of
exposure, percent gain and percent offset were set for each probe as follows: 130ms exposure, 29.21% gain and 16.85% offset for CRH mRNA; 120ms exposure, 33.14% gain and 18.53% offset for AVP mRNA; and 165ms exposure, 30.11% gain and 30.18% offset for GnRH mRNA.

CRH mRNA in the parvocellular cells of the PVN was measured by outlining one half of the PVN, ensuring that the rostral-caudal level was consistent. Background, where no specific CRH mRNA hybridization was detected, lateral to the area of interest and within a fixed window (circle, height = 1.00 inches, width = 1.00 inches) was then subtracted. AVP mRNA at a consistent rostral-caudal level of the PVN was measured by outlining a fixed window (circle, height = 0.50 inches, width = 0.50 inches) which encompassed only the dorsomedial parvocellular portion of the PVN. Background medial to the area of interest and of the same dimensions was then subtracted. For both CRH and AVP mRNA, measurements were expressed in pixel area by mean gray value. GnRH mRNA was measured in the most rostral MPOA section of each animal, between the anterior commissure and optic chiasm, by outlining neurons containing labeled GnRH mRNA and using Image J to analyze number of neurons, the pixel area of each neuron and the mean gray value of each neuron. Minimum pixel size for analyzing number of neurons was set to 35. GnRH mRNA measurements were expressed as pixel area by mean gray value per neuron.

2.8 STATISTICAL ANALYSES:

All data were analyzed using analyses of variance (ANOVA) for factors of prenatal treatment (E, PF and C), testing condition (basal and stress) and surgical
treatment (Sham, OVX, OVX-L, OVX-H) using the PC software Statistica 6.0v (StatSoft Inc., Tulsa, OK, USA). Plasma ACTH, CORT and E2 were further analyzed using ANOVAs for factors of prenatal treatment, testing condition and stage of estrous cycle (metestrus, diestrus, proestrus, estrus). Significant main and interaction effects were analyzed by Newman-Keuls post hoc analysis. Values were expressed as mean ± standard error of the mean (SEM) and outliers of two standard deviations or more were excluded. Pearson's product-moment correlations ($r^2$) were used to correlate basal plasma E2 to body and organ weights. Statistical significance was set at $p<0.05$. 
Figure 2: Diagrammatic Representation of the Paraventricular Nucleus

The parvocellular (light gray) and magnocellular PVN (dark gray) within the hypothalamus (Paxinos & Watson, 1997) (a) and representative nuclear emulsion-dipped sections of CRH mRNA (b) and AVP mRNA (c) expression in the PVN. Only the dorsomedial parvocellular portion of the PVN was analyzed for AVP mRNA expression (outlined).
Figure 3: Diagrammatic Representation of the Medial Preoptic Area

The medial preoptic area (MPOA) (gray) within the hypothalamus (Paxinos & Watson, 1997) (a) and representative nuclear emulsion-dipped section of GnRH mRNA expression in the MPOA (b).
CHAPTER 3: Results

3.1 DEVELOPMENTAL DATA:

a. Ethanol Intake and Blood Alcohol Levels

Ethanol intake by pregnant dams averaged 11.37±0.32, 15.37±0.45 and 15.96±0.51 g/Kg body weight/day of gestation for weeks 1, 2 and 3, respectively. These intake levels resulted in blood alcohol levels (BAL) of 192.45±4.12 mg/dl.

b. Maternal Body Weights and Gestational Length

A repeated measures ANOVA revealed that maternal weight increased throughout gestation (G) \[F(3,114) = 814.02, p<0.001\] (Figure 4) and that there was a significant effect of prenatal treatment \[F(2,38) = 4.10, p<0.05\] as well as a significant prenatal treatment X gestation day interaction \[F(6,114) = 17.07, p<0.001\]. Post-hoc analysis revealed that there were no significant differences among prenatal treatment groups on G1 to G14, but that E and PF dams weighed less than C dams on G21 (p<0.01).

Analysis of maternal weights during lactation (Figure 5) revealed a significant effect of postnatal (PN) day \[F(3,138) = 138.22, p<0.001\] as well as a significant prenatal treatment X postnatal day interaction \[F(6,138) = 8.65, p<0.001\]. All dams gained weight over the first two weeks of lactation and weights decreased by weaning on PN22. There were no significant differences among prenatal treatment groups on any postnatal day. However, further inspection of Figure 5 suggests that the prenatal treatment X postnatal day interaction could be due to differential rates of weight gain.
Analysis of percent increase over initial weights (Table 1) revealed that C dams had a higher percent weight gain than PF and E dams during gestation \([F(2, 38)=29.941, p<0.001]\) and a lower percent weight gain during lactation \([F(2, 46)=12.583, p<0.001]\). This latter finding supports the suggestion that differential rates of maternal weight gain among prenatal treatment groups may underlie the prenatal treatment \(\times\) gestation day interaction during lactation.

Analysis of gestation length indicated a significant effect of prenatal treatment \([F(2,39) = 4.0, p<0.05]\); gestation length was greater for E \((\bar{x}=22.0\pm0.09)\) dams compared to PF \((\bar{x}=21.59\pm0.14)\) and C \((\bar{x}=21.64\pm0.15)\) dams \((p<0.05)\).

c. **Pup Data**

A repeated measures ANOVA on pup weight gain (Figure 6a and 3b) revealed a significant effect of prenatal treatment \([F(2, 91)=4.8798, p<0.01]\), sex \([F(1, 91)=12.042, p<0.001]\), post-natal (PN) day \([F(3, 273)=6387.3, p<0.001]\) and a post-natal day \(\times\) sex interaction \([F(3, 273)=6.3721, p<0.001]\). Post-hoc analyses indicated that E and PF pups weighed less than C pups \((p<0.05)\). Both male and female pups in all prenatal treatment groups increased in weight from PN1 to PN22 \((p'<0.001)\) and males weighed significantly more than females on PN22 \((p<0.05)\). When male and female offspring were analyzed separately using a repeated measures ANOVA, there was a significant effect of postnatal day on both male \([F(3, 138)=2278.9, p<0.001]\) and female \([F(3, 135)=6127.1, p<0.001]\) offspring.
Analysis of number of live and stillborn offspring and number of males and females per litter (Table 2) did not reveal any significant differences among prenatal treatment groups.

d. **Age of Vaginal Opening**

The onset of puberty in female offspring, as measured by the age of vaginal opening (Table 3), did not differ significantly among the three prenatal treatment groups as measured using both post-natal and gestational age. A one-way ANOVA revealed a significant effect of prenatal treatment on body weight at vaginal opening \[F(2, 235)=6.4390, p<0.005\]; C female pups were heavier than E and PF female pups at vaginal opening \(p<0.005\).

### 3.2 EXPERIMENTAL DATA:

a. **Body Weight Pre- and Post-Testing**

A repeated measures ANOVA indicated that there was a significant change in body weight (BW) in the interval between surgery and testing \[F(1, 167)=469.67, p<0.001\] as well as an effect of surgical treatment \[F(3, 167)=13.004, p<0.001\] and a weight X surgical treatment interaction \[F(3, 167)=287.58, p<0.001\]. Post-hoc analysis revealed that Sham, OVX and OVX-L females gained weight from between surgery to testing, while OVX-H females lost weight \(p's<0.001\). Pre-testing BW comparisons revealed that OVX females weighed more than females in all other surgical treatment groups \(p's<0.001\) while OVX-H females weighed the least \(p's<0.05\).
Analysis of change in BW (Figure 7) similarly revealed an effect of surgical treatment $[F(3, 167)=287.58, p<0.001]$ with OVX females gaining more weight ($p's<0.001$) and OVX-H females losing more weight ($p's<0.001$) than females in all other surgical treatment groups. Further analysis of the OVX-H females revealed an effect of prenatal treatment $[F(2, 47)=3.8550, p<0.05]$ and post-hoc analysis indicated that E females lost less weight than PF and C females ($p<0.05$).

b. **Organ Weights at Testing**

i. **Adrenal Weight**

Although an overall ANOVA for absolute adrenal weight did not reveal any significant effects of prenatal treatment, surgical treatment or testing condition (Figure 8), an overall ANOVA for adrenal weight adjusted for BW (adrenal:BW ratios) indicated a significant effect of surgical treatment $[F(3, 167)=16.753, p<0.001]$ (Figure 9). Post-hoc analysis revealed that OVX females had lower adrenal:BW ratios compared to females in all other surgical treatment groups ($p's<0.001$) and that Sham females had lower adrenal:BW ratios compared to OVX-H females ($p<0.05$). No significant differences among E, PF and C females were found.

ii. **Thymus Weight**

An overall ANOVA indicated a significant effect of surgical treatment for absolute thymus weight $[F(3, 168)=187.85, p<0.001]$ (Figure 10) and thymus weight adjusted for BW (thymus:BW ratios) $[F(3, 168)=140.19, p<0.001]$ (Figure 11). Post-hoc analysis revealed OVX females had higher thymus weights ($p's<0.001$) and thymus:BW ratios ($p's<0.001$) and OVX-H females had lower thymus weights ($p's<0.001$) and
thymus:BW ratios (p's<0.001) than females in all other surgical treatment groups. Also, OVX-L females had higher thymus weights (p<0.05) and thymus:BW ratios than Sham females (p<0.01). No significant differences among E, PF and C females were found.

iii. Uterus Weight

An overall ANOVA indicated a significant effect of surgical treatment [F(3, 168)=245.72, p<0.001], testing condition [F(1, 168)=4.0109, p<0.05] and prenatal treatment [F(2, 168)=3.5150, p<0.05] on absolute uterus weight (Figure 12). Post-hoc analysis revealed that OVX females had lower uterus weights (p's<0.001) and OVX-H females had higher uterus weights (p's<0.001) than females in all other surgical treatment groups, and that OVX-L females had lower uterus weights than Sham females (p<0.001). Furthermore, E and PF females had lower uterus weights than C females (p<0.05) overall.

An overall ANOVA also indicated a significant effect of surgical treatment [F(3, 168)=245.87, p<0.001] and an effect of prenatal treatment [F(2, 168)=2.9611, p = 0.05447] on uterus weight adjusted for BW (uterus:BW ratios) (Figure 13). Like absolute uterus weights, post-hoc analysis revealed that OVX females had a lower uterus:BW ratio (p's<0.001) and OVX-H females had a higher uterus:BW ratio (p's<0.001) than females in all other surgical treatment groups, and that OVX-L females had a lower uterus:BW ratio than Sham females (p<0.001). In addition, overall, E females had a lower uterus:BW ratio than C females (p<0.05).
c. **Vaginal Cytology at Testing**

As expected, an overall ANOVA indicated a significant effect of surgical treatment \(F(3, 167)=40.168, p<0.001\) on vaginal cytology at the time of testing (Figure 14). Post-hoc analysis revealed that females in the OVX condition showed a predominance of diestrus/metestrus cytology \(p's<0.001\) and high E2 replacement resulted in a predominance of estrus/proestrus cytology \(p's<0.001\) compared to the other surgical treatment groups. No significant differences among E, PF and C females were found.

d. **Plasma Hormones**

i. **Plasma CORT**

An overall ANOVA revealed significant effects of testing condition \(F(1, 156)=1579.2, p<0.001\) and surgical treatment \(F(3, 156)=8.9481, p<0.001\), and a testing condition X surgical treatment interaction \(F(3, 156)=4.8447, p<0.005\) for plasma CORT levels (Figure 15). As expected, plasma CORT levels were significantly higher following 30 min restraint stress than under basal conditions for all surgical treatment groups \(p<0.001\).

Separate analyses of basal and stress CORT levels revealed a significant effect of surgery under both basal \(F(3, 72)=4.0167, p<0.05\) and stress \(F(3, 84)=8.0524, p<0.001\) conditions. Basal CORT levels were reduced in OVX compared to Sham females \(p=0.054\), and E2 replacement at both low (OVX-L) and high (OVX-H) levels brought CORT back to Sham levels \(OVX-L=OVX-H=Sham>OVX, p's<0.05\). Further analysis of basal CORT following OVX revealed a significant effect of prenatal treatment
C females had higher plasma CORT than E (p<0.05) and PF (p=0.05) females. Similar to the results for basal CORT, stress CORT levels were lower in OVX than in all other surgical treatment groups (p's<0.05). Replacement with low E2 levels increased CORT over that in OVX females (p<0.05), although levels were still lower than those in Sham (OVX-L<Sham, p<0.05), and replacement with high E2 brought CORT close to Sham levels (OVX-H>OVX, p<0.01; OVX-H<Sham, p=0.073).

Separate analyses of basal and stress CORT levels also revealed a significant effect of estrous cycle stage [F(3, 84)=3.5408, p<0.05] on plasma CORT under stress conditions (Figure 17); metestrus plasma CORT levels were marginally lower than proestrus (p=0.063) and estrus (p=0.070) and diestrus plasma CORT levels were marginally lower than proestrus (p=0.089). No significant differences among E, PF and C females were found.

ii. Plasma ACTH

An overall ANOVA revealed significant effects of prenatal treatment [F(2, 159)=3.4821, p<0.05], testing condition [F(1, 159)=295.00, p<0.001], and surgical treatment [F(3, 159)=13.877, p<0.001], as well as a testing condition X surgical treatment interaction [F(3, 159)=14.727, p<0.001] for plasma ACTH levels (Figure 16). As expected, plasma ACTH levels were higher following 30 min restraint stress than under basal conditions for all surgical treatment groups (p<0.001).

Separate analysis of basal and stress ACTH levels revealed effects of prenatal treatment under both basal [F(2, 78)=3.2329, p<0.05] and stress [F(2, 81)=3.0886, p=0.05097] conditions, as well as a significant effect of surgical treatment [F(3, 81)=14.892, p<0.001] following stress. Post-hoc analyses indicated that overall, E
(p<0.05) and PF (p=0.069) females had lower plasma ACTH levels than C females under basal conditions, and plasma ACTH in E females was marginally lower than C (p=0.079) females under stress conditions. Furthermore, following stress, OVX-H females had lower plasma ACTH compared to females in all other surgical treatment groups (p’s<0.05) and Sham females had lower plasma ACTH levels compared to OVX females (p<0.001).

Separate analyses of basal and stress ACTH levels also revealed a significant effect of estrous cycle stage \([F(3, 83)=6.8776, p<0.001]\) on plasma ACTH under stress conditions (Figure 17); metestrus and diestrous plasma ACTH levels were higher than proestrus and estrus levels (p’s<0.05).

One-way ANOVAs for each prenatal treatment group across surgical conditions revealed a significant effect of surgical treatment for stress ACTH levels in PF \([F(3, 27)=6.3492, p<0.005]\) and C \([F(3, 27)=7.9977, p<0.001]\) females and a marginal effect \([F(3,27)=2.69, p<0.066]\) for E females. Post-hoc analyses indicated that OVX significantly increased ACTH levels in PF (p<0.05) and C (p<0.05) females but not in E (p>0.17) females. For E females, neither OVX nor E2 replacement groups differed from Sham, although ACTH in both OVX-L (p=0.086) and OVX-H (p=0.052) was marginally reduced below that in OVX females. For PF females, replacement with high E2 levels was needed to bring ACTH back to Sham levels, whereas for C females, replacement with low E2 normalized ACTH and replacement with high E2 suppressed ACTH (OVX-H<Sham, p<0.05) below Sham levels.

Finally, an overall ANOVA on the ACTH stress increment (stress minus basal) revealed a significant effect of surgery \([F(3, 78)=14.314, p<0.001]\); OVX and OVX-L
animals (p's<0.05) had the greatest stress increment and OVX-H animals had the lowest stress increment compared to animals in all other surgical treatment groups (p's<0.05).

iii. Plasma E2

An overall ANOVA revealed significant effects of testing condition [F(1, 163)=4.5669, p<0.05] and surgical treatment [F(3, 163)=91.579, p<0.001], and a prenatal treatment X testing condition interaction [F(2, 163)=3.2036, p<0.05] for plasma E2 levels (Figure 18). Post-hoc analyses indicated that overall, plasma E2 was lower under basal conditions than following 30 min restraint stress (p<0.05). Furthermore, C but not E or PF females had significantly higher plasma E2 levels following stress compared to basal conditions (p<0.01). Further analysis within prenatal treatment groups revealed a surgical treatment X testing condition interaction [F(3, 53)=4.3801, p<0.01] for C females, such that stress plasma E2 levels were higher than basal levels in Sham (p<0.01) and OVX-H (p<0.001) C females.

When basal and stress E2 levels were analyzed separately, there were significant effects of surgical treatment under both basal [F(3, 81)=44.209, p<0.001] and stress [F(3, 82)=48.302, p<0.001] conditions. Post-hoc analyses indicated that, not surprisingly, OVX-H females had higher plasma E2 levels compared to females in all other surgical treatment groups under both basal and stress conditions (p's<0.001). In addition, both basal and stress plasma E2 levels were reduced following OVX (p<0.06 and p<0.01, respectively), normalized to Sham levels by low E2 replacement under basal but not stress conditions (OVX-L<Sham, p<0.01), and increased above Sham levels with high E2 replacement under both basal and stress conditions (p’s<0.001).
An overall ANOVA also revealed a significant effect of estrous cycle stage \( [F(3, 161)=26.740, p<0.001] \) on plasma E2 (Figure 19); as expected, plasma E2 levels were higher during proestrus than during metestrus and diestrus \( (p's<0.001) \) and highest during estrus \( (p's<0.01) \) compared to all other stages.

iv. Plasma LH

An overall ANOVA revealed a significant effect of surgical treatment \( [F(3, 167)=245.33, p<0.001] \) and a testing condition X surgical treatment interaction \( [F(3, 167)=8.1415, p<0.001] \) for plasma LH levels (Figure 20). Post-hoc analyses indicated that plasma LH was higher following stress than under basal conditions for OVX females \( (p<0.001) \) and the opposite was true for OVX-L females \( (p<0.05) \). When basal and stress LH levels were analyzed separately, there was a significant effect of surgery under both basal \( [F(3, 83)=143.95, p<0.001] \) and stress \( [F(3, 84)=118.75, p<0.001] \) conditions. Post-hoc analyses indicated that, as expected, under both basal and stress conditions, OVX females had higher plasma LH compared to all other surgical treatment groups \( (p's<0.001) \) and OVX-L females had higher plasma LH compared to Sham and OVX-H females \( (p's<0.001) \). No significant differences among E, PF and C females were found.

e. In situ hybridizations

i. CRH mRNA

An overall ANOVA revealed significant effects of prenatal treatment \( [F(2, 103)=6.0520, p<0.005] \) and surgical treatment \( [F(3, 103)=2.9361, p<0.05] \) for CRH mRNA levels in the PVN (Figure 21). Post-hoc analyses indicated that CRH mRNA was higher in PF compared to E and C females \( (p's<0.01) \). In addition, one way ANOVAs
within prenatal treatment group revealed that for C females [\(F(3, 34)=2.3534,\) 
\(p=0.08939\)], OVX marginally increased CRH mRNA compared to that in Sham females 
\((p=0.06)\). Overall, high E2 replacement decreased CRH mRNA compared to that in OVX 
females (OVX-H<OVX, \(p<0.05\)). There was no significant effect of stress on CRH 
mRNA. This was not unexpected as termination occurred at 30 min post-stress.

ii. AVP mRNA

An overall ANOVA revealed a significant effect of prenatal treatment [\(F(2,\) 
\(93)=3.2058, \ p<0.05\)] for AVP mRNA levels in the PVN (Figure 22). Post-hoc analysis 
indicated that AVP mRNA levels were higher in E and PF compared to C females 
\((p’s<0.05)\). There were no significant effects of surgical treatment or stress on AVP 
mRNA.

iii. GnRH mRNA

An overall ANOVA did not reveal any significant effects of prenatal treatment, 
testing condition or surgical treatment on number of GnRH mRNA expressing neurons, 
total amount of GnRH mRNA expression, as expressed by pixel area by mean gray value, 
or GnRH mRNA expression per neuron. When basal and stress animals were analyzed 
separately, however, there was a marginal effect of prenatal treatment on stress levels of 
GnRH mRNA expression per neuron [\(F(2, 47)=3.0533, \ p=.05666\)] (Figure 23). Post-hoc 
analysis indicated that GnRH mRNA expression per neuron was lower in E compared to 
PF females \((p=0.05)\).
f. Correlations

All correlations between hormone levels and body or organ weights utilized basal hormone levels. There were significant negative correlations between change in BW and plasma E2 for E (r = -0.57, p<0.001), PF (r = -0.43, p<0.05) and C (r = -0.66, p<0.001) females (Figure 24). There was a significant positive correlation between adrenal:BW ratios and plasma E2 for C females (r = 0.37, p<0.05) (Figure 25). There were significant negative correlations between thymus:BW ratios and plasma E2 for E (r = -0.59, p<0.001), PF (r = -0.37, p<0.05) and C (r = -0.65, p<0.001) females (Figure 26). There was a significant positive correlation between uterus:BW ratios and plasma E2 for E (r = 0.72, p<0.001), PF (r = 0.65, p<0.001) and C (r = 0.74, p<0.001) females (Figure 27).
Table 1: Percent Increase over Initial Weights of Dams during Gestation & Lactation (mean ± SEM)

Percent weight gain of E, PF and C dams during gestation and lactation (n = 14-18 per group). C dams had a higher percent weight gain than PF and E dams during gestation (\(^a\) \(p<0.001\)) and a lower percent weight gain during lactation (\(^b\) \(p<0.001\)).

<table>
<thead>
<tr>
<th>Group</th>
<th>During Gestation (%)</th>
<th>During Lactation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>31.81 ± 2.15</td>
<td>12.95 ± 1.73</td>
</tr>
<tr>
<td>PF</td>
<td>30.83 ± 1.59</td>
<td>14.61 ± 1.70</td>
</tr>
<tr>
<td>C</td>
<td>53.19 ± 2.51(^a)</td>
<td>2.62 ± 2.02(^b)</td>
</tr>
</tbody>
</table>
Table 2: Birth Data (mean ± SEM)

Total number of liveborn and stillborn E, PF and C male and female pups (n = 14-18 litters per group). Analysis of number of live and stillborn offspring and number of males and females per litter did not reveal any significant differences among prenatal treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total M</th>
<th>Total F</th>
<th>M:F</th>
<th>Total Liveborn</th>
<th>Stillborn</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>8.47 ± 0.65</td>
<td>6.82 ± 0.53</td>
<td>1.43 ± 0.20</td>
<td>15.29 ± 0.70</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td>PF</td>
<td>7.64 ± 0.41</td>
<td>7.57 ± 0.53</td>
<td>1.14 ± 0.15</td>
<td>15.21 ± 0.47</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>C</td>
<td>8.11 ± 0.46</td>
<td>7.22 ± 0.43</td>
<td>1.22 ± 0.11</td>
<td>15.33 ± 0.44</td>
<td>0.28 ± 0.14</td>
</tr>
</tbody>
</table>
Table 3: Vaginal Opening (mean ± SEM)

Post-natal and gestational age and body weight at vaginal opening for E, PF and C female offspring (n = 67-90 per prenatal treatment group). There was a significant effect of prenatal treatment on body weight at vaginal opening (p<0.005). C female offspring were heavier at vaginal opening than E and PF female offspring (a p<0.005).

<table>
<thead>
<tr>
<th>Group</th>
<th>Postnatal Age (d)</th>
<th>Gestational Age (d)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>33.53 ± 0.25</td>
<td>55.53 ± 0.25</td>
<td>108.44 ± 1.21</td>
</tr>
<tr>
<td>PF</td>
<td>33.78 ± 0.27</td>
<td>55.33 ± 0.26</td>
<td>106.80 ± 1.63</td>
</tr>
<tr>
<td>C</td>
<td>33.69 ± 0.20</td>
<td>55.27 ± 0.22</td>
<td>113.44 ± 1.35a</td>
</tr>
</tbody>
</table>
Figure 4: Dam Body Weights (g) (mean ± SEM) During Gestation

Body weights of E, PF and C dams during gestation (G) (n = 14-19 per group). There was a significant effect of prenatal treatment (p<0.05), gestation day (p<0.001) and a prenatal treatment X gestation day interaction (p<0.001). E and PF dams weighed less than C dams on G21 (\(^a\) p<0.01).
Figure 5: Dam Body Weights (g) (mean ± SEM) During Lactation

Body weights of E, PF and C dams during lactation (n = 14-18 per group). There was a significant effect of post-natal (PN) day (p<0.001) and prenatal treatment X post-natal day interaction (p<0.001). Maternal body weights increased until PN15 (p<0.001) and then decreased by PN22 (p<0.001).
Figure 6: Postnatal Male and Female Pup Body Weights (g) (mean ± SEM)

Body weights of E, PF and C male (top panel) and female (bottom panel) offspring on postnatal (PN)1, PN8, PN15 and PN22 (n = 14-18 litters per group). E and PF pups weighed less than C pups overall (p<0.05). Both male and female pups in all prenatal treatment groups increased in weight from PN1 to PN22 (p’s<0.001), and males weighed more than females on PN22 (p<0.05).
Figure 7: Change in Body Weight Between Surgery and Testing (g) (mean ± SEM)

Change in body weight (BW) between surgery and testing for E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups (n = 16 per surgical treatment group). Overall, OVX females gained more weight (a p's<0.001) and OVX-H females lost more weight (b p's <0.001) than females in all other surgical treatment groups. Under OVX-H surgical treatment conditions, E females lost less weight than PF and C females (c p<0.05).
Figure 8: Absolute Adrenal Weight (g) (mean ± SEM) at Testing

Absolute adrenal weights of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). There were no significant effects of prenatal treatment, testing condition or surgical condition on absolute adrenal weights.
**Figure 9: Adrenal:Body Weight Ratios (X10^5) (mean ± SEM) at Testing**

Adrenal:body weight ratios of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). Overall, OVX females had lower adrenal:body weight ratios compared to females in all other surgical treatment groups (\(^a\) p's<0.001) and Sham females had lower adrenal:body weight ratios compared to OVX-H females (\(^b\) p<0.05).
Figure 10: Absolute Thymus Weight (g) (mean ± SEM) at Testing

Absolute thymus weights of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). Overall, OVX females had higher thymus weights ($^a_p's<0.001$) and OVX-H females had lower thymus weights ($^b_p's<0.001$) than females in all other surgical treatment groups. Also, OVX-L females had higher thymus weights than Sham females ($^c_p<0.05$).
Figure 11: Thymus:Body Weight Ratios (X10^4) (mean ± SEM) at Testing

Thymus:body weight ratios of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). Overall, OVX females had higher thymus:body weight ratios (a p’s<0.001) and OVX-H females had a lower thymus:body weight ratio (b p’s<0.001) than females in all other surgical treatment groups. Also, OVX-L females had a higher thymus:body weight ratio than Sham females (c p<0.01).
**Figure 12: Absolute Uterus Weight (g) (mean ± SEM) at Testing**

Absolute uterus weights of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). Overall, OVX females had lower uterus weights ($a \ p's<0.001$) and OVX-H females had higher uterus weights ($b \ p's<0.001$) than females in all other surgical treatment groups, and OVX-L females had lower uterus weights than Sham females ($c \ p<0.001$). Furthermore, E and PF females had lower uterus weights than C females ($d \ p<0.05$) overall.
Figure 13: Uterus:Body Weight Ratios (X10^4) (mean ± SEM) at Testing

Uterus:body weight ratios of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups at testing (n = 16 per surgical treatment group). Overall, OVX females had a lower uterus:body weight ratio (a p’s<0.001) and OVX-H females had a higher uterus:body weight ratio (b p’s<0.001) than females in all other surgical treatment groups, and OVX-L females had a lower uterus:body weight ratio than Sham females (c p<0.001). In addition, overall, E females had a lower uterus:body weight ratio than C females (d p<0.05).
Figure 14: Vaginal Cytology at Testing

Percentage of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups in each of the four estrous cycle stages – metestrus, diestrus, proestrus and estrus – at testing (n = 8 per group). Overall, females in the OVX conditions showed a predominance of diestrus/metestrus cytology (p’s < 0.001) and high E2 replacement resulted in a predominance of estrus/proestrus cytology (p’s < 0.001) compared to the other surgical treatment groups.
Figure 15: Plasma CORT levels (ug/dl) (mean ± SEM)

Plasma CORT levels of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal (top panel) conditions and following 30 min restraint stress (bottom panel) (n = 7-8 per group). Overall, plasma CORT was higher following stress than under basal conditions (a p<0.001). Plasma CORT was lower in OVX compared to females in all other surgical treatment groups under both basal (b p’s<0.05) and stress (c p’s<0.05) condition. Stress plasma CORT levels were also lower in OVX-L compared to Sham females (d p<0.05). C females had higher plasma CORT than E and PF females under basal OVX conditions (e p<0.05).
Figure 16: Plasma ACTH Levels (pg/ml) (mean ± SEM)

Plasma ACTH levels of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal (top panel) conditions and following 30 min restraint stress (bottom panel) (n = 7-8 per group). Overall, plasma ACTH was higher following stress than under basal conditions (a p<0.001). C females had higher basal plasma ACTH than E (b p<0.05) and PF (c p=0.069) females and there was a similar trend for lower ACTH in E compared to C females (d p=0.079) under stress conditions.

Following stress, E, PF and C females had different plasma ACTH levels across surgical treatment. However, the overall trend was for lower plasma ACTH levels in OVX-H compared to females in all other surgical treatment groups (e p's<0.05) and lower plasma ACTH levels in Sham compared to OVX females (f p<0.001).
Figure 17: Plasma CORT (ug/dl) and ACTH (pg/ml) Levels (mean ± SEM) by Estrous Cycle Stage

Plasma CORT (top panel) and ACTH (bottom panel) levels of E, PF and C females by estrous cycle stage under basal and stress conditions (n = 3-13 per stage). There were no differences in plasma CORT and ACTH across the estrous cycle under basal conditions. Under stress conditions, metestrus plasma CORT levels were marginally lower than proestrus (a p=0.063) and estrus (b p=0.070) and diestrus plasma CORT levels were marginally lower than proestrus (c p=0.089). Furthermore, metestrus and diestrus plasma ACTH levels were higher than proestrus (d p’s<0.05) and estrus (e p’s<0.05) levels.
Figure 18: Plasma E2 Levels (pg/ml) (mean ± SEM)

Plasma E2 levels of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal (top panel) conditions and following 30 min restraint stress (bottom panel) (n = 7-8 per group). Overall, plasma E2 was lower under basal conditions than following stress ($^a p<0.05$). In addition, C but not E and PF females had significantly higher plasma E2 levels following stress compared to basal conditions ($^b p<0.01$). Under basal conditions, OVX-H females had higher plasma E2 levels compared to females in all other surgical treatment groups ($^c p's<0.001$). Following stress, OVX-H females had higher plasma E2 compared to females in all other surgical treatment groups ($^d p's<0.001$) and Sham females had higher plasma E2 compared to OVX and OVX-L females ($^e p's<0.001$).

Note: $^b$ = significant difference between basal and stress conditions
Figure 19: Plasma E2 Levels (pg/ml) (mean ± SEM) by Estrous Cycle Stage

Plasma E2 levels of E, PF and C females by estrous cycle stage with basal and stress groups collapsed (n = 7-25 per stage). Overall, plasma E2 levels were higher during proestrus than during metestrus and diestrus ($^{a}$ p’s<0.001) and highest during estrus ($^{b}$ p’s<0.01) compared to all other stages.
Figure 20: Plasma LH Levels (ng/ml) (mean ± SEM)

Plasma LH levels of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal (top panel) conditions and following 30 min restraint stress (bottom panel) (n = 7-8 per group). As expected, under both basal and stress conditions, plasma LH was higher in OVX-L females compared to Sham and OVX-H females (a p’s<0.001) and highest in OVX females (b p’s<0.001). Plasma LH was higher following stress than under basal conditions for OVX females (c p<0.001) and the opposite was true for OVX-L females (d p<0.05).
Figure 21: CRH mRNA Levels (pixel area X mean gray value) (mean ± SEM)

CRH mRNA levels in the PVN of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups (n = 9-13 per group). There was no significant effect of testing condition, so basal and stress groups were collapsed. Overall, CRH mRNA was higher in PF females compared to E and C females (a p’s<0.01) and lower in OVX-H females than OVX females (b p<0.05). In addition, one-way ANOVAs within prenatal treatment group revealed that for C females, OVX marginally increased CRH mRNA compared to that in Sham females (c p=0.06).
AVP mRNA levels in the PVN of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups (n = 8-13 per group). There was no significant effect of testing condition, so basal and stress groups were collapsed. Overall, AVP mRNA was lower in C females compared to E and PF females (*p’s<0.05).
Figure 23: GnRH mRNA expression per neuron (pixel area X mean gray value/neuron) (mean ± SEM)

GnRH mRNA expression per neuron in the MPOA of E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal (top panel) conditions and following 30 min restraint stress (bottom panel) (n = 4-6 per group). Under stress conditions, GnRH mRNA expression per neuron was lower in E compared to PF females overall (a p=0.05).
Figure 24: Correlation between Change in Body Weight from Surgery to Testing and Plasma E2

Correlations between change in body weight (BW) from surgery to testing and plasma E2 for E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal conditions (n = 30-32 per group). There was a significant negative correlation between change in BW and plasma E2 for E (r = -0.57, p<0.001), PF (r = -0.43, p<0.05) and C (r = -0.66, p<0.001) females.
Figure 25: Correlation between Adrenal:Body Weight Ratios and Plasma E2

Correlations between adrenal:body weight (BW) ratios and plasma E2 for E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal conditions (n = 30-32 per group). There was a significant positive correlation between adrenal:BW ratios and plasma E2 for C females (r = 0.37, p<0.05).
Figure 26: Correlation between Thymus:Body Weight Ratios and Plasma E2

Correlations between thymus:body weight (BW) ratios and plasma E2 for E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal conditions (n = 30-32 per group). There was a significant negative correlation between thymus:BW ratios and plasma E2 for E (r = -0.59, p<0.001), PF (r = -0.37, p<0.05) and C (r = -0.65, p<0.001) females.
Correlations between uterus:body weight (BW) ratios and plasma E2 for E, PF and C females in Sham, OVX, OVX-L and OVX-H surgical treatment groups under basal conditions (n = 30-32 per group). There was a significant positive correlation between uterus:BW ratios and plasma E2 for E (r = 0.72, p<0.001), PF (r = 0.65, p<0.001) and C (r = 0.74, p<0.001) females.
CHAPTER 4: Discussion

The aim of the present study was to determine the role of estradiol in the altered HPA response to stress in female rats prenatally exposed to ethanol. We hypothesized that prenatal ethanol exposure would alter HPG development and activity, and that the altered HPA activity in E females may be due, at least in part, to altered HPA axis sensitivity to E2. Although we found no evidence of delayed HPG development, as measured by the age of vaginal opening, E females had decreased uterine sensitivity to E2 compared to C females and lower GnRH mRNA expression/neuron in the MPOA following stress compared to PF females. Furthermore, acute stress failed to increase plasma E2 in E and PF females, as it did in controls.

HPA activity and responsiveness to E2 was also altered in E females. Following OVX, E and PF females had lower basal plasma CORT levels compared to C females. In addition, under both basal and stress conditions, E females had lower plasma ACTH levels compared to C females overall. Moreover, under stress conditions, plasma ACTH levels in E females did not change across surgical treatment while PF and C females had increased ACTH levels following OVX, which returned to Sham levels with E2 replacement. Also, under resting conditions, AVP mRNA expression was higher in E compared to C females and both CRH and AVP mRNA expression were elevated in PF compared to C females. Thus, the HPA axis in E females appears to be less responsive to the effects of E2 and manipulations of the HPG axis. Additionally, E and PF females have increased HPA activity at the level of the PVN, although the mechanisms underlying this appear to be different between the two prenatal treatment groups. Taken together, these data indicate that E females may have decreased tissue responsiveness to
E2 and altered HPG sensitivity to acute stress at various levels of the axis compared to controls. In addition, E females appear to have altered HPG and HPA activity under both basal and stress conditions, as well as altered bidirectional interactions between the two axes compared to control females.

4.1 EFFECT OF ETHANOL ON DEVELOPMENT:

In the present study, ethanol intake by pregnant female dams ranged from 11.37 to 16.36 g/Kg body weight during the three weeks of gestation. This high level of maternal ethanol intake resulted in mean peak BALs of 192.45 mg/dl. E and PF dams had significantly lower body weights and percent weight gains during gestation compared to C dams. This suggests that the lower maternal weight in E dams is at least partly mediated by ethanol-induced nutritional effects. During lactation, dams in all prenatal treatment groups gained weight; however, E and PF dams had a significantly higher percent weight gain than C dams, suggesting that the effects of ethanol and the effects of a restricted diet do not permanently alter dam weight and metabolism and that E and PF dams may show compensatory increases in body weights following parturition. E dams had a longer gestation period than PF and C dams, indicating a direct effect of ethanol on parturition. There were no differences in litter size among E, PF and C dams, but E and PF pups weighed less than C pups, again, suggesting a role for ethanol-induced nutritional effects. These data are consistent with previous data from our lab (Weinberg, 1985, 1989, 1995b) and others (Reyes et al., 1983; Kotkoskie & Norton, 1988; Norton et al., 1988; Kelce et al., 1989; Chen et al., 2004) demonstrating the adverse effects of
maternal ethanol consumption, as well as a restricted diet, on maternal weights and pregnancy outcome.

4.2 EFFECT OF ETHANOL ON HPG DEVELOPMENT & FUNCTION:

One marker of reproductive function is the timing of sexual maturation, which is measured by the age of vaginal opening in females. At birth, the rodent vaginal canal is closed by a covering of stratified epithelial cells (Gitlin, 1974). At the onset of puberty, the vagina responds to rising estrogen levels and the solid cells of the vaginal canal are deleted, resulting in vaginal opening (Elger, 1977; Ojeda et al., 1980). Development and maturation of the HPG axis is disrupted by prenatal ethanol exposure. A number of studies (Boggan et al., 1979; Esquifino et al., 1986; McGivern et al, 1992a, 1992b) have reported that E females are delayed in the onset of puberty, suggesting that in E females, the rise in endogenous estrogen at puberty or the sensitivity of estrogen responsive tissues, such as the vagina, may be altered. In the present study, there were no significant differences among prenatal treatment groups in the age of vaginal opening, which occurred at approximately 33 days of age. This age is similar to data reported in the literature (Lewis et al., 2002). Delays in vaginal opening may occur but it does not appear to be a robust phenomenon. The lack of prenatal ethanol effects on sexual maturation in this study was somewhat surprising as BALs in ethanol consuming dams were high, indicating a high level of ethanol exposure. Methodological differences or differences among experimental paradigms between this and previous studies may provide some explanation for the differences in results. The ethanol diet in our study was especially formulated to provide adequate nutrition to pregnant dams, and thus the adverse effects of
ethanol might have been slightly alleviated compared to those in pregnant dams receiving an unfortified ethanol diet (Esquifino et al., 1986). Also, McGivern et al. (1992a) used reproductively experienced dams, which may have altered behavioral and endocrine functions compared to those in virgin females (Mann & Bridges, 1992).

Another marker of reproductive function is the estrous cycle. In female rodents, the estrous cycle consists of four phases – proestrus, estrus, metestrus and diestrus – across which sex steroids and vaginal cytology fluctuate. Proestrus cytology consists mainly of nucleated epithelial cells, while estrus is characterized by an abundance of cornified cells. Both estrogen and progesterone levels are elevated during these two phases. In metestrus, vaginal cytology consists of an equal number of nucleated epithelial cells, cornified cells and leukocytes, while diestrus is characterized primarily by an abundance of leukocytes. During these two phases, circulating estrogen and progesterone levels are low.

In the present study, vaginal cytology was examined by vaginal lavage on the day of testing. As expected, animals in the Sham condition, regardless of prenatal treatment, could be found at all stages of the estrous cycle on the test day, and mean basal plasma E2 levels in these females ranged from 7.0 to 16.3 pg/ml. In the OVX condition, animals had either metestrus or diestrus cytology that reflected an atrophic vaginal epithelium, as has previously been reported in the literature (Montes & Luque, 1988), and mean basal plasma E2 levels were near zero. Low E2 replacement returned vaginal cytology to roughly the same proportions as seen in Sham animals, with mean basal plasma E2 levels ranging from 5.1 to 6.6 pg/ml. As expected, high E2 replacement resulted in mainly estrus cytology and high mean basal plasma E2 levels of 34.1 to 46.7 pg/ml. Analysis of
plasma E2 by stage of estrous cycle indicated that plasma E2 levels were higher during proestrus than during metestrus and diestrus, and highest during estrus. These data are consistent with those of Allen and Doisy (1923) and others (Montes & Luque, 1988; Chateau & Boehm, 1996) who reported that estrus is induced when circulating estrogen levels are at their peak (Figure 1). E females did not differ from control females in vaginal cytology or mean basal plasma E2 levels under any surgical condition. This was not surprising, as we have previously shown that E females have regular estrous cycles (Yamashita et al., 2003) and E females have been reported to be reproductively competent (Lam et al., 2000).

There was an overall effect of stress on plasma E2 levels, such that plasma E2 was higher following stress than under basal conditions. This effect was most prominent in Sham females whose plasma E2 increased as much as 3-fold compared to basal levels. Shors et al. (1999) reported similar increases in plasma E2 in intact female rats following acute exposure to tailshock or swim stress. The effects of stress on plasma E2 levels may be mediated at multiple levels of the HPG axis. The ovary has been reported to be a glucocorticoid target organ. High-affinity glucocorticoid binding sites, presumed to be GR, are present in rat granulosa cells (Schreiber et al., 1982), and glucocorticoids act directly to modulate gonadotropin action on these cells in vitro (Hsueh & Erickson, 1978; Schoonmaker & Erickson, 1983). Recently, Tetsuka et al. (1999) found GR and MR mRNA in the corpus luteum and granulosa cells of the rat ovary, confirming a role for glucocorticoids in mediating ovarian function. The pituitary may also mediate the stress-induced increase in plasma E2. Acute stress has been shown to increase LH secretion in estrogen primed female rats (Paris et al., 1973; Briski & Sylvester, 1988) and both acute
ACTH and CORT treatment have been reported to facilitate LH and FSH secretion in estrogen primed female rats (Brann et al., 1990, 1991b). Increased gonadotropin secretion would then result in elevated plasma E2. There was also a stress-induced increase in plasma E2 levels in OVX, OVX-L and OVX-H females, who lack ovaries. This increase is likely due to an adrenal source of estrogens (Wasada et al., 1978). The adrenals have been shown to increase estrogen production with administration of ACTH and acute stress increased plasma ACTH levels in all females in this study (Wasada et al., 1978).

In the present study, C but not E and PF females had significantly higher plasma E2 levels following stress, especially under Sham and OVX-H conditions. Since plasma CORT levels were similar among prenatal treatment groups under Sham and E2 replaced conditions, the lack of a stress-induced increase in plasma E2 in E and PF females may be due to altered ovarian sensitivity to glucocorticoids or decreased pituitary responsiveness to the facilitative effects of stress on LH and FSH secretion. Furthermore, C females had higher plasma ACTH under basal and stress conditions compared to E females, suggesting that C females may have had greater facilitation of LH and FSH release as well as adrenal estrogen secretion following stress.

The uterus, which is an estrogen responsive tissue, also provides an index of reproductive function. Estrogens increase uterine weight through increased cell division (Kaye et al., 1972; Stormshak et al., 1976) and the magnitude of the response is dose dependent (O’Connor et al., 1996). As expected, mean absolute uterus weights and uterus:BW ratios paralleled plasma E2 levels. Both absolute weight and organ weight:BW ratios were examined. In general, a normal large rat would be expected to have proportionally larger organs than a smaller rat, thus most investigators adjust for
body weight when reporting organ weights. However, in this study, OVX rats were used and it has been established that OVX rats tend to increase food intake and gain more weight compared to intact rats. However, the increase in weight is primarily due to a disproportionate increase in body fat due to changes in appetite and metabolism (Shinoda et al., 2002). An increase in fat, does not lead to an increase in organ weights, thus absolute organ weights were also reported for this study.

Both absolute uterus weight and uterus:BW ratios decreased with OVX, returned to Sham levels with low E2 replacement, and increased with high E2 replacement. In addition, females in all prenatal treatment groups had a significant positive correlation between uterus:BW ratios and plasma E2 under basal conditions. Miskowiak et al. (1988) also found that uterine weight decreased with OVX and increased with E2 replacement. Importantly, our data indicate that E females had lower uterus:BW ratios than C females and both E and PF females had lower absolute uterus weights than C females overall.

Since plasma E2 levels were similar between E and control females, differences in uterus:BW ratios may be due to altered uterine responsiveness to E2. The actions of steroid hormones are mediated through specific steroid receptors, such as estrogen (ER) and progesterone (PR) receptors. In the uterus, estrogen treatment decreases ER levels and increases PR levels (Manni et al., 1981; Shupnik et al., 1989). Additionally, PR levels are body weight dependent, increasing as body weight increases (O’Connor et al., 1996). Our data show that although E, PF and C animals had similar body weights post-surgery, E animals lost less weight and thus had higher pre-testing body weights under OVX-H conditions compared to PF and C females. Therefore, E females may have had higher uterine PR expression compared to C females. Furthermore, E2 appears to
potentiate plasma CORT and since progesterone is produced in the adrenal glands as a precursor to CORT (Yen & Jaffe, 1986), progesterone levels may increase as plasma CORT increases (Pellegrini et al., 1998). Progesterone binding to PR then inhibits estrogen-induced hyperplasia of luminal and glandular epithelium (Graham & Clarke, 1997; Moutsatsou & Sekeris, 2003). Thus this may be an explanation for the lower uterus:body weight ratios found in E females. Another possible explanation may be differences in plasma CORT levels between prenatal treatment groups. CORT has been shown to inhibit E2-stimulated uterine growth (Rabin et al., 1990). Thus, although plasma CORT was not elevated in E compared to C females at the time point measured in this study, the lower uterus weights and uterus:BW ratios overall in E compared to C females suggest that if tested at a different time point we may have seen higher basal HPA activity or HPA hyperresponsiveness to stressors in E compared to C females.

Plasma LH levels provide a third index of reproductive function. As expected, plasma LH levels were highest following OVX due to the loss of gonadal steroid negative feedback (Yen & Jaffe, 1986). E2 replacement decreased the elevated plasma LH levels attained by OVX animals, but high E2 replacement was required to return plasma LH levels back to those of Sham females. This suppressive effect of E2 on LH secretion is consistent with previous reports in the literature (Leipheimer et al., 1986; Ratka & Simpkins, 1990). Following stress, plasma LH increased under OVX conditions, and decreased under OVX-L conditions, suggesting mediation of the LH response to stress by E2. Indeed, E2 has been reported to enhance the stress-induced suppression of LH (Cates et al., 1999; Li et al., 2003). CORT and CRH have been suggested as mediators of the effect of E2 on LH secretion, but the mechanism has yet to be elucidated (Li et al., 2003).
The increase in plasma LH following stress under OVX conditions may be due to the lack of E2, and therefore the lack of enhanced LH suppression by E2. Additionally, studies have indicated that AVP may stimulate LH release (Palm et al., 2001). Ono et al. (1985) demonstrated that injection of AVP antiserum into the third ventricle of OVX rats reduced LH release in response to ether stress, indicating a stimulatory effect of AVP on LH release. Since AVP mRNA levels did not differ among surgical treatment groups, the combination of AVP stimulation and the lack of enhanced suppression by E2 on LH release may have resulted in increased LH levels in OVX females. There were no significant differences among E, PF and C females in plasma LH levels. Therefore, despite reports in the literature of decreased and/or altered patterns of plasma LH release in adult rats prenatally exposed to ethanol (Guerri et al., 1984; Morris et al., 1989; Creighton & Rudeen, 1990; Creighton-Taylor & Rudeen, 1991a; Wilson et al., 1995), E females in this experimental paradigm appear to have normal LH secretion, at least at the time point assessed.

GnRH mRNA in the MPOA was measured as an index of central regulation of HPG activity. There were no significant effects of surgical treatment or prenatal treatment on the number of GnRH expressing neurons, total amount of GnRH mRNA expression or GnRH mRNA expression per neuron under basal conditions. Advis et al. (1980) showed that GnRH content in the suprachiasmatic-medial preoptic region peaked at 10d post-OVX, and was significantly reduced following E2 administration. The lack of surgical treatment effects on GnRH mRNA expression in the present study may be due to several possibilities. 1) Only the anterior MPOA was examined in this experiment; thus differences in GnRH expression and neuron number may not have been captured within
the selected area. We are currently in the process of examining several sections throughout the MPOA, which will provide a more complete picture of GnRH expression, distribution and neuron number. 2) Changes may have occurred in excitability or firing patterns of GnRH neurons (Moenter et al., 2003) that may not have appeared as differences in GnRH mRNA expression. 3) Differences among groups may have occurred in neuron distribution rather than absolute number of neurons and this could only be determined if the entire MPOA was sectioned. Ongoing examination of the MPOA may reveal differences among groups. 4) Advis et al. (1980) reported that peak GnRH expression was found 10d after OVX, while our animals were tested 14d post-OVX, and thus we may have missed the period of maximal GnRH expression following OVX. 5) GnRH mRNA may not change in parallel with GnRH protein. Therefore, there may be differences among prenatal treatment groups in post-transcriptional events, which may result in differences in GnRH protein levels, but not in GnRH mRNA expression.

In contrast, we found an effect of prenatal treatment under stress conditions, such that GnRH mRNA expression per neuron was lower in E compared to PF females. This suggests possible deficits in GnRH mRNA production, stability, release, degradation or storage, altered sensitivity to the activational effects of E2 on GnRH release or altered feedback/feedforward mechanisms to GnRH neurons in E animals. Conversely, this may reflect altered GnRH mRNA expression that could possibly lead to deficits in the synthesis and secretion of GnRH in PF females. The effects of pair-feeding on offspring HPG axis activity have not been studied. Gavin et al. (1994) demonstrated that acute prenatal ethanol exposure decreased GnRH-positive cell numbers in male and female rats on gestation day 22 but by postnatal day 60, E animals were no different from controls.
Wilson et al. (1995) found no differences in the distribution of GnRH mRNA expressing cells or grain density/cell in adult OVX E females compared to controls. These reports suggest that postnatal catch-up in GnRH neuron number can occur, which may be why there were no differences in the number of GnRH expressing neurons in our adult females. Interestingly, Morris et al. (1989) found decreased hypothalamic GnRH content in peripubertal female rats prenatally exposed to ethanol. Thus, ethanol exposure during the developmental and/or maturational stages of hypothalamic growth may have altered the synthesizing capabilities of the GnRH neurons present. Morris et al. (1989) and Creighton-Taylor and Rudeen (1991) reported decreased pituitary responsiveness to GnRH in vitro in E animals compared to controls. Thus it is possible that alterations in responsiveness to GnRH may occur even in the face of similar GnRH neuron numbers or mRNA expression. This remains to be tested in our animals. These findings suggest that prenatal ethanol exposure may be disruptive to GnRH neurons but that the negative effects of ethanol become less prominent with age. Furthermore, under our conditions, differences in GnRH mRNA expression among E, PF and C animals were seen only following stress. Kam et al. (2002) found that GnRH mRNA expression in the MPOA decreased following immobilization stress. Thus perhaps, GnRH neurons in E animals are more susceptible to stress-induced alterations in function than those in PF and C animals.

4.3 EFFECT OF ETHANOL ON HPA FUNCTION:

In the present study, OVX altered body, thymus and adrenal weights, which were then normalized with E2 replacement. Body weights of females in all prenatal treatment
groups increased significantly with OVX and decreased in a dose-dependent manner with E2 replacement, indicating a role for E2 in the maintenance of body weight. In addition, under basal conditions, there was a significant negative correlation between change in body weight from surgery to testing and plasma E2 in females of all prenatal treatment groups. Estrogen has been reported to have anorectic effects, which may be mediated by CRH (Wade, 1975; Dagnault & Richard, 1997; McCormick et al., 2002; Liang et al., 2002). Acute stress-induced anorexia was reversed with a CRH receptor antagonist, and chronic icv infusion of CRH resulted in decreased weight gain, indicating that CRH itself has anorectic effects (Krahn et al., 1986; Buwalda et al., 1997). Dagnault et al. (1993) found that E2 reduced food intake in castrated females and this effect was eliminated with icv administration of a CRH antagonist. Furthermore, Dagnault and Richard (1997) reported that ER and CRH are colocalized in neurons of the MPOA and adjacent areas, such as the bed nucleus of the stria terminalis. Liang et al. (2002) also reported a decrease in food intake and BW following E2 administration, and that this effect was mediated by ERβ, which is found in the MPOA. The PVN, which also expresses ERβ and contains CRH neurons, may be another site for CRH-mediated anorectic effects of E2 (Krahn et al., 1988; Laflamme et al., 1998). Thus the anorectic effects of E2 appear to involve CRH at the level of the hypothalamus, although the mechanism remains to be elucidated. While females in the OVX-H condition lost the most weight compared to females in all other surgical treatment groups, E females lost less weight than C females. CRH mRNA expression in the MPOA was not measured, but CRH mRNA expression in the PVN showed no significant differences between E and C females. Therefore, it is possible that in E animals, CRH expressing neurons may be less responsive to E2, perhaps due to
down-regulation of ERβ or altered ERβ function, resulting in decreased anorectic effects of E2. This possibility remains to be investigated.

Like body weight, absolute thymus weight and thymus weight corrected for body weight, increased with OVX and returned to Sham levels with E2 replacement. Young et al. (2001) similarly found that thymus weight decreased in response to estrogen. Since the thymus is a GR containing tissue, and thus provides a sensitive index of total CORT levels, decreased thymus weight in response to E2 replacement may be due to E2 facilitation of CORT release (Young et al 2001). In the present study, under basal conditions, thymus:BW ratios were negatively correlated with plasma E2 for females in all prenatal treatment groups. This supports the suggestion of a possible facilitative role for E2 on HPA axis activity (Kitay, 1963; Viau & Meaney, 1991; Burgess & Handa, 1992; Carey et al., 1995). There were no significant differences among prenatal treatment groups in thymus:BW ratios or plasma CORT. Thus, E animals appear to be similar to C in thymus sensitivity to circulating E2 and CORT.

There was no significant effect of surgical treatment on absolute adrenal weights, however, adrenal weight corrected for BW decreased with OVX and increased with E2 replacement. This finding should be taken with a grain of salt, since the decrease in adrenal:BW ratios with OVX is likely due to an increase in body weight, as discussed earlier, as opposed to a significant change in adrenal weight. Nevertheless, the adrenal:BW ratios are in keeping with what is found in the literature. Young et al. (2001) found increased adrenal weights following E2 treatment. Similarly, McCormick et al. (2002) reported increased adrenal weights following E2 treatment and females had higher adrenal weights than males. OVX has been shown to decrease, and E2 to increase CORT.
content of the adrenals (Nicola et al., 1962; Kitay et al., 1965; Malendowicz & Mlynarczyk, 1982), and ERβ is reported to be expressed in the adrenal gland (Saunders et al., 1997). Furthermore, Nowak et al. (1995) reported that E2 enhanced basal, but not ACTH-stimulated, CORT secretion by adrenocortical cells in vitro. Thus E2 appears to induce adrenal hypertrophy and hyperplasia, which would result in increased adrenal weight and enhanced adrenal steroidogenesis. In the present study, we found that under basal conditions, adrenal:BW ratios were positively correlated with plasma E2 for C females. Thus the effect of surgical treatment on adrenal:BW ratios is likely due to altered adrenal steroidogenesis and possibly to a stimulatory effect of E2 on HPA axis activation at the level of the adrenal. In E females, although there were no differences in adrenal:BW ratios compared to controls, there was also no significant positive correlation with plasma E2. Thus E animals may be less sensitive to E2 enhanced adrenal CORT production. The desensitization of the adrenals to E2 may be a compensatory mechanism for the increased ovarian weight, and possible increase in plasma E2, reported in adult E females (McGivern et al. 1992a).

Overall, the plasma CORT data support the adrenal and thymus weight data in suggesting a stimulatory role for E2 on HPA axis activity. Under both basal and stress conditions, plasma CORT levels were lower in OVX compared to Sham females, and E2 replacement returned CORT to Sham levels. E2 enhancement of plasma CORT has been widely reported in the literature (Kitay, 1963; Burgess & Handa, 1992; Carey et al., 1995). Furthermore, plasma CORT has been shown to be elevated during the proestrus phase of the estrous cycle, when E2 levels are at their peak (Raps et al., 1971; Buckingham et al., 1978; Viau & Meaney, 1991; Carey et al., 1995; Atkinson & Waddell,
1997). We similarly found elevated plasma CORT levels during proestrus and estrus when E2 levels were high, and low plasma CORT levels during diestrus and metestrus, when circulating plasma E2 levels were low, following stress.

C females had higher basal CORT than E and PF females under OVX conditions. This may be due to the higher basal ACTH levels seen in C compared to E females. It is also possible that E females have a greater dependence on plasma E2 to maintain basal HPA activity. Alternatively, it may reflect decreased adrenal sensitivity to E2 in E females. Although not significant, E females appeared to have higher basal plasma E2 levels under Sham conditions. Furthermore, increased ovarian weights have been reported in E females (McGivern et al. 1992a). Thus decreased adrenal sensitivity to E2 may be a compensatory mechanism against elevated circulating plasma E2 levels in intact E females, which is only revealed following OVX.

Contrary to plasma CORT, plasma ACTH data suggest an inhibitory role for E2 in stress responsiveness. E2 did not affect resting plasma ACTH, but under stress conditions, OVX females had higher plasma ACTH compared to Sham females and E2 replacement decreased the ACTH response to stress in a dose-dependent manner. In addition, following stress, plasma ACTH levels were higher in animals in metestrus and diestrus phases of the estrous cycle, when E2 levels are low, compared to animals in proestrus and estrus, when E2 levels are high. Furthermore, when the difference between basal and stress ACTH levels were analyzed, Sham females had a lower stress increment than OVX and OVX-L females, and OVX-H females had the lowest stress increment. Since basal ACTH levels were comparable among surgical treatment groups, this suggests E2 has a dampening effect on the ACTH response to stress. It is likely that basal
E2 levels maintain normal HPA axis responsiveness to stressors. Thus, eliminating E2 leads to an exaggerated ACTH response to stress, while high E2 levels suppress the normal ACTH response to stress. Since plasma CORT levels were decreased with OVX and increased with E2 replacement, the effect of E2 on plasma ACTH may be via decreased CORT negative feedback with OVX and increased CORT negative feedback with E2 replacement. This may also explain the pattern of plasma ACTH across the estrous cycle following stress. Plasma ACTH levels were higher during metestrus and diestrus, when E2 levels are low and thus E2 potentiated CORT negative feedback would be at a minimum, than during proestrus and estrus, when high E2 levels lead to increased CORT negative feedback.

Young et al. (2001) also found that physiological levels of E2 decreased ACTH release in response to stress and that E2 antagonism resulted in exaggerated stress responsiveness. Furthermore, Miskowiak et al. (1988) found that E2 markedly lowered pituitary ACTH release when calculated per mg of gland. The inhibitory effects of E2 were only found in plasma ACTH, not CORT, suggesting that E2 may have differential effects at various levels of the HPG axis. E2 enhancement of plasma CORT may be due to the direct effects of E2 on adrenal steroidogenesis (Kitay et al., 1965; Malendowicz & Mlynarczyk, 1982; Nowak et al., 1995), instead of effects at the level of the hypothalamus and pituitary. On the other hand, the lack of corresponding ACTH and CORT data may be explained, at least in part, by the nature of these hormones themselves, rather than differential effects of E2. ACTH and CORT have different peaks following stress activation (Kovacs & Sawchenko, 1996). ACTH peaks 5-10 min after the onset of stress while CORT peaks at 15-30 min. In this study, we terminated animals
30 min after the onset of stress, at a point where ACTH levels were recovering, and thus we may have missed differences among surgical treatment groups that were only visible at the peak. Also, only a small amount of ACTH is required to increase CORT secretion (Kaneko et al. 1981), and thus ACTH and CORT do not necessarily fluctuate in parallel.

There were several differences in plasma ACTH levels among prenatal treatment groups. Under basal conditions, E and PF females had lower plasma ACTH than C females overall, and there was a similar trend for lower plasma ACTH levels in E compared to C females following stress. In addition, stress ACTH levels of PF and C females increased with OVX and decreased with E2 replacement in a dose-dependent manner while stress ACTH levels of E females did not change significantly across surgical treatment groups, although plasma ACTH levels in both OVX-L and OVX-H females were marginally reduced below that in OVX females. It is possible that E females may have increased adrenal responsiveness to ACTH, such that lower levels of ACTH are required to maintain resting plasma CORT levels and to elicit an increase in plasma CORT levels in response to stressors. If indeed E females have altered adrenal responsiveness to ACTH, this effect appears to depend more on the presence of E2 than in C females, since differences in basal plasma CORT levels among prenatal treatment groups were more prominent under OVX conditions. At the level of the pituitary, the stress response in E females may actually be less dependent on E2, as stress plasma ACTH levels of E females did not change significantly among surgical treatment groups.

Overall, the data on CRH mRNA expression in the PVN also suggest an inhibitory role for E2 on HPA axis activity. CRH mRNA expression was lower in OVX-H females compared to OVX females and in C females, CRH mRNA expression was
higher following OVX compared to Sham levels. In addition, we found that CRH mRNA was lower in E and C compared to PF females. Paulmyer-Lacroix et al. (1996) similarly found decreased CRH mRNA levels in OVX rats treated with E2 and this effect was eliminated with ADX. This suggests that E2 potentiates CORT release from the adrenals and thus CORT inhibition of CRH mRNA expression is potentiated. This may also explain E2’s inhibitory effects on plasma ACTH. E2 may act directly on the adrenal gland to increase CORT secretion (Malendowicz & Mlynarczyk, 1982; Nowak et al., 1995), which would then feedback to the PVN to suppress CRH mRNA. Decreased CRH mRNA levels would then result in decreased pituitary ACTH, but since only a small amount of ACTH is required for the CORT response to stress (Kaneko et al., 1981), plasma CORT levels may not appear to be altered.

AVP mRNA expression in the PVN was not influenced by plasma E2. However, there was an effect of prenatal treatment on AVP mRNA expression, such that C females had lower AVP mRNA expression compared to E and PF females. The lack of E2 effects on AVP mRNA expression is contrary to the reports of Patchev et al. (1995), who found increased AVP mRNA in the PVN of E2 treated OVX rats compared to non-treated female or male rats. Ferrini et al. (1997) also found higher AVP mRNA levels in the PVN of estrogen-treated OVX females compared to intact males under basal conditions. However, both Patchev et al. (1995) and Ferrini et al. (1997) did not replace their females with E2 immediately following OVX, but injected E2 two to four days prior to testing. Thus, the sudden rise in plasma E2 may have been more stimulatory to the PVN than chronic steady physiological levels of E2, as was used in our model. In addition, AVP is a co-secretagogue of CRH, and therefore it may not be the major target for E2.
modulation. Thus AVP mRNA expression may not be as sensitive as CRH to changes in plasma E2 levels.

Increased AVP mRNA expression in E and PF females and increased CRH mRNA expression in PF females compared to controls suggest either increased drive to the PVN or decreased CORT feedback resulting in increased HPA activity in both E and PF females. Different mechanisms may underlie the hyperresponsiveness often seen in E and PF females since both CRH and AVP mRNA expression were elevated in PF females, whereas only AVP mRNA expression was increased in E females. This shift toward increased AVP expression has also been reported in animals following chronic or repeated stress (Ma et al., 1997, 1998; Leo, 2002). The increase in AVP is thought to maintain HPA responsiveness to stress despite the adaptation or desensitization of the HPA axis resulting from chronic or repeatedly elevated plasma CORT. Thus, in E females, a shift toward increased AVP mediation of HPA activity may be a mechanism to enhance pituitary ACTH production by CRH, without increasing CRH production itself. The lack of higher plasma ACTH and CORT in E females compared to controls under basal or stress conditions may have been due to the time-point that was chosen. Testing at 30 min following the onset of stress may have been too early or too late to reveal prenatal treatment differences in peak plasma ACTH and too early to reveal differences in plasma ACTH and CORT recovery. E animals have, however, been shown to have increased elevations of CORT and/or ACTH, and/or a delayed return to basal levels in response to a variety of stressors including restraint (Weinberg, 1988), cardiac puncture (Taylor et al., 1982b), noise and shaking (Taylor et al., 1982b), footshock (Nelson et al., 1986), novel environments (Weinberg, 1988), cold (Angelogianni & Gianoulakis, 1989) and ether
(Weinberg, 1982; Angelogianni & Gianoulakis, 1989). HPA hyperresponsiveness appears to be a robust phenomenon, but observation of differential stress responsiveness in E compared to control animals may depend on the stressor as well as, the time or hormonal end point measured (Weinberg et al., 1996).

PF females also showed increased HPA activity at the level of the hypothalamus, which appears to be mediated via a different mechanism than that in E females. As discussed in the introduction, pair-feeding, in addition to serving as a nutritional control group, is itself a treatment condition. PF animals are in a state of constant hunger because they are restricted in their diet intake. In addition, this restriction results in consumption of the entire daily ration within a short time after it is presented, resulting in a “meal feeding” regimen, which has been shown to alter basal HPA activity and the pattern of HPA responsiveness to stressors in PF dams (Weinberg & Gallo, 1992). Thus we hypothesize that restricted feeding of the dam may act as a prenatal stressor. Prenatal stress with stressors such as restraint, crowding, noise, saline injection or conditioned avoidance training has been reported to result in HPA hyperresponsiveness to stressors (McCormick et al., 1995; Weinstock, 1997). Rats prenatally exposed to stress have been shown to have higher plasma CORT under basal and stress conditions, higher plasma ACTH levels in response to stress, and higher levels of CRH in the amygdala (Weinstock et al., 1992; Cratty et al., 1995; McCormick et al., 1995). Jezova et al. (2002), however, found no differences in CRH mRNA levels in the PVN following prenatal stress induced by gestational food restriction. This may be because in their experimental paradigm, pregnant dams were only food restricted from day 13 to 20 of gestation and they had a small sample size. Thus it appears that, in general, prenatal stress is capable of markedly
altering HPA activity, although the mechanism may be different from that occurring with prenatal ethanol exposure. This may explain the increased HPA activity at the level of the hypothalamus in PF females.

Although the effect of plasma progesterone was not studied in this experiment, it is important to keep in mind that it too may be playing a role in HPA-HPG interactions. The role of progesterone in the modulation of the HPA axis is unclear in the literature. Progesterone has been reported to be inhibitory (Buckingham, 1982; Jones & Hillhouse, 1976) or have no effect (Carey et al., 1995) on HPA activity. Progesterone may be exerting its effects on the HPA axis through two different mechanisms. It may have an antagonistic effect on estrogen, or it may bind with high affinity to glucocorticoid receptors in a competitive manner. Since OVX not only eliminates the ovarian source of E2 but also progesterone, differences among prenatal treatment groups may be due to differential responsiveness to progesterone, or altered interactions between progesterone and E2. Furthermore, only E2 was replaced following OVX; therefore, the observed effects of E2 may have been exaggerated without the inhibitory effects of progesterone on HPA activity. Thus, the effects of progesterone, alone or in combination with E2, on HPA activity remain to be examined.

4.4 SUMMARY

In summary, E2 modulates HPA axis activity, and the bi-directional interaction between the HPA and HPG axes appears to be altered in E females (Figure 28). E2 acts directly on the adrenal glands to facilitate both basal and stress-induced secretion of plasma CORT. E2 may also play a role in facilitating CRH-mediated anorectic effects at
the level of the hypothalamus. However, by enhancing plasma CORT secretion, E2 also potentiates CORT inhibition of CRH in the PVN and ACTH secretion by the anterior pituitary. Thus, in this study, plasma CORT levels were higher in the presence of E2 while CRH mRNA and plasma ACTH levels were lower.

Acute stress increased plasma E2 levels. However, E2 has been reported to facilitate stress-induced inhibition of HPG activity (Cates et al., 1999). Thus plasma CORT, released in response to acute stress, likely enhances E2 secretion, but in combination with E2, inhibits GnRH and LH/FSH secretion. CRH and AVP have also been reported to inhibit GnRH and LH/FSH secretion (Petraglia et al., 1987; Cates et al., 1999).

E females appeared to have altered HPA responsiveness to E2. E and PF females had lower basal CORT levels following OVX compared to controls, suggesting altered adrenal responsiveness to E2. E females also had lower basal and stress-induced plasma ACTH levels compared to controls. Since plasma CORT levels were similar among prenatal treatment groups, this suggests altered adrenal responsiveness to ACTH in E females. Furthermore, while PF and C females had higher stress ACTH levels following OVX and lower stress ACTH levels following E2 replacement, E females showed little change across surgical treatment conditions. Thus, E females may also have altered pituitary responsiveness to E2. There was also a suggestion that CRH expressing neurons may be less sensitive to E2 in E females, perhaps due to down-regulation of ERs or altered ER function. E females showed increased HPA activity at the level of the PVN compared to controls, such that resting AVP mRNA expression in the PVN was higher than that in C females. However, this increased AVP mRNA expression does not appear
to be mediated by E2. E females also had altered HPG responsiveness to modulation by the HPA axis. Acute stress did not increase plasma E2 as it did in controls, suggesting altered ovarian sensitivity to CORT in E and PF females. Furthermore, GnRH mRNA expression/neuron was decreased following stress in E females, indicating increased susceptibility to stress-induced alterations to GnRH neuron function.

We hypothesized that the altered HPA activity in E females may be due to altered HPA axis sensitivity to E2. These data indicate that E females have altered HPA axis responsiveness to E2 and altered HPG responsiveness to acute stress. Thus, further experimentation using different time-points and manipulations of the HPA and HPG axes, as well as analyzing other hormones involved in the two axes will develop a more complete picture of the alterations that occur within the HPA and HPG axes following prenatal ethanol exposure. It would also be interesting to see if the alterations to the HPA and HPG axes, as well as the interactions between the two, seen in E females exist prepubertally, which would then allow us to determine what the effects of prenatal ethanol exposure are on organizational and activational events.
Figure 28: Bidirectional Interaction Between the HPA & HPG Axes

The HPA and HPG axes interact in a bidirectional manner and both axes are altered by prenatal ethanol exposure. This is a diagrammatic representation of the interactions that may be taking place between the two axes to explain the data presented in this study. Solid lines represent activation while dashed lines represent inhibition. Diamonds denote areas that may be altered with prenatal ethanol exposure in this experimental paradigm.
CHAPTER 5: SUMMARY & FUTURE DIRECTIONS

5.1 GENERAL SUMMARY:

Sexual dimorphism of the HPA axis has been well established in the literature. Furthermore, prenatal ethanol exposure has been shown to differentially alter the normal sexual dimorphism of the HPA axis in males and females. Therefore, the focus of the present study was to begin to examine the role of E2 in the HPA response to stress in female rats prenatally exposed to ethanol. We hypothesized that prenatal ethanol exposure would alter HPG development and activity and that altered HPA activity in E females may be due, at least in part, to altered HPA axis sensitivity to E2. The data presented here suggest that E females have altered HPG axis activity and HPA responsiveness to stressors as well as altered bidirectional interactions between the HPA and HPG axes. Overall, E females appear to be less responsive to the effects of E2, both in reproductive and non-reproductive measures, and HPG sensitivity to acute stress was altered at various levels of the axis.

5.2 FUTURE DIRECTIONS:

In order to elucidate the complex interactions between the HPA and HPG axes in animals prenatally exposed to ethanol, further experimentation is required. One area to examine would be CORT negative feedback mechanisms. E2 has been shown to modulate glucocorticoid receptor expression and binding. Carey et al. (1995) reported an E2-induced decrease in hippocampal MR mRNA expression and binding. Burgess & Handa (1993) reported a decrease in steady-state levels of MR and GR mRNA.
in the anterior pituitary, hypothalamic preoptic area and hippocampus of OVX-E2 treated rats. Peiffer et al. (1991) reported an increase in GR mRNA in the hypothalamus following OVX. Since the anterior pituitary, hypothalamus and hippocampus are all sites for CORT negative feedback, the increase in HPA activity at proestrus and in estrogen primed animals may be attributed to the negative effect of E2 on GR and MR activity. In E animals, GR and MR sensitivity to the negative effects of E2 may be altered. Therefore, HPA hyperresponsiveness to stressors may be due to increased GR and MR sensitivity to E2, resulting in decreased GR and MR expression or binding capacity and decreased CORT negative feedback. Altered GR and MR binding capacity rather than expression is more likely, since decreased hippocampal glucocorticoid receptor expression does not appear to underlie the possible feedback deficits seen in E animals (Weinberg & Petersen 1991; Kim et al. 1999). Glavas et al. (2000, 2001) found that hippocampal MR and GR mRNA expression in E males and females did not differ from their PF and C counterparts in either SHAM or ADX surgical conditions. This suggests, at least at the receptor level, CORT feedback via the hippocampus is normal in E animals. CORT replacement, however, was less effective in normalizing ADX-induced increases in MR and GR mRNA in E males in several hippocampal subfields. In addition, E females exhibited an overall greater expression of MR mRNA in the CA3 subfield due to higher levels in the ADX and ADX+CORT conditions. Together these data suggest that MR and GR mRNA may be less sensitive to CORT regulation in E compared to PF and C animals.

To compliment investigations into feedback mechanisms in E females, using ADX animals would reveal effects of E2 on HPA drive alone. By removing the adrenals,
CORT negative feedback would be eliminated. Thus in combination with OVX and E2 replacement, the effects of E2 on HPA activation could be studied. Glavas et al. (2001) found plasma ACTH levels were increased in E rats compared to controls following ADX. This suggests either enhanced stimulatory inputs to the hypothalamus and/or increased pituitary sensitivity to secretagogues, which may be mediated by altered responsiveness to gonadal steroids. Indeed, anterior pituitary sensitivity to CRH containing hypothalamic extracts was increased in the presence of estrogen (Coyne & Kitay, 1971) and CRH mRNA transcription was increased in the parvocellular neurons of the hypothalamus in response to estrogen (Lightman & Young, 1989). Thus, E females may have a greater increase in CRH and ACTH secretion in the presence of E2, when CORT negative feedback is eliminated. Furthermore, the adrenals are capable of synthesizing progesterone, estrogens and androgens (Wasada et al., 1978; Yen & Jaffe, 1986), therefore, ADX would also eliminate non-ovarian sources of gonadal hormones.

Finally, in order to determine whether E2 has direct effects on the tissues involved in the stress response, E2 receptors (ER) should be examined. Two types of ER have been discovered – ERα and ERβ. ERα regulates the differentiation and maintenance of neural, skeletal, cardiovascular and reproductive tissues, while the role of ERβ is still unknown (Korach 1994; Smith et al. 1994). It has been suggested that ERβ may modulate ERα-mediated gene expression in the presence of ERα, and partially replace ERα in its absence (Toran-Allerand 2004). The two receptor subtypes appear to be complementary but not redundant, thus ERβ may be a good candidate for direct E2 regulation of non-reproductive functions, such as mediation of HPA activity. Both receptor subtypes are expressed throughout the brain and in reproductive and non-reproductive tissues.
(Laflamme et al. 1998; Gustafsson 2003). Of interest are reports of high ERβ expression in the PVN (Kuiper et al. 1998). Furthermore, Isgor et al. (2003) found that injection of a pure ER antagonist into the PVN inhibited CORT secretion in response to restraint stress and ADX resulted in down regulation of ERβ mRNA in the PVN, while CORT replacement recovered this effect in a dose-dependent manner. These data suggest that ERβ in the PVN may influence the HPA axis response to stress. Also, ERα, but not ERβ, is expressed in the pituitary (Pelletier et al. 2000) while both receptor subtypes are expressed in the adrenal gland (Saunders et al. 1997; Kuiper et al. 1997). Thus, E2 may exert its facilitative effects on HPA activity at the level of the hypothalamus or adrenal. In E animals, the expression or binding capacity of ERα and ERβ may be altered in the neuroendocrine tissues involved in the stress response, resulting in increased or decreased E2 facilitation of HPA activity.

5.3 CLINICAL IMPLICATIONS:

Acute activation of the stress response results in a number of metabolic, neuroendocrine and autonomic adaptations that are beneficial to the organism in times of threatened homeostasis. Chronic activation or hyperresponsive HPA activity leads to many adverse effects on the organism, including myopathy, fatigue, hypertension, gastrointestinal ulceration, immunosuppression, psychogenic dwarfism, osteoporosis, steroid diabetes, weight loss, neuronal death and reproductive dysfunction (Sapolsky 1993; Chrousos et al. 1998). In addition, HPA hyperresponsiveness has been associated with numerous psychiatric disorders. For example, major depressive disorder is associated with chronic HPA hyper-drive leading to emotional dyregulation, cognitive
problems, severe disturbances in sleep, appetite regulation, sexual drive and autonomic regulation, as well as an increased risk for osteoporosis and cardiovascular disorders (Claes 2004). Furthermore, the prevalence of major depressive disorder is higher in women than men, suggesting a role for gonadal steroids in modulating stress-induced psychiatric disorders (Shansky et al. 2004). Other psychiatric disorders associated with HPA hyperresponsiveness include dysthymia, posttraumatic stress disorder, eating disorders, personality disorders, and panic disorder (Claes 2004).

HPA hyperresponsiveness to stressors following prenatal ethanol exposure has been extensively reported in the animal literature (Taylor et al. 1982b; Nelson et al. 1986; Weinberg 1982, 1988; Angelogianni & Gianoulakis 1989). Hyperresponsiveness to stressors has also been reported in humans prenatally exposed to ethanol. Jacobson et al. (1999) found that infants who had been exposed to heavy alcohol both at conception and during pregnancy had higher basal cortisol levels as well as higher cortisol levels following a blood draw than infants who were born to light alcohol users or abstainers. Thus prenatal ethanol exposure may put an individual at greater risk for the adverse effects of HPA hyperresponsiveness. Indeed individuals prenatally exposed to ethanol have been reported to have attention deficits, hyperactivity, personality disorders, eating disorders, posttraumatic stress disorder, panic disorder, depression and anxiety (Mattson & Riley 1998b; Famy et al. 1998). In addition, decreased immune function, weight deficiency and a slight delay in puberty have been found in individuals prenatally exposed to ethanol (Streissguth et al. 1991; Chiappeli & Taylor 1995). The present findings support reports of HPA hyperresponsiveness following ethanol exposure in utero and suggest a role for gonadal hormones in the modulation of HPA axis activity. Further
research into the mechanisms responsible for HPA hyperresponsiveness and the influence of gonadal hormones on HPA activity may lead to the development of pharmacological interventions for individuals prenatally exposed to alcohol. For example, antalarmin, a CRH receptor antagonist, was found to reverse CRH-induced pituitary-adrenal activation and hypertension, and block the acquisition and expression of conditioned fear (Webster et al. 1996; Deak et al. 1999; Briscoe et al. 2000). Thus, CRH receptor antagonists are currently being investigated for the pharmacotherapy of pathological anxiety and depression and may be useful in the treatment of these mental illnesses in individuals prenatally exposed to ethanol.
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