THE EFFECT OF GONADECTOMY ON
THE HYPOTHALAMIC-PITUITARY-ADRENAL RESPONSIVENESS
IN MALE RATS PRENATALLY EXPOSED TO ETHANOL

by

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ABSTRACT

Prenatal ethanol (E) exposure has many adverse effects on offspring development. Exposure to chronic high levels of alcohol can result in Fetal Alcohol Syndrome (FAS), one of the leading known causes of mental retardation in the Western world. Prenatal exposure to lower doses of alcohol can result in partial FAS or Alcohol-Related Neurodevelopmental Disorder (ARND), characterized primarily by physiological and behavioral abnormalities; and Alcohol-Related Birth Defects (ARBD), characterized primarily by physical abnormalities. Work in our lab has focused on the effects of prenatal ethanol exposure on the hypothalamic-pituitary-adrenal (HPA) axis, a critical system for the stress response. Previous studies have shown that prenatal ethanol exposure reprograms the fetal HPA axis such that E offspring are hyperresponsive to stressors in adulthood.

Rats prenatally exposed to ethanol (E rats) show increased HPA activation and/or delayed or deficient recovery to basal levels following stress. Previous data in our lab and others showed that there are marked sex differences in stress responsiveness and HPA regulation at different levels of the axis in E rats, and also, that E males and females differ from their control counterparts, suggesting that the balance between HPA drive and feedback is differentially altered in E males and females compared to their control counterparts. The aim of the present study was to investigate the influence of gonadal hormones on HPA responsiveness in E compared to control males. Experiments were carried out on adult (~70d) male offspring from E, pair-fed (PF) and ad-lib fed control (C) dams. All the animals were subjected to either sham-gonadectomy (intact) or gonadectomy
(GDX). Animals were tested at the circadian trough at 0 (basal), 30 (stress) or 90 min (recovery) after the onset of a 30 min restraint stress. Plasma testosterone, luteinizing hormone (LH), corticosterone (CORT), and adrenocorticotropin (ACTH) levels were measured as an index of hypothalamic-pituitary-gonadal (HPG) and HPA function. Testosterone concentrations in GDX rats were below the detectable range. In intact rats, PF and C rats had higher testosterone at 30 than at 0 min, whereas E rats did not differ in testosterone across time. As expected, GDX markedly increased LH levels. In intact rats, overall, LH level increased at 30 min and decreased to basal levels at 90 min. This increase at 30 min mainly reflects a significant increase in LH in intact C rats, whereas E and PF males showed no significant change over time. Following GDX, LH levels were higher at 0 min than at 30 min, and higher at 30 min than at 90 min in all prenatal groups. There were no significant differences among E, PF and C across time in GDX condition. CORT levels were higher at 30 min and 90 min than at 0 min, and higher at 30 min than at 90 min in all animals under both sham and GDX treatments. ACTH levels were higher in 30 min than in 0 and 90 min in all animals, under both sham and GDX treatments. Importantly, intact E rats had significantly higher ACTH levels than C rats at 30 min, and the difference was eliminated by GDX. There was a strong negative correlation between the testosterone and CORT response to restraint stress at 30 min in intact E but not PF and C males. Furthermore, following GDX, there was a strong positive correlation between the LH and ACTH responses to stress in PF and C but not E male rats.

These data indicated that the stimulatory effect of GDX on stress-induced ACTH
release is impaired in E rats, suggesting a reduced effect of androgens on HPA responsiveness or a decreased sensitivity to androgens in E males. The findings from the present study support the hypothesis that HPA alterations are mediated, at least in part, by ethanol induced changes in androgens regulation or HPA sensitivity to androgens.

HPA hyperresponsiveness results in prolonged or chronic CORT elevations, with concomitant adverse physiological and behavioral consequences that could compromise health and even survival. Early life events (ie. prenatal ethanol) that result in greater reactivity to stress and increased CORT levels throughout the life span can increase the vulnerability to illnesses later in life. Therefore, research aimed at elucidating the mechanisms underlying this hyperresponsiveness of prenatal ethanol exposure, will significantly advance our understanding of the long term adverse consequences of prenatal ethanol exposure, and will have important implications for the development of interventions and treatments.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARBD</td>
<td>alcohol-related birth defect</td>
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<tr>
<td>ARND</td>
<td>alcohol-related neurodevelopmental disorder</td>
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<td>AVP</td>
<td>arginine vasopressin</td>
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<td>BAL</td>
<td>blood alcohol level</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>C</td>
<td>control</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CORT</td>
<td>corticosterone</td>
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<td>CRH</td>
<td>corticotrophi releasing hormone</td>
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<td>d</td>
<td>day</td>
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<tr>
<td>E</td>
<td>prenatally exposed to ethanol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FAS</td>
<td>fetal alcohol syndrome</td>
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<tr>
<td>g</td>
<td>gram</td>
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<td>G</td>
<td>gestation day</td>
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<td>GC</td>
<td>glucocorticoid</td>
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<td>GR</td>
<td>glucocorticoid receptor</td>
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<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
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<tr>
<td>hnRNA</td>
<td>heteronuclear ribonucleic acid</td>
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<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>reduced form of NAD</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>PF</td>
<td>pair-fed</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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</table>
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CHAPTER 1 INTRODUCTION

A. Fetal Alcohol Syndrome (FAS)

The first research data on the adverse outcomes from prenatal alcohol exposure in humans (Sullivan, 1899; Templeman, 1892) and animal models (Fere, 1895) were published in the 1890s. However, the association between maternal consumption of ethanol and abnormal development of the offspring was not brought to our attention until Lemoine and colleagues in France (Lemoine et al., 1968) and Jones and colleagues in Seattle (Jones et al., 1973) independently reported a pattern of craniofacial, limb and cardiovascular defect with prenatal-onset growth deficiency and developmental delay in children born to alcoholic mothers. It was not until 1973 that the term Fetal Alcohol Syndrome (FAS) was coined to describe this cluster of malformation in children prenatally exposed to alcohol (Jones and Smith, 1973; Jones et al., 1973). As a result of the identification of FAS, there followed considerable interest in the teratogenicity of ethanol.

FAS can be diagnosed by three major criteria (Sokol and Clarren, 1989): (1) growth deficits, including prenatal and postnatal growth deficiency; (2) alterations in the central nervous system (CNS), including microcephaly, delayed or altered intellectual development, and behavioural deficits, such as hyperactivity, poor attention span, impaired habituation, impulsivity, lack of inhibition (Shaywitz et al., 1980); (3) a characteristic facial dysmorphology including: a narrow forehead, short palpebral fissures, small nose, small midface, a smooth philtrum and a thin vermilion upper lip (Jones et al., 1973).

Considered the most common nonhereditary cause of mental retardation (Abel and
Sokol, 1986), FAS occurs in approximately one-third of infants born to alcoholic women, the remaining two-third showing effects ranging from severe disabilities to only minimal deficits. If the full diagnostic criteria of FAS are not met, then alcohol-related effects are termed partial FAS (Abel, 1984). Children with significant prenatal alcohol exposure can lack the characteristic facial defects or growth deficiency of FAS but still have alcohol-induced mental impairments that are just as serious as those in children with FAS. Children displaying only physical abnormalities are referred to as having alcohol-related birth defects (ARBD) (Sokol and Clarren, 1989; Stratton et al., 1996). Children who display behavioural or functional abnormalities are described as displaying alcohol-related neurodevelopmental disorders (ARND) (Stratton et al., 1996).

Longitudinal studies on offspring with FAS suggest that prenatal ethanol alcohol may produce long-term alterations or consequences that persist throughout life span. The effects range from physical abnormalities to altered cognitive and behavioral function, compromising an individual's ability to adapt to his/her environment, for example, to recognize and act upon significant stimuli, to inhibit responses to irrelevant stimuli, and to adapt to new and changing external environments (Baer et al., 2003; Bookstein et al., 2002; Spohr et al., 1993; Streissguth et al., 1994).

1. Occurrence of FAS

The literature on the epidemiology of FAS and ARBD or ARND is extensive and complicated. The incidence and prevalence rates vary widely depending on the methodologies and definitions in case finding and diagnosis, wide variation in the types of
populations studied, prospective versus retrospective methods of data gathering et al (Stratton et al., 1996). Various studies reporting the occurrence of FAS range from 0.5 to 3 per 1000 live births in most populations and the combined rate of FAS and ARND was estimated to be at least 9.1 per 1000 (Sampson et al., 1997).

2. Factors that Influence the Teratogenic Effect of Ethanol

Ethanol is of particular interest as a teratogen because of its wide use, and the wide range of effects on the developing fetus. There is convincing evidence that alcohol directly affects the fetus because it readily across the placental and blood brain barriers (Waltman and Iniquez, 1972). The metabolic product of ethanol, acetaldehyde, is also both teratogenic and embryolethal if administered to pregnant rats.

FAS does not occur in all infants born to heavily drinking mothers suggesting that factors other than the dose/duration of alcohol exposure alone are involved. Factors that may contribute to the pathogenesis include the critical periods of alcohol exposure in pregnancy, peak blood alcohol level due to drinking pattern, physiological variables that affect absorbance and metabolism of alcohol, general maternal health, nutritional factors, use of other drugs, et al (Schenker et al., 1990). Here I will focus on discussing two of the most important factors: temporal vulnerability and peak blood alcohol level.

a. Temporal Vulnerability

The timing of alcohol exposure is a key risk factor that influences the type or extent of damage. The first trimester is the critical period of organogenesis, when the major organs form and the facial and skull bones develop. Exposure during this time can produce facial
dysmorphology similar to that associated with FAS (Sulik et al., 1981). The second trimester is a developmental stage when the nerve cells in the neocortex are generated and migrate to the appropriate brain regions. Alcohol appears to affect the timing and pattern of nerve cell generation, both delaying the process and altering the number of cells that are produced. Alcohol exposure during this period can produce neuronal loss (Barnes and Walker, 1981; Miller and Potempa, 1990). The third trimester is the brain growth spurt, a time of very rapid brain development that occurs in part during the third trimester in humans and postnatal period in rats. This is an especially vulnerable period for the brain in response to alcohol insult. Brain weight reduction is a consistent finding in rat models of prenatal alcohol exposure during this trimester. Other anatomical changes include reduced number of cells in certain regions of the hippocampus and the cerebellum which are related to abnormal behavior later in life, such as hyperactivity and learning deficits observed in animals that appear to be physically normal (Goodlett et al., 1990; West and Goodlett, 1990).

b. Peak Blood Alcohol Level

The type and extent of the alcohol-induced fetal damage is partly related to the level and pattern of fetal alcohol exposure. The peak blood alcohol level is a key factor in determining the severity of brain damage (West et al., 1990). Maternal binge drinking (ie., consumption of five or more standard drinks per occasion) [one standard drink is defined as one 12-ounce can of beer or wine cooler, one 5-ounce glass of wine, or 1.5 ounces of distilled spirits (Gabriel et al., 1998)] during pregnancy is one of the strongest predictors of
later neurodevelopmental deficits in children with alcohol-induced damage (Streissguth et al., 1989). The correlation between alcohol damage and blood alcohol levels has been shown for both structural and functional studies (Bonthius et al., 1988; Bonthius and West, 1990; Goodlett et al., 1991).

3. **Animal Models of FAS**

There is evidence in both human and animal models that ethanol is a teratogen and can cause malformations, intrauterine death, growth retardation, central nervous system abnormalities, and behavioral deficits. However, much information is still needed to determine the circumstances that increase the risk and severity of fetal alcohol-induced brain damage and to identify the mechanisms underlying such damage. Animal research has been used to address these issues because, for the most part, they are unapproachable experimentally in humans.

Rodent models are the major subjects of investigation for biochemical, neuroanatomical, and behavioral effects resulting from prenatal alcohol insult. Physical alterations observed in children with FAS also have been shown to occur in rodent models of prenatal ethanol exposure, where physical alterations include retarded pre- and postnatal growth and development, and physical malformations (Abel and Dintcheff, 1978; Sulik et al., 1981). Rodents also show CNS abnormalities, including learning deficits (Bond and Digiusto, 1977) and a range of behavioural deficits, such as behavioural hyperactivity and hyperresponsiveness (Abel, 1979; Diaz and Samson, 1980). Animals prenatally exposed to ethanol (E) exhibit behavioral hyperactivity in a variety of tasks, such as increased open
field and running wheel activity, increased exploratory behavior and startle reactivity (Becker et al., 1996; Bond and Di Giusto, 1976). They also show deficits in using environmental cues (Bond and Di Giusto, 1977; Weinberg, 1992b). Furthermore, E animals have deficits in passive avoidance learning, suggesting an impairment in response inhibition (Abel, 1982; Driscoll et al., 1982; Gallo and Weinberg, 1982), and defects in Morris water maze performance, suggesting defects in spatial learning and memory (Byrnes et al., 2003; Gabriel et al., 2002; Kim et al., 1997; Wainwright et al., 1993; Westergren et al., 1996). These findings relate to the behavioral abnormalities observed in alcohol-exposed children, such as poor impulse control, poor habituation and lack of response inhibition (Driscoll et al., 1990). Prenatal ethanol induced physiological abnormalities such as altered endocrine and immune competence have also been observed in animal models (Jerrells and Weinberg, 1998; Redei et al., 1993; Weinberg, 1994). For example, hormonal hyperresponsiveness to stressors (Nelson et al., 1986; Taylor et al., 1982; Taylor et al., 1984; Weinberg et al., 1996) and immune challenges (Kim et al., 1999b), suppressed splenic T-cell proliferative response to the mitogenic lectin Concanavalin-A (Con-A) (Chang et al., 1994; Norman et al., 1989; Redei et al., 1989; Weinberg and Jerrells, 1991).

Several methods of ethanol administration have been developed to investigate the effects of prenatal ethanol exposure, including intraperitoneal injection, inhalation, oral intubation, and placing alcohol in the drinking water or a liquid diet. Although administering ethanol via injection or intubation can cause high blood alcohol levels, and researcher can keep the same dose in each animal, both of them are invasive techniques that
result in a fair amount of stress to the pregnant dam, and thus may lead to prenatal stress effects in the offspring. Prenatal stress in itself has been shown to produce hyperresponsiveness of the HPA axis (Suchecki and Palermo Neto, 1991). Putting alcohol in the drinking water is non-invasive, and this is the same route of exposure that occurs in humans. However, most rodents will not consume alcohol voluntarily and will reduce their fluid intake and therefore their food intake as well, resulting in lower blood alcohol levels due to the reduced amount of alcohol digested. Exposing animals to alcohol vapors is also non-invasive and may achieve high blood alcohol levels. However, this is not representative of the way humans consume ethanol. Adding alcohol to a liquid diet and providing this as the only source of nutrition is simple, nontraumatic, and easy to administer. It achieves a level of alcohol consumption that is of clinical relevance, while maintaining dietary control and providing adequate nutrition, and results in high BALs (Lieber and DeCarli, 1989). However, there are a number of issues with this method as well: animals do not all consume the same amount of alcohol; animals consume greater amount of water with liquid diet than they would with pelleted diet; and, of using a commercial liquid diet like sustecal, the presence of flavorings and preservatives that may have toxicological effects is another disadvantage of this method.

Alcohol-derived calories replace the calories supplied by food, so that ethanol diets won’t produce adequate nutrition. Inclusion of a pair-fed (PF) group is necessary to control for the reduced food intake typically found in alcohol-fed animals. Animals in the PF group are fed the amount consumed by an E partner (g/kg body wt/day of gestation), using a
liquid control diet, with Maltose-dextrin isocalorically substituted for ethanol. Although this provides a nutritional control in which nutritional intake is matched with that of the prenatal ethanol group, it can never completely control for alcohol’s effects on digestion, absorption and utilization of nutrients. Furthermore, pair-feeding itself is a type of experimental treatment because pair-fed animals typically get less food than they want and are constantly hungry (Weinberg, 1984). Thus pair-feeding may actually be a kind of prenatal stress. Prenatal stress affects the hormonal and behavioural development of offspring (Kofman, 2002). Because of the problems inherent in pair-feeding, it is important to include an ad libitum fed standard lab chow control (C) group in all studies of fetal ethanol exposure. If a difference is found between the ethanol group and both the PF and C groups, but there are no differences between the two control groups, then it is probably reasonable to assume that effects are alcohol related.

**B. Hypothalamic-Pituitary-Adrenal (HPA) Axis**

Stress was originally defined by Selye as the nonspecific response of the body to any demand, characterized by a pathological triad (adrenal enlargement, gastrointestinal ulceration, and thymiclymphatic involution) (Selye, 1973). Chrousos and Gold introduced the concept of stress as a state of disharmony or of threatened homeostasis evoking both specific and nonspecific responses (Chrousos and Gold, 1992). A challenge to homeostasis (stressor) induces activation of the HPA axis and the sympathetic nervous system (SNS), resulting in a series of neural and endocrine adaptations known as the "stress response" or "stress cascade". The stress response is responsible for allowing the body to make the
necessary physiological and metabolic changes required to cope with the demands of a homeostatic challenge, such as energy mobilization, increase in cardiovascular and respiratory tone, suppression of anabolic processes, sharpened cognition, increased arousal and alertness, focused attention, and altered sensory threshold. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances for survival. Acute stress responses are advantageous for the survival of the organism; however, prolonged or chronic stress may be maladaptive and can lead to a variety of pathological conditions. The sympathetic nervous system, also called the locus coeruleus (LC)/autonomic nervous system, mediates the stress response via the release of noradrenaline from postganglionic nerve terminals, while preganglionic innervation of the adrenal medulla results in increased secretion of adrenaline, into the peripheral circulation (Goldstein, 1987). The SNS is involved in the “fight or flight” response and enables the organism to react rapidly to the stressor.

The ability to respond appropriately to stress is an important basic adaptive mechanism, and HPA activation is a central feature of this response. The stressor initiates the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular division of the paraventricular nucleus (PVN) of the hypothalamus. CRH and AVP reach the anterior pituitary via the hypothalamic-hypophyseal portal vessels, regulates the synthesis of proopiomelanocortin (POMC), and stimulates the release of POMC-derived peptides such as adrenocorticotropic hormone (ACTH) and β-endorphin (β-EP) from the anterior pituitary into general circulation. It is well recognized that AVP acts
synergistically with CRH to enhance ACTH release. ACTH then acts on the adrenal cortex to stimulate the synthesis and release of the end-product of the HPA axis, species-specific glucocorticoid [corticosterone (CORT) in rodents; cortisol in human] into the systemic circulation. Glucocorticoids act in a negative feedback fashion on various areas of the brain and different levels of the axis to terminate the stress response.

The actions of CORT are mediated through its binding to specific intracellular receptors. There are two major classes of corticosteroid receptors: Type I (MR, mineralocorticoid receptor) and Type II (GR, glucocorticoid receptor), which differ in binding affinity to various endogenous and synthetic ligands as well as distribution throughout the brain (Reul and de Kloet, 1985). Both MR and GR mediate CORT negative feedback upon the HPA axis. MRs bind with high affinity to CORT, suggesting that they are involved mainly in regulating basal levels of CORT, whereas GRs bind with about 10-fold less affinity to CORT and are occupied predominantly during high levels of circulating CORT, such as during stress or the circadian peak. GRs are widely distributed throughout the brain with high concentrations in hippocampus and septum, PVN and supraoptic nuclei of the hypothalamus, cerebral cortex, and anterior pituitary. MRs are located primarily within limbic structures such as hippocampus, septum and amygdala.

C. Effects of Prenatal Ethanol Exposure on the HPA axis

The endocrine system plays a critical role in maintaining a successful pregnancy. Prenatal ethanol exposure directly affects the mother's own health and thus indirectly affects fetal development by disrupting the normal hormonal interactions between the
mother and the fetus (Anderson, 1981). The development of fetal endocrine function, as well as fetal metabolic or physiological function, could thus be affected in utero. Furthermore, ethanol directly affects fetal development because ethanol freely crosses the placental barrier (Waltman and Iniquez, 1972) and can be distributed in the amniotic fluid and multiple fetal tissues, so that it has direct effects on fetal endocrine glands and brain.

Maternal ethanol intake alters many aspects of offspring endocrine function, including the HPA axis (Lee and Rivier, 1996; Rivier, 1996; Taylor et al., 1982; Taylor et al., 1984; Weinberg et al., 1996). Clinical studies on children prenatally exposed to alcohol showed that alcohol has stimulatory effects on the HPA axis of the newborn (Binkiewicz et al., 1978). Animal studies strongly support the concept that alcohol exposure in utero can have major effects on the development and function of the offspring HPA axis. At birth, neonates exposed to alcohol in utero have elevated plasma and brain concentrations of CORT (Kakihana et al., 1980). During the first 3 weeks of life, E offspring exhibit suppressed CORT responses to a wide range of stressors including ether, novel environment, saline injection, and cold stress, as well as to drugs such as alcohol and morphine (Taylor et al., 1986; Weinberg, 1989). Following weaning, E animals are typically hyperresponsive to stressors. Enhanced pituitary-adrenal activation to footshock, ether, and drugs such as alcohol and morphine, as well as increased β-EP responses to cold and ether stress, have been reported in E animals (Angelogianni and Gianoulakis, 1989; Kim et al., 1999a; Nelson et al., 1986; Weinberg et al., 1996). E offspring showed prolonged CORT elevations following stress, suggesting deficits in adrenocortical response inhibition or recovery to
basal levels (Weinberg, 1988).

Previous data in our lab (Weinberg, 1988; Weinberg, 1992a) and others (Lee and Rivier, 1996) show that there are marked sex differences in stress responsivenesness and HPA regulation at different levels of the axis in E males and females, and also, that E males and females differ from their control counterparts, suggesting that the balance between HPA drive and feedback is differentially altered in E compared to their PF and C counterparts. This raises the possibility that the gonadal hormones may play a role in mediating prenatal ethanol effects on HPA activity.

D. Hypothalamic-Pituitary-Gonadal (HPG) Axis

Gonadotropin releasing hormone (GnRH) neurons in the hypothalamic region of the brain provide the central neural drive that directs all aspects of the reproductive activity. GnRH neurons are neuroendocrine cells in the preoptic area of the hypothalamus. Their axons terminate on blood vessels within the median eminence. GnRH is released in bursts from terminals in the median eminence into the hypophysial portal system and travels a short distance to the anterior pituitary, regulates the synthesis and release of two gonadotropic glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), together referred as gonadotropins. LH and FSH are released from the pituitary into the systemic circulation in response to GnRH, and they travel to the gonads, where they direct gamete production, as well as gonadal hormone production. The secretion and/or action of GnRH is influenced by feedback actions of sex steroids and inhibin which are produced by the gonads.
In males, LH stimulates testosterone production in the Leydig cells of the testes. FSH and testosterone then act in coordination to simulate spermatogenesis in the testicular seminiferous tubules. In addition, testosterone supports the sexual organs of the male, including the penis, prostate, and seminal vesicles.

In females, LH and FSH act at the ovary. FSH stimulates ovarian follicle development at the beginning of each ovarian cycle, and FSH and LH together stimulate ovulation in the middle of each cycle. FSH and LH also stimulate the production of the ovarian steroid hormones, estrogen and progesterone. These ovarian hormones, in turn, support the sexual organs of the female, including the uterus, cervix, and fallopian tubes. Estrógen, in particular, also acts on other body tissues to cause breast development and increase subcutaneous deposition of fat.

**E. Effects of Prenatal Ethanol Exposure on the HPG axis**

Previous work has shown that prenatal ethanol exposure has marked effects on the development of the HPG axis in both males and females. Here I will focus on the adverse effect of prenatal alcohol on the male HPG function. Alcohol exposure in utero has marked adverse effects on the fetal testis. Decreased numbers of Leydig cells, the presence of vacuoles in the seminiferous tubules, and insensitivity to LH have been reported in E compared to control fetuses (McGivern et al., 1988). Both the pre- and postnatal testosterone surges are also suppressed in E fetuses/neonates (McGivern et al., 1993; McGivern et al., 1988; Ward et al., 2003). At birth, E pups exhibit decreased brain and plasma testosterone, and deficits in testicular steroidogenic enzyme activity compared with

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controls (Kelce et al., 1990; Kelce et al., 1989). E neonates also exhibit a decreased anogenital distance compared to controls, suggesting feminization (Udani et al., 1985). However, some studies of prenatal ethanol on anogenital distance report no effect in some experimental models (McGivern et al., 1992; McGivern et al., 1988). The developmental pattern of LH secretion also is altered in E males, which could contribute to later changes in reproductive function (Handa et al., 1985). Male rats prenatally exposed to ethanol showed decreased testicular weight, and severely altered morphology of seminiferous tubules, ie. absence of reticulin fibers in the peritubular tissue of seminiferous tubules at PND 42 (Fakoya and Caxton-Martins, 2004). In adulthood, rats prenatally exposed to ethanol show reduced weights of testes, prostate and seminal vesicles, decreased serum testosterone and LH levels, and altered neurotransmitter responses to testosterone, suggesting central dysregulation of HPG activity (Udani et al., 1985). Handa et al. reported that E males exhibited lower mean levels of LH, in addition to a decrease in pulse amplitude and frequency. They also found that GDX E male rats had significantly reduced plasma LH titers as compared to those of PF controls (Handa et al., 1985). Deficits in sexual behavior, as well as feminization of a variety of nonsexual sexually dimorphic behaviors have also been reported in E males (Barron et al., 1995; McGivern et al., 1984). However, normal LH and testosterone levels in adult E males have also been reported (Ward et al., 1996).

F. Interaction of HPA and HPG Axes

1. The Effects of Gonadal Hormones on HPA Regulation

A wide body of evidence showed that gonadal hormones are involved in the
regulation of HPA axis. In general, estradiol (E₂) activates, and androgen inhibits HPA
function, mainly by changing the production and secretion of CRH in the PVN of the
hypothalamus. There are sex differences at different levels of HPA axis, such as females
have higher basal and stress levels of ACTH and CORT than males. As well, higher levels
of CRH protein and CRH mRNA in the PVN has been found in females than males in both
human and animal models (Watts and Swanson, 1989).

An effect of gonadal hormones on HPA function has been demonstrated at different
levels of the axis. For example, androgen inhibits hypothalamic CRH peptide (Almeida et
al., 1992; Bingaman et al., 1994) as well as restraint-induced c-fos mRNA, CRH
heteronuclear RNA (hnRNA), and AVP hnRNA expression in the PVN (Lund et al., 2004).
CRH mRNA levels are higher during proestrous in female rats, when E₂ levels are highest
(Bohler et al., 1990). In addition, androgen decreased GR mRNA expression in the
hippocampus (Kerr et al., 1996), and increased GR levels in the medial preoptic area
(mPOA) (Viau and Meaney, 1996).

Gonadectomy (GDX) on adult male rats increased the ACTH as well as the CORT
response to physical and psychological stressors (Handa et al., 1994). GDX male rats
showed greater stress-induced Fos expression and higher AVP hnRNA levels. Both
responses are negatively correlated with plasma testosterone levels (Viau et al., 2003). This
enhanced response of the HPA axis to stress is not accompanied by changes in anterior
pituitary sensitivity to CRH, suggesting a central site of action. In addition, the GDX effect
can be inhibited by the administration of the non-aromatizable androgen,
dihydrotestosterone (DHT), implicating androgen receptors in the regulatory pathway (Handa et al., 1994).

2. The Effects of HPA Hormones on HPG Regulation

   It is conventional knowledge that stress has inhibitory effects on reproductive behavior and HPG hormones. Stress could affect the secretion of the gonadotropins through mechanisms that modify synthesis and/or secretion of GnRH, the responsiveness of the gonadotrophs to the actions of GnRH or the feedback actions of gonadal hormones. Although there are various pathways involved, the HPA axis is the most likely candidate for the mediation of stress-induced gonadal inhibition (Rivier and Rivest, 1991; Tilbrook et al., 2000; Tilbrook et al., 2002). In the rodent, central injections of CRH inhibits the synthesis of GnRH and plasma LH (Petraglia et al., 1987; Rivier and Vale, 1984). It has been shown that CRH either directly or indirectly acts on GnRH neurons in the mPOA of the hypothalamus (Rivier and Vale, 1984). GCs generally disrupts all aspects of HPG function, including reproductive behavior, GnRH expression, plasma LH secretion and sex steroid synthesis and release (Tilbrook et al., 2000; Tilbrook et al., 2002). Adrenalectomy reverses stress-induced suppression of LH secretion in long-term ovariectomized rats, indicating a primary role of GCs in the stress-induced HPG function (McGivern and Redei, 1994). While direct effects of stress on the gonads is possible, it is generally accepted that the predominant impact is to influence the secretion of GnRH from the brain and of the gonadotrophins from the gonadotrophs of the anterior pituitary gland (Rivier and Rivest, 1991; Tilbrook et al., 2000; Tilbrook et al., 2002). Central administration of ACTH caused a
dose-related rise in LH levels in intact male rats (Mann et al., 1986). GCs suppress gonadotropin release not only by acting at the level of pituitary, but also at the hypothalamic level (Calogero et al., 1999). However, there are also many reports indicating that acute and chronic stress may induce different states of gonadal activity. Lemaire et al. showed that HPG axis is activated during social interactions even when the HPA axis is stimulated (Lemaire et al., 1997), suggesting that stress does not always inhibit gonadal function.

The sex differences in HPA responsiveness to stress are due principally to the influence of the peripheral sex steroids. Similarly, the effect of stress on the HPG axis also differs between sexes and is affected by sex hormones. Androgens potentiate the effect of GCs on GnRH release (Calogero et al., 1999). Acutely elevated GCs induced by psychosocial or immune stress suppresses LH pulse amplitude in ovariectomized ewes by inhibiting pituitary responsiveness to GnRH (Breen and Karsch, 2004; Debus et al., 2002). Cortisol does not acutely inhibit GnRH pulsatility in the absence of gonadal steroids (Breen and Karsch, 2004).

It has been reported that both independent and interactive effects of testosterone and CORT play a role in HPA function (Viau, 2002). As the adrenal and gonadal axes develop in parallel and interact in a bidirectional manner, alterations in HPG function may play a role in the differential HPA hyperresponsiveness in E males and females.

**G. Thesis Objective**

The ability to respond appropriately to stress is an important basic adaptive
mechanism, and HPA activation is a central feature of this response. Rats prenatally exposed to ethanol are typically hyperresponsive to stress in adulthood. However, prenatal ethanol differentially affects HPA responsiveness in male and female offspring compared to their PF and C counterparts, suggesting that the gonadal hormones may play a role in mediating prenatal ethanol effects on HPA activity.

How and where stress and gonadal steroids interact in the brain to affect HPA regulation is an important question in view of the marked functional consequences of altered hormone activity. The objective of this study was to investigate the influence of gonadal hormones on HPA responsiveness in E compared to control males. We tested the hypothesis that the differential alterations in HPA drive and feedback observed in E males compared to their control counterparts are mediated, at least in part, by ethanol induced changes in androgens regulation or HPA sensitivity to androgens.

Experiments were carried out on adult (~70d) male offspring from E, PF and C dams. All the animals were subjected to either sham-gonadectomy (intact) or gonadectomy. Animals were tested at the circadian trough at 0 (basal), 30 (stress) or 90 min (recovery) after the onset of a 30 min restraint stress. Plasma ACTH, CORT, LH and testosterone levels were measured as an index of HPA and HPG function.
CHAPTER 2: MATERIALS AND METHODS:

A. Animals and Breeding

Male (275-300 g, n=18) and female (230-275 g, n=50) Sprague-Dawley rats were obtained from Charles Rivers Laboratories (Montreal, PQ, Canada). Rats were pair- or triple-housed by sex in the Animal Care Center (Vancouver Hospital, the University of British Columbia Site, Vancouver, BC, Canada) and maintained on a 12 : 12 hr light/dark cycle (lights on at 06:00 hr), with controlled temperature (21-22 °C), and ad libitum access to standard lab chow and water. One – two weeks following arrival, males were placed singly in stainless steel suspended cage (25 × 18 × 18 cm) with mesh front and floor and provided with ad libitum access to standard lab chow and water. Three days later females were randomly assigned to a male, with the heavier females being placed into the hanging cages first. Wax paper was placed under the hanging cages and was checked daily before 11 AM for the appearance of vaginal plugs. The presence of a vaginal plug indicated day 1 of gestation (G1). All animal use and care procedures were in accordance with the National Institutes of Health and Canadian Council on Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee.

B. Diets and Feeding

On G1, females were singly housed in polycarbonate cages (24 × 16 × 46 cm) and randomly assigned to one of three treatment groups: 1) Ethanol (E), liquid ethanol diet containing 36% ethanol-derived calories, and water, ad libitum (n=17); 2) Pair-fed (PF), liquid control diet with maltose-dextrin isocalorically substituted for ethanol. The amount
provided was matched to the amount consumed by an E female partner (g/kg body weight) on the same day of gestation, and animals had ad lib access to water (n=14); 3) Control (C): standard lab chow and water, ad libitum (n=18). E females were introduced to the ethanol diet by gradually increasing the ratio of ethanol: control diet, with 1/3, 2/3, and 100% ethanol diet on G1, G2 and G3, respectively. The liquid diets (Dyets Inc., Bethlehem, PA, USA) were formulated in our lab to provide adequate nutrition to pregnant female rats. All animals were provided with fresh diet within 1.5 hr of lights off to prevent a shift of CORT circadian rhythms, which may occur in PF animals who are on a restricted diet (Gallo and Weinberg, 1981). Feeding bottles were removed and weighed daily to determine the amount consumed by each animal. E and PF dams were provided with fresh liquid diet until G22 when they were provided ad libitum access to standard lab chow and water, which they received throughout lactation. Pregnant dams were handled only on G1, G7, G14 and G21 for cage changing and weighing. On postnatal day 1 (PND1), pups were weighed and were culled to a litter size of 10 (5 males and 5 females). If necessary, cross fostering of pups occurred with pups from the same prenatal treatment group born on the same day, to maintain a litter size of 10 (5 males and 5 females). Dams and pups were weighed on PND1, PND8, PND15 and PND22. On PND22, pups were weaned and group-housed by litter and sex. All pups were ear-punched to indicate litter number. Male pups were pair-housed starting around PND40.

C. Blood Alcohol Level (BAL) Measurements

To determine the maximal BAL achieved by E dams, tail blood samples from 3
randomly chosen E dams were taken at around G15, two hours after lights off, when major
eating bouts occur. Blood was sampled via removal of the tip of the tail with a razor blade,
and collected into 600 µl Eppendorf tubes. The blood sample was allowed to coagulate for
two hours at room temperature and then spun down at 3000 rpm for 20 min at 4 °C. Serum
was collected and stored at −20 °C until the time of assay. BALs were measured using
Pointe Scientific Inc. Alcohol Reagent Set (Lincoln Park, MI, USA). The assay is based on
the change in absorbance at a wavelength of 340 nm which occurs when alcohol
dehydrogenase (ADH) catalyzes the oxidation of the 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.

D. Surgery

All animals were 53-58 days of age at the time of surgery. Male offspring from the 3
prenatal groups were randomly assigned to two weight-matched groups: 1) sham-
gonadectomized (Intact) group; 2) gonadectomized (GDX) group. Testes were extracted
under halothane anesthesia via a longitudinal scrotal incision, the testicular vessels were
ligated, and scrotal incision was closed with 4-0 nonabsorbable suture. Sham gonadectomy
was done by making an incision and then suturing the scrotum, without touching the testes.
All the animals received a 0.1 cc intramuscular injection of the antibiotic Duplocillin
(Intervet Canada Inc., Whitby, ON, Canada, DIN 01983377). Surgery was done between
0900-1200 hr each day. Animals were weighed right after the surgery.
E. Testing and Sampling

Rats were weighed one day before testing, which occurred 14 d after the surgery. Rats from the two treatment groups (sham-GDX and GDX) were decapitated at 0, 30, and 90 min after the onset of a 30 min restraint stress. The number of rats per group, time condition and surgical treatment is 7-12. Stress testing achieved by placing rats into polyvinyl chloride restraint tube for a 30 min period. The restraint tubes were 5.5 × 20 cm (inner diameter × length) for rats weighed less than 380 g and 7.5 × 20 cm (inner diameter × length) for rats weighed more than 380 g at testing. The tubes 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 a 1.5 cm opening for the tail. Restraint is primarily a psychological stressor, and caused no pain or injury. Basal control rats (0 min) were terminated immediately after removal from their home cage. Following restraint stress, rats were terminated either immediately upon releasing from the tube (30 min time point) or were returned to their home cage for recovery for another 30 min and then terminated.

Animals were terminated by decapitation in order to get trunk blood for plasma hormone measures. All sampling were done at circadian trough between 0930 – 1200 hr.

F. Radioimmunoassay (RIA)

At the time of sampling, trunk blood was collected into ice-chilled polystyrene tubes 200 μl 0.5 M EDTA (to prevent blood from coagulating) and 4 μg/ml aprotinin (to protect ACTH from denaturation). Blood sample was centrifuged under 3200 rpm for 10 min at 0 °C. Plasma was transferred into 600 μl Eppendorf tubes and stored at -80 °C until
assayed for CORT, ACTH, T and LH by RIA.

1. Corticosterone (CORT)

Total CORT (bound and free) levels were measured via RIA in plasma extracted in 95% ethanol (Weinberg and Bezio, 1987), adapted from (Kaneko et al., 1981). 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 antiserum cross-reacts 100% for CORT, 2.3% for desoxycorticosterone, 0.47% for testosterone, 0.17% for progesterone and 0.05% for aldosterone. The minimum detectable CORT concentration of the assay was 0.25 μg/dl and the intra- and inter-assay coefficients of variation were 1.55% and 4.26% respectively.

2. Adrenocorticotropicin (ACTH)

Plasma ACTH levels were measured using an adaptation of an ACTH RIA kit (Diasorin Inc., Stillwater, MS, USA) with [125I] ACTH as tracer. The ACTH antibody cross-reacts 100% with porcine ACTH1-39 and human ACTH1-24, but not with α-melanocyte-stimulating hormone (MSH), β-endophin, β-lipotropin (<0.1%). All reagents were halved and 50 μl of each plasma sample was used to determine ACTH concentrations. The minimum detectable ACTH concentration of the assay was 20 pg/ml and the intra- and inter-assay coefficients of variation were 3.9% and 6.5% respectively.

3. Testosterone (T)
Plasma T levels were measured using an adaptation of the T RIA kit of ICN Biomedicals (Carson, CA) with \([^{125}\text{I}] T\) as tracer. The T antibody (solid phase) cross-reacts slightly with 5α-DHT (3.4%), 5α-androstane-3β, 17β-diol (2.2%) and 11-oxotestosterone (2%) but does not cross-react with progesterone, estrogen, or the glucocorticoids (all <0.01%). All reagents were halved and 25 μl of each plasma sample were used to determine T concentrations. The minimum detectable T concentration of the assay T was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were 4.6 % and 7.5 % respectively.

4. Luteinizing Hormone (LH)

Plasma LH levels were measured by RIA in the laboratory of National Hormone and Peptide Program, A.F. Parlow. (Harbor-UCLA Medical Center, California, USA). The minimum detectable LH concentration of the assay was 0.1 ng/ml.

G. Statistical Analysis

The data were analyzed using three-way analyses of variance (ANOVA) for the factors of prenatal treatment group (E, PF and C), surgical treatment (intact and GDX) and testing time point (0, 30, 90 min) with Fisher LSD post hoc analysis performed on significant main and interaction effects. The error term was adjusted appropriately for two-way ANOVAs. We used the PC software Statistica 6.0v (StatSoft Inc., Tulsa, OK, USA). Values were expressed as Mean ± Standard Error (SE) of the Mean.
CHAPTER 3: RESULTS

A. Developmental Data

1. Ethanol Intake and Blood Alcohol Levels

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 9.51 ± 0.35, 11.93 ± 0.36, 12.08 ± 0.34 g ethanol/kg BW for weeks 1, 2 and 3 of gestation, respectively. This level of intake resulted in peak blood ethanol levels of 192.45 ± 4.123 mg/dl.

2. Maternal Body Weights during Gestation and Lactation

Repeated measures ANOVA indicated that maternal weight increased throughout gestation (G) (Figure 1). There was a significant main effect of group \([F(2,38)= 4.10, \ p<0.05]\), day \([F(3,114)= 814.02, \ p<0.001]\) and a group x day interaction \([F(6,114)= 17.07, \ p<0.001]\) for E, PF and C dams during gestation. Post-hoc analysis indicated that E and PF dams did not differ from C dams on G1 but weighed significantly less than C dams (*) \((p<0.01)\) on G21. C dams showed significant weight gains on G7, G14 and G21 \((p<0.001)\), although E and PF dams did not increase in weight on G7, these dams gained weight on G14 and G21 compared to G1.

Repeated measures ANOVA on maternal weight during lactation indicated a main effect of day \([F(3,138)= 138.22, \ p<0.001]\) and a group x day interaction \([F(6,138)= 8.65, \ p<0.001]\) (Figure 2). Post-hoc analysis revealed that all dams increased in weight on L8 and L15 compared to L1 \((p<0.001)\). On L22, E and PF but not C dams weighed significantly more than that on L1 \((p<0.001)\). There were no differences in weight among groups during
3. Gestation Length

One-way ANOVA revealed a significant effect of group on gestation length (Table 1) \([F(2,39)= 4.0, p<0.05]\). Post-hoc analysis indicated a longer gestation length for E compared to PF and C dams \((p< 0.05)\).

4. Postnatal Body Weights of Female and Male Pups

Pup weights were measured at postnatal day (PND) 1, 8, 15 and 22 (Figure 3). Repeated measures ANOVA revealed a significant effect of day for both female \([F(3,135)= 6127.06, p<0.001]\) and male pups \([F(3,138)= 2278.89, p<0.001]\). Both female and male pups in all prenatal treatment groups increased in weight from PND1 through to PN22 \((p's<0.001)\). There was also a trend for an effect of group for male pups \([F(2,138)= 2.855, p=0.068]\) but not for females. One-way ANOVAs revealed significant main effects of group on female and male pup weight on PND 1 \{females: \([F(2,46)= 12.38, p<0.001]\); males: \([F(2,46)= 13.70, p<0.001]\}\) and PND 8 \{females: \([F(2,46)= 5.69, p<0.05]\); males: \([F(2,46)= 3.80, p<0.05]\}\}. On PND1, E and PF female and male pups weighed less than their C counterparts \((p's<0.001)\). On PND8, C female and male pups weighed more than E counterparts \((p's<0.05)\); C female weighed more than PF female \((p<0.01)\) whereas C male weighed marginally more than PF males \((p=0.057)\). There were no differences among groups on PND15 and PND22.

B. Experimental Data

1. Body Weight at Post-surgery and Pre-testing

All animals were 53-58 days of age at the time of surgery. Animals were tested 2
weeks after the surgery. All animals were weighed right after surgery (post-surgery) and one day before testing (pre-testing). Repeated measures ANOVA indicated a significant effect of day \[F(1,156)= 6205.35, p<0.001\], surgical treatment \[F(1,156)= 10.50, p<0.005\] and a day x treatment interaction \[F(1,170)= 152.16, p<0.001\] (Table 2), as well as a trend for an effect of group \[F(2,156)= 2.46, p=0.089\]. Post-hoc analysis revealed that all animals increased in weight 2 weeks after the surgery \(p<0.001\). While there are no differences in weight between intact and GDX animals at immediately post-surgery, GDX rats had significantly lower weights than intact rats prior to testing \(p<0.001\). Furthermore, overall, E rats weighed marginally higher than PF \(p=0.067\) and C \(p=0.052\).

2. Plasma CORT levels (μg/dl)

Plasma CORT levels of intact and GDX E, PF and C males were measured at 0, 30 or 90 min after the onset of a 30 min restraint stress. An overall 3 way ANOVA (group*treatment*time) indicated a main effect of time \[F(2,153)= 90.12, p<0.001\] (Figure 4). Post hoc analysis revealed that CORT levels were higher at 30 min and 90 min than at 0 min, and higher at 30 min than at 90 min in all animals. There was no overall GDX effect on the CORT response, and there were no significant differences among E, PF and C males across time.

3. Plasma ACTH levels (pg/ml)

An overall 3-way ANOVA (group*treatment*time) on plasma ACTH levels indicated main effects of treatment \[F(1,153)= 4.84, p<0.05\] and time \[F(2,153)= 107.91, p<0.001\]. Post hoc analysis (Figure 5.1) revealed that ACTH levels were higher at 30 min
than at 0 and 90 min in all animals under both sham and GDX treatment. In addition, ACTH levels were significantly higher in GDX than in intact males. Further analysis by separate 2-way ANOVAs (group*time) for intact and GDX animals indicated that intact E rats had significantly higher ACTH levels than C rats at 30 min (p<0.01), and the difference was eliminated by GDX. Further 2-way ANOVAs (treatment*time) for each prenatal group (Figure 5.2) revealed that only C rats showed a significant effect of GDX [F(1,54)=3.58, p<0.05], ie. ACTH in GDX was higher than in intact males, whereas PF rats showed a trend for a GDX effect [F(1,45)=2.86, p=0.076], and GDX E rats were not significantly different from their intact counterparts.

4. Plasma testosterone (T) levels (ng/ml)

Plasma T levels in GDX rats were in the undetectable range. A 2-way ANOVA (group*time) for intact animals indicated a main effect of time [F(2,78)=5.52, p<0.01] (Figure 6). Post hoc analysis revealed that PF rats had higher T at 30 than at 0 and 90 min (p<0.05), and C rats had higher T at 30 than at 0 min (P<0.05), whereas E rats showed no significant increase in T levels over time. However, there were no significant differences among E, PF and C males at any time point.

5. Plasma LH levels (ng/ml)

An overall 3-way ANOVA (group*treatment*time) indicated main effects of treatment [F(1,149)=962.50, p<0.001] and time [F(2,149)=40.84, p<0.005] and a treatment by time interaction [F(2,149)=30.46, p<0.001] (Figure 7). As expected, post hoc analysis revealed that GDX markedly increased LH levels. Further 2-way ANOVAs
(group*time) for intact and GDX males indicated a main effect of time (p<0.001) in intact rats. Overall, LH level increased at 30 min (P< 0.005), and decreased to basal levels at 90 min (p<0.001). However, this increase at 30 min mainly reflects a significant increase in LH in intact C rats (p<0.005), but not in E and PF rats. In contrast, following GDX, LH levels were higher at 0 min than at 30 min, and higher at 30 min than at 90 min in all prenatal groups. There were no significant differences among E, PF and C males across time in the GDX condition.

6. Correlations between HPA and HPG hormones

6.1. Correlations between T and CORT levels at 30 min (Figure 8)

There was a strong negative correlation between the T and CORT responses to restraint stress at 30 min in intact E (r = -0.7936, p<0.005) but not PF (r = -0.5814, P= 0.1) and C males (r = 0.0672).

6.2. Correlations between LH and ACTH levels at 30 and 90 min (Figure 9)

There was a strong positive correlation between the LH and ACTH responses to stress in GDX PF (r = 0.7371, p<0.001) and C (r = 0.5007, p< 0.05) but not E (r = 0.1818) male rats.
Table 1  Gestation data of E, PF and C Dams

Gestation length (mean ± SEM), number of live-born pups (mean ± SEM) and number of stillborn pups (mean ± SEM) of E, PF and C Dams (n= 14-18 per group). There was a significant effect of group on gestation length. Gestation length of E dams were significantly greater compared to PF and C dams (* p< 0.05). However, There were no significant differences among E, PF and C dams in the number of live-born pups and stillborn pups.

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>gestation length</th>
<th>live-born</th>
<th>stillborn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>22.0±0.09*</td>
<td>15.3±0.7</td>
<td>0.23±0.11</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>21.59±0.14</td>
<td>15.2±0.47</td>
<td>0.07±0.07</td>
</tr>
<tr>
<td>Control</td>
<td>21.64±0.15</td>
<td>15.5±0.44</td>
<td>0.28±0.14</td>
</tr>
</tbody>
</table>
All animals were 53-58 days of age at the time of surgery (n= 21-36 per group and surgical treatment). Animals were tested 2 weeks after the surgery. All animals were weighed right after surgery (post-surgery) and one day before testing (pre-testing). There were significant effects of day (p<0.001), surgical treatment (p<0.005) and day x treatment interaction (p<0.001) as well as a trend for an effect of group (p=0.089). All animals increased in weight at pre-testing compared to post-surgery (*p<0.001). While there are no differences in weight between intact and GDX animals at immediately post-surgery, GDX rats had significantly lower weights than intact rats prior to testing (*p<0.001).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INT</th>
<th>GDX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>332.2 ± 5.1</td>
<td>328.9 ± 4.7</td>
</tr>
<tr>
<td>PF</td>
<td>316.3 ± 5.1</td>
<td>317.4 ± 4.1</td>
</tr>
<tr>
<td>C</td>
<td>321.4 ± 4.8</td>
<td>317.4 ± 4.8</td>
</tr>
<tr>
<td>MEAN ± SEM</td>
<td>323.9 ± 2.9</td>
<td>321.4 ± 2.7</td>
</tr>
<tr>
<td>Pre-testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>418.7 ± 6.1</td>
<td>388.9 ± 5.4</td>
</tr>
<tr>
<td>PF</td>
<td>405.4 ± 7.0</td>
<td>383.9 ± 5.5</td>
</tr>
<tr>
<td>C</td>
<td>408.4 ± 5.3</td>
<td>380.8 ± 5.2</td>
</tr>
<tr>
<td>MEAN ± SEM</td>
<td>411.0 ± 3.5*</td>
<td>384.6 ± 3.1*#</td>
</tr>
</tbody>
</table>
Figure 1  Maternal body weights (g) (mean ± SEM) during gestation

Maternal body weights of E, PF and C dams during gestation (G) (n= 14-18 per group).

There were significant main effects of group (p<0.05), day (p<0.001) and group x day interaction (p<0.001) for dam weights during gestation. E and PF dams weighed significantly less than C dams (* p<0.01) on G21. C dams gained weights from G1 through to G21. Although E and PF dams did not increased in weight on G7, these dams gained weight on G14 and G21 compared to G1.
Maternal body weights (g) (mean ± SEM) during lactation

Maternal body weights of E, PF and C Dams during lactation (L) (n= 14-18 per group).

There were significant main effects of day (p<0.001) and group x day interaction (p<0.001). All dams increased in weight on L15, L8 compared to that on L1 (* p’s<0.001). On L22, E and PF dams but not C dams weighed significantly higher than that on L1 (# p’s<0.001). There were no differences in weight among groups during lactation.
Figure 3  Postnatal pup body weights (g) (mean ± SEM)

Postnatal body weights of E, PF and C female (upper panel) and male (lower panel) pups on PND1, PND8, PND15 and PND22 (n= 14-18 litters per group). There were significant main effects of day for both female (p<0.001) and male pups (p<0.001) in all prenatal treatment groups. Both female and male pups in all prenatal treatment groups increased in weight from PND1 through to PND22 (p’s<0.001). There was also a trend for an effect of group for male pups (p=0.068) but not for females. One-way ANOVA revealed significant main effects of group on female and male pup weight on PND1 (p’s<0.001) and PND8 (p’s<0.05). On PND1, E and PF female and male pups weighed less than their C counterparts (* p’s<0.001). On PND8, C female and male pups weighed more than E counterparts (# p’s<0.05); C female weighed more than PF female (^ p<0.01) whereas C male weighed marginally more than PF males (p=0.057). There were no differences among groups on PND15 and PND22.
Plasma CORT levels (μg/dl) (mean ± SEM)

Plasma CORT levels in intact and GDX E, PF and C males were measured at 0, 30 or 90 min after the onset of a 30 min restraint stress (n= 7-12 per group, surgical treatment and time point). There was a main effect of time (p<0.001). Overall CORT levels were higher at 30 min and 90 min than at 0 min, and higher at 30 min than at 90 min in all animals under both sham and GDX treatment ("30 min > *90 min > 0 min, p<0.001). There were no GDX effects at any time points in any prenatal treatment group and no significant differences among E, PF and C across time points.
Figure 5.1  Plasma ACTH levels (pg/ml) (mean ± SEM)

Plasma ACTH levels of intact and GDX E, PF and C males were measured at 0, 30 or 90 min after the onset of a 30 min restraint stress (n= 7-12 per group, surgical treatment and time point). There are main effects of treatment (p<0.05) and time (p<0.001). Overall ACTH levels were significantly higher after GDX (" p<0.05) compared to those in intact males. ACTH levels were higher at 30 min than at 0 min and 90 min in all animals under both sham and GDX conditions (* 30 min > 0 min = 90 min, p<0.001). Intact E rats had significantly higher ACTH levels than C rats at 30 min (" p<0.01), and the difference was eliminated by GDX.
Figure 5.2  Plasma ACTH levels (pg/ml) (mean ± SEM)

Treatment*time analyses for each prenatal group further demonstrates that gonadectomy had marked effects of PF and C but not E males (\# GDX > intact overall, p< 0.05). GDX C rats had significantly higher ACTH levels than intact C rats at 30 min (@ p<0.01), and PF rats showed a trend for a GDX effect (& GDX > intact overall, p=0.076; ^ GDX > intact at 30 min, p=0.072). However, ACTH responses to stress were similar in intact and GDX E rats.
Plasma T levels of intact and GDX E, PF and C males were measured at 0, 30 or 90 min after the onset of a 30 min restraint stress (n= 7-12 per group, surgical treatment and time point). T levels in GDX rats were under detectable range. For intact animals, there was a main effect of time (p<0.01). Overall T levels were significantly higher at 30 min than 0 min and at 90 min (* 30 min > 0 min, p<0.005; # 30 min > 90 min, p<0.05). However, further analysis indicated that this overall effect of time reflected a significant increase from basal levels only in PF and C males. PF rats had higher T at 30 than at 0 and 90 min (& p<0.05), and C rats had higher T at 30 than at 0 min (^ P<0.05). E males showed no significant change in T levels over time. In addition, there were no significant differences among E, PF and C across time points.
Plasma LH levels of intact and GDX E, PF and C males were measured at 0, 30 or 90 min after the onset of a 30 min restraint stress (n= 7-12 per group, surgical treatment and time point). There are main effects of treatment (p<0.001) and time (p<0.005) and a treatment by time interaction (p<0.001). GDX markedly increased LH levels (* p<0.001). In intact rats, overall, LH level increased at 30 min (@ 30 min > 0 min, P < 0.005), and decreased to basal levels at 90 min (# 30 min > 90 min, p<0.001). This increase at 30 min mainly reflects a significant increase in LH in intact C rats (^ p<0.005), whereas E and PF rats showed no significant change in levels over time. In contrast, following GDX, LH levels decreased over time (# 0 min > & 30 min > 90 min, p<0.001). However, there were no significant differences among E, PF and C across time.
There was a strong negative correlation between the T and CORT responses to restraint stress at 30 min in intact E but not PF and C males.
There was a strong positive correlation between the LH and ACTH responses to stress in GDX PF and C but not E male rats.
CHAPTER 4: DISCUSSION

The objective of this thesis was to investigate if HPA activity varies differentially as a function of androgens in E compared to control males. The study follows up previous work in our laboratory showing that there are marked sex differences in stress responsiveness and HPA regulation at different levels of the axis in E rats, and also, that E males and females differ from their control counterparts. This raises the possibility that gonadal steroids may play a role in mediating prenatal ethanol effect on HPA activity.

There are two major findings in this study. 1) There were adverse effects of ethanol on pregnancy outcome. E dams had a longer gestational length compared to PF and C dams. E and PF female and male pups had significantly lower weights than C pups at birth. However, on PND8, only E male pups weighed less than C. 2) We detected an effect of GDX on HPA responsiveness in male offspring from E, PF and C dams under basal, stress and recovery conditions. Results from this study showed that plasma ACTH levels were differentially altered in E compared to PF and C male rats following GDX, indicating that HPA hyperresponsiveness in E male rats is mediated, at least in part, by ethanol induced changes in androgens regulation or HPA sensitivity to androgens. This is reflected in the finding that GDX eliminated the differences between intact E and C rats following a 30 min restraint stress. These data suggest that the stimulatory effect of GDX on stress-induced ACTH release was impaired in E rats, indicating a reduced effect of androgens on HPA responsiveness or a decreased sensitivity to androgens in E males.
A. The Effect of Ethanol on Pregnancy Outcome

In this breeding, ethanol intake of pregnant females was consistently high throughout gestation, averaging 9.51 ± 0.35, 11.93 ± 0.36, 12.08 ± 0.34 g ethanol/kg BW for weeks 1, 2 and 3 of gestation, respectively. This level of ethanol intake results in peak blood ethanol levels of 192.45 ± 4.123 mg/dl (the equivalent of two-fold of the drunk driving standard: 80–100 mg/dl).

E and PF dams failed to gain weight during the first week of gestation and weighed significantly less than C dams by G21, whereas C dams gained weight each week from G1 through to G21. E and PF dams had similar defect in body weight gain but for different reasons. Ethanol administration may directly affect nutrient intake and has the potential to adversely affect maternal nutritional health. E dams consume less food because ethanol provides high energy value (7.1 kcal/g) and may readily displace other nutrients in the diet. Ethanol also causes secondary malnutrition due to its deleterious effects at almost every level of the gastrointestinal tract, as well as direct toxic effects on liver, pancreas etc. Thus ethanol administration may alter metabolism, transport and utilization of almost every essential nutrient (Weinberg, 1984). PF dams were fed a reduced ration (i.e., equivalent to that consumed by their E partners) so that they did not get enough food to meet their daily energy requirements. The relative undernutrition of E dams could further exaggerate the effect of ethanol on the developing fetuses. A longer gestational length seen in E compared to PF and C dams indicates that ethanol delays parturition. These data are consistent with previous data from our lab (Weinberg, 1985; Weinberg et al., 1995).
There were no significant differences in weight among E, PF and C dams during lactation. However, on L22, E and PF but not C dams weighed significantly more than that on L1. This suggests that E and PF dams showed “catch up” weight gain after parturition, to compensate for the reduced food intake and weight gains during gestation.

In the present study, we found E and PF female and male pups had significantly lower weights than C pups at birth, suggesting an ethanol-related nutritional effect. However, on PND8, only E male pups weighed less than C, indicating a special effect of prenatal ethanol on postnatal growth. These data are consistent with previous findings from our lab (Weinberg, 1985; Weinberg et al., 1995) and others (Abel, 1978; Taylor et al., 1981; Tritt et al., 1993), and further support that alcohol has adverse effects on the developing fetus.

**B. HPA Hormone Levels in Intact and GDX Male Rats**

In the literatures on restraint stress, it has been shown that CORT and ACTH levels typically peak at 15–30 min, then decline towards/to basal levels during recovery (Lund et al., 2004; Viau and Meaney, 1996). In this study, 30 min (stress) and 90 min (recovery) time points were chosen to investigate whether E animals are hyperresponsive to stressors and/or deficient in recovery compared to their control counterparts. We found that HPA axis in intact E male rats did show hyperresponsiveness at 30 min, with significantly higher ACTH levels than C rats. Importantly, this difference was eliminated by GDX, suggesting that the stimulatory effect of GDX on stress-induced ACTH release was attenuated in E rats. That is, there was a reduced inhibitory effect of androgens on HPA responsiveness, or possibly a
decreased sensitivity to androgens in E males. CORT levels increased at 30 min and decreased toward basal at 90 min in all animals. This is consistent with the previous finding in our lab (Weinberg, 1988; Weinberg, 1992a) and others (Slone and Redei, 2002; Taylor et al., 1986), which showed that there were no significant differences in CORT levels at 30 min post-stress among E, PF and C males. However, during prolonged (4 hr) restraint stress, E males showed a greater and more prolonged CORT elevation over the 4-hour period (Weinberg, 1992a). Because of the marked variability in CORT levels during the recovery period in the present study (ranging from $1.75 \sim 52.74 \mu g/dl$ in intact rats and $1.88 \sim 79.99 \mu g/dl$ in GDX rats at 90 min time point), it is quite possible that the effect of GDX or prenatal treatment on CORT levels could not be observed.

There was an overall GDX effect on the ACTH response. This is mainly due to higher ACTH in GDX C compared to intact C males. Our analysis revealed that only C rats showed a significant GDX effect in ACTH, whereas PF rats showed a trend for a GDX effect, and GDX E rats were not significantly different from their intact counterparts. Although there was no overall GDX effect on the CORT response, inspection of Fig 4 reveals that changes in CORT levels were similar in pattern to those seen for ACTH, that is, at 30 min, C males showed the greatest CORT increase following GDX. These data further suggest that GDX has a stimulatory role on HPA responsiveness to stress, and this effect is attenuated in E and PF males.

The effects of gonadal hormones on HPA function have been demonstrated at different levels of the axis. Androgen inhibits hypothalamic CRH peptide and mRNA
(Almeida et al., 1992; Bingaman et al., 1994; Viau et al., 2001), as well as restraint-induced CRH and AVP hnRNA (Lund et al., 2004; Viau et al., 2003). Gonadectomy (GDX) on adult male rats increased the ACTH as well as the CORT response to physical and psychological stressors (Handa et al., 1994). Androgen decreased GR mRNA expression in the hippocampus (Kerr et al., 1996), and increased GR levels in the mPOA (Viau and Meaney, 1996). In the stress paradigm here, we showed androgens overall have inhibitory effects on the ACTH response to stress. Importantly, there is a reduced effect of androgens on HPA responsiveness or a possibly decreased sensitivity to androgens in E males.

C. HPG Hormone Levels in Intact and GDX Male Rats

Stress appears to have a biphasic effect on the HPG axis. Chronic stress typically inhibits HPG function. However, data indicated that acute stress elicits variable patterns of LH and testosterone release in male rats. Acute psychological stressors [handling, novel environments, visual (rats were exposed to a flashing bright lights for 2 min at a frequency of 4 sec) or audiogenic stimulation (exposure to a bell ringing at a noise level of 1.9 dB for 2 or 4 min)] consistently result in transient elevations of plasma LH levels (Briski and Sylvester, 1987a; Briski and Sylvester, 1987b; Krulich et al., 1974; Siegel et al., 1981; Turpen et al., 1976). Effects of acute stressors that have both a psychological and a physical component (cold, restraint, immobilization, swim, foot shock) are more variable. Increase (Briski and Sylvester, 1987b; Ruisseau et al., 1978), decrease (Rivier et al., 1986) or no change (Charpenet et al., 1982; Mann and Orr, 1990) in LH levels following acute physical stressors has been reported.
In this study, LH levels were significantly increased after 30 min restraint stress in intact C but not E and PF males. It has been demonstrated that stress can affect LH secretion through different pathways. Indirect pathways involve central neuronal changes that regulate GnRH secretion and/or release (Brann and Mahesh, 1991), whereas direct pathways are mediated by effects of circulating adrenal GCs on pituitary gonadotropes or alterations in the responsiveness of the gonadotrophs to GnRH (Briski and Sylvester, 1991). Since CORT levels were similar among groups in the present study, the elevated LH levels may due to increased GnRH secretion and/or release. Thus, further investigation of central measures is warranted.

It has been demonstrated that the stimulation vs. inhibition of pituitary LH release during acute stress may be correlated with the magnitude of adrenal glucocorticoid secretion (Briski, 1996). Previous studies showed that the magnitude of stress-induced increases in circulating CORT is proportional to stressor intensity (Armario et al., 1986; Hennessy and Levine, 1978; Kant et al., 1983). Although we did not find significant differences in CORT levels among prenatal treatment groups, only intact C rats showed higher LH levels after 30 min stress. This may indicate that the effect of CORT on LH is differentially altered in E and PF rats despite the fact that CORT levels were similar to those of C rats.

As we expected, GDX markedly increased LH levels due to removal of androgens feedback. Interestingly, LH levels decreased over time in GDX rats. However, there were no significant differences among E, PF and C across time. Previous studies have shown that
stress inhibits the LH response in the ovariectomized ewe (Breen and Karsch, 2004; Debus et al., 2002), or in the gonadectomized male and female rats (Euker et al., 1975; McGivern and Redei, 1994). Since data indicate that GnRH content fall significantly to 57% of control levels in hypothalami 14-21 days following GDX (Emanuele et al., 1996), and adrenalectomy reverses stress-induced suppression of LH secretion in ovariectomized rats (McGivern and Redei, 1994), it is likely that a reduced stimulatory GnRH effect on LH release and an increased inhibitory GCs effect on pituitary responsiveness to GnRH contribute to the suppressed LH levels following stress in GDX animals. A further decrease in LH levels during the recovery period was found in this study, and ACTH and CORT levels were also lower during recovery than during stress. Interestingly, a previous study reported that central administration of ACTH caused a dose-related rise in LH levels in intact male rats (Mann et al., 1986). Furthermore, it is known that stress-induced LH decrease is also modulated by both opioid and noradrenergic influences (Armario et al., 1987; Briski et al., 1984; Cicero et al., 1979; Van Vugt et al., 1981; Van Vugt and Meites, 1980). However, effects of ACTH, and the interaction of neurotransmitters with HPA components within the brain and the pituitary to modulate LH release following GDX is unknown, as it is likely that hormones and neurotransmitters will have differential effects in intact vs. GDX animals. For example, previous studies showed that the effects of stress on the HPG axis depend on the gonadal status and are affected by sex hormones, ie. androgens potentiate the effects of GCs on GnRH release (Calogero et al., 1999), and cortisol does not acutely inhibit GnRH pulsatility in the absence of gonadal steroids (Breen and Karsch,
We found that testosterone levels in intact rats were significantly increased at 30 min, and decreased to basal levels at 90 min. Further analysis indicated that this overall increase reflected the fact that a significant increase in testosterone actually occurred only in PF and C rats. PF rats had higher testosterone at 30 than at 0 and 90 min. C rats had higher testosterone at 30 than at 0 min, whereas E rats did not differ in testosterone levels across time. As previously mentioned, acute stress elicits variable patterns of testosterone release in male rats. Although stress can potentially affect all levels of the HPG axis, it is now recognized that stress mainly influences the secretion of GnRH at the level of hypothalamus and gonadotropin at the level of pituitary (Rivier and Rivest, 1991; Tilbrook et al., 2000). Testosterone synthesis and secretion are influenced by several mechanisms that regulate testosterone release through hormones and neurosecretagogues that are present in the systemic circulation and/or synthesized and secreted within the testis and directly influence steroidogenic enzymes. These mechanisms include the well-studied LHRH-LH pathway (Saez, 1994), sympathetic and parasympathetic innervation of the male gonad, and CRF-like peptides, opiates, catecholamines, growth factors and cytokines that can regulate testicular steroidogenesis independently of gonadotropins (Saez, 1994). Furthermore, it has been recently proved that there is an inhibitory neural hypothalamic-testicular pathway independent of the pituitary in male Sprague-Dawley rats (Lee et al., 2002), and alcohol can inhibit Leydig cell activity through this pathway (Selvage et al., 2004). In this study, the testosterone responses to stress are in parallel with the changes of LH. These results are
consistent with previous findings that acute stress elicits a small and transient increase in both plasma LH and testosterone levels. Only PF and C rats had higher testosterone levels following stress, indicating that testosterone regulation might be altered in E males compared to controls.

In the stress paradigm used in the present study, the stressor is mild enough to elicit increased LH levels in C rats, thus causing increased testosterone levels. Catecholamines acting peripherally and directly on the testis may increase testosterone concentrations (Sapolsky, 1986). Although we did not find differences in testosterone levels among prenatal treatment groups, reduced testosterone levels in adult (PND 55) E male rats have been reported previously (Udani et al., 1985). However, the data were obtained from Wistar rats, and ethanol was given from gestation day 12 until 10 days after delivery of the pups. Since the adverse effects of prenatal ethanol on the fetus depend on the length of the period of ethanol exposure as well as genetic factors (Coles, 1994; Sokol et al., 1986), withdraw ethanol on G21, as in the present study, appears to attenuate some of the adverse effect of ethanol

**D. Correlations between HPA and HPG hormones**

A strong negative correlation between testosterone and CORT responses to 30 min restraint stress was found only in intact E animals, indicating that the HPA hyper-responsiveness to stressors in E compared to their control counterparts may be due, at least in part, to relatively lower testosterone levels following stress. Furthermore, following GDX, PF and C but not E animals showed positive correlation between post-stress LH and
ACTH levels, mainly due to relatively lower ACTH levels in E rats at 90 min, indicating that GDX had less of a stimulatory effect on ACTH responses in E animals compared to controls, despite similar effects on plasma LH levels. Altered HPA-HPG cross-talk in E compared to control animals indicates a possible role for androgens in the differential HPA responsiveness in E males.

Taken together, these data suggest that androgens may have less of an inhibitory effect on HPA function in E males compared to controls.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

A. Summary

The objective of this study was to investigate if HPA activity varies differentially as a function of androgens in E compared to control males. Differential effects of GDX on the HPA responsiveness in male offspring from E, PF and C dams under basal, stress and recovery conditions were observed. We found that intact E rats had significantly higher ACTH levels than C rats at 30 min, and the difference was eliminated by GDX. Furthermore, PF and C rats had higher testosterone at 30 than at 0 min, whereas E rats did not differ in testosterone across time. Similarly, in intact C but not E and PF rats, LH level increased at 30 min and decreased to basal levels at 90 min. In contrast, following GDX, LH levels were higher at 0 min than at 30 min, and higher at 30 min than at 90 min in all prenatal groups. In addition, there was a strong negative correlation between the testosterone and CORT response to restraint stress at 30 min in intact E but not PF and C males, whereas following GDX, there was a strong positive correlation between the LH and ACTH response to stress in PF and C but not E male rats.

These data suggest that the stimulatory effect of GDX on stress-induced ACTH release was impaired in E rats, indicating a reduced effect of androgens on HPA responsiveness or a decreased sensitivity of ACTH responsiveness to androgens in E males. Results from this study support the hypothesis that HPA hyperresponsiveness in E male rats are mediated, at least in part, by ethanol induced changes in androgens regulation or HPA sensitivity to androgens.
B. Future Directions

The present study is the first step to elucidate how HPA and HPG interaction changes in males prenatally exposed to ethanol. The data indicate that E males had higher ACTH levels compared to C males following 30 min restraint stress, and this difference was eliminated by GDX. The time points we chose for the present study appear to be optimal for measuring the peak of the ACTH response, but were not optimal for revealing possible differences in CORT. Since Weinberg showed that E males had a greater and more prolonged CORT elevation over a 4-hour restraint period (Weinberg, 1992a), further research on longer restraint times is warranted to determine if there is a GDX effect on CORT levels and if GDX will also play a role in CORT the difference between E and control males.

As the data from this study may indicate a reduced effect of androgens on HPA responsiveness or a decreased sensitivity to androgens in E males, measuring androgen receptor levels in mPOA is warranted. As well, human chorionic gonadotropin (hCG)-induced androgens secretion can be used to test the sensitivity of Leydig cell to pituitary secretogogue of HPG axis.

In this study, LH levels were significantly higher after 30 min stress in intact C rats, whereas E and PF did not show difference cross time. Since stress can affect LH secretion through central neuronal changes that regulate GnRH secretion and/or release (Brann and Mahesh, 1991) or by circulating adrenal GCs on pituitary gonadotropes or the responsiveness of the gonadotrophs to GnRH (Briski and Sylvester, 1991), and CORT
levels were similar among groups, the elevated LH levels are likely due to increased GnRH secretion and/or release. Thus, further investigation of GnRH mRNA in the mPOA is warranted. As well, central measures of HPA activity are also required to further demonstrate the role of GDX on the HPA responsiveness to stress; these include measures of CRH and AVP hnRNA and mRNA levels in the PVN; and c-fos mRNA and protein in the PVN and mPOA.

A testosterone replacement study is warranted to confirm that the role of testosterone in the HPA responsiveness to stress is differentially altered in male rats prenatally exposed to ethanol compared to controls. Viau reported that the HPA response to stress varies inversely as a function of individual plasma testosterone levels, and also higher physiological testosterone levels are required to suppress HPA responses to stress (Viau and Meaney, 1996). Inclusion of a high (5~7 ng/ml) and a low (~1 ng/ml) testosterone replacement group is needed to access the dose-related effects of testosterone on HPA activity.

C. Clinical Implications

Children born to alcoholic mothers show behavioral deficits, including hyperactivity, attentional deficits, impaired habituation and cognitive and perceptual problems (Shaywitz et al., 1980). Children with FAS also exhibit lack of inhibition, impulsivity and poor sensitivity to social cues (Streissguth et al., 1991; Streissguth et al., 1986; Streissguth et al., 1985), which may occur in the absence of intellectual impairment. In fact, offspring activity level may be a more sensitive indicator of alcohol’s teratogenicity than physical features.
(Landesman-Dwyer, 1982; Shaywitz et al., 1980). Attentional deficits may reflect the child’s inability to withhold a response (Streissguth et al., 1986). Previous data in our lab have shown that deficits in pituitary-adrenal response inhibition or recovery following stress could accompany these behavioral deficits, and further affect the child’s ability to respond appropriately to challenging or stressful situations. To date few clinical studies have focused on possible sexual dimorphism in HPA function. In a 10-year follow-up study, Spohr et al. reported that boys were affected more by intrauterine ethanol exposure than girls were, in terms of three morphometric variables: height, weight and head circumference (Spohr et al., 1993).

The ability to respond appropriately to stress is an important basic adaptive mechanism, and HPA activation is a central feature of this response. HPA hyperresponsiveness results in prolonged or chronic CORT elevations, with concomitant adverse physiological and behavioral consequences that could compromise health and even survival. Therefore, early life events (ie. prenatal ethanol) that result in greater reactivity to stress and increased CORT levels throughout the life span can increase the vulnerability to illnesses later in life. Previous work in our lab (Weinberg, 1988; Weinberg, 1992a) and others (Lee and Rivier, 1996) showed that the balance between HPA drive and feedback is differentially altered in E males and females. The imbalance impairs the ability to maintain homeostasis in E animals and progressively creates a condition of neuroendocrine dysregulation and impaired behavioral adaptation. These changes have implications for the development of secondary disabilities in children with FAS.
D. Conclusions

In conclusion, we found the effect of androgens on HPA responsiveness or the sensitivity of ACTH responsiveness to androgens in E males was decreased compared to controls. Results from this study support the hypothesis that HPA hyperresponsiveness in E male rats are mediated, at least in part, by ethanol induced changes in androgens regulation or HPA sensitivity to androgens.
REFERENCES


Gen Psychiatry 60(4):377-85.


Bingaman EW, Magnuson DJ, Gray TS, Handa RJ (1994) Androgen inhibits the increases in hypothalamic corticotropin-releasing hormone (CRH) and CRH-immunoreactivity following gonadectomy. Neuroendocrinology 59(3):228-34.


Brann DW, Mahesh VB (1991) Role of corticosteroids in female reproduction. Faseb J


Overview of physical and behavioral homeostasis. Jama 267(9):1244-52.


McGivern RF, Raum WJ, Handa RJ, Sokol RZ (1992) Comparison of two weeks versus one week of prenatal ethanol exposure in the rat on gonadal organ weights, sperm


Rivier C, Rivest S (1991) Effect of stress on the activity of the hypothalamic-pituitary-


Van Vugt DA, Meites J (1980) Influence of endogenous opiates on anterior pituitary


