ROLE OF TESTOSTERONE IN MEDIATING PRENATAL ETHANOL EFFECTS ON HYPOTHALAMIC-PITUITARY-ADRENAL ACTIVITY IN MALE RATS

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ABSTRACT

Prenatal ethanol (E) exposure has marked effects on development of the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes. E rats show HPA hyperresponsiveness to stressors and altered reproductive function in adulthood. Importantly, prenatal ethanol differentially alters stress responsiveness in adult males and females, raising the possibility that gonadal hormones play a role in mediating ethanol effects on HPA function. To address this possibility, two studies were conducted to test the hypothesis that the differential alterations in HPA activity observed in E compared to control males are mediated, at least in part, by ethanol-induced changes in HPG effects on HPA regulation.

The first study compared the effects of gonadectomy (GDX) on HPA and HPG activity in adult male offspring from prenatal E, pair-fed (PF) and ad libitum-fed control (C) dams. There were no differences among groups in basal testosterone levels under intact conditions. However, E males showed increased adrenocorticotropin but blunted testosterone and luteinizing hormone (LH) responses to restraint stress compared to PF and/or C rats, and no stress-induced elevation in arginine vasopressin (AVP) mRNA levels. GDX eliminated these differences among groups. The second study explored dose-related effects of testosterone on HPA regulation. Testosterone had less of an inhibitory effect on stress-induced CORT and LH increases in E than in PF and C males. Furthermore, testosterone had a reduced effect on central corticotropin-releasing hormone pathways, but

an increased effect on central AVP pathways in E compared to PF and/or C males. Importantly, reduced androgen receptor (AR) mRNA levels, possibly reflecting downregulation of AR in key brain areas, may counteract the increased inhibitory AVP signals upstream from the paraventricular nucleus, and thus contribute to the HPA hyperresponsiveness seen in E males.

Together these findings suggest that central regulation of both the HPA and HPG axes are altered by prenatal ethanol exposure. The capacity of testosterone to regulate HPA activity is altered in E males, with some effects mediated by the nutritional effects of ethanol. These changes would impair the ability to maintain homeostasis in E animals and have implications for the development of secondary disabilities in children with Fetal Alcohol Spectrum Disorder.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ANOVA	analysis of variance
AR	androgen receptor
ARBD	alcohol-related birth defect
ARND	alcohol-related neurodevelopmental disorder
AVP	arginine vasopressin
BAL	blood alcohol level
BNST	bed nucleus of stria terminalis
BNST fu	fusiform nucleus of the BNST
BW	body weight
C	control
CeA	central nucleus of amygdala
CNS	central nervous system
CORT	corticosterone
CRH	corticotrophin-releasing hormone
CRH-R1	CRH receptor 1
d	day
DG	dentate gyrus
DTT	dithiothreitol

E	prenatal ethanol
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FAS	fetal alcohol syndrome
FSH	follicle stimulating hormone
g	gram
G	gestation day
GABA	γ-aminobutyric acid
GDX	gonadectomy
GDX-H	GDX with high testosterone replacement
GDX-L	GDX with low testosterone replacement
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
hnRNA	heteronuclear ribonucleic acid
hr	hour(s)
ir	immunoreactive
LH	luteinizing hormone
MeA	medial nucleus of amygdala

min	minute(s)
MPOA	medial preoptic area
MPN	medial preoptic nucleus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
NE	norepinephrine
OD	optical density
PF	pair-fed
PN	postnatal day
POMC	proopiomelanocortin
PVN	paraventricular nucleus
RIA	radioimmunoassay
SEM	standard error of the mean
wt	weight

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DEDICATION

This thesis is dedicated to my dearest son, Zijian Han, who has been “without mom” for a fairly long time of his childhood. I deeply regret for not being around and giving the best care for him during that time.

CHAPTER I: GENERAL INTRODUCTION

A. Fetal Alcohol Syndrome

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

Considered the most common nonhereditary cause of mental retardation in the western world (Abel and Sokol 1986), FAS occurs in approximately one-third of infants born to alcoholic women, with 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 a number of categories of alcohol-related effects can be identified. Children with significant prenatal alcohol exposure who lack the full characteristic facial defects or growth deficiency of FAS but still have alcohol-induced mental impairments or neurobehavioral abnormalities are identified as having partial FAS (Abel 1984). Children displaying only physical abnormalities are referred to as having alcohol-related birth defects (ARBD) (Sokol and Clarren 1989; Stratton, Howe et al. 1996). Children who display primarily behavioural or functional abnormalities are referred to as having alcohol-related neurodevelopmental disorder (ARND) (Stratton, Howe et al. 1996). The umbrella term Fetal Alcohol Spectrum Disorder (FASD) is now used to refer to the wide range of abnormalities or deficits that result from prental alcohol exposure (Chudley, Conry et al. 2005; Hoyme, May et al. 2005; Riley and McGee 2005). FASD is not a threshold condition. It is a continuum ranging from mild intellectual and behavioural issues to the extreme that often leads to profound disabilities or premature death.

Longitudinal studies on offspring with FAS suggest that prenatal 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, and may

compromise 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 (Spohr, Willms et al. 1993; Streissguth, Barr et al. 1994; Mattson, Schoenfeld et al. 2001; Bookstein, Sampson et al. 2002; Baer, Sampson et al. 2003). As well, children with FASD often develop secondary disabilities, such as mental health problems; disrupted school experience; trouble with the law; confinement in prison; inappropriate sexual behaviour; alcohol and drug problems; inability to live independently; and employment problems (Streissguth, Barr et al. 1996).

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, for example, on the methodologies and definitions in case finding and diagnosis, wide variation in the types of populations studied, and prospective versus retrospective methods of data gathering (Stratton, Howe et al. 1996). Various estimates of 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, Streissguth et al. 1997). The incidence of FASD in the general US population ranges from 0.7 to 10 cases per 1000 live births annually (Eustace, Kang et al. 2003). In Canada, the incidence of FASD has been estimated to be 10 in 1000 live

births and the cost of FASD is estimated $4 billion a year (Stade, Stevens et al. 2006; Stade, Ungar et al. 2007).

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 ethanol directly affects the fetus because it readily acrosses 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 absorption and metabolism of alcohol, general maternal health, nutritional factors, use of other drugs, et al (Schenker, Becker et al. 1990). The most important factors are temporal vulnerability and peak blood alcohol level.

a. Temporal vulnerability

The timing of ethanol 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, Johnston 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. Ethanol 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 the early postnatal period in rats. This is an especially vulnerable period for the brain in response to ethanol insult. Brain weight reduction is a consistent finding in rat models of prenatal ethanol exposure during this trimester. Other anatomical changes include reduced numbers 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, Marcussen et al. 1990; West and Goodlett 1990) .

b. Peak blood alcohol level (BAL)

There is no known safe level of alcohol consumption during pregnancy. 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, Goodlett et al. 1990). Maternal binge drinking (ie. consumption of five or more standard drinks per occasion) [one standard drink is defined as one half ounce of absolute alcohol included in one 12-ounce can of beer or wine cooler, one 5-ounce glass of wine, or 1.5 ounces of distilled spirits] during pregnancy is one of the strongest predictors of later neurodevelopmental deficits in children with alcohol-induced damage (Streissguth, Sampson et al. 1989). The correlation between alcohol damage and blood alcohol levels has been shown for both structural and functional studies (Bonthius, Goodlett et al. 1988; Bonthius and West 1990; Goodlett, Thomas 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 (Jones and Smith 1973; Jones, Smith et al. 1973; Abel and Dintcheff 1978; Sokol and Clarren 1989; Zajac and Abel 1992; Weinberg 1993; Abel and Berman 1994; Stratton, Howe et al. 1996). 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, including retarded pre- and postnatal growth and development, and physical malformations (Abel and Dintcheff 1978; Sulik, Johnston et al. 1981). Rodents also show CNS abnormalities, including learning deficits (Bond and Di Giusto 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 (Bond and Di Giusto 1976; Becker, Diaz-Granados et al. 1996). They also show deficits in using environmental cues appropriately (Bond and Digiusto 1977; Weinberg 1992). Furthermore, E animals have deficits in passive avoidance learning, suggesting an impairment in response inhibition (Abel 1982; Driscoll, Chen et al. 1982; Gallo and Weinberg 1982), and defects in Morris water maze performance, suggesting defects in spatial learning and memory (Wainwright, Levesque et al. 1993; Westergren, Rydenhag et al. 1996; Kim, Kalynchuk et al. 1997; Gabriel, Johnston et al. 2002; Byrnes, Reynolds et al. 2003). These findings relate to the behavioural abnormalities observed in alcohol-exposed children, such as poor impulse control, poor habituation and lack of response inhibition (Driscoll, Streissguth et al. 1990). Prenatal ethanol-induced physiological abnormalities such as altered endocrine and immune

competence have also been observed in animal models (Taylor, Branch et al. 1982a; Taylor, Branch et al. 1982b; Taylor, Branch et al. 1983; Redei, Clark et al. 1989; Redei, Halasz et al. 1993; Weinberg 1994; Jerrells and Weinberg 1998; Kim, Turnbull et al. 1999; Kim, Yu et al. 1999; Lee, Schmidt et al. 2000; Zafar, Shelat et al. 2000). For example, hypothalamic-pituitary-adrenal (HPA) hyperresponsiveness to stressors such as cardiac puncture, noise, footshock, restraint, or lipopolysaccharide (LPS) injection (Taylor, Branch et al. 1982b; Taylor, Nelson et al. 1984; Nelson, Taylor et al. 1986; Weinberg, Taylor et al. 1996; Lee, Schmidt et al. 2000) and to immune challenges such as LPS, IL-1β or IL-2 (Jerrells and Weinberg 1998; Kim, Turnbull et al. 1999), and suppressed splenic T-cell proliferative response to the mitogenic lectin Concanavalin-A (Con-A) (Norman, Chang et al. 1989; Redei, Clark et al. 1989; Weinberg and Jerrells 1991; Chang, Yamaguchi et al. 1994) have been observed in ethanol-exposed compared to control animals.

Several methods of ethanol administration have been developed to investigate the effects of prenatal ethanol exposure, including intraperitoneal injection, inhalation, oral intubation, and placing ethanol in the drinking water or a liquid diet. Although administering ethanol via injection or intubation can cause high blood ethanol levels, and the researcher can achieve 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 ethanol 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 ethanol voluntarily and will reduce their fluid intake and therefore their food intake as well, resulting in lower blood ethanol levels due to the reduced amount of ethanol digested. Exposing animals to ethanol vapors is also non-invasive and may achieve high blood ethanol levels (Kang, Cole et al. 2004). However, this is not representative of the way humans consume alcohol. Adding ethanol 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 ethanol consumption that is of clinical relevance, while maintaining dietary control and providing adequate nutrition, and results in relatively high blood ethanol levels (Lieber and DeCarli 1989; Weinberg 1989). However, there are a number of issues with this method as well: animals do not all consume the same amount of ethanol; animals consume greater amount of water with liquid diet than they would with pelleted diet; and, if using a commercial liquid diet like Sustecal, the presence of flavorings and preservatives that may have toxicological effects is another disadvantages of this method.

Animal models of FASD have allowed researchers to study the mechanisms underlying the deleterious effects of alcohol on fetal developent. Choosing the appropriate model for a specific study is important in order to fulfill the criterion of face validity; that is, we must attempt to closely match in our model what is observed in humans with FASD. In our laboratory, we study the effects of prenatal ethanol exposure on offspring HPA and HPG function. Therefore, we believe that administering ethanol using a liquid diet, which closely

matches the route of exposure that occurs in humans, is an appropriate model in our studies.

Ethanol-derived calories replace the calories supplied by food, so that ethanol diets will not produce adequate nutrition. Inclusion of a pair-fed (PF) group is necessary to control for the reduced food intake typically found in ethanol-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 ethanol'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 (Koehl, Darnaudery et al. 1999; Kofman 2002; Matthews 2002; Seckl 2004). Because of the problems inherent in pair-feeding, it is important to include an *ad libitum* fed control (C) group, fed either standard lab chow or liquid control diet, *ad libitum*, 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 reasonable to assume that effects are alcohol related. If the E and PF groups are similar, then one must conclude that the effects are, at least partially, mediated by the nutritional effects of prenatal

ethanol exposure, although other factors such as prenatal stress may also influence outcome, as noted above.

B. HPA Axis and Stress Response

A common working definition of the term “stress” is that it is a state of threatened homeostasis (Chrousos 1998). A challenge by either intrinsic or extrinsic adverse forces (or stressors) to homeostasis 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 allows the body to make the necessary physiological and metabolic changes required to cope with the demands of a homeostatic challenge (Chrousos 1998; Miller and O'Callaghan 2002). The stressor can be physical or psychological (imposition or perception of environmental change). 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. Behavioral adaptations include increased arousal and alertness, increased recognition, vigilance and focused attention, enhanced analgesia and elevations in core temperature, as well as suppression of appetite, feeding and reproductive behavior. Depending on the stressor and the memory of the organism, they may also includes euphoria or dysphoria (Chrousos 1998). Concomitantly, physical adaptations involve redirection of energy: oxygen and nutrients to the CNS and the stressed body sites, where they are needed most. Altered cardiovascular tone, increasd blood

pressure, heart rate, respiratory rate, gluconeogenesis and lipolysis, work in concert to promote vital substrates availability. Digestive function and growth, reproduction, and immunity are inhibited (Chrousos 1998). Acute stress responses are advantageous for survival; however, prolonged or chronic stress may be maladaptive and can lead to a variety of pathological conditions. Inappropriate regulation of stress has been implicated in the pathogenesis of systemic disease (eg. hypertension), affective disease (eg. depression, post-traumatic stress disorder), and neurodegenerative disease (eg. Alzheimer's disease) (Charney, Deutch et al. 1993; McEwen and Stellar 1993; Parker, Schatzberg et al. 2003; De Kloet, Joels et al. 2005; Dinan and Scott 2005; Bao, Meynen et al. 2007).

The SNS 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 (Goldstein 1987) and is involved in the "fight or flight" response and enables the organism to react rapidly to the stressor.

The HPA axis (Figure 1) involves a cascade of responses. The stressor initiates the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the medial parvocellular dorsal division (mpd) of the paraventricular nucleus (PVN) of the hypothalamus, which summate excitatory and inhibitory inputs into a net secretary signal at the pituitary gland (Herman and Cullinan 1997). CRH and AVP reach the anterior pituitary via the hypothalamic-hypophyseal portal vessels, regulate the synthesis of proopiomelanocortin (POMC), and stimulate the release of POMC-derived peptides such as

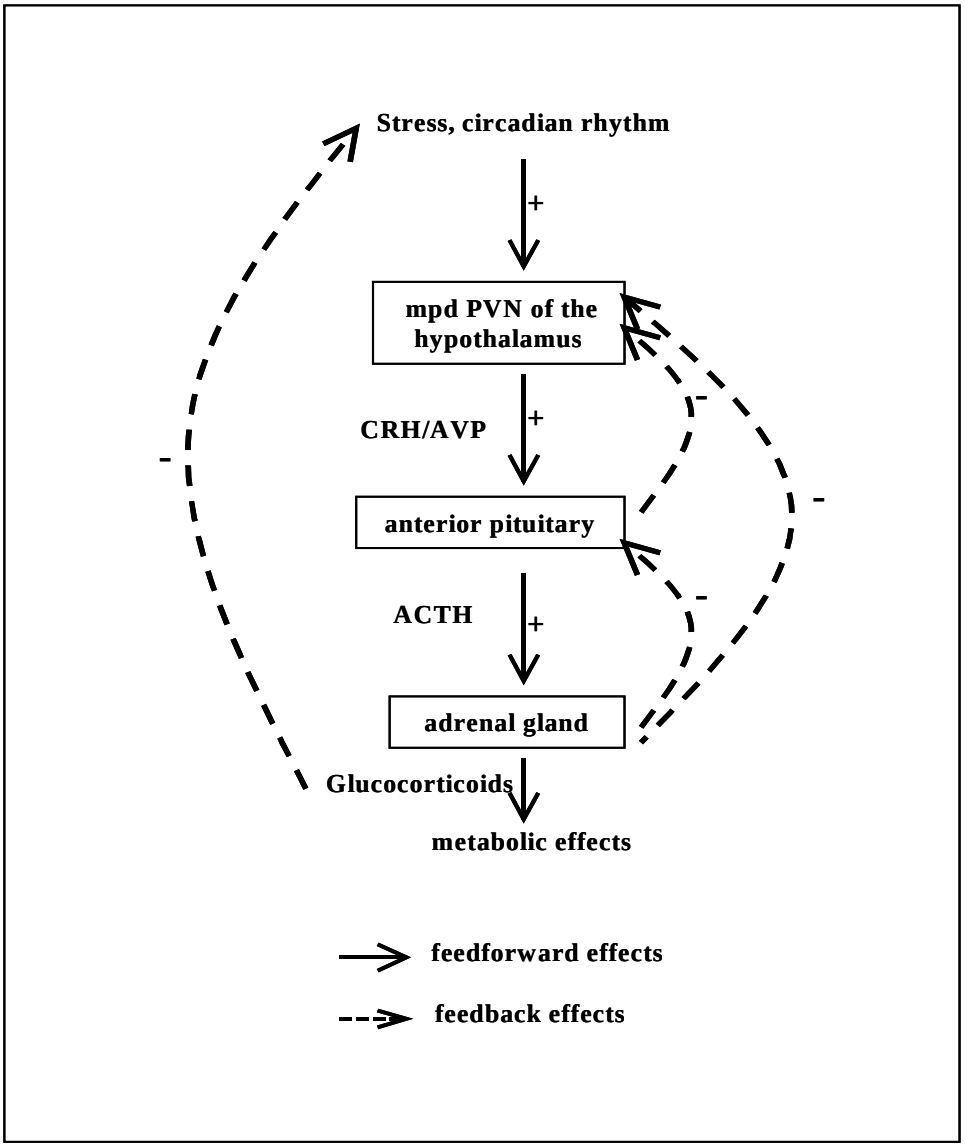


Figure 1. Schematic of the hypothalamic-pituitary-adrenal axis

adrenocorticotropin (ACTH) and β-endorphin from the anterior pituitary into the general circulation. AVP acts synergistically with CRH to enhance ACTH release (Jones and Gillham 1988; Antoni 1993; Whitnall 1993). Lesion of PVN markedly reduces portal CRH levels and stress-induced ACTH and corticosterone (CORT) secretion (Makara 1992). ACTH then acts on the adrenal cortex to stimulate the synthesis and release of species-specific glucocorticoids [CORT in most rodents; cortisol in humans] 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 (De Kloet, Vreugdenhil et al. 1998; Aguilera, Kiss et al. 2007).

The actions of CORT are mediated through its binding to specific intracellular receptors. The nonactivated glucocorticoid receptor resides in the cytosol in the form of a heterooligomer with heat-shock proteins and immunophilins. Upon ligand binding, the glucocorticoid receptors dissociate from the rest of the heterooligomer and translocate into the nucleus, where they interact as homodimers with specific glucocorticoid-responsive elements (GREs) within the DNA to transactivate appropriate effective genes. There are two major classes of glucocorticoid 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. MRs bind with high affinity to CORT, suggesting that they are involved mainly in regulating basal levels (De Kloet, Vreugdenhil et

al. 1998), but they may also play a role in feedback regulation following exposure to mild stressors (Ratka, Sutanto et al. 1989; Bradbury, Akana et al. 1994). GRs bind with about 10-fold less (5 - 10 nM) affinity to CORT and are occupied predominantly during high levels of circulating CORT, such as during stress or at the circadian peak, suggesting a role in suppression of stress-induced HPA activity (De Kloet, Vreugdenhil et al. 1998; Sapolsky, Romero et al. 2000). 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, whereas MRs are located primarily within limbic structures such as hippocampus, septum and amygdala. The balance between MR- and GR-mediated feedback is critical for maintaining homeostasis (Ratka, Sutanto et al. 1989; Bradbury, Akana et al. 1994; De Kloet, Vreugdenhil et al. 1998). Both MR and GR are self-regulated genes, such that removal of ligand (i.e. adrenalectomy, ADX) increases levels of both receptors in the hippocampus (Herman 1993).

Besides the important role of glucocorticoids in feedback regulation, the HPA axis is also susceptible to glucocorticoid-independent inhibition from various neuronal sources. The PVN is richly innervated by GABAergic neurons from multiple brain regions, including the bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), dorsomedial hypothalamus, lateral hypothalamic area and peri-PVN region (Cullinan, Herman et al. 1993; Roland and Sawchenko 1993; Cullinan, Helmreich et al. 1996; Herman, Figueiredo et al. 2003).

The densest PVN projections originate from the intrafascicular, transverse, and anterodorsal nuclei of BNST (Ju and Swanson 1989a; Ju, Swanson et al. 1989b; Cullinan, Herman et al. 1993). These BNST nuclei highly express gonadal steroid receptors, whereas expression of these receptors is low or absent in the mpd PVN (Simerly, Chang et al. 1990; Zhou, Blaustein et al. 1994; Shughrue, Lane et al. 1997; Bingham, Williamson et al. 2006). Thus, effects of gonadal steroids on the HPA axis may be also be modulated by receptive neurons in these regions. The anterior and posterior divisions of the BNST contain CRH- and AVP-projecting cells to the PVN (Moga and Saper 1994; Champagne, Beaulieu et al. 1998), and exert a stimulatory (CRH-projecting) and inhibitory (AVP-projecting) influence on CRH and AVP mRNA expression in the PVN, respectively (Herman, Cullinan et al. 1994).

The BNST regions make bidirectional contacts between the amygdala and mpd PVN (Lee, Imaki et al. 1990; Prewitt and Herman 1998; Dong, Petrovich et al. 2001a; Dong, Petrovich et al. 2001b). The amygdala is known to prompt behavioral and cardiovascular responses to stress and is activated during stress primarily by ascending catecholaminergic neurons in the brainstem or by emotional stressors (Davis 1992). The influence of the amygdala on the HPA system is excitatory and largely mediated by the medial (MeA) and central (CeA) amygdaloid nuclei (Swanson and Petrovich 1998). Lesions of the MeA or CeA reduce ACTH and/or CORT secretion following stress (Allen and Allen 1974; Beaulieu, Di Paolo et al. 1986; Feldman, Conforti et al. 1994; Dayas and Day 2002), whereas stimulation of the amygdala increases HPA activity (Matheson, Branch et al. 1971; Redgate and

Fahringer 1973; Dunn and Whitener 1986). The ventrolateral regions of the BNST may be of importance in relaying information from the CeA to the PVN (Dong, Petrovich et al. 2001a).

The amygdala is also a potential target for glucocorticoids. Both the MeA and CeA express GR (Aronsson, Fuxe et al. 1988; Ahima and Harlan 1990) and MR (Arriza, Simerly et al. 1988). CRH neurons in the CeA respond positively to glucocorticoids (Makino, Gold et al. 1994a; Makino, Gold et al. 1994b). The activation of these neurons leads to anxiety, fear, and stress system activation.

There are other limbic areas that also have influences on neurosecretory neurons in the PVN, including medial prefrontal cortex, ventral subiculum, and lateral septum. Neurons in these areas project directly to the brain stem, hypothalamic nuclei and BNST that in turn, innervate the neurosecretory neurons in the mpd PVN. The inhibitory feedback effect of hippocampus on the HPA function is relayed via ventral subiculum, in which neurons contains glutamate, and stimulate the inhibitory interneurons in the MPOA or BNST (Herman and Cullinan 1997; Herman, Figueiredo et al. 2003)**.**

C. Effects of Prenatal Ethanol Exposure on the HPA Axis

Clinical studies in humans have shown that prenatal exposure to alcohol results in HPA hyperactivity at 2 (Ramsay, Bendersky et al. 1996) and 13 months (Jacobson and Jacobson 1999) of age. Animal studies strongly support and extend these data, indicating that alcohol exposure in utero can alter the development and function of the offspring HPA axis (Taylor, Branch et al. 1982a; Taylor, Branch et al. 1982b; Taylor, Nelson et al. 1984; Lee,

Imaki et al. 1990; Lee and Rivier 1996; Rivier 1996; Weinberg, Taylor et al. 1996; Lee, Schmidt et al. 2000). At gestation day (G) 19, E rat fetuses show reduced CORT levels (Weinberg 1989; Revskoy, Halasz et al. 1997), whereas at birth, E neonates exhibit elevated plasma and brain levels of CORT, and increased plasma but decreased pituitary levels of β-endorphin (Kakihana, Butte et al. 1980; Taylor, Branch et al. 1983; Weinberg, Nelson et al. 1986; Angelogianni and Gianoulakis 1989; Weinberg 1989). During the pre-weaning stress hyporesponsive period, E offspring exhibit even greater blunting of CORT responses to stressors than controls (Weinberg and Gallo 1982; Taylor, Branch et al. 1986; Weinberg 1989) as well as delayed maturation of the HPA axis (Halpert, Lan et al. 2004), and delayed onset of puberty (Esquifino, Sanchis et al. 1986; McGivern and Yellon 1992; Yamashita, Lan et al. 2003) compared to controls. In contrast, following weaning and throughout adulthood, E animals are typically hyperresponsive to stressors. Enhanced and/or prolonged CORT, ACTH and β-endorphin responses to stressors including footshock, ether, restraint, cold and immune challenges, and to drugs such as alcohol and morphine, have been reported in E rats (Weinberg and Gallo 1982; Nelson, Taylor et al. 1986; Weinberg 1988; Angelogianni and Gianoulakis 1989; Weinberg 1989; Weinberg 1992; Lee and Rivier 1996; Osborn, Kim et al. 1996; Weinberg, Taylor et al. 1996; Kim, Giberson et al. 1999; Lee, Schmidt et al. 2000; Lan, Yamashita et al. 2006), and both behavioral and HPA hyperresponsiveness to maternal separation stress have been observed in non-human primates (Schneider, Moore et al. 2004).

The mechanisms underlying hyperresponsiveness of the HPA axis are beginning to be elucidated and appear to involve alterations at multiple levels of the axis, and changes in both HPA drive and feedback regulations. A number of studies suggest the possibility of increased HPA drive, i.e. increased central secretagogue levels (Redei, Halasz et al. 1993; Osborn, Kim et al. 1996; Gabriel, Yu et al. 2000; Glavas, Ellis et al. 2000; Lee, Schmidt et al. 2000; Glavas, Hofmann et al. 2001; Gabriel, Glavas et al. 2005). That E males show increased basal CRH mRNA in the PVN has been observed in weanlings at 21 days of age (Lee, Imaki et al. 1990) and in adult E males, who also exhibit increased pituitary POMC mRNA levels (Redei, Halasz et al. 1993; Glavas, Ellis et al. 2000; Gabriel, Glavas et al. 2005). E animals showed increased immediate early gene and CRH heteronuclear RNA (hnRNA) responses in the PVN following footshock and endotoxemia compared to controls (Lee, Schmidt et al. 2000). Furthermore, following ADX, basal ACTH levels are higher in E compared to control males, and CRH mRNA levels are increased in both E males and females compared to their control counterparts (Glavas, Hofmann et al. 2001; Glavas, Ellis et al. 2007). Data from the Weinberg laboratory also indicate increased sensitivity to secregogogues. Increased ACTH responses to CRH following dexamethasone (DEX) blockade are found in E compared to controls rats (Osborn, Kim et al. 1996; Osborn, Yu et al. 2000). However, without DEX blockade, Rivier group found pituitary responsiveness to CRH was unchanged, while responsiveness to AVP was marginally increased in female rats (Lee, Schmidt et al. 2000).

Deficits in feedback regulation in E animals have also been observed. Both E males and E females demonstrate significantly greater CORT and/or ACTH responses to ether stress 3 - 6 hr following DEX blockade, suggesting deficits in the intermediate (2 - 10 hr) feedback time domain (Osborn, Kim et al. 1996). However, E animals do not show deficits in fast feedback (Hofmann, Glavas et al. 1999). Furthermore, HPA responses to both MR and GR blockade are differentially altered in E and control females (Glavas, Yu et al. 2006). CORT replacement at low basal levels is ineffective in normalizing ACTH levels in E females and MR mRNA levels in E males, suggesting a decreased sensitivity to CORT feedback regulation in E animals (Glavas, Ellis et al. 2007).

Importantly, however, while HPA hyperresponsiveness is a robust phenomenon, occurring in both male and female offspring, data indicate that differential effects of prenatal ethanol exposure may be observed in male and female offspring, depending on the nature and intensity of the stressor, and the time course and hormonal endpoint examined. As noted, in adulthood, E males and females both exhibit increased CORT, ACTH and/or β-endorphin responses to stressors such as repeated restraint, footshock and immune challenges (Taylor, Branch et al. 1988; Weinberg 1993; Kim, Osborn et al. 1996; Lee and Rivier 1996; Weinberg, Taylor et al. 1996; Kim, Giberson et al. 1999; Kim, Yu et al. 1999; Lee, Schmidt et al. 2000). Both E males and females also show increased immediate early gene and CRH mRNA level s following stress (Lee, Schmidt et al. 2000), as well as deficits in habituation to repeated restraint (Weinberg, Taylor et al. 1996). In contrast, in response to prolonged restraint or cold

stress, HPA hyperactivity is seen primarily in E males (Weinberg 1992; Kim, Giberson et al. 1999), whereas in response to acute restraint, or acute ethanol or morphine challenge, increased hormone responses occur primarily in E females (Taylor, Branch et al. 1982b; Taylor, Branch et al. 1983; Weinberg, Nelson et al. 1986; Taylor, Branch et al. 1988; Weinberg 1988). These findings raise the possibility that ethanol-induced alterations in the gonadal hormones and/or in HPA-hypothalamic-pituitary-gonadal (HPG) interactions may play a role in mediating prenatal ethanol effects on HPA activity in adulthood.

D. Hypothalamic-Pituitary-Gonadal Axis

The HPA axis develops in parallel, and interacts in a bidirectional manner, with the HPG axis (Figure 2). The HPG axis controls the reproductive system, as well as non-reproductive sexually dimorphic functions. 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 where it regulates the synthesis and release of two gonadotropic glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), together referred to as gonadotropins. LH and FSH are released from the pituitary into the systemic circulation in response to

GnRH. They then travel to the gonads, where they direct gamete production, as well as gonadal hormone production. The secretion and/or action of GnRH are 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 development, growth and differentiation of 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. Estrogen, in particular, also acts on other body tissues to cause breast development and increase subcutaneous deposition of fat.

Testosterone and its metabolite, 5α-dihydrotestosteone (DHT), are the major circulating androgens in males. Androgens exert their actions by binding to and activating androgen receptors (AR) in target cells. ARs are ligand-dependent transcriptional factors and regulate gene expression through interaction with androgen response element (ARE) of

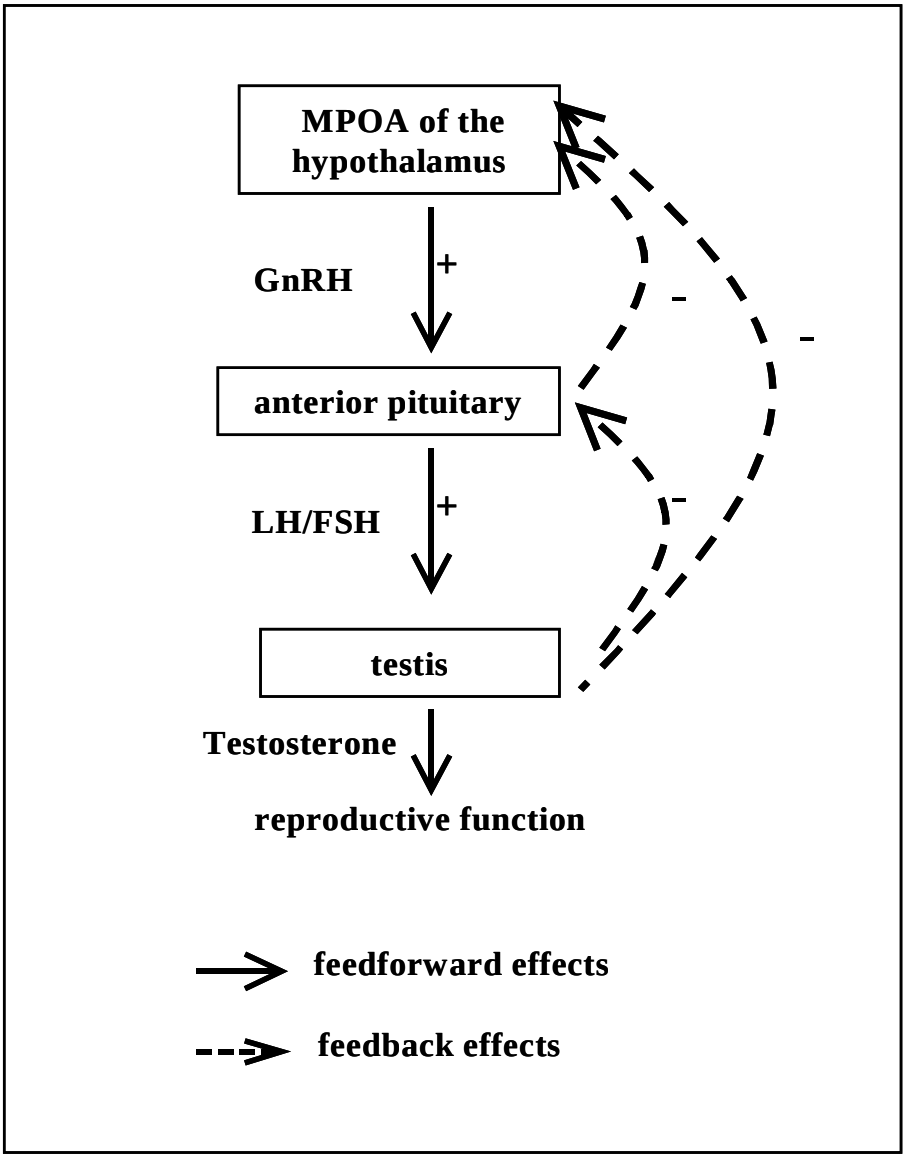


Figure 2. Schematic of the hypothalamic-pituitary-gonadal axis

specific genes to trigger a cascade of transcriptional events. ARs have been localized in brain areas that mediate reproductive behaviors and function, such as the hypothalamus (Sar, Lubahn et al. 1990; Burgess and Handa 1993; Kerr, Allore et al. 1995; Bingham, Williamson et al. 2006; Williamson and Viau 2007) , as well as areas that not classically associated with reproduction, such as the hippocampus (Simerly, Chang et al. 1990; Clancy, Bonsall et al. 1992; McAbee and DonCarlos 1998; Williamson and Viau 2007), indicating that androgens have influences on non-reproductive function, such as learning and memory.

E. Effects of Prenatal Ethanol Exposure on the HPG Axis

In addition to effects on HPA activity, prenatal ethanol exposure has marked effects on the development and activity of the HPG axis in both males and females. In males, 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, Raum et al. 1988). Both the pre- and postnatal testosterone surges are suppressed in E fetuses/neonates (McGivern, Raum et al. 1988; McGivern, Handa et al. 1993; Ward, Ward et al. 2003), and at birth, E pups exhibit decreased brain and plasma testosterone (Kakihana, Butte et al. 1980), and deficits in testicular steroidogenic enzyme activity (Kelce, Rudeen et al. 1989; Kelce, Ganjam et al. 1990) compared with controls. E neonates also exhibit a decreased anogenital distance compared to controls, suggesting feminization (Udani, Parker et al. 1985). However, some studies report no effect of prenatal ethanol on anogenital distance in some experimental

models (McGivern, Raum et al. 1988; McGivern, Raum et al. 1992). The developmental pattern of LH secretion is also altered in E males, which could contribute to later changes in reproductive function (Handa, McGivern et al. 1985), such as spermatogenesis and sexual behavior. In adulthood, E males have a significantly reduced volume of the sexually dimorphic nucleus of the preoptic area (Barron, Gagnon et al. 1988) and show decreased testicular weight (Parker, Udani et al. 1984), reduced weights of prostate and seminal vesicles, decreased serum testosterone and LH levels, and altered neurotransmitter responses to testosterone, suggesting central dysregulation of HPG activity (Udani, Parker et al. 1985). Altered morphology of the seminiferous tubules has also been reported, eg. absence of reticulin fibers in the peritubular tissue of seminiferous tubules at postnatal day (PN) 42 (Fakoya and Caxton-Martins 2004). Furthermore, E males were shown to have lower mean levels of LH, in addition to a decrease in pulse amplitude and frequency. They also found that gonadectomized E male rats had significantly reduced plasma LH titers as compared to those of PF controls (Handa, McGivern et al. 1985). However, normal LH and testosterone levels in adult E males have also been reported (Ward, Ward et al. 1996).

Similarly, E females have lower basal plasma LH levels both pre- and postpubertally (Handa, McGivern et al. 1985; Esquifino, Sanchis et al. 1986; Morris, Harms et al. 1989), and show reduced LH secretion following GnRH treatment in adulthood (Creighton-Taylor and Rudeen 1991b). Furthermore, sexual maturation (vaginal opening) (Boggan, Randall et al. 1979; Esquifino, Sanchis et al. 1986; Creighton-Taylor and Rudeen 1991a; McGivern and

Yellon 1992), onset of FSH secretion (Wilson and Handa 1997), and onset of puberty (McGivern and Yellon 1992) may be delayed in E females. E females also have decreased hypothalamic GnRH content (Creighton-Taylor and Rudeen 1991b), altered distribution of GnRH neurons (McGivern and Yellon 1992; Gavin, Kates et al. 1994; Wilson, Marshall et al. 1995) and decreased anterior pituitary LH release in response to GnRH and estrogen stimulation *in vitro* (Morris, Harms et al. 1989). Deficits in sexual behavior, as well as feminization or masculinization of a variety of nonsexual sexually dimorphic behaviors have also been reported in both E males and females (McGivern, Clancy et al. 1984; Barron, Razani et al. 1995).

F. Interaction of HPA and HPG Axes

1. The effects of gonadal hormones on HPA regulation

Sex differences have been shown in HPA responses, indicating that gonadal hormones are involved in the regulation of the HPA axis. Females have higher basal and stress levels of ACTH and CORT than males (Le Mevel, Abitbol et al. 1978; Le Mevel, Abitbol et al. 1979; Handa, Burgess et al. 1994; Young 1995), and the differences can be abolished by ovariectomy (OVX) and restored by estradiol replacement (Le Mevel, Abitbol et al. 1978). As well, higher levels of CRH protein and CRH mRNA in the PVN have been found in females than males (Watts and Swanson 1989). In general, estradiol activates, and androgens inhibit HPA function (Bohler, Zoeller et al. 1990; Almeida, Hassan et al. 1992; Bingaman,

Magnuson et al. 1994; Patchev, Hayashi et al. 1995; Roy, Reid et al. 1999; Lund, Munson et al. 2004). For example, androgens inhibited hypothalamic CRH peptide (Almeida, Hassan et al. 1992; Bingaman, Magnuson et al. 1994). Gonadectomy (GDX) of adult male rats increased the ACTH as well as the CORT responses to physical and psychological stressors (Handa, Nunley et al. 1994), and the effect was reversed with testosterone or DHT replacement (Handa, Nunley et al. 1994; Viau, Lee et al. 2003). GDX male rats showed greater stress-induced Fos expression and higher AVP hnRNA levels than intact males, and both responses in intact males were shown to be negatively correlated with plasma testosterone levels (Viau, Lee 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, 5α-DHT, implicating androgen receptors in the regulatory pathway (Handa, Nunley et al. 1994). On the other hand, for females, significantly higher ACTH and CORT levels following stress were found during proestrus compared to other estrous phases, when estradiol levels are high. Estradiol replacement that mimicked the proestrus phase also enhanced stress-induced ACTH, β-endorphin, and CORT (Viau and Meaney 1991). CRH mRNA levels are also higher during proestrous in female rats, when estradiol levels are highest (Bohler, Zoeller et al. 1990). In addition, androgens decrease GR mRNA expression in the hippocampus (Kerr, Beck et al. 1996), and increase GR levels in the MPOA (Viau and Meaney 1996).

As mentioned earlier, the mpd PVN that have CRH- and AVP-expressing neurosecretory neurons, is the final pathway that integrates multiple excitatory and inhibitory inputs from other brain areas regulating the HPA axis. The medial parvocellular ventral division of the PVN (mpv PVN) houses parvicellular neurosecretory, magnocellular neurosecretory, and autonomic premotor neurons (Viau and Sawchenko 2002). Mapping studies have demonstrated that ARs are not localized in the mpd PVN, but restricted to the mpv PVN, periventricular and dorsal parvocellular part of the PVN (Simerly, Chang et al. 1990; Zhou, Blaustein et al. 1994; Shughrue, Lane et al. 1997; Bingham, Williamson et al. 2006). These findings suggest that androgens can directly act on the PVN to regulate autonomic function, but act upstream from the PVN to regulate HPA output. Candidate brain areas that mediate androgenic effects on the PVN are the medial preoptic area [where ARs are densest in the medial preoptic nucleus (MPN)], BNST, amygdala, and hippocampus, which contain high densities of AR (Simerly, Chang et al. 1990; Lisciotto and Morrell 1994; Zhou, Blaustein et al. 1994; Kerr, Allore et al. 1995; Shughrue, Lane et al. 1997; Romeo, Diedrich et al. 2000; Bingham, Williamson et al. 2006; Williamson and Viau 2007).

2. The Effects of HPA Hormones on HPG Regulation

Conversely, stress has inhibitory effects on reproductive behavior and HPG hormone activity. 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, Turner et al. 2000; Tilbrook, Turner et al. 2002). In female rats, central injections of CRH inhibit the synthesis of GnRH and plasma LH (Rivier and Vale 1984; Petraglia, Sutton et al. 1987). CRH either directly or indirectly acts on GnRH neurons in the MPOA of the hypothalamus (Rivier and Vale 1984). Central administration of ACTH caused a dose-related rise in LH levels in intact male rats (Mann, Evans et al. 1986). Glucocorticoids generally disrupt all aspects of HPG function, including reproductive behavior, GnRH expression, plasma LH secretion and sex steroid synthesis and release (Tilbrook, Turner et al. 2000; Tilbrook, Turner et al. 2002).

Adrenalectomy reverses stress-induced suppression of LH secretion in long-term OVX rats, indicating a primary role of glucocorticoids in the stress-induced HPG function (McGivern and Redei 1994). In vitro studies show that glucocorticoids directly inhibit testosterone production by Leydig cells, via inhibition of testosterone biosynthetic enzyme gene transcription (Hales and Payne 1989; Payne and Sha 1991; Orr, Taylor et al. 1994). Indeed, glucocorticoid receptors have been found in Leydig cells (Stalker, Hermo et al. 1989; Stalker, Hermo et al. 1991). Glucocorticoids have been shown to induce Leydig cell apoptosis, thus reducing Leydig cells numbers per testis (Hardy, Gao et al. 2005). Glucocorticoids also 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, Turner et al. 2000; Tilbrook, Turner et al. 2002). Glucocorticoids suppress gonadotropin release not only by acting at the level of pituitary, but also at the hypothalamic level (Calogero, Burrello 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. (Lemaire, Taylor et al. 1997) showed that HPG axis is activated during social interactions even when the HPA axis is stimulated, suggesting that stress does not always inhibit gonadal function.

The sex differences in HPA responsiveness to stress are due in part, 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 glucocorticoids on GnRH release (Calogero, Burrello et al. 1999). Acutely elevated glucocorticoids induced by psychosocial or immune stress suppress LH pulse amplitude in ovariectomized ewes by inhibiting pituitary responsiveness to GnRH (Debus, Breen et al. 2002; Breen and Karsch 2004). Cortisol does not acutely inhibit GnRH pulsatility in the absence of gonadal steroids (Breen and Karsch 2004).

Both CORT and testosterone exert inhibitory effects on HPA function, therefore it is important to elucidate how CORT and testosterone act and interact on PVN function. An elegant study by Viau and colleagues demonstrated that both independent and interactive effects of testosterone and CORT play a role in HPA function by manipulating both HPA and HPG axes simultaneously in the male rat (Viau, Chu et al. 1999). Under basal conditions,

CRH is regulated primarily by CORT dependent effects, whereas AVP is regulated primarily by testosterone dependent effects. In contrast, following stress, ACTH release appears to be determined by an interaction of CORT and testosterone acting primarily on drive to the PVN.

G. Rationale and Thesis Objectives

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. As the adrenal and gonadal axes develop in parallel and interact in a bidirectional manner, and because HPA function depends on gonadal status [reviewed in (Handa, Burgess et al. 1994; Dallman, Viau et al. 2002; Viau 2002)], ethanol-induced changes in HPG activity could play a role in altering the HPA response to stress.

The main objective of this thesis was to investigate the influence of gonadal steroids, particularly testosterone, on HPA responsiveness in E compared to control males. To this end, we examined basal and stress-induced changes in HPG and HPA activity in adult E, PF and C

male rats. We also examined the effects of GDX, with or without testosterone replacement, in unmasking the extent to which prenatal ethanol influences HPA-HPG interactions. Our experimental questions were: 1) Do E males differ from controls in adrenal and gonadal hormone levels under intact conditions, following GDX or following GDX with low or high testosterone replacement? 2) Do differences between E and controls with different circulating testosterone levels occur under both basal and stress conditions? 3) Do central measures of HPA and HPG activity under different circulating testosterone levels differ in E compared to controls? And 4) Are activities of central testosterone-sensitive pathway that regulate CRH and AVP neurosecretory neurons altered in E males compared to controls? Two studies were conducted to test the overall hypothesis that the alterations in HPA activity and regulation observed in E males compared to their controls counterparts are mediated, at least in part, by ethanol-induced changes in HPG effects on HPA regulation. The first study examined if the normal testicular influence on HPA activity is altered in E males. We compared the effects of GDX on HPA and HPG activity in adult male offspring from prenatal E, PF and C dams. We tested the hypothesis that a decreased testicular influence could contribute to the HPA hyperresponsiveness typically observed in E males. Animals were gonadectomized (GDX) or received Sham surgery (intact) and terminated under basal conditions or 30 or 90 min following restraint stress. The data suggest that the normal testicular influences on HPA function are significantly reduced in E males. The second study explored dose-related effects of testosterone on HPA regulation. Animals were subjected to Sham surgery, to GDX alone,

or to GDX with testosterone replacement at low or high basal levels. Animals were terminated under basal conditions or following 30 min restraint stress. The data suggest that overall, testosterone has a reduced effect on central CRH pathways, but an increased effect on central AVP pathways in E compared to PF and/or C males. Importantly, reduced AR mRNA levels, possibly reflecting downregulation of AR in key brain areas, may counteract the increased inhibitory AVP signals upstream from the paraventricular nucleus, and thus contribute to the HPA hyperresponsiveness seen in E males. Together these findings suggest that central regulation of both the HPA and HPG axes are altered by prenatal ethanol exposure. The capacity of testosterone to regulate HPA activity is altered in E males, with some effects mediated by the nutritional effects of ethanol.

H. Significance

HPA hyperresponsiveness results in prolonged or chronic CORT elevations. The HPA hormones play key roles in mediating both physiological (Sapolsky et al., 2000) and behavioral (Koob 1999; Koob and Heinrichs 1999) responses to stressors. Thus prolonged CORT elevations will have adverse consequences that could compromise health and even survival. Therefore, early life events (ie. prenatal ethanol exposure) that result in greater reactivity to stress and increased CORT levels throughout the life span can increase the vulnerability to illnesses later in life. Testosterone normally attenuates HPA responses to stress, thus serving a protective role during acute stress to attenuate the pathological effects

of elevated glucocorticoid levels (Williamson, Bingham et al. 2005). The thesis study investigated if prenatal ethanol-induced changes in HPG regulation play a role in the alterations in HPA activity observed in E males compared to controls. Altered HPA-HPG interactions could impair the ability to maintain homeostasis in E animals and progressively create a condition of neuroendocrine dysregulation and impaired behavioral adaptation. These changes have implications for the development of secondary disabilities in children with FASD, and may further affect the child's ability to respond appropriately to challenging or stressful situations.

CHAPTER II: GENERAL METHODS

A. Animals and Breeding

For both studies, male (275 - 300 g) and female (230 - 275 g) Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, PQ, Canada). Rats were group-housed by sex 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 (Jamieson's Pet Food Distributors Ltd., Delta, BC, Canada) and water. One to two weeks following arrival, males were placed singly in stainless steel suspended cage (25 × 18 × 18 cm), with mesh front and floor, together with a female. Wax paper under the cages was checked daily, and the presence of a vaginal plug indicated day 1 of gestation (G 1). All animal use and care procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the 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) with pine shavings bedding and randomly assigned to one of three treatment groups: 1) Ethanol (E), liquid ethanol diet (36 % ethanol-derived calories) and water, *ad libitum*; 2) Pair-fed (PF), liquid control diet with maltose-dextrin isocalorically substituted for ethanol, and intake matched to the amount consumed by an E partner (g/kg body weight/ gestation d), and water

ad libitum; 3) Control (C): standard lab chow and water, *ad libitum*. E females were gradually introduced to the ethanol diet by providing 1/3 ethanol : 2/3 PF diet on G 1, 2/3 ethanol : 1/3 PF diet on G 2, and 100% ethanol diet on G 3. The liquid diets (Dyets Inc., Bethlehem, PA) were formulated in the Weinberg laboratory to provide adequate nutrition to pregnant rats regardless of ethanol intake. All animals were provided with fresh diet daily within 1.5 hr prior to lights off to prevent a shift of CORT circadian rhythms, which may occur in PF animals who are on a restricted feeding schedule (Gallo and Weinberg 1981). Feeding bottles were removed and weighed daily to determine the amount consumed by each animal. Experimental diets were continued through G 21, and beginning on G 22 animals were provided *ad libitum* access to standard lab chow and water, which they received throughout lactation. Pregnant dams were handled only on G 1, G 7, G 14 and G 21 for cage changing and weighing. On postnatal day 1 (PN 1), pups were weighed and litters were randomly culled to 10 (5 males and 5 females when possible). If necessary, pups from the same prenatal treatment group born on the same day, were fosterd to in to a litter to maintain the litter size. Dams and pups were weighed on PN 1, PN 8, PN 15 and PN 22. On PN 22, pups were weaned, and group-housed by litter and sex. Male pups were pair-housed starting around PN 40.

C. Blood Alcohol Level (BAL) Measurement

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. Gonadectomy (GDX) and Testosterone Replacement

Surgery occurred at 53 - 58 d of age. Male offspring from the 3 prenatal groups were weight-matched and randomly assigned to Sham GDX (Intact), GDX or GDX with low or high testosterone replacement conditions. To control for litter effects, no more than one animal from any one litter was used per test condition. Testes were removed under halothane anaesthesia via a longitudinal scrotal incision, the testicular vessels were ligated, and the

scrotal incision was closed with 4-0 nonabsorbable suture. Sham surgery was done by making an incision and then suturing the scrotum, without touching the testes. Testosterone was provided by subcutaneous implantation of silastic capsules (2.5 cm length; 0.062 inch i.d., 0.125 inch o.d.) packed with crystalline testosterone designed to provide circulating testosterone concentrations approximating low (~ 1 ng/ml) and high (4 ~ 6 ng/ml) physiological basal levels. All animals received a 0.1 cc intramuscular injection of the antibiotic Duplocillin (Intervet Canada Inc., Whitby, ON, Canada, DIN 01983377). Surgery was done at 0900 - 1200 hr each day, and animals were weighed prior to surgery and again 1 d before testing.

E. Restraint Stress, Sampling and Tissue Collection

Rats were weighed one day before testing, which occurred 14 d after the surgery. This recovery period was based on previous published studies from our collaborator (Viau and Meaney 1996). Stress testing was 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 that weighed less than 380 g and 7.5 × 20 cm (inner diameter × length) for rats that 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 60 min and then terminated. Animals were decapitated within 30 seconds of touching the cage or the restraint tube.

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

F. Radioimmunoassays (RIA)

Blood samples were centrifuged at 3200 rpm for 10 min at 0 ˚C. Plasma or serum was transferred into 600 µl Eppendorf tubes and stored at - 80 ˚C until assayed.

Corticosterone. The following RIA protocol was used to measure CORT levels in Chapter III: Total corticosterone (bound plus free) levels were measured by RIA [adapted from (Kaneko, Kaneko et al. 1981)] in plasma extracted in absolute ethanol (Weinberg and Bezio 1987). Antiserum was obtained from MP Biomedicals (Orangeburg, NY, USA), tritiated corticosterone tracer from Mandel Scientific (Guelph, ON, Canada) and corticosterone for standards from Sigma Chemical Co (St. Louise, MO, USA). Dextran-treated charcoal (Fisher Scientific Ltd., Nepean, ON, Canada) was used to absorb free corticosterone after incubation. The antiserum cross-reacts 100 % for corticosterone, 2.3 % for desoxycorticosterone, 0.47 % for testosterone, 0.17 % for progesterone and 0.05 % for

aldosterone. The minimum detectable corticosterone concentration was 0.25 μg/dl and the intra- and inter-assay coefficients of variation were 1.55 % and 4.26 % respectively.

The following RIA protocol was used to measure CORT levels in Chapter IV: Total corticosterone (bound plus free) levels were measured using an RIA kit from MP Biomedicals (Solon, OH) with [^{125}I] corticosterone as tracer. The antiserum cross-reacts 100 % for corticosterone, 0.34 % for deoxycorticosterone, 0.10 % for testosterone, 0.10 % for cortisol, but not cross-reacts for progesterone and estrogens (< 0.01%). The minimum detectable corticosterone concentration was 0.63 μg/dl and the intra- and inter-assay coefficients of variation were 1.55 % and 4.26 % respectively.

Adrenocorticotrophin (ACTH). Plasma ACTH levels were measured using an adaptation of an ACTH RIA kit (Diasorin Inc., Stillwater, MS, USA) with [^{125}I] ACTH as tracer and all reagent volumes halved. The ACTH antibody cross-reacts 100 % with porcine $ACTH_{1-39}$ and human $ACTH_{1-24}$, but not with α-melanocyte-stimulating hormone (MSH), β-endorphin, β-lipotropin (< 0.1 %). The minimum detectable ACTH concentration was 20 pg/ml, and the intra- and inter-assay coeffeicients of variation were 3.9 % and 6.5 % respectively.

Testosterone. Plasma testosterone levels were measured using an adaptation of the testosterone RIA kit of MP Biomedicals (Solon, OH) with [^{125}I] testosterone as tracer and all reagent volumes halved. The testosterone 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 %), and 25 µl of each plasma sample were used to determine testosterone concentrations. The minimum detectable testosterone concentration was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were 4.6 % and 7.5 % respectively.

Luteinizing Hormone (LH). Plasma LH levels were measured by RIA in the laboratory of Dr. A.F. Parlow, NIDDK, National Hormone and Peptide Programme (Harbor-UCLA Medical Centre, California, USA). The cross-reactivity with other pituitary hormones was negligible. The minimum detectable LH concentration of the assay was 0.1 ng/ml. The intra- and inter-assay coefficients of variation were less than 10% (Attademo, Sanchez-Borzone et al. 2004).

G. In situ Hybridization

1. Brain preparation

In the first study, brains were sectioned coronally into 20 µm sections through the rostral medial preoptic area (Bregma - 0.12 mm), and 14 µm sections through the paraventricular nucleus (Bregma - 1.80 mm) (Paxinos and Watson 2005) of the hypothalamus. In the second study, brains were sectioned into five one-in-five series of 30 µm coronal sections from the nucleus of the vertical limb of the diagonal band (Bregma 0.48

mm) to the medial nucleus of amygdala (Bregma - 3.36 mm) (Paxinos and Watson 2005). Frozen sections were thaw-mounted onto gelatin-coated slides and stored at - 80 ˚C.

2. Oligonucleotide probes and labeling

Oligonucleotide probes 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 as follows: antisense CRH (5'-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3') (Jingami, Mizuno et al. 1985; Young, Mezey et al. 1986), antisense AVP (5'-GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG-3') (Ivell and Richter 1984; Young, Mezey et al. 1986) and antisense GnRH (5'-TTC AGT ATT TCT CTT CCC CCC AGG GCG CAA CCC ATA GGA CCA GTG CTG-3') (Zoeller, Seeburg et al. 1988). Sense oligos for CRH, AVP and GnRH mRNA were used as negative controls. Probes were 3' tail labeled with ^{35}S-dATP (Amersham Biosciences, NJ, USA) using terminal deoxytransferase (New England Biolabs Inc., ON, Canada) as per supplier protocol. Probes were purified using Roche DNA G-25 Sephadex Columns (Roche Scientific, IN., USA). 1 M dithiothreitol (DTT) was added to prevent oxidation.

3. Ribonucleotide probes and labeling

Ribonucleotide probes were used to measure MR (Cullinan, Herman et al. 1995), GR (Cullinan, Herman et al. 1995), AR (Chang and Kokontis 1988; Simerly, Chang et al. 1990) mRNA (probes are identical to the probes used in reference paper). Probes were labeled with ^{35}S-UTP (Amersham Biosciences, NJ, USA) using Promega Riboprobe System (Promega Corp., Madison, WI, USA). All probes were purified using Roche RNA G-50 Sephadex Columns (Roche Scientific, IN., USA). 1 M DTT was added to prevent oxidation.

4. Hybridization with oligonucleotide probes

Sections were thawed (20 min) and went through prehybridisation as follows: formalin (30 min), 1 × PBS (10 min) twice, 0.1M triethanolamine-hydrochloride - 0.9 % NaCl + 0.25 % acetic anhydride (10 min), 2 × SSC (5 min), dehydrated through a graded series of ethanol, chloroform (5 min) followed by 100 % ethanol, and then air-dried. Hybridisation buffer (50 % formamide, 3 × SSC, 1 × Denhardt's solution, 100 μg/ml yeast tRNA, 25 mM sodium phosphate buffer (pH 7.4), 10 % dextran sulphate, 55 mM DTT, 30 % deionized water) was applied and covered with hybrislips (Sigma-Aldrich Canada Ltd., ON, Canada). Sections were incubated overnight at 40 °C in 50 % formamide humidified containers. Hybrislips were removed and slides were washed in 2 × SSC (20 min) twice, 2 × SSC/0.01 M DTT (45 °C, 20 min), 1 × SSC (45 °C, 15 min), 1 × SSC/50 % formamide (45

°C, 30 min), 1 × SSC (10 min), 0.5 × SSC (10 min). Sections were dipped briefly in water five times then plunged into 70 % ethanol (5 min), then air dried overnight. Sections for CRH and AVP mRNA were exposed to Kodak BioMax MR film (Eastman Kodak Co., NY, USA). All slides were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H_2O) in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak Polymax T fixer at 14 °C, then counterstained with Cresyl Violet. Coverslips were mounted with Permount (Fisher Scientific Ltd., ON, Canada).

5. Hybridization with ribonucleotide probes

Sections were thawed (20 min) and went through prehybridisation as follows: formalin (30 min), 1 × PBS (10 min) twice, digested by proteinase K (100 μg/L, at 37 °C for 9 min) and then washed in DEPC-treated deionized water (5 min), 0.1M triethanolamine-hydrochloride - 0.9 % NaCl + 0.25 % acetic anhydride (10 min), 2 × SSC (5 min, twice), dehydrated through a graded series of ethanol, chloroform (5 min) followed by 100 % ethanol, then air-dried. Hybridization buffer (75 % formamide, 3 × SSC, 1 × Denhardt's solution, 200 μg/ml yeast tRNA, 50 mM sodium phosphate buffer (pH 7.4), 10 % dextran sulphate, 10 mM DTT) was applied (probe activity for MR: 2.8×10^5 cpm/section; GR: 3.2×10^5 cpm/section; AR: 2.7×10^5 cpm/section) and covered with hybrislips (Sigma-Aldrich Canada Ltd, ON, Canada). Sections were incubated overnight at 55 °C in 75 % formamide

humidified containers. Hybrislips were removed and slides were washed in 2 × SSC (20 min) twice, RNAse A solution (25 μg/ml, 37 °C, 90 min), 2 × SSC/0.01 M DTT (10 min), 1 × SSC (15 min), 0.5 × SSC (15 min), 0.1 × SSC/0.01 M DTT (60 °C, 20 min), and 0.1 × SSC (5 min). Sections were dehydrated through 50%, 70 %, 95% and 100% ethanol (2 min each), then air dried overnight. Sections were exposed to Kodak BioMax MR film (Eastman Kodak Co., NY, USA) for 6 d for the GR, 3 d for the MR, 18 d for the AR, respectively. AR in situ slides were then dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H_2O) and exposed for 124 d for AR in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak Polymax T fixer at 14 °C. Coverslips were mounted with Permount (Fisher Scientific Ltd., ON, Canada)

CHAPTER III: EFFECTS OF GONADECTOMY ON HYPOTHALAMIC-PITUITARY-ADRENAL ACTIVITY IN MALE RATS PRENATALLY EXPOSED TO ETHANOL[1]

A. Introduction

Prenatal ethanol exposure has marked effects on the development of the HPA and HPG axes. While E offspring typically exhibit blunted responses to stressors during the pre-weaning period (Weinberg and Gallo 1982; Taylor, Branch et al. 1986; Weinberg 1989), following weaning and throughout adulthood, E animals are typically hyperresponsive to stressors. As noted, however, while HPA hyperresponsiveness is a robust phenomenon, data (Weinberg 1988; Weinberg 1992; Lee and Rivier 1996) indicate that differential effects of prental ethanol exposure may be observed in males and females depending on the nature and intensity of the stressor, and the time course and hormonal endpoint examined (Weinberg 1988; Weinberg 1992; Zhang, Sliwowska et al. 2005). These findings raise the possibility that ethanol-induced alterations in the gonadal hormones and/or in HPA-HPG interactions may play a role in mediating prenatal ethanol effects on HPA activity in adulthood.

In intact male rats, the HPA response to stress varies negatively as a function of individual plasma testosterone levels (Viau and Meaney 1996). To examine if the normal testicular influence on HPA activity is altered in E males, we compared the effects of GDX

[1] These data have been published in: Lan, N., F. Yamashita, A. G. Halpert, L. Ellis, W. K. Yu, V. Viau and J. Weinberg (2006). "Prenatal ethanol exposure alters the effects of gonadectomy on hypothalamic-pituitary-adrenal activity in male rats." J Neuroendocrinol 18(9): 672-84

on HPA basal and stress responses as well as HPG regulation of adult E, PF and C male offspring. We test the hypothesis that a decreased testicular influence could contribute to the HPA hyperresponsiveness typically observed in E males.

B. Methods

1. Breeding and Feeding

Male (275 - 300 g, n = 18) and female (230 - 275 g, n = 50) Sprague-Dawley rats (17 E dams, 14 PF dams and 18 C dams) were used for this breeding. The breeding and feeding procedures were described in Chapter II. Only male offspring were used for this study (total N = 162, consisting of n = 7 per prenatal groupand surgical treatment under basal conditions, and n = 12 per prenatal group and surgical treatment under stress and recovery conditions).

2. Gonadectomy

Surgery procedures were described in Chapter II. There are two surgical treatment groups in this study: Sham GDX (Intact) or GDX.

3. Testing

Testing occurred 14 d after surgery. Rats were decapitated at 0, 30, and 90 min after the onset of a 30 min restraint stress. Trunk blood was collected into ice-chilled polystyrene

tubes containing 200 μl 0.5 M EDTA (to prevent coagulation) and 4 μg/ml aprotinin (to protect ACTH from denaturation). Brains were rapidly removed and immediately frozen on dry ice, then stored at - 80 ˚C until sectioning.

4. Radioimmunoassays (RIA)

Blood samples were centrifuged at 3200 rpm for 10 min at 0 ˚C. Plasma was transferred into 600 μl Eppendorf tubes and stored at - 80 ˚C until assayed.

RIA for CORT, ACTH, testosterone, and LH was described in Chapter II.

5. In situ Hybridization

a. Brain preparation

Brains were sectioned coronally into 20 μm sections through the rostral medial preoptic area (Bregma - 0.12 mm), and 14 μm sections through the paraventricular nucleus (Bregma - 1.80 mm) (Paxinos and Watson 2005) of the hypothalamus. Frozen sections were thaw-mounted onto gelatin-coated slides and stored at - 80 ˚C.

b. Oligonucleotide probes and labeling

Oligonucleotide probes and labeling procedures were described in Chapter II.

c. Hybridization with oligonucleotides

Hybridization procedures were described in Chapter II. Probe activities are: CRH: 1×10^5 cpm/section; AVP: 5×10^4 cpm/section; GnRH: 4.5×10^5/section.

Sections for CRH and AVP mRNA were exposed to Kodak BioMax MR film (Eastman Kodak Co., NY, USA) for 6 d and 1 hr, respectively. All slides were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H_2O) and exposed for 19 days for CRH, overnight for AVP, and 24 days for GnRH mRNA in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak Polymax T fixer at 14 °C, then counterstained with Cresyl Violet. Coverslips were mounted with Permount (Fisher Scientific Ltd., ON, Canada)

d. Densitometric analysis

CRH, AVP and GnRH mRNA positive cells were visualized with 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 1.33v software (National Institutes of Health, Bethesda, MD). The mean optical density (OD) of hybridisation signal was measured under dark-field illumination (Fig 5). Densitometric analysis was conducted over the medial parvocellular dorsal division (mpd) of the PVN for

both CRH and AVP (Swanson and Sawchenko 1983). The mpd PVN was traced by outlining a fixed circle (0.75 in diametre; scale 300 pixels/inch) under bright-field. Illumination was then switched to dark-field, the tracing was restored over the image, and OD measurements were taken. AVP sections were matched for rostrocaudal level based on the signal pattern, and CRH measurement was carried out on the section adjacent to the AVP section. CRH OD was measured concurrently with AVP, such that the area of measurement used for AVP was then superimposed on the corresponding CRH image. Background signal was measured over a region immediately lateral to each side of the PVN. The corrected grey levels from both the left and right sides of two sections of the PVN were averaged to obtain a mean corrected grey level of the four measurements for each animal.

For GnRH mRNA, two anatomically matched sections were evaluated from each brain, corresponding to the region of the rostral MPOA that surrounds the rostral tip of the 3rd ventricle. Signal was measured by outlining clusters of labeled neurons and measuring the pixel area and the mean OD (grain density measurements over individual clusters of neurons). Total GnRH mRNA levels for each section were calculated by pixel area × mean OD for each cluster and then summing the values of all the clusters. Values from the two sections were averaged to represent the total GnRH mRNA levels of each animal.

6. Statistical analysis

Developmental and body weight data were analyzed using repeated measures ANOVA and Newman-Keuls *post hoc* tests. To examine effects of GDX, hormone and mRNA data were first analyzed using 3-way ANOVAs for the factors of prenatal group (E, PF, C), surgical treatment (intact, GDX) and time (0, 30, 90 min), followed by Newman-Keuls *post hoc* tests on significant main or interaction effects. Further analyses to test the a priori hypothesis that ethanol exposure would alter androgen mediated HPA responsiveness utilized 2-way ANOVAs followed by Fisher's Least Significant Difference (LSD) *post hoc* tests, with the error term adjusted to reflect the error term from the 3-way ANOVA. Statistical significance was set at $P < 0.05$. P values between 0.05 and 0.10 were considered a statistical trend.

C. Results

1. Developmental Data

a. Ethanol intake and blood alcohol levels

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 9.56 ± 0.32, 12.02 ± 0.36, 12.36 ± 0.27 g ethanol/kg BW for weeks 1, 2 and 3 of gestation, respectively, and resulting in blood alcohol levels of 192.45 ± 4.123 mg/dl measured 2 hr after lights off.

b. Maternal body weights during gestation and lactation

Analysis of maternal body weights during gestation indicated significant main effects of group ($F_{(2,38)} = 4.10$; $P < 0.05$) and day ($F_{(3,114)} = 814.02$; $P < 0.001$), and a group × day interaction ($F_{(6,114)} = 17.07$; $P < 0.001$) (Table 1). E and PF dams did not differ from C dams on G1 but weighed significantly less than C dams ($P < 0.01$) by G 21. For lactation (L) body weights, a main effect of day ($F_{(3,138)} = 138.22$; $P < 0.001$) and a group × day interaction ($F_{(6,138)} = 8.65$; $P < 0.001$), reflected the finding that E and PF but not C dams weighed significantly more on L 22 than L 1 ($P < 0.001$), suggesting catch up weight gain in E and PF dams

c. Gestational length

One-way ANOVA revealed a significant effect of group on gestation length (Table 1) ($F_{(2,39)} = 4.0$; $P < 0.05$). E dams had a longer gestation (by approximately 12 hr) than PF and C dams ($P < 0.05$). There were no significant differences among prenatal groups for litter size or number of live born or stillborn pups.

d. Postnatal Body Weights of Female and Male Pups

Significant group effects for birth weights in both male ($F_{(2,46)} = 12.073$; $P < 0.001$) and female ($F_{(2,46)} = 11.448$; $P < 0.001$) offspring indicated that E and PF pups weighed less

than their C counterparts at birth ($P < 0.001$) (Table 2). All pups gained weight from PN 1 to PN 22 (males, ($F_{(3,138)} = 2278.89$; $P < 0.001$; females, $F_{(3,135)} = 6127.06$; $P < 0.001$), with catch up growth in E and PF animals such that there were no significant differences among groups at weaning on PN 22.

2. Body weight before surgery and 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 prior to the surgery and one day before testing. Analysis of adult body weights indicated significant effects of surgery ($F_{(1,156)} = 10.50$; $P < 0.005$) and day ($F_{(1,156)} = 6205.35$; $P < 0.001$), and a surgery × day interaction ($F_{(1,170)} = 152.16$; $P < 0.001$). There were no differences among E, PF and C males either prior to surgery or testing. Intact rats gained significantly more weight than GDX in the 2 weeks between surgery and testing such that at testing, intact males (411.35 ± 3.5) weighed more than GDX males (384.6 ± 3.1) ($P < 0.001$).

3. HPA responses to restraint

a. Plasma CORT levels

A main effect of time ($F_{(2,153)} = 90.12$; $P < 0.001$) (Figure 3 A, B) indicated that CORT levels increased significantly over basal (0 min) levels at 30 min ($P < 0.001$), and

decreased from 30 to 90 min ($P < 0.001$), but remained elevated over basal levels ($P < 0.001$) in all animals. There were no effects of GDX on the CORT response, and no significant differences among E, PF and C males across time.

b. Plasma ACTH levels

The 3-way ANOVA indicated main effects of surgery ($F_{(1,153)} = 4.84$; $P < 0.05$) and time ($F_{(2,153)} = 107.91$; $P < 0.001$) (Figure 3 C, D). Overall, ACTH levels increased at 30 min and recovered to basal levels at 90 min (30 > 0, 90 min, Ps < 0.001), and were significantly higher in GDX than intact males ($P < 0.001$). However, further analyses revealed differential effects of GDX on E and control animals. Under intact conditions, ACTH responses to restraint were significantly greater in E than in C males ($P < 0.01$). GDX resulted in a significant increase in the ACTH stress response in C males ($P < 0.05$). The ACTH response of GDX PF males approached but did not reach significance ($P < 0.08$), nor was there a significant change in the ACTH response of E males ($P = 0.77$) compared to their respective intact counterparts. Thus, the normal testicular influence on ACTH responsiveness appears to be attenuated in E and PF males, and thus GDX eliminated the group differences that were observed in intact males.

c. CRH and AVP mRNA in the mpd PVN

There were no significant effects of prenatal treatment or GDX on CRH mRNA levels (Figure 4 A, B).

In contrast, AVP mRNA levels were not different among intact E, PF and C males at 0 or 30 min, but were lower in intact E and PF than C rats at 90 min post-stress onset (Ps < 0.01) (Figure 4 C, D). Importantly, these differences among intact males were eliminated by GDX.

Representative bright-field and dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating CRH (Panels A, B) and AVP (Panels C, D) mRNA patterns in the PVN were shown in Figure 5.

4. HPG responses to restraint

a. Plasma testosterone levels

As expected, plasma testosterone levels in GDX rats were in the undetectable range. Analysis of testosterone levels in intact animals indicated a main effect of time ($F_{(2,78)} = 5.52$; $P < 0.01$) (Figure 6 A). Importantly, however, only PF and C males showed a significant testosterone increase following a 30 min restraint stress (PF: 30 min > 0, 90 min, Ps < 0.05; C: 30 min > 0 min, P < 0.05), whereas E males showed no significant change in testosterone levels over time.

b. Plasma LH levels

Main effects of surgery ($F_{(1,148)} = 1096.10; P < 0.001$) and time ($F_{(2,148)} = 41.97; P < 0.001$), and a surgery × time interaction ($F_{(2,148)} = 31.29; P < 0.001$), indicated that intact and GDX animals showed different patterns of LH secretion (Figure 6 B, C). Separate ANOVAs for intact and GDX animals revealed that in intact males, only C rats ($P < 0.05$) showed a significant LH increase after 30 min restraint stress (main effect of time ($F_{(2,75)} = 10.55; P < 0.001$). In contrast, a main effect of time for GDX males ($F_{(2,73)} = 38.30; P < 0.001$) reflects the finding that LH levels were high at 0 min and decreased over time (0 min > 30 min > 90 min, Ps < 0.001). Importantly, at 90 min post-stress, LH levels were higher in E than in PF males ($P < 0.05$).

c. GnRH mRNA in the rostral MPOA

The overall ANOVA indicated a significant surgery × time interaction ($F_{(2,92)} = 3.15; P < 0.05$), and a prenatal treatment × surgery interaction that approached significance ($F_{(2,92)} = 2.93; P < 0.06$) (Figure 7). Separate ANOVAs revealed that E, PF and C males showed differential patterns of response over time. Under intact conditions, E males showed no change in GnRH mRNA levels over time, and the trend toward an increase in GnRH mRNA in PF ($P < 0.07$) and C ($P < 0.10$) males after 30 min restraint stress did not reach significance. Following GDX, there were no significant changes in GnRH mRNA levels over time in any

group, but E males had significantly higher basal (0 min) GnRH mRNA levels than C males ($P < 0.05$).

Table 1 Maternal body weights (g) during gestation (days 1 and 21) and lactation (day 1); Gestation Length

	Gestation Day 1	**Gestation Day 21**	Lactation Day 1	Gestation Length
E	283.4 ± 3.6	373.7 ± 8.3*	305.4 ± 6.4	22.0 ± 0.09†
PF	279.8 ± 4.4	366.1 ± 7.3*	298.9 ± 6.7	21.59 ± 0.14
C	269.5 ± 2.8	411.5 ± 7.5	321.2 ± 4.1	21.64 ± 0.15

Values represent the mean ± SEM of 14-18 rats per group.

* $P < 0.001$ compared to C; † $P < 0.05$ compared to PF and C

Table 2 Body weights (g) of male and female offspring at birth (postnatal day 1, PN 1) and weaning (PN 22)

	Males		**Females**	
	PN 1	**PN 22**	**PN 1**	**PN 22**
E	5.81 ± 0.14*	50.79 ± 1.17	5.45 ± 0.12*	48.42 ± 0.96
PF	5.74 ± 0.13*	51.54 ± 0.95	5.41 ± 0.16*	48.25 ± 0.91
C	6.58 ± 0.11	53.89 ± 1.74	6.20 ± 0.11	50.33 ± 1.0

Values represent the mean ± SEM of the average male or female pup body weight per litter in 14-18 litters per group. * $P < 0.001$ compared to C.

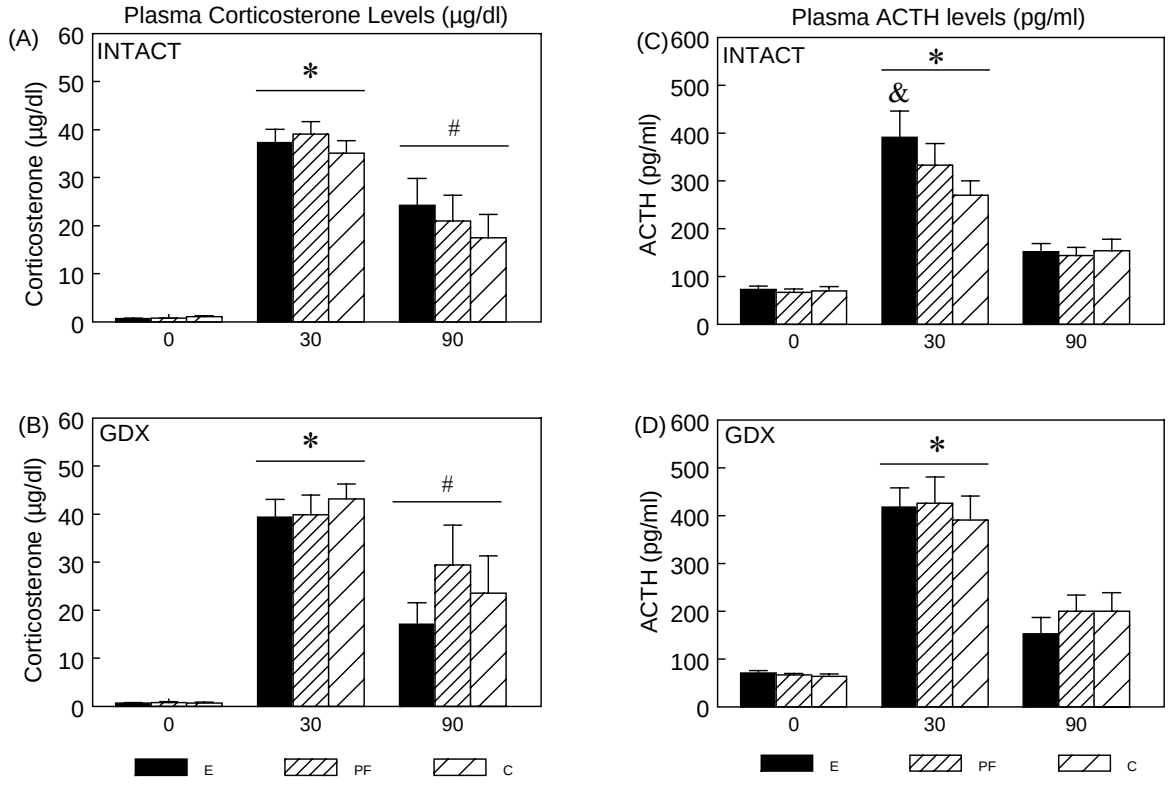


Figure 3 Plasma CORT (µg/dl) (Panels A, B) and ACTH (pg/ml) (Panels C, D) levels (mean ± SEM, n = 7 - 12 per group) in intact and GDX E, PF and C males at 0, 30 and 90 min after stress onset. Corticosterone: * 30 min > $^{\#}$ 90 min > 0 min, Ps < 0.001; ACTH: * 30 min > 0, 90 min, Ps < 0.001. In addition, under intact conditions, ACTH levels in E > C at 30 min ($^{\&}$ P < 0.01). Following GDX, the ACTH stress response was significantly increased in C (overall GDX > INT, P < 0.05), but not in PF (P < 0.08) or E (P = 0.77) rats compared to their intact counterparts.

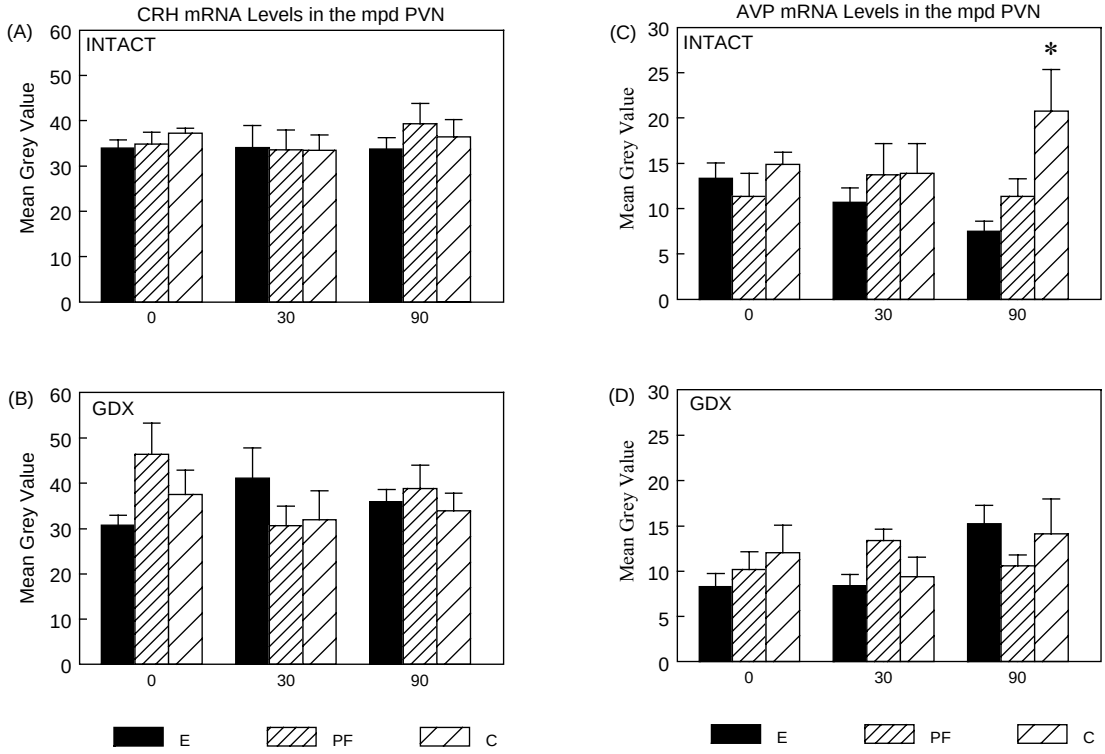


Figure 4 CRH (Panels A, B) and AVP (Panels C, D) mRNA levels (mean ± SEM, n = 5 - 6 per group) in the medial parvocellular dorsal (mpd) PVN (outlined in circle) in intact and GDX E, PF and C males at 0, 30 and 90 min after stress onset. For CRH mRNA: no significant differences among groups. For AVP mRNA: Under intact conditions, at 90 min, AVP mRNA lower in intact E and PF compared to intact C rats (* E = PF < C, P < 0.01). GDX eliminated the differences seen among intact males.

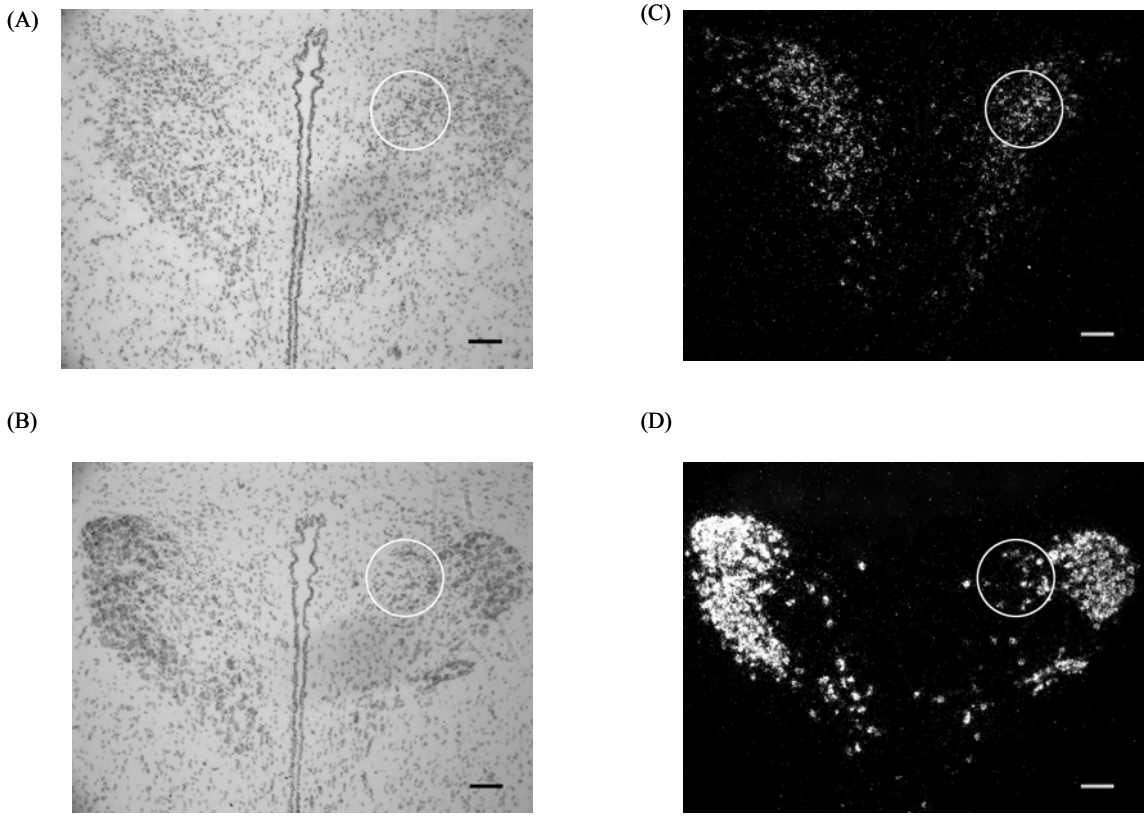


Figure 5 Representative bright-field and dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating CRH (Panels A, B) and AVP (Panels C, D) mRNA patterns in the PVN. Scale bar = 0.1 mm

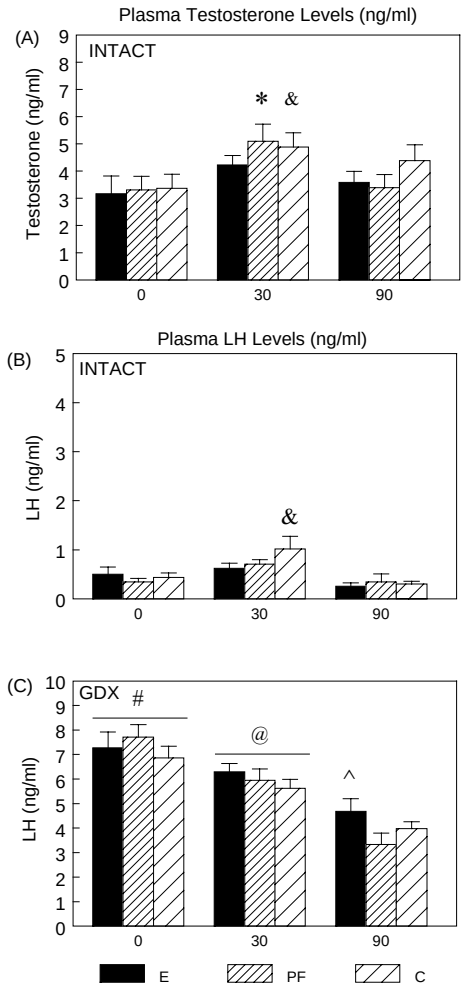


Figure 6 Plasma testosterone (ng/ml) in intact males (Panel A), and LH (ng/ml) in intact (Panel B) and GDX (Panel C) E, PF and C males (mean ± SEM, n = 7 - 12 per group). Under intact conditions, PF and C showed a significant testosterone response to 30 min restraint (* PF: 30 min > 0, 90 min, Ps < 0.05; $^{\&}$ C: 30 min > 0 min, P < 0.05); no significant change in testosterone in E males over time. Similarly, LH levels increased at 30 min in intact C rats only ($^{\&}$ P < 0.005). Following GDX: LH levels significantly elevated over those of intact rats (P < 0.001) and decreased over time ($^{\#}$ 0 min > $^{@}$ 30 min > 90 min, Ps < 0.001). LH levels higher in E than in PF males ($^{\wedge}$ P < 0.05) at 90 min post-stress.

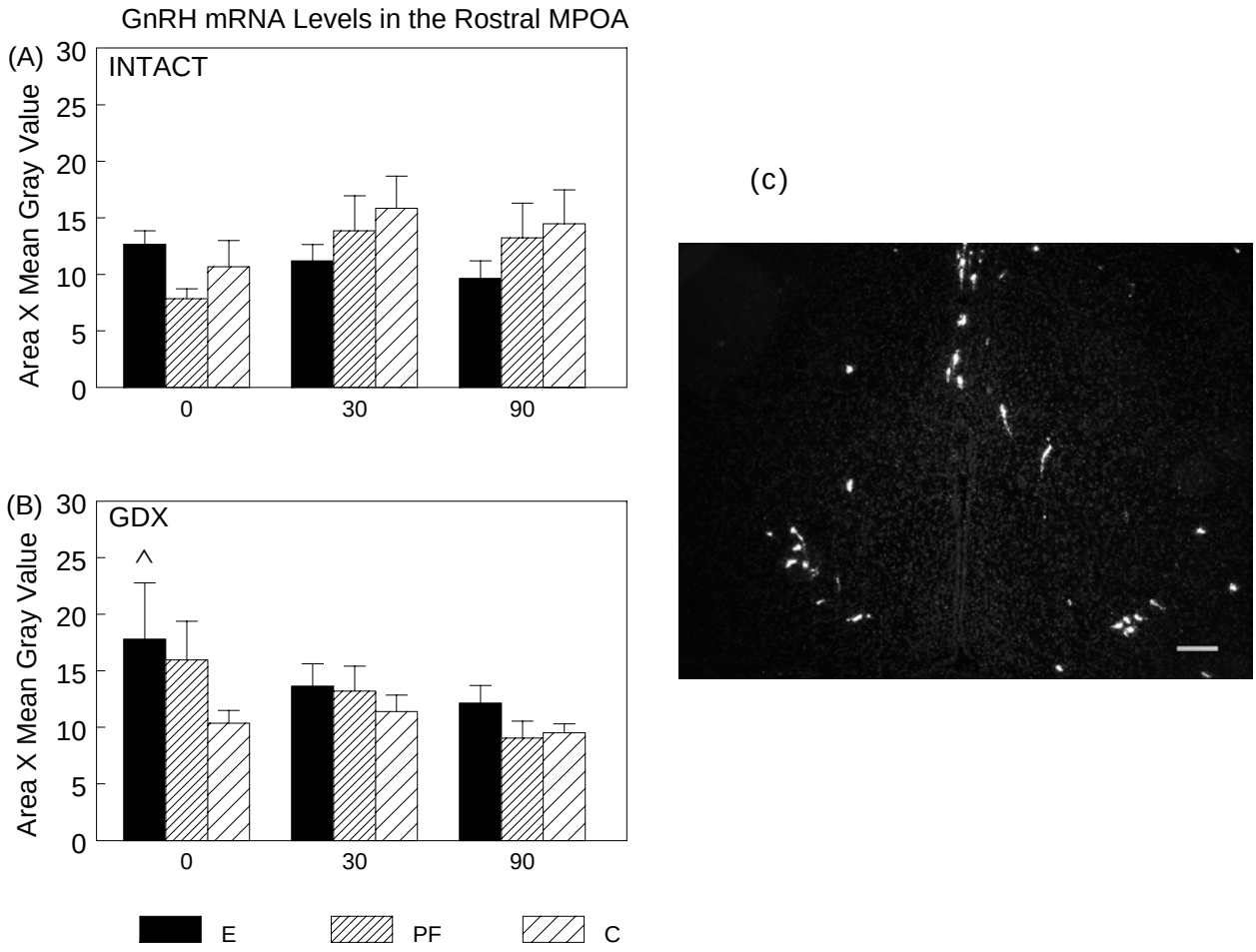


Figure 7 GnRH mRNA levels (Mean ± SEM, n = 5 - 6 per group) in the rostral MPOA in intact (Panel A) and GDX (Panel B) E, PF and C males and representative dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating GnRH mRNA pattern in the rostral MPOA (panel C). Under intact conditions: no change in GnRH mRNA levels in E ($P = 0.66$), PF ($P < 0.08$) or C ($P < 0.10$) males over time. Following GDX: basal GnRH mRNA levels in E > C males (^ $P < 0.05$); no change in GnRH mRNA levels in any group over time. Scale bar = 0.2 mm

D. Discussion

Study 1 tested the hypothesis that prenatal ethanol-induced changes in gonadal hormone activity could play a role in differentially altering the HPA response to stress in adult E compared to control males. Consistent with previous studies from Weinberg laboratory (Weinberg 1985; Weinberg, Kim et al. 1995) and others (Abel 1978; Taylor, Branch et al. 1981; Tritt, Tio et al. 1993), the results demonstrate the adverse effects of prenatal ethanol exposure on pregnancy outcome, including maternal body weights during gestation and lactation, gestation length and pup body weights. Importantly, our data also indicate significant effects of ethanol on both HPA and HPG activity, and suggest that the normal testicular influences on HPA function are significantly reduced in E males. These data support and extend previous work in the Weinberg laboratory (Weinberg 1988; Weinberg 1992a; Kim, Giberson et al. 1999) and others (Taylor, Branch et al. 1983; Taylor, Branch et al. 1988; Lee and Rivier 1996) showing that E males and females show different patterns of HPA response to stressors compared to their control counterparts, suggesting a marked sexual dimorphism in fetal ethanol effects and a possible role for the gonadal steroids in mediating HPA hyperresponsiveness in E males and females.

Detailed discussion of Study 1.

1. Ethanol effects on pregnancy outcome

Prenatal ethanol exposure had significant adverse effects on pregnancy outcome. E dams had a longer gestation length than PF and C dams. In addition, E and PF pups had significantly lower weights than C pups at birth, and while PF pups showed catch up growth in the first week of life, E pups did not. Thus we found both specific effects of ethanol on developmental outcome, as well as some effects that are, at least in part, nutritionally mediated. Ethanol administration may directly affect nutrient intake and has the potential to adversely affect maternal nutritional health. 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 the liver and pancreas. By contrast, PF dams are fed a reduced ration, equivalent to that consumed by their E partners, such that their caloric intake is less than that of control dams. Thus, in addition to controlling for the reduced food intake of ethanol-consuming dams, pair-feeding is also a treatment in itself. Although the relative undernutrition of E dams could further exacerbate the effects of ethanol on the developing fetus, pair-feeding introduces some level of prenatal stress that itself may play a role in the outcomes observed in PF offspring.

2. Ethanol effects on HPA activity

Previous studies indicate that CORT and ACTH levels following restraint stress typically peak at approximately 15 - 30 min and 5 - 10 min, respectively, then decline towards/to basal levels during recovery (Viau and Meaney 1996; Lund, Munson et al. 2004). In the present study, we found that CORT levels increased at 30 min and decreased toward basal at 90 min in all animals. This is consistent with previous findings in Weinberg laboratory (Weinberg 1988; Weinberg 1992) and others (Taylor, Branch et al. 1986; Slone and Redei 2002), showing that although E males may show more prolonged CORT elevations than controls during prolonged (4 hr) restraint stress, significant differences in CORT levels among E, PF and C males are not always observed following acute stress. It is possible that the variability in CORT levels during the 1 hr recovery period in the present study masked the effect of prenatal treatment on CORT levels. By contrast, we found significantly higher ACTH levels in E than in C rats at 30 min, suggesting increased responsiveness to restraint stress in E males at the level of the pituitary.

With regard to central HPA regulation, we saw no significant differences in CRH mRNA levels across time in any group, a phenomenon that might be due, in part, to the abundance of CRH mRNA under basal conditions and/or the short time frame of the study. This finding is consistent with data from other studies showing that CRH mRNA fails to increase over basal levels 3 hr after acute ethanol administration (Rivier and Lee 1996),

following 60 min restraint stress (Ma, Lightman et al. 1999) or until 60 min post injection of metyrapone, a glucocorticoid synthesis blocker (Herman, Schafer et al. 1992).

Previous studies in the Weinberg laboratory (Gabriel, Glavas et al. 2005; Zhang, Sliwowska et al. 2005) and others (Redei, Halasz et al. 1993) have shown that basal CRH mRNA levels may be higher in E than in C males, although not all studies have shown such alterations in basal CRH mRNA (Kim, Giberson et al. 1999; Lee, Schmidt et al. 2000). Importantly, however, Lee et al. (Lee, Schmidt et al. 2000) found that PVN CRH hnRNA levels were significantly higher in E than in control offspring at 15 min following mild footshock stress, indicating that E rats exhibit increased neuronal activity of CRH perikarya in the PVN after stress. In the present study, under intact conditions, we found no differences among prenatal groups in CRH mRNA levels but lower AVP mRNA levels in E and PF compared to C rats at 90 min post-stress onset. Importantly, these differences were eliminated by GDX. AVP is a weak ACTH secretagogue on its own, but acts synergistically with CRH and plays an important role in sustaining pituitary responsiveness during chronic stress (Gillies, Linton et al. 1982; Aguilera 1994) or to a novel heterotypic stressor following repeated stress (Ma, Lightman et al. 1999). Moreover, it has been shown that the inhibitory effects of testosterone on stress-induced ACTH levels vary strongly and negatively with the resting-state of AVP but not CRH content in the median eminence (Viau and Meaney 1996). Thus CRH biosynthesis in the PVN is CORT-dependent, whereas AVP biosynthesis is testosterone-dependent (Viau 2002). Explored in greater detail below, data from the present

study indicate that intact C males have a greater propensity to utilize AVP than E and PF males, suggesting a reduced testicular influence on HPA function in E and PF males at the level of PVN.

3. Ethanol effects on HPG activity

In general, stress appears to have a biphasic effect on the HPG axis. Chronic stressors typically inhibit HPG function, whereas acute stressors elicit variable patterns of LH and testosterone release in male rats (Rivier and Rivest 1991). Acute psychological stressors [handling, novel environments, visual (flashing bright lights) or audiogenic stimulation (ringing bell)] consistently result in transient elevations of plasma testosterone and LH levels (Krulich, Hefco et al. 1974; Turpen, Johnson et al. 1976; Siegel, Weidenfeld et al. 1981; Briski and Sylvester 1987; Briski and Sylvester 1987). However, effects of acute stressors that have both a psychological and a physical component (cold, restraint, immobilization, swim, foot shock) are more variable. Increased (Ruisseau, Tache et al. 1978; Briski and Sylvester 1987), decreased (Rivier, Rivier et al. 1986) or no change (Charpenet, Tache et al. 1982; Mann and Orr 1990) in LH levels following acute stressors have been reported.

In the present study, we found that testosterone levels were significantly increased following stress in intact PF and C males, whereas E rats show blunted testosterone responses to restraint stress, indicating that testosterone regulation is altered in E compared to

control males under gonadal intact conditions. C males also showed a significant increase in LH levels following 30 min restraint, whereas E and PF males showed no significant change in LH levels over time. These results are consistent with previous findings that acute stress elicits a small and transient increase in both plasma LH and testosterone levels (Krulich, Hefco et al. 1974; Turpen, Johnson et al. 1976; Siegel, Weidenfeld et al. 1981; Briski and Sylvester 1987; Briski and Sylvester 1987) in normal male rats, and support the hypothesis that HPG regulation is altered in E rats. Testosterone synthesis and secretion are influenced by several mechanisms including the well-studied GnRH-LH pathway (Saez 1994), sympathetic and parasympathetic innervations of the male gonad, and CRH-like peptides, opiates, catecholamines, growth factors and cytokines that can regulate testicular steroidogenesis independently of gonadotropins (Saez 1994). Furthermore, it has recently been shown that there is an inhibitory neural hypothalamic-testicular pathway independent of the pituitary in male Sprague-Dawley rats (Lee, Miselis et al. 2002), and that ethanol can inhibit Leydig cell activity through this pathway (Selvage, Hales et al. 2004). It is not possible at present to determine which of these mechanisms may underlie the altered testosterone responsiveness in E animals.

Stress could affect GnRH regulation of LH secretion through a number of different pathways. Indirect pathways involve central neuronal changes that regulate GnRH secretion and/or release (Brann and Mahesh 1991), whereas direct pathways involve effects of circulating adrenal glucocorticoids on pituitary gonadotrophs or alterations in the

responsiveness of the gonadotrophs to GnRH (Briski and Sylvester 1991). We found that under both gonadal intact and GDX conditions, there were no statistically significant changes in GnRH mRNA levels over time in any prenatal group. It was not unexpected that the increases in GnRH mRNA levels were not significant within the short 30 min time frame of this study. While there are few if any reports on the time course of GnRH mRNA changes following acute stress, what previous data do show is that acute mild stress stimulates the HPG axis (Krulich, Hefco et al. 1974; Turpen, Johnson et al. 1976; Ruisseau, Tache et al. 1978; Siegel, Weidenfeld et al. 1981; Briski and Sylvester 1987; Briski and Sylvester 1987). Importantly, despite the absence of stress effects on GnRH mRNA levels, both PF and/or C males showed stress-induced increases in testosterone and LH levels, whereas stress failed to stimulate the HPG axis in E males at any level. Acute stress-associated increases in plasma levels of LH and testosterone would afford some protection of reproductive function (Handa, Burgess et al. 1994), which would obviously be less in E than in control males. Together, these data indicate that the regulation of the HPG axis is altered at several levels, and that androgen has differential effects on central regulation of HPG activity in E (and to some extent in PF) males compared to controls.

4. Ethanol effects on HPA-HPG interactions

The adrenal and gonadal axes develop in parallel and interact bidirectionally. Stress-induced HPA activation has been shown to modulate HPG activity and reproductive function.

For example, acute stressors typically cause a brief and transient activation of the axis (Krulich, Hefco et al. 1974; Turpen, Johnson et al. 1976; Siegel, Weidenfeld et al. 1981; Briski and Sylvester 1987; Briski and Sylvester 1987), while chronic stressors may delay puberty (Ramaley 1974; Almeida, Petenusci et al. 2000), and suppress LH and testosterone levels (Charpenet, Tache et al. 1981; Almeida, Anselmo-Franci et al. 1998). Conversely, the effects of gonadal hormones on HPA function have been demonstrated at all levels of the axis. For example, GDX of adult male rats increases both ACTH and CORT responses to physical and psychological stressors (Handa, Nunley et al. 1994; Viau and Meaney 1996; Seale, Wood et al. 2004). GDX males also show greater stress-induced Fos expression and higher AVP hnRNA levels in the PVN than intact males, both of which are negatively correlated with plasma testosterone levels (Viau, Lee et al. 2003). Furthermore, it has been reported that androgens inhibit hypothalamic CRH peptide and mRNA (Almeida, Hassan et al. 1992; Bingaman, Magnuson et al. 1994; Viau, Soriano et al. 2001), as well as restraint-induced increases in CRH and AVP hnRNA (Viau, Lee et al. 2003; Lund, Munson et al. 2004).

In the present study, gonadectomy allowed us to unmask differential testicular influences on HPA activity as well as differential responsiveness of central components of the HPG axis to androgens in E compared to control males. We found a selective GDX effect on the ACTH response to stress in C males. That is, GDX significantly increased the ACTH response to stress in C males such that the differences between intact E and C males were

eliminated. The CORT pattern of response was similar to that of ACTH, but differences among groups were not statistically significant. In addition, we found that the differences in AVP mRNA levels seen among intact E, PF and C males were eliminated by GDX. The finding that intact E and PF males appear to utilize AVP to a lesser degree than intact C males could be explained by a shift away from gonadal dependence in E and PF animals. These data support the suggestion that normal testicular influences on HPA responsiveness appear to be reduced in E and PF males.

In addition to effects on HPA regulation, GDX also significantly altered LH and GnRH responses in E males. Under gonadal intact conditions, only C males showed a LH increase to 30 min restraint stress. Following GDX, basal LH levels were dramatically increased in all animals and levels then decreased over time. However, LH levels decreased more slowly in E compared to PF and C males, and at 90 min post-stress, E males had higher LH levels than PF males. Furthermore, basal GnRH mRNA levels were significantly higher in E than C males following GDX. These data suggest a potential shift in how testosterone regulates GnRH transcription [and potentially post-translational processing (see (Kalra, Simpkins et al. 1984)] as well as stress-induced LH responses in E compared to control males. Testosterone exerts a permissive effect on stress-induced stimulation of LH synthesis in C males. In contrast, this permissive role of testosterone is absent or attenuated in E and/or PF males. Since there are no differences in basal testosterone levels among groups, it is possible that ARs might play a role in the altered HPA-HPG interaction seen in E males. Although

there are no reports of prenatal ethanol effects on ARs levels in the brain, Kaiser et al. (Kaiser, Kruijver et al. 2003; Kaiser, Kruijver et al. 2003) showed that prenatal stress upregulates both ARs and estrogen receptors (ERs) in female offspring, and downregulates ARs in male offspring. It is possible that prenatal ethanol may alter AR expression and thus induce altered HPA response to testosterone.

Together these findings suggest that regulation of both the HPA and HPG axes are altered by prenatal ethanol exposure, with normal testicular influences on HPA function markedly reduced in E animals. While some effects (plasma LH and AVP mRNA responses) may be, at least partially, nutrition-mediated, there are also major effects (e.g. plasma ACTH and testosterone, and GnRH mRNA responses) that are specific to ethanol. Our data suggest that a decreased sensitivity to the inhibitory effects of androgens could contribute to the HPA hyperresponsiveness typically observed in E males. Furthermore, our current findings provide a clear indication that gonadal status plays an important role in ethanol-induced changes in neuroendocrine function and are an excellent starting point for examining the effects of androgen replacement and/or receptor blockade on HPA activity and regulation in E males.

CHAPTER IV: EFFECTS OF TESTOSTERONE ON HYPOTHALAMIC-PITUITARY-ADRENAL ACTIVITY IN MALE RATS PRENATALLY EXPOSED TO ETHANOL[2]

A. Introduction

In our previous study, we found that intact E rats showed no differences in basal testosterone levels under intact conditions. However, they did show increased ACTH but blunted testosterone and LH responses to restraint stress, and no stress-induced elevation in AVP mRNA levels compared to those observed in PF and/or C rats. GDX significantly increased ACTH responses to stress in C but not E and PF males, eliminated differences among groups in plasma ACTH and AVP mRNA levels, and altered LH and GnRH responses in E males. These findings indicated that central regulation of both the HPA and HPG axes is altered by prenatal ethanol exposure, with normal testicular inhibitory influences on HPA function markedly reduced in E males. The present study was undertaken to determine if the differential HPA responsiveness in E and control males depends on testosterone, in particular, a decreased capacity of testosterone to regulate HPA activity. Therefore, we explored the dose-related effects of testosterone on HPA regulation and responsiveness. We extended our previous work in several important ways. First, we examined the CRH and AVP mRNA expression in PVN-projecting neurons, including the BNST and amygdala. This is important because mapping studies have demonstrated that ARs are not localized in mpd PVN which

[2] These data have been submitted to *Psychoneuroendocrinology*: Lan, N., Hellemans, K.G.C., Ellis, L., Viau, V., and Weinberg, J., Role of Testosterone in Mediating Prenatal Ethanol Effects on Hypothalamic-Pituitary-Adrenal Activity in Male Rats.

directly regulates HPA activity (Simerly, Chang et al. 1990; Zhou, Blaustein et al. 1994; Shughrue, Lane et al. 1997; Bingham, Williamson et al. 2006), suggesting that androgens act upstream from the PVN to regulate HPA output. The BNST and amygdala contain high densities of ARs, indicating that the CRH and AVP circuits within these areas are androgen sensitive such that the expression of these secretogugues is subject to changes in circulating testosterone levels. Second, we measured AR mRNA expression in the brain areas that regulate HPA function. Third, we measured hippocampal mRNA levels of MR and GR to assess prenatal ethanol effect on CORT feedback regulation as previous studies suggest that both HPA drive and feedback are altered by prenatal ethanol exposure (Lee, Schmidt et al. 2000; Zhang, Sliwowska et al. 2005; Sliwowska, Zhang et al. 2006; Glavas, Ellis et al. 2007). Our experimental questions were: 1) Do E males differ from controls in adrenal and gonadal hormone levels under intact conditions, following GDX or following GDX with low or high testosterone replacement? 2) Do differences between E and control animals with different circulating testosterone levels occur under both basal and stress conditions? 3) Do central measures of HPA and HPG activity under different circulating testosterone levels differ in E rats compared to controls? and 4) Are activities of the central testosterone-sensitive pathways that regulate CRH and AVP neurosecretory neurons altered in E males compared to controls?

Adult E, PF and C males were subjected to 1 of 4 treatments: 1) Sham GDX (INT); 2) GDX; 3) GDX and subcutaneous implantation of silastic capsules packed with crystalline testosterone designed to provide circulating testosterone concentration approximating low (~

1 ng/ml) (GDX-L), or 4) high (4 ~ 6 ng/ml) (GDX-H) physiological levels. Animals were tested before or immediately after a 30 min restraint stress. Trunk blood samples and brains were collected. We measured serum levels of testosterone, LH and CORT. Brains were sectioned from the nucleus of the vertical limb of the diagonal band (Bregma 0.48 mm) to the medial nucleus of amygdala (Bregma - 3.36 mm) at 30 μm thickness. We measured basal mRNA levels of CRH, AVP, and AR in the BNST, PVN and amygdala to assess the biosynthesis of these peptides in areas related to HPA function, as well as potential changes in response to androgen in these brain areas. In addition, we measured hippocampal mRNA levels of MR and GR to assess CORT feedback regulation.

By comparing HPA responsiveness in intact males, in males where testes are absent, and in the presence of low vs. high basal testosterone levels, we investigated if the differential HPA activity in E and control males depends on the levels of testosterone or alterations in the capacity of testosterone to regulate HPA activity. We tested the hypothesis that the differential alterations in HPA activity observed in E males compared to their control counterparts are mediated, at least in part, by ethanol-induced changes in HPA sensitivity to testosterone.

B. Methods

1. Breeding and feeding

Male (275 - 300 g, n = 18) and female (230 - 275 g, n = 46) Sprague-Dawley rats were used (16 E dams, 15 PF dams and 15 C dams) for this breeding. The breeding and feeding procedures were described in Chapter II. Only male offspring were used for this study (total N = 166, consisting of n = 6 per prenatal group and surgical treatment under basal conditions and n = 8 per prenatal group and surgical treatment under stress conditons).

2. Gonadectomy and testosterone replacement

Surgery procedures were described in Chapter II. There are four surgical treatment conditions in this study: Sham GDX (Intact), GDX, GDX with low or high testosterone replacement.

3. Testing

Testing occurred 14 d after surgery. Rats were decapitated at 0 and 30 min after the onset of a 30 min restraint stress. Trunk blood was collected into ice-chilled polystyrene tubes and was allowed to coagulate overnight at 4˚C and then centrifuged. For the animals subjected to 30 min stress, 200 µl blood samples were collected from the tail right after the

rats went into the restraint tubes. Tail blood samples were drawn within 1 min of touching the animal's cage to remove it for testing. These tail blood samples allow us to measure pre-stress testosterone levels. Brains were rapidly removed and immediately frozen on dry ice, then stored at - 80 ˚C until sectioning.

4. Radioimmunoassays (RIA)

Blood samples were centrifuged at 3200 rpm for 10 min at 0 ˚C. Serum was transferred into 600 µl Eppendorf tubes and stored at - 80 ˚C until assayed.

RIA for CORT, testosterone, and LH was described in Chapter II.

5. In situ hybridization

a. Brain preparation

Brains were sectioned into five one-in-five series of 30 µm coronal sections from the nucleus of the vertical limb of the diagonal band (Bregma 0.48 mm) to the medial nucleus of amygdala (Bregma - 3.36 mm) (Paxinos and Watson 2005). Frozen sections were thaw-mounted onto precleaned colorfrost/plus microscope slides (Fisher Scientific, AB, CA) and stored at - 80 ˚C.

b. Oligonucleotide probes and labeling

Oligonucleotide probes for CRH and AVP mRNA and labeling protocol were described in Chapter II.

c. Ribonucleotide probes and labeling

Ribonucleotide probes for MR, GR and AR mRNA and labeling protocol were described in Chapter II.

d. Hybridization with oligonucleotides

Hybridization procedures were described in Chapter II. Probe activities are: CRH: 1.03×10^5 cpm/section; AVP: 1.17×10^5 cpm/section. Sections were dipped in Kodak NTB2 autoradiography emulsion (Eastman Kodak Co.) diluted 1:1 (Deionized H_2O) and exposed for 20 d for CRH in the PVN, 101 d for CRH in the CeA, 94 d for CRH in the anterior division of the BNST (aBNST); 24 hr for AVP in the PVN, 16 d for AVP in the MeA and the posterior division of the BNST (pBNST) in desiccated, light tight boxes at 4 °C. Slides were developed with Kodak D-19 developer at 14 °C and fixed with Kodak Polymax T fixer at 14 °C. Coverslips were mounted with Permount (Fisher Scientific Ltd., ON, Canada).

e. Hybridization with ribonucleotides

Hybridization procedures were described in Chapter II.

f. Densitometric analysis

In situ signals were visualized with 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 1.33v software (National Institutes of Health, Bethesda, MD). The mean OD of hybridization signal, corrected by background subtraction, was taken at 150 μm intervals under dark-field illumination. Semiquantitative densitometric analysis for CRH and AVP in the mpd PVN was described in Chapter III. Semiquantitative densitometric analysis of CRH mRNA in the aBNST and CeA, AVP mRNA in the pBNST and the MeA, AR mRNA in the MPN, the principal nucleus of pBNST and the MeA was assisted by redirected sampling of dark-field autoradiographic images aligned to corresponding Nissl stained sections. CRH mRNA expression within the aBNST were measured within two cell groups, the oval and fusiform BNST nuclei, at the decussation of the anterior commissure (Bregma -0.12 mm ~ - 0.48 mm) (Paxinos and Watson 2005). The oval nucleus of the aBNST was traced by outlining a fixed rectangle (Width = 0.45; Height = 0.87; scale 300 pixels/inch). The fusiform nucleus of the aBNST was traced by free-hand

draw surround the CRH mRNA signal. The CeA was traced by outlining a fixed oval (Width = 0.45; Height = 0.71; scale 300 pixels/inch). The MeA was traced by outlining a fixed triangle (length: 1.65, 0.60, and 1.36; angle: 280.0, 48.5, 120.0; scale 300 pixels/inch for the left side. length: 1.45, 0.55, and 1.60; angle: 232.5, 316.0, 72.6; scale 300 pixels/inch for the right side). The MPN was traced by outlining a fixed square (0.33 X 0.33, scale 300 pixels/inch). The principal nucleus of pBNST was traced by free-hand draw surround the AR mRNA signal. Background signal was measured over a region immediately lateral to each side of the interested area. The corrected grey levels from both sides of two to three sections of each region were averaged to obtain a mean corrected grey level of the four to six measurements for each animal.

The majority of AVP mRNA signals were found in the transverse and intrafascicular nuclei of the pBNST (Bregma – 0.6 mm to – 0.84 mm) (Paxinos and Watson 2005). Because AVP mRNA is widely distributed in the pBNST, signal was measured by outlining neurons containing labeled AVP mRNA and using Image J to analyze the pixel area and the mean grey value of each signal cluster. Total grey value from both sides of the two sections of the pBNST was calculated by pixel area × mean grey value of each cluster and then summing the measured grey values for each brain.

In the dorsal hippocampus, a representative section (Bregma –3.00 mm) (Paxinos and Watson 2005), in which the two arms of the dentate gyrus (DG) were equal in length, was chosen for analyzing. Measurements were taken from the subfields CA1, CA2, CA3 and DG

from 2 sections for each animal. Background signal was measured from the molecular layer of each side of the hippocampus. The corrected grey levels from both sides of each subfield were averaged to obtain a mean corrected grey level of the 4 measurements for each animal.

6. Statistical analysis

Developmental and pup body weight data were analyzed using repeated measures ANOVA and Newman-Keuls post hoc tests. The hormone data were analyzed using 3-way between-factors ANOVA with the factors of prenatal group (E, PF, C), surgical treatment (INT, GDX, GDX-L, and GDX-H) and time (0 and 30 min). For serum LH levels, 2-way ANOVAs within each surgical treatment group were performed. As adult body weight, organ weight, and brain mRNA levels of neuropeptides and receptors were measured under basal conditions (0 min) only, these data were analyzed using two-way between-factors ANOVA (prenatal group and surgical treatment). Significant main effects and interactions were further analyzed using Fisher's LSD for factors with only 3 levels, and pairwise comparisons with a Šidák correction for factors with > 3 levels. To test the a priori hypothesis that testosterone mediates the effect of prenatal ethanol exposure on HPA and HPG responsiveness, planned comparisons using a Šidák correction were carried out for all comparisons (Cardinal and Aitken 2006).

Statistical significance was set at $P < 0.05$. P values between 0.05 and 0.10 were

considered a statistical trend.

C. Results

For all Figures in this section, Figure Legends are included on the same page of the Figure if possible or on the page following the Figure if it cannot fit on the same page.

1. Developmental Data

a. Ethanol intake and blood alchol levels

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 13.43 ± 0.36, 16.94 ± 0.41, 16.43 ± 0.45 g ethanol/kg BW for weeks 1, 2 and 3 of gestation, respectively, and resulting in blood ethanol levels of 166.98 ± 4.715 mg/dl measured 2 hr after lights off.

b. Maternal body weights during gestation and lactation

Analysis of maternal body weights during gestation indicated significant main effects of prenatal group ($F_{(2,41)} = 20.30$; $P < 0.01$) and day ($F_{(3,123)} = 1732.09$; $P < 0.001$), and a group × day interaction ($F_{(6,123)} = 36.34$; $P < 0.001$) (Table 3). E and PF dams did not differ from C dams on G1 but weighed significantly less than C dams ($P < 0.001$) by G 21. For lactation (L) body weights, main effects of prenatal group ($F_{(2,37)} = 3.88$; $P < 0.05$) and day ($F_{(3,111)} = 117.36$; $P < 0.001$), and a group × day interaction ($F_{(6,111)} = 2.83$; $P < 0.05$), reflected

the finding that E and PF dams weighed less than C dams on L 1 (E: $P < 0.05$; PF: $P < 0.06$), but did not differ from C dams on L 22, suggesting catch up weight gain in E and PF dams.

c. Gestational length

One-way ANOVA revealed a significant effect of group for gestation length (Table 3) ($F_{(2,40)} = 2.4$; $P < 0.01$). E dams had a longer gestation (by approximately 6 hr) than PF dams ($P < 0.05$). There were no significant differences among prenatal groups for litter size or number of live born or stillborn pups.

d. Postnatal body weights of female and male pups

Significant prenatal group effects for body weights in both male ($F_{(2,38)} = 4.59$; $P < 0.05$) and female ($F_{(2,38)} = 3.66$; $P < 0.05$) offspring indicated that E and PF pups weighed less than their C counterparts at birth (Ps < 0.01) (Table 4). All pups gained weight from PN 1 to PN 22 (males, ($F_{(3,114)} = 2978.41$; $P < 0.001$; females, $F_{(3,114)} = 3437.38$; $P < 0.001$), with catch up growth in PF but not E animals on PN 22 (E < C, Ps < 0.05).

2. Adult body and organ weight

a. Body weight before surgery and testing

Analysis of adult body weights prior to surgery indicated a significant main effect of prenatal group ($F_{(2,154)} = 4.0$; $P < 0.05$); E and PF had lower weights than C males. Analysis of weight gain over the 2 wks between surgery and testing (Figure 8 A) indicated significant effects of surgical treatment ($F_{(3,154)} = 12.839$; $P < 0.001$). Overall, INT males gained the most weight and GDX and GDX-H males gained the least weight (INT > GDX = GDX-H, Ps < 0.05; INT > GDX-L, $P < 0.07$; GDX-L > GDX, $P < 0.05$). Furthermore, low testosterone replacement restored body weights in PF and C, but not in E males (GDX-L < INT in E, $P < 0.05$).

b. Thymus weight

A significant effect of surgical treatment ($F_{(3,154)} = 78.5$; $P < 0.001$) (Figure 8 B) indicated that GDX rats had the highest, and GDX-H rats had the lowest thymus weight corrected for body weight (thymus wt/BW ratio) among the 4 surgical treatments (GDX > all, GDX-H < all, Ps < 0.001). In addition, planned comparisons revealed that GDX E showed a lower thymus wt/BW ratio than GDX C males ($P < 0.05$). Low testosterone replacement was sufficient to restore thymus wt/BW ratio to that of intact animals among all prenatal groups.

c. Adrenal weight

Analysis of adrenal weight corrected for body weight (adrenal wt/BW ratio) indicated a significant effect of surgical treatment ($F_{(3,149)} = 4.05; P < 0.01$) and a trend for a group X treatment interaction ($F_{(6,149)} = 2.087; P < 0.06$) (Figure 8 C). Adrenal/BW ratios were higher in GDX C males compared to C males in all other surgical treatments, whereas surgical treatment did not differentially affect adrenal weights in E and PF males (C males: GDX > INT = GDX-H, Ps < 0.01; GDX > GDX-L, P < 0.09; GDX-L > GDX-H, P = 0.05).

3. HPA data

a. Serum CORT levels

As expected, a main effect of time ($F_{(1,126)} = 1985.33; P < 0.001$) (Figure 9) indicated that CORT levels increased significantly over basal (0 min) levels at 30 min (P < 0.001). There was trend for a main effect of surgical treatment ($F_{(3,126)} = 2.452; P < 0.07$) and a significant treatment X time interaction ($F_{(3, 126)} = 2.621$, P = 0.05). Prenatal treatment had no effect on basal hormone levels, but differentially altered the stress-induced CORT response among surgical treatment conditions. For E males, CORT levels were similar in intact and GDX conditions, whereas GDX increased CORT levels for PF and C males (PF males: GDX > INT, P < 0.06; C males: GDX > INT = GDX-L = GDX-H, Ps < 0.01). In addition, low and high testosterone replacement restored CORT levels for PF and C males, whereas high

testosterone levels were needed to restore CORT levels for E males. Indeed, low testosterone replacement was actually somewhat suppressive to CORT in E males (E males: INT > GDX-L, P = 0.05; GDX > GDX-L = GDX-H, Ps < 0.05).

b. Basal CRH mRNA levels in the mpd PVN, aBNST and CeA

Two-way ANOVA (group X surgical treatment) indicated a significant effect of surgical treatment ($F_{(3,58)} = 3.009$; $P < 0.05$) on basal CRH mRNA in the mpd PVN. Overall, GDX increased basal CRH mRNA levels in the PVN whereas testosterone replacement restored CRH mRNA to levels in intact animals (GDX > INT, $P < 0.05$) (Figure 10 A). Furthermore, planned comparisons revealed that basal CRH mRNA levels changed across surgical treatment in PF and C only (PF: GDX > INT, $P < 0.09$; GDX > GDX-H, $P < 0.05$; C: GDX > INT, $P < 0.05$), whereas E animals did not differ in CRH mRNA levels across surgical conditions.

Analyses indicated no significant main effects or interactions on basal CRH mRNA levels in the aBNST or MeA. Planned comparion did not reveal any differences among prenatal groups or surgical treatment in the oval nucleus of the aBNST (data not shown). However, INT E had marginally lower CRH mRNA levels compared to INT C in the fusiform nucleus of the aBNST ($P < 0.06$) (Figure 10 B). Furthermore, planned comparison

indicated that following high dose testosterone replacement, E males had significantly higher CRH mRNA in the CeA than C males ($P < 0.05$) (Figure 10 C).

c. Basal AVP mRNA levels in the mpd PVN, aBNST and CeA

There were no significant effects of prenatal ethanol or surgical treatment on basal AVP mRNA levels in the PVN (Figure 11 A) or the MeA (Figure 11 C). However, two-way ANOVA indicated an effect of surgical treatment ($F_{(2,40)} = 3.194$; $P = 0.05$) on AVP mRNA levels in the pBNST (Figure 11 B). Planned comparisons indicated that E and PF but not C males had increased AVP mRNA levels in the pBNST following high testosterone replacement (E males: GDX-H > INT, $P = 0.05$; GDX-H > GDX-L, $P < 0.05$; PF males: GDX-H > INT, $P < 0.05$). INT E males also had marginally higher AVP mRNA in the MeA than INT C males ($P < 0.06$) (Figure 11 C). The AVP mRNA signals in the pBNST and the MeA were undetectable in GDX animals.

d. Basal MR and GR mRNA levels in the hippocampus

An effect of surgical treatment for MR mRNA levels (Ps < 0.05) (Figure 12) in the CA1, CA2 and CA3 subfields reflected the fact that GDX animals had higher MR mRNA levels compared to GDX-L and GDX-H animals (Ps < 0.05). Thus testosterone replacement

normalized MR mRNA levels to those of intact animals. There were no differences in MR mRNA among the three prenatal treatment groups.

By contrast, analysis of GR mRNA levels indicated main effects of group ($F_{(2,53)}$ = 3.756; $P < 0.05$) and surgical treatment ($F_{(3,53)}$ = 6.35; $P < 0.001$) in the CA1 subfield (Figure 13). GDX animals had higher GR mRNA levels compared to INT and GDX-H animals (Ps < 0.01). In addition, E and PF males had higher GR mRNA levels overall in the CA1 subfield compared to C males (E > C, $P < 0.08$; PF > C, $P < 0.05$), and under intact conditions, E males had higher GR mRNA levels than C males in the CA3 ($P < 0.08$) and the DG ($P < 0.05$). There were no effects of prenatal or surgical treatment on GR mRNA levels in the CA2.

4. HPG Data

a. Serum testosterone levels

As expected, serum testosterone levels in GDX rats were in the undetectable range. Analysis of testosterone levels in other groups indicated a main effect of surgical treatment ($F_{(2,103)}$ = 78.072; $P < 0.001$) (Figure 14). As expected, overall, GDX-H > INT > GDX-L, Ps < 0.001. There were no differences in testosterone levels across time.

b. Serum LH levels

Main effects of surgical treatment ($F_{(3,135)}$ = 345.697; P < 0.001) and time ($F_{(1,139)}$ = 3.290; P < 0.07), indicated that, not surprisingly, GDX animals had higher LH levels compared to animals in the other 3 surgical treatment groups (Ps < 0.001) (Figure 15). Separate ANOVAs within each surgical treatment revealed that LH levels did not change following stress in GDX or GDX-H animals. By contrast, INT males in all prenatal groups showed a significant stress-induced increase in LH levels (P < 0.05) [main effect of time ($F_{(1,35)}$ = 29.116; P < 0.001)], whereas in the GDX-L condition, effects of prenatal group ($F_{(2,35)}$ = 3.613; P < 0.05) and time ($F_{(1,35)}$ = 4.064; P = 0.05) indicated that only E males showed an LH increase following 30 min stress, and thus at 30 min, E had higher LH levels than PF and C males (Ps < 0.05).

c. Basal AR mRNA levels in the MPN, pBNST, MeA and hippocampus

Analyses revealed similar patterns of AR mRNA expression in the MPN and the principal nucleus of pBNST. Although there were no significant main effects of prenatal group or surgical treatment, planned comparisions revealed that E and PF males showed no changes in AR mRNA levels in either the MPN (Figure 16A) or the the principal nucleus of pBNST (Figure 16 B) across surgical conditions, whereas C males had higher AR mRNA levels in the INT condition compared to the other surgical conditions (MPN: INT > all, Ps <

0.05; pBNST: INT > GDX, $P < 0.05$; INT > GDX-H, $P < 0.06$). Furthermore, under intact conditions, E and PF animals had lower AR mRNA expression in both the MPN and the principal nucleus of pBNST than C males ($Ps < 0.05$).

There are no main effects of prenatal group or surgical treatment on the AR mRNA levels in the MeA (Figure 16 C). Planned comparisons indicated a trend for GDX to decrease AR mRNA levels relative to intact rats in E but not PF and C animals (GDX E < INT E, $P < 0.08$), whereas PF and C males did not differ in AR mRNA levels across surgical treatments. Furthermore, following GDX, E had lower AR mRNA levels than GDX PF males ($P < 0.05$).

There were no effects of prenatal treatment or surgical condition on AR mRNA levels in any hippocampal subfield (Figure 17).

d. Correlations between pre-stress testosterone and post-stress CORT levels

In intact animals, negative correlations between pre-stress testosterone and post-stress CORT levels were found in C ($r^2 = 0.6982$, $P < 0.05$) but not E and PF males (Figure 18).

Table 3 Maternal body weights (g) during gestation (days 1 and 21) and lactation (day 1); Gestation Length

	Gestation Day 1	**Gestation Day 21**	Lactation Day 1	Gestation Length
E	262.6 ± 2.2	355.6 ± 3.9*	285.5 ± 4.5^	22.0 ± 0.00†
PF	262.1 ± 1.8	357.1 ± 4.5*	291.9 ± 3.9^	21.73 ± 0.12
C	258.8 ± 2.8	405.2 ± 5.4	312.5 ± 5.8	21.83 ± 0.11

Values represent the mean ± SEM of 12-16 rats per group.

* $P < 0.001$ compared to C; ^ $P < 0.01$ compared to C; † $P < 0.05$ compared to PF

Table 4 Body weights (g) of male and female offspring at birth (postnatal day 1, PN 1) and weaning (PN 22)

	Males		**Females**	
	PN 1	**PN 22**	**PN 1**	**PN 22**
E	$6.11 \pm 0.14^{*}$	$46.74 \pm 1.00^{*}$	$5.76 \pm 0.12^{*}$	$46.52 \pm 0.86^{\#}$
PF	$6.02 \pm 0.16^{*}$	$49.13 \pm 1.61^{\wedge}$	$5.41 \pm 0.16^{*}$	48.54 ± 1.58
C	6.69 ± 0.15	53.89 ± 1.74	6.32 ± 0.14	51.03 ± 1.19

Values represent the mean ± SEM of the average male or female pup body weight per litter in 12-16 litters per group. * $P < 0.01$ compared to C; $^{\wedge}$ $P < 0.06$ compared to C; $^{\#}$ $P < 0.05$ compared to C.

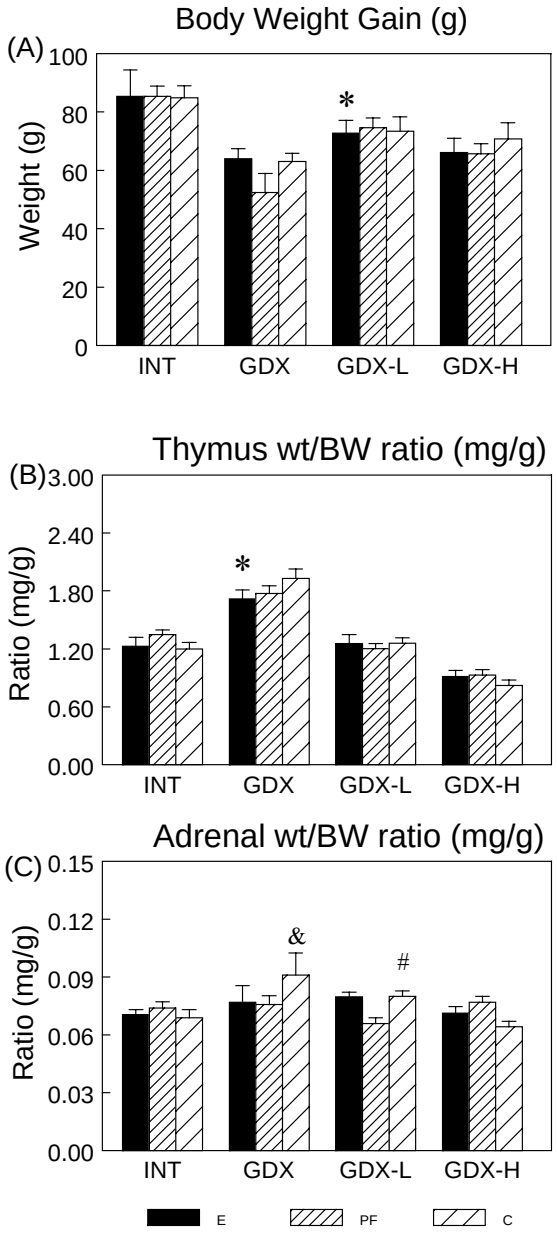


Figure 8 Body weight (BW) gain (g) (Panel A) and organ wt/BW ratio (mg/g) (Panels B, C) (mean ± SEM, n = 6 - 8 per group) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment groups. BW gain: overall INT > GDX = GDX-H, Ps < 0.05; GDX-L > GDX, P < 0.05; * GDX-L < INT in E males only, P < 0.05. Thymus wt/BW ratio: * E < C, P < 0.05 in GDX rats. Adrenal wt/BW ratio: $^{\&}$ GDX > INT = GDX-H, Ps < 0.005; $^{\#}$ GDX-L > GDX-H, P = 0.05 in C males.

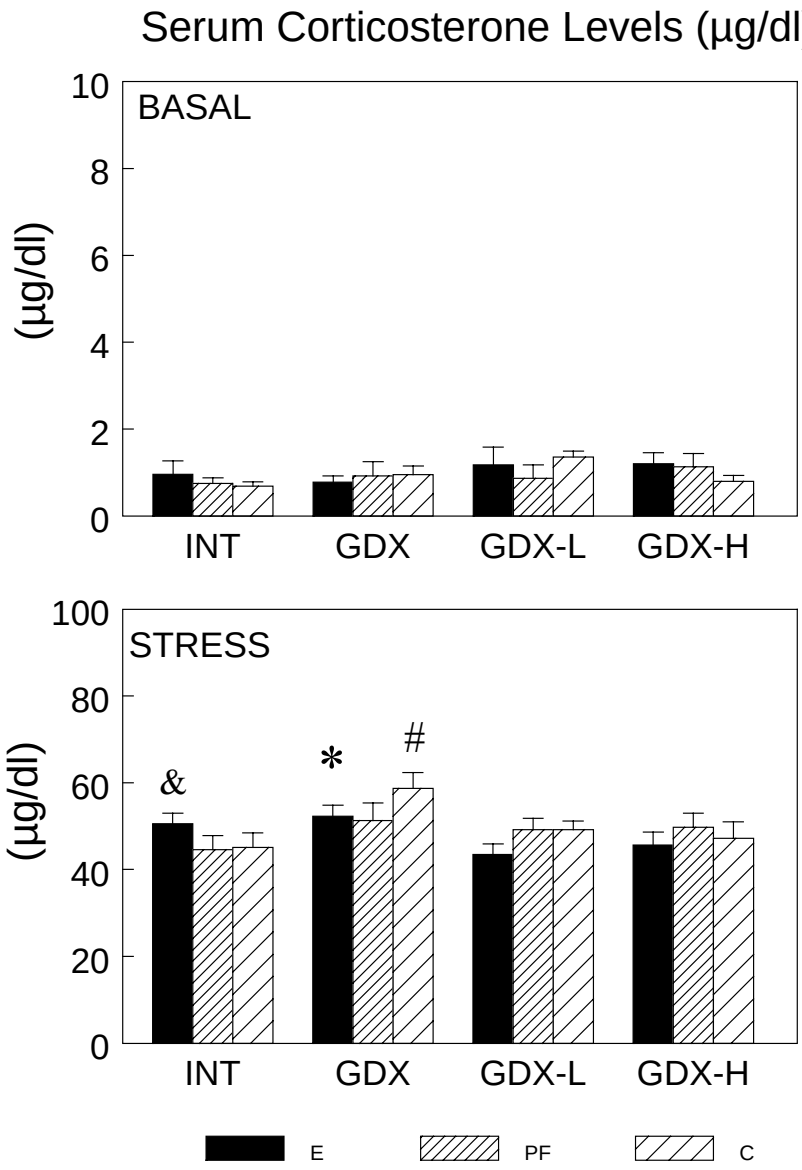
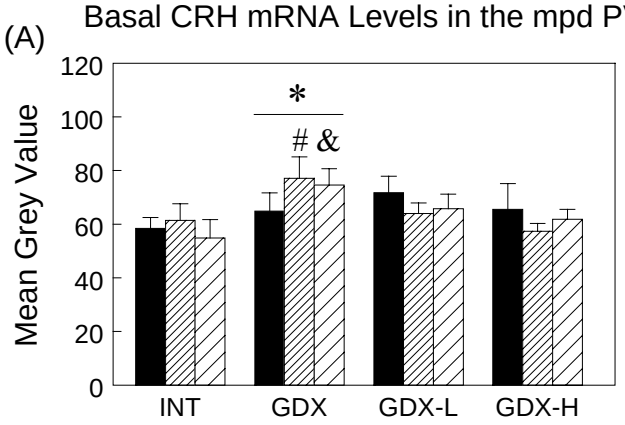
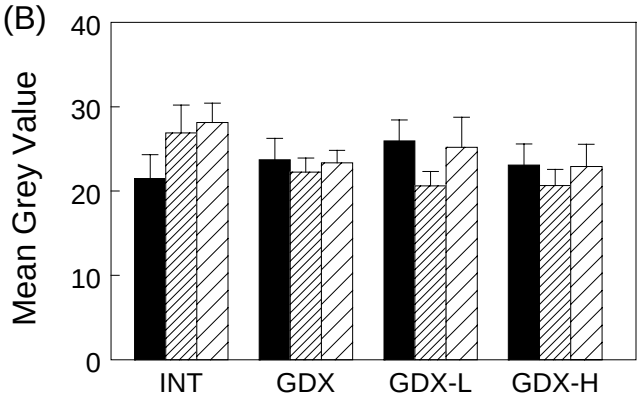


Figure 9 Serum CORT (µg/dl) levels (mean ± SEM, n = 7 - 12 per group) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment groups at 0 and 30 min after stress onset. Overall, 30 min > 0 min, Ps < 0.001; E males: * GDX > GDX-L = GDX-H, Ps < 0.05; & INT > GDX-L, P = 0.05; C males: # GDX > all, Ps < 0.01; PF males: GDX > INT, P < 0.06.

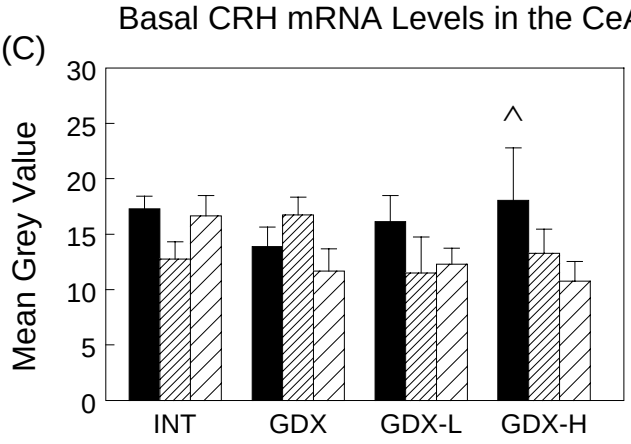
(A)


Basal CRH mRNA Levels in the mpd PVN
Mean Grey Value
120
100
80
60
40
20
0
*
&
INT
GDX
GDX-L
GDX-H

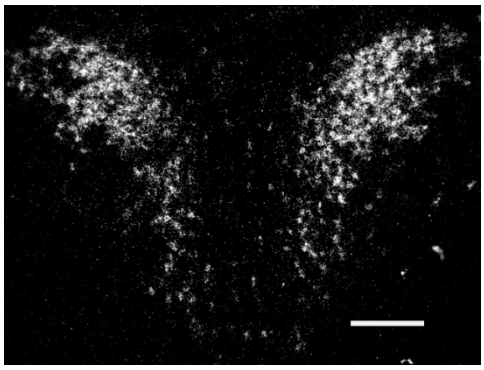
(B)


Basal CRH mRNA Levels in the aBNST fu
Mean Grey Value
40
30
20
10
0
INT
GDX
GDX-L
GDX-H

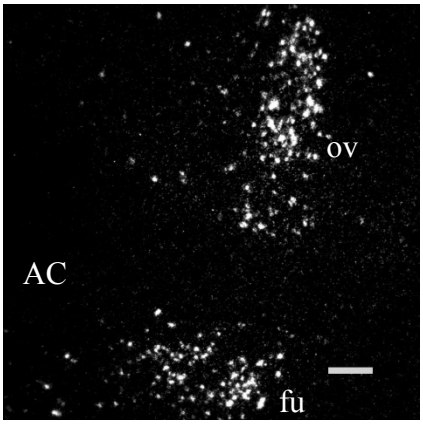
(C)


Basal CRH mRNA Levels in the CeA
Mean Grey Value
30
25
20
15
10
5
0
^
INT
GDX
GDX-L
GDX-H

(D)



(E)


ov
AC
fu

(F)

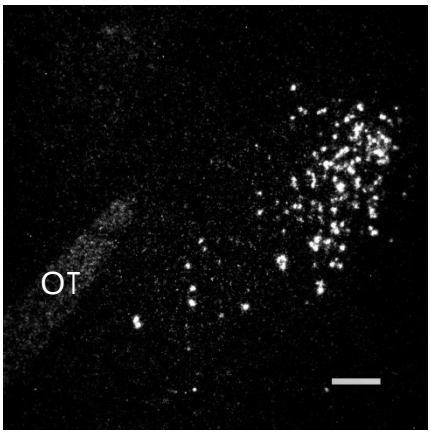
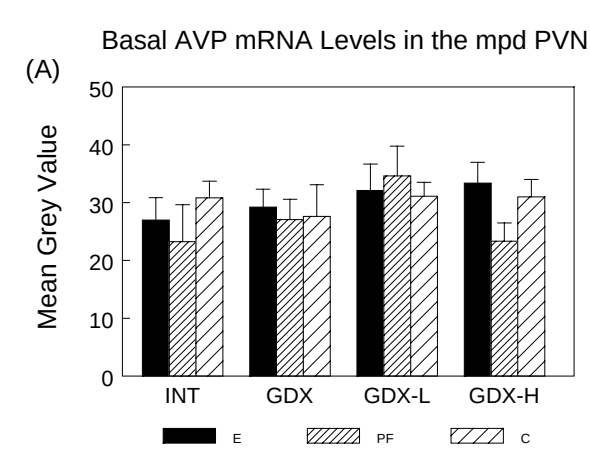

OT

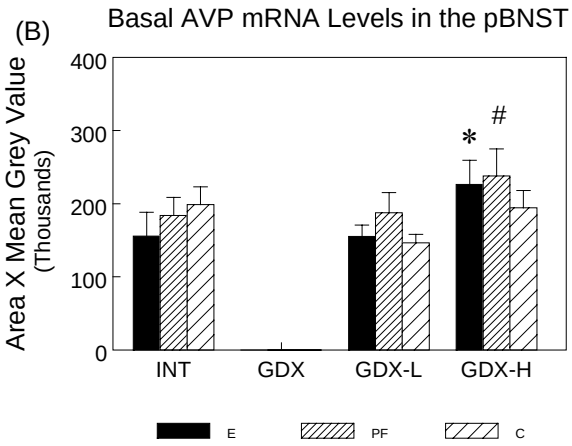
Figure 10 Basal CRH mRNA levels (mean ± SEM, n = 5 - 6 per group) in the mpd PVN (Panel A), fusiform nucleus of the aBNST (aBNST fu) (Panel B) and CeA (Panel C) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group and representative dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating CRH mRNA patterns in the mpd PVN (Panel D), oval and fusiform nucleus of the aBNST (Panel E) and CeA (Panel F). Scale bar = 0.2 mm. AC: anterior commisure; ov: oval nucleus of the aBNST; fu: fusiform nucleus of the aBNST; OT: optic tract.

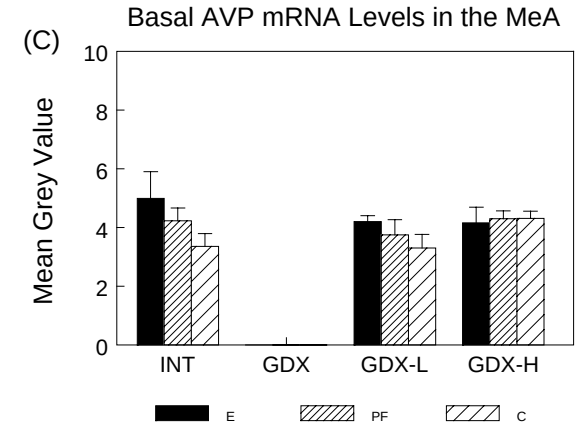
mpd PVN: Overall * GDX > INT, $P < 0.05$; PF males: $^{\#}$ GDX > GDX-H, $P < 0.05$; GDX > INT, $P < 0.09$; C males: $^{\&}$: GDX > INT, $P < 0.05$.

aBNST fu: E < C in intact males, $P < 0.06$.

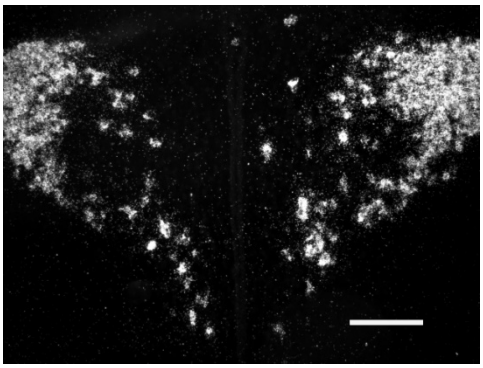
CeA: $^{\wedge}$ E > C in GDX-H males, $P < 0.05$.


Basal AVP mRNA Levels in the mpd PVN
(A)
Mean Grey Value
50
40
30
20
10
0
INT
GDX
GDX-L
GDX-H
E
PF
C

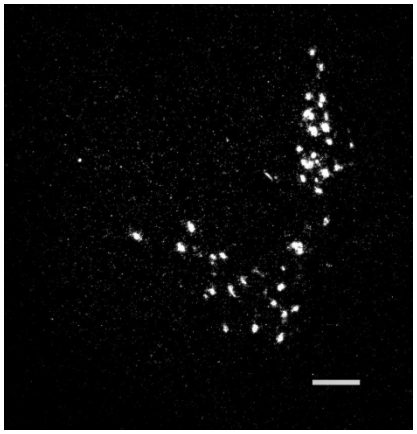

Basal AVP mRNA Levels in the pBNST
(B)
Area X Mean Grey Value
(Thousands)
400
300
200
100
0
*
#
INT
GDX
GDX-L
GDX-H
E
PF
C


Basal AVP mRNA Levels in the MeA
(C)
Mean Grey Value
10
8
6
4
2
0
INT
GDX
GDX-L
GDX-H
E
PF
C

(D)



(E)



(F)

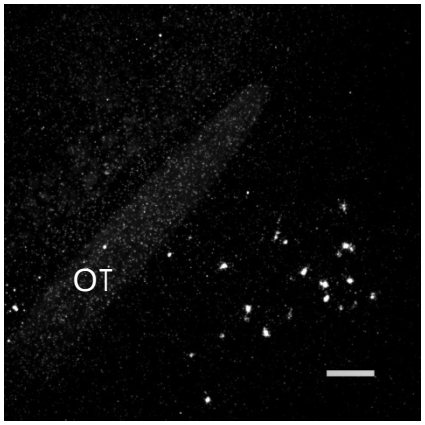

OT

Figure 11 Basal AVP mRNA levels (mean ± SEM, n = 5 - 6 per group) in the mpd PVN (Panel A), pBNST (Panel B) and MeA (Panel C) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group and representative dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating AVP mRNA patterns in the mpd PVN (Panel D), pBNST (Panel E) and MeA (Panel F). Scale bar = 0.2 mm. OT: optic tract.

mpd PVN: There were no significant effects of prenatal ethanol or surgical treatment on AVP mRNA levels in the mpd PVN.

pBNST: E males: * GDX-H > GDX-L, $P < 0.05$. PF males: $^{\#}$ GDX-H > INT, $P < 0.05$. The AVP mRNA signals in the pBNST were undetectable in GDX animals.

MeA: E > C in INT males, $P < 0.06$. The AVP mRNA signals in the pBNST were undetectable in GDX animals.

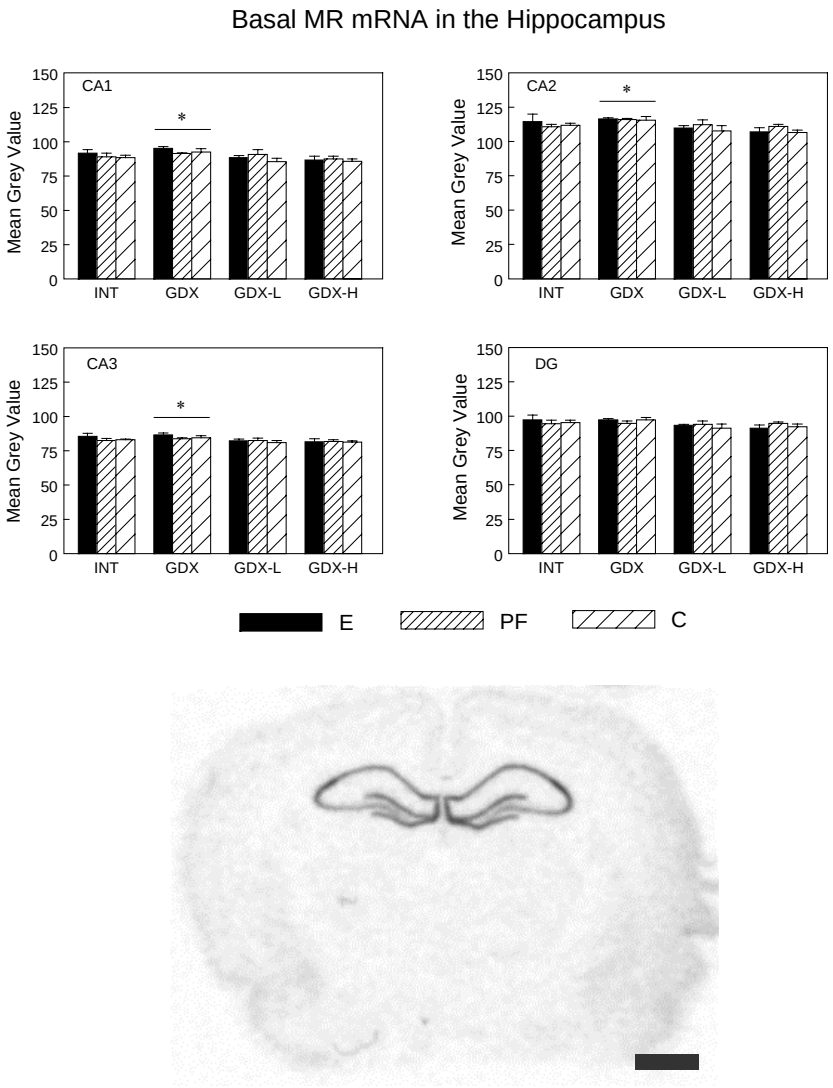


Figure 12 Basal MR mRNA levels (mean ± SEM, n = 5 - 6 per group) in the CA1, CA2, CA3 and DG subregions of the dorsal hippocampus in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group (upper panel) and representative autoradiography of sections labeled for MR mRNA in the dorsal hippocampus (lower panel). Scale bar = 2 mm. *GDX > GDX-L = GDX-H, Ps < 0.05. There were no differences in MR mRNA among the three prenatal treatment groups.

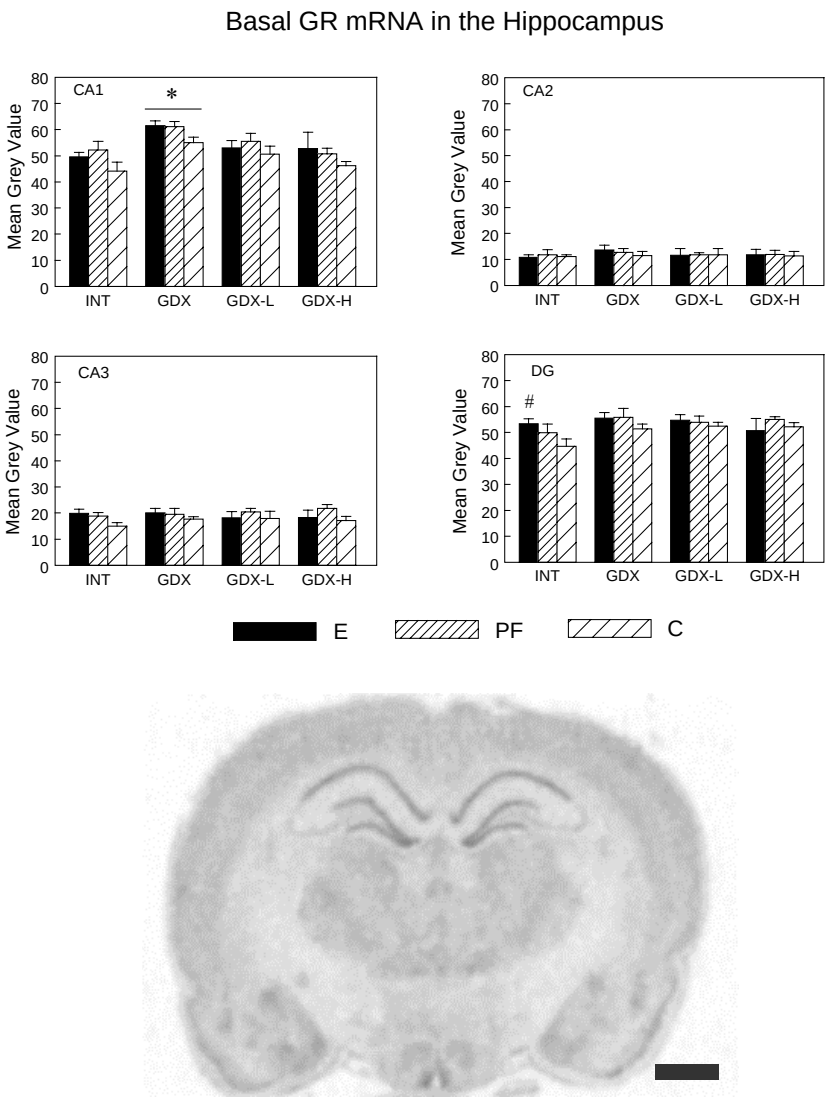


Figure 13 Basal GR mRNA levels (mean ± SEM, n = 5 - 6 per group) in the CA1, CA2, CA3 and DG subregions of the dorsal hippocampus in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group (upper panel) and representative autoradiography of sections labeled for GR mRNA in the dorsal hippocampus (lower panel). Scale bar = 2 mm. CA1: Overall, E > C, $P < 0.08$; PF > C, $P < 0.05$; * GDX > INT = GDX-H, Ps < 0.01; E > C in INT males in DG ($^{\#}P < 0.05$) and in the CA3 ($P < 0.08$).

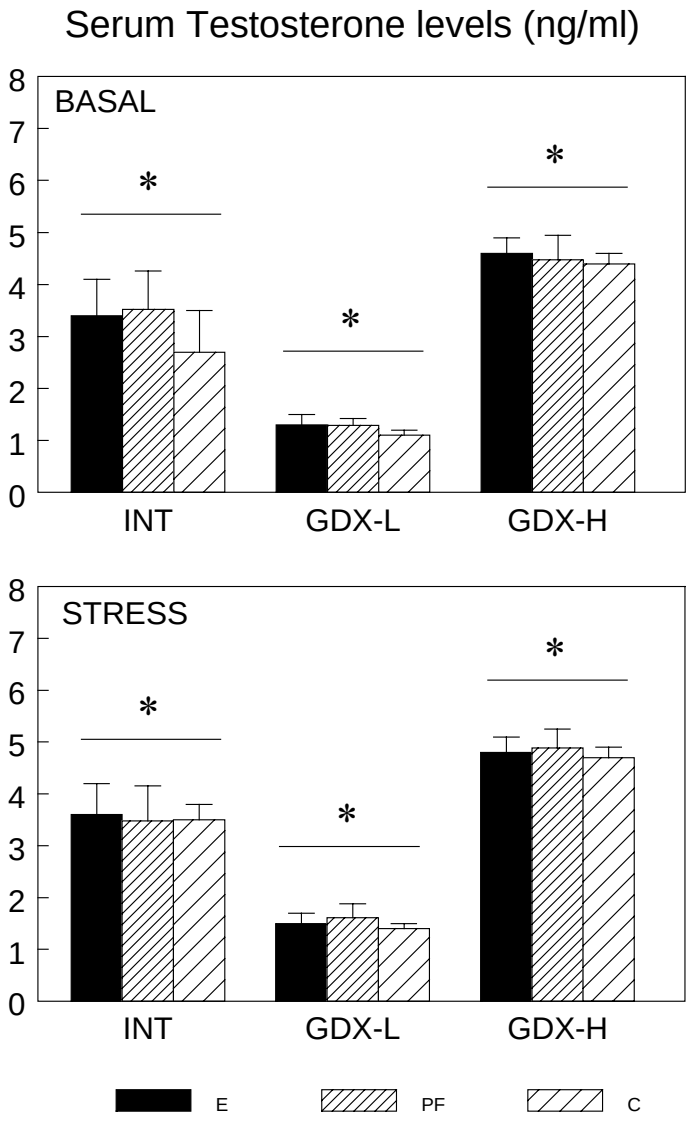


Figure 14 Serum testosterone (ng/ml) levels (mean ± SEM, n = 7 - 12 per group) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment groups at 0 and 30 min after stress onset.

* GDX-H > INT > GDX-L, Ps < 0.001.

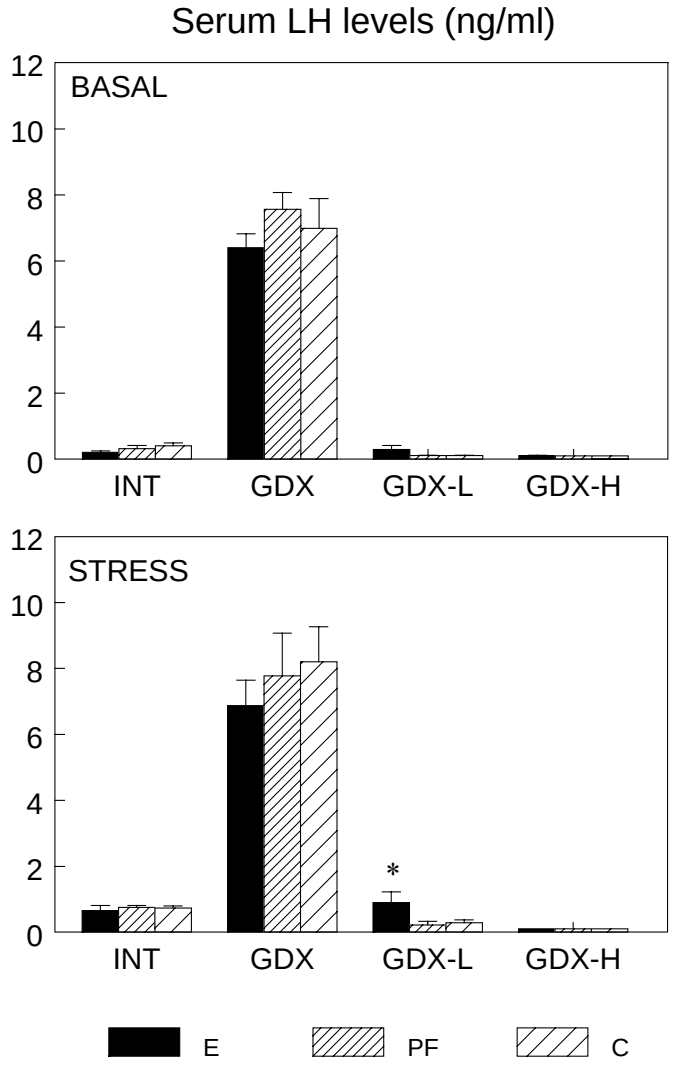
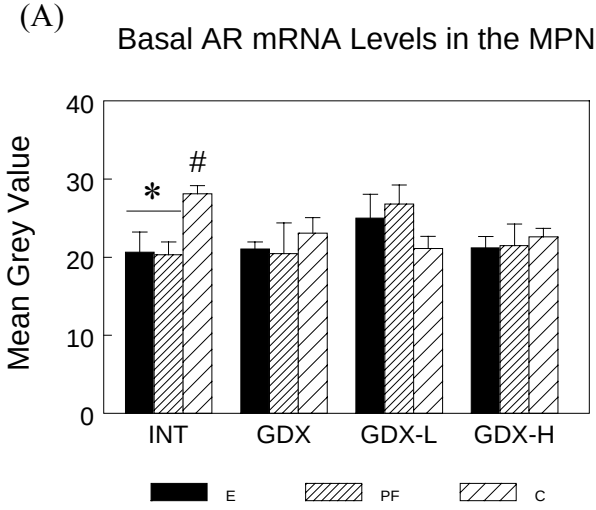
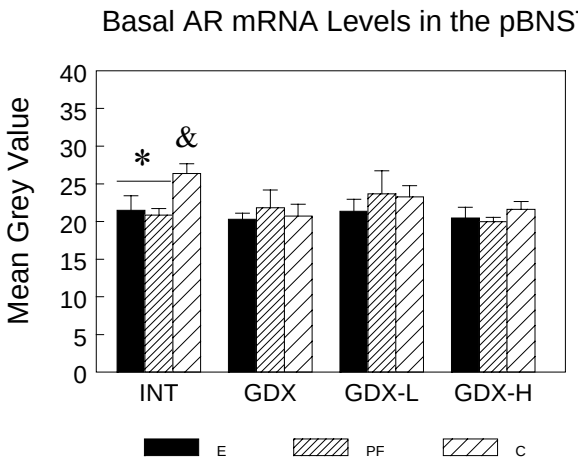


Figure 15 Serum LH (ng/ml) levels (mean ± SEM, n = 7 - 12 per group) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment groups at 0 and 30 min after stress onset. * E > PF = C in GDX-L animals at 30 min post-stress, Ps < 0.05

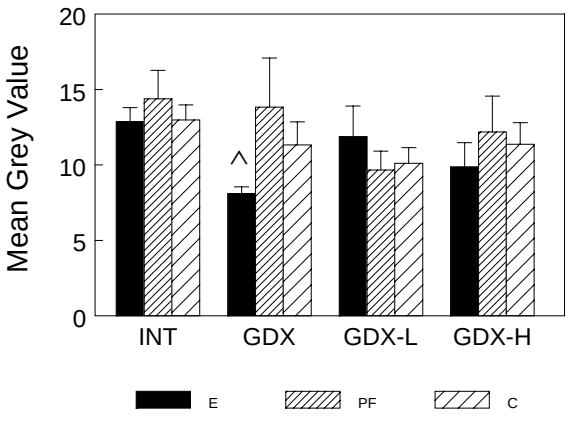
(A)


Basal AR mRNA Levels in the MPN
Mean Grey Value
40
30
20
10
0
*
#
INT
GDX
GDX-L
GDX-H
E
PF
C

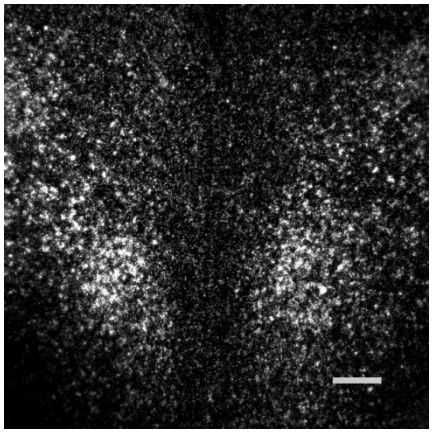
(B)


Basal AR mRNA Levels in the pBNST
Mean Grey Value
40
35
30
25
20
15
10
5
0
*
&
INT
GDX
GDX-L
GDX-H
E
PF
C

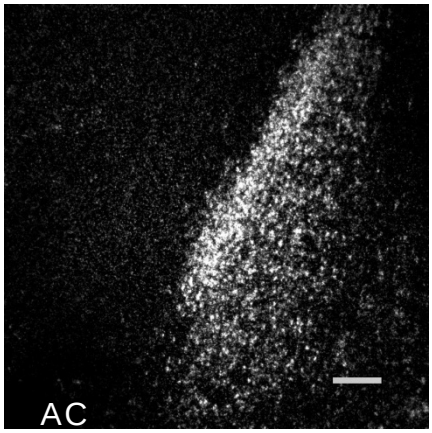
(C)


Basal AR mRNA Levels in the MeA
Mean Grey Value
20
15
10
5
0
^
INT
GDX
GDX-L
GDX-H
E
PF
C

(D)



(E)


AC

(F)

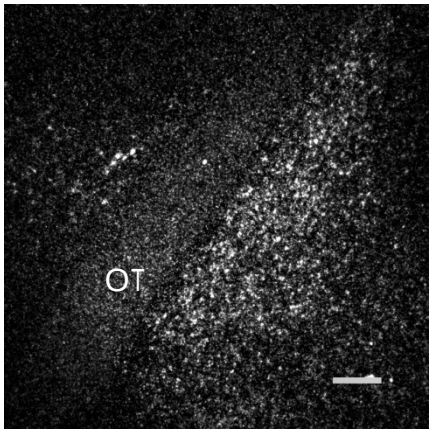

OT

Figure 16 Basal AR mRNA levels (mean ± SEM, n = 5 - 6 per group) in the MPN (Panel A), principal nucleus of pBNST (Panel B) and MeA (Panel C) in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group and representative dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating AR mRNA patterns in the MPN (Panel D), principal nucleus of pBNST (Panel E) and MeA (Panel F). Scale bar = 0.2 mm. AC: anterior commissure; OT: optic tract.

MPN: * E = PF < C in INT males, Ps < 0.05; # C: INT > all, Ps < 0.05.

pBNST: * E = PF < C in INT males, Ps < 0.05; & C: INT > GDX, P < 0.05;.

MeA: E: INT > GDX, P < 0.09; whereas PF and C males did not differ in AR mRNA levels across surgical treatment. ^ E < PF in GDX males, P < 0.05.

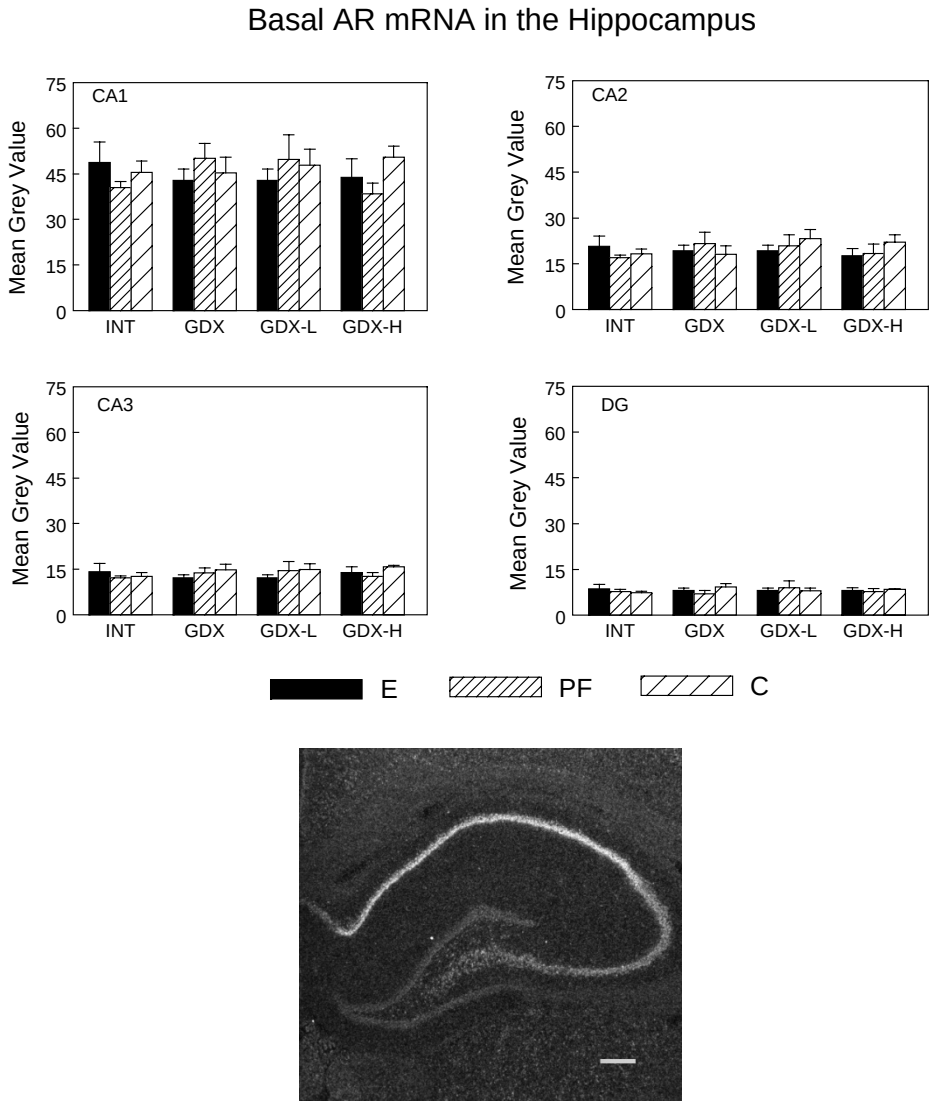


Figure 17 Basal AR mRNA levels (mean ± SEM, n = 5 - 6 per group) in the CA1, CA2, CA3 and DG subregions of the dorsal hippocampus in E, PF and C males from intact, GDX, GDX-L and GDX-H treatment group (upper panel) and representative dark-field photomicrographs of nuclear emulsion-dipped sections demonstrating AR mRNA patterns in the dorsal hippocampus (lower panel). Scale bar = 0.4 mm, There were no significant effects of prenatal ethanol or surgical treatment on basal AR mRNA levels in the dorsal hippocampus.

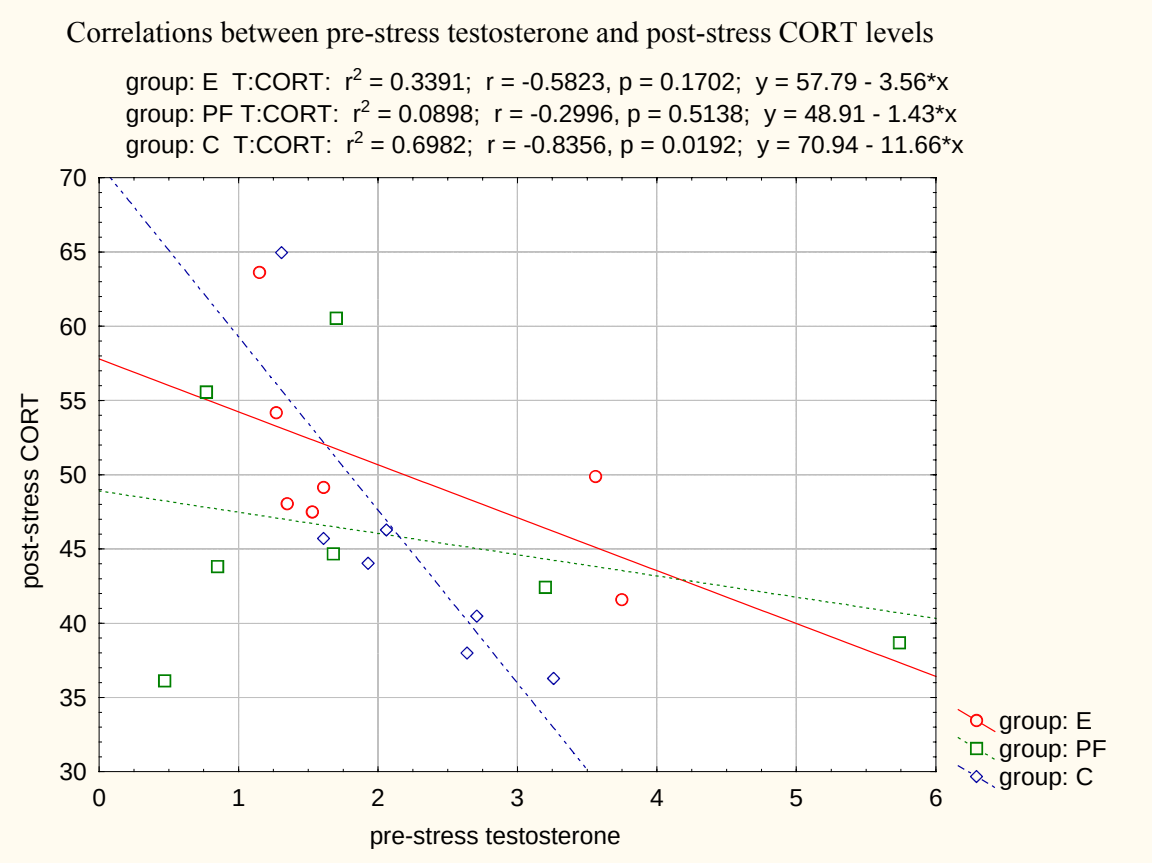


Figure 18 Correlations between pre-stress testosterone and post-stress CORT levels (mean ± SEM, n = 6 - 7 per group) in E, PF and C males from intact animals. Negative correlations between pre-stress testosterone and post-stress CORT levels were found in C ($P < 0.05$) but not E and PF males.

D. Discussion

In the present study we tested the hypothesis that the differential alterations in HPA activity observed in E males compared to their control counterparts are mediated, at least in part, by ethanol-induced changes in HPA sensitivity to testosterone. To this end, we explored dose-related effects of testosterone on HPA regulation and responsiveness. Our findings support and extend those of our previous study in providing strong evidence that regulation of both the HPA and HPG axes is altered by prenatal ethanol exposure, and that E males show altered sensitivity to the inhibitory effects of testosterone. Our previous data suggested that HPA sensitivity to androgens is reduced in E compared to control males (Lan, Yamashita et al. 2006). However, examination of central CRH/AVP expression profiles in the present study demonstrates that the story is more complex than that. Indeed, testosterone appears to have a reduced effect on central CRH pathways, but an increased effect on central AVP pathways in E compared to PF and/or C animals.

HPA activity in E males showed a number of alterations that relate specifically to testosterone status. Low dose testosterone replacement restored body weight in PF and C but not E males. Sensitivity of both the thymus and adrenal to circulating testosterone was reduced in E rats. A negative correlation between pre-stress testosterone and post-stress CORT levels in C but not in E and PF males further demonstrates the reduced adrenal sensitivity to testosterone in E and PF males. Furthermore, prenatal ethanol altered the responsiveness of central CRH and AVP pathways to testosterone. Basal CRH mRNA levels

in the mpd PVN were increased by GDX in PF and C but not E males. INT E also had lower CRH mRNA levels in the fusiform nucleus of the aBNST than INT C males, but higher CRH mRNA levels under high testosterone replacement conditions in the CeA. Together, these findings suggest reduced capacity of testosterone to regulate the central CRH pathway in E males. In contrast, testosterone appeared to have greater effects on central AVP pathway in E and/or PF compared to C males. Both E and PF males showed higher AVP mRNA levels in the pBNST following high testosterone replacement compared to either intact or low testosterone replacement conditions, respectively, and E males had higher AVP mRNA levels under intact conditions in the MeA compared to C males. Furthermore, while testosterone had an inhibitory role in both MR and GR mRNA levels in the hippocampus across all prenatal groups, prenatal ethanol exposure appeared to differentially regulate basal hippocampal GR expression, with E males showing GR upregulation compared to PF and/or C males.

HPG regulation was also altered by prenatal ethanol exposure. Testosterone had less of an inhibitory effect on stress-induced LH increases in E than in PF and C males. In addition, AR mRNA levels in the MPN and the principal nucleus of pBNST were lower in E and PF compared to C males under intact conditions. If decreased AR mRNA levels in key brain areas reflects downregulation of AR levels, this may counteract the increased inhibitory AVP signals upstream from the paraventricular nucleus, and thus contribute to the HPA hyperresponsiveness observed in E males.

Together, these data suggest that altered HPA activity in E males represents a complex balance between reduced effects of testosterone on central CRH pathways and enhanced effects of testosterone on central AVP pathways, with some effects possibly mediated by the nutrional effects of ethanol exposure.

Detailed discussion of Study 2.

1. Developmental effects of ethanol

Overall, the developmental data from this study were consistent with previous studies in our laboratory [i.e. (Weinberg 1988; Weinberg 1989; Lan, Yamashita et al. 2006)]. Briefly, E and PF dams did not differ from C dams on G1 but weighed significantly less than C dams by G21 and on L1. In addition, E and PF pups had significantly lower weights than C pups at birth, and while PF pups showed catch up growth on PN22, E pups did not.

2. Ethanol effects on body weight, organ weight, and peripheral hormone levels

We found that overall, INT males gained the most weight and GDX rats gained the least weight, consistent with previous data from Weinberg laboratory (Lan, Yamashita et al. 2006) and others (Wade 1976) showing that GDX decreased food intake and weight gain. Importantly, low dose testosterone replacement was sufficient to restore body weights in PF and C but not E males, and high dose testosterone replacement was not better than GDX-L,

and if anything was slightly worse in restoring body weights. This was not surprising in view of previous studies showing that the decrease in weight gain and food intake can be reversed by testosterone treatment, but that effects of testosterone are dose-dependent. For example, low concentrations of testosterone restored metabolism in GDX rats to normal, whereas higher doses of testosterone decreased weight gain and food intake (Gentry and Wade 1976). In many tissues, testosterone can be metabolized to estradiol by aromatase and to 5α-DHT by 5α-reductase. The effect of low dose testosterone on weight gain is due to its anabolic action on lean body mass, whereas high dose testosterone causes fat loss, mediated by aromatization to estradiol (Dallman, Viau et al. 2002). In the present study, weight gain in the GDX-H group was slightly but not significantly lower than that in the GDX-L group. This might be due to the fact that our high dose of testosterone was within the physiological basal range. Importantly, however, the finding that low dose testosterone replacement did not restore body weight in E males supports our hypothesis that E males had decreased sensitivity to testosterone.

All males had increased thymus wt/BW ratios following GDX, consistent with previous reports (Viau, Chu et al. 1999; Oner and Ozan 2002; Tanriverdi, Silveira et al. 2003). Androgens exert considerable effects on the size and composition of the thymus. Removal of androgens by castration results in thymic enlargement even in old rats and androgen replacement reversed this effect (Greenstein, Fitzpatrick et al. 1986). Importantly, we found that GDX E males had a lower thymus/BW ratio than GDX C males. Furthermore,

GDX increased adrenal wt/BW ratio in C but not E and PF males, indicating that the capacity of circulating testosterone to regulate the thymus and adrenal is reduced in E and PF rats. In line with these results, we also found that prenatal treatment differentially altered the stress CORT response depending on surgical treatment conditions. For E males, CORT levels were similar in intact and GDX conditions, whereas GDX increased CORT levels for PF and C males, indicating that E males were less sensitive to the effect of androgens removal compared to controls. In addition, low testosterone replacement restored CORT levels for PF and C males, whereas high testosterone levels were needed to restore CORT levels for E males. Indeed, low testosterone replacement was actually somewhat suppressive to CORT in E males. The finding that a negative correlation exists between pre-stress testosterone and post-stress CORT levels in C but not in E and PF males further demonstrates the reduced adrenal sensitivity to testosterone in E and PF males.

As expected, testosterone replacement at low and high basal concentrations resulted in low (~1 ng/ml) and high (4 - 6 ng/ml) physiological testosterone levels in all males, and there were no effects of prenatal treatment or time on testosterone levels. In addition, GDX animals had higher LH levels compared to animals in the other three surgical treatment groups due to the absence of testosterone feedback. In addition, intact males from all prenatal treatment groups showed a significant LH increase following 30 min restraint stress. These results were consistent with previous reports that acute stress elicits a small and transient increase in plasma LH levels in male rats (Krulich, Hefco et al. 1974; Turpen, Johnson et al.

1976; Siegel, Weidenfeld et al. 1981; Briski and Sylvester 1987; Briski and Sylvester 1987). Among animals in the GDX or GDX-H condition, there were no effects of prenatal treatment or stress on LH levels. In contrast, only E males in the GDX-L condition showed an LH increase following 30 min stress and thus, at 30 min, LH levels were higher in E than in PF and C males. These data suggest that testosterone had less of an inhibitory effect on stress-induced LH increases in E than in PF and C males.

3. Ethanol effects on central CRH and AVP expression profiles

The synthesis and secretion of CRH and AVP are key events that integrate multiple influences from the CNS and the environment to influence HPA activation (Antoni 1986; Antoni 1993; Whitnall 1993). CRH and AVP are not only expressed in the PVN, but also distributed widely in the brain through central CRH and AVP circuits that project to the PVN. The influence of the amygdala on neurosecretory PVN neurons involve a CRH-communicating pathway from the CeA to and through the fusiform nucleus of the aBNST (Champagne, Beaulieu et al. 1998), and an AVP-communicating pathway from the MeA to the principal nucleus of the pBNST (Herman and Cullinan 1997; Herman, Figueiredo et al. 2003). The amygdala and BNST contain high densities of ARs, indicating that the CRH and AVP circuits within these areas are androgen sensitive such that the expression of these secretogugues is subject to changes in circulating testosterone levels (Walker, Toufexis et al. 2003). It is also reported that 90.5% and 91.2% of AVP-immunoreactive (ir) neurons in the

BNST and MeA, respectively, contain ARs, suggesting that androgens may influence AVP expression by acting directly on these neurons (Zhou, Blaustein et al. 1994). Importantly, there are no ARs or either isoforms of estrogen receptors (ERs) present in the neurosecretory neurons of the PVN that project to the median eminence (Simerly, Chang et al. 1990; Zhou, Blaustein et al. 1994; Williamson, Bingham et al. 2005; Bingham, Williamson et al. 2006; Williamson and Viau 2007), suggesting that testosterone likely has its effects on HPA output on neurons upstream from the PVN. Thus, we examined prenatal ethanol effects on basal CRH and AVP gene expression in the mpd PVN, the BNST and the amygdala.

In the present study, analysis of the mpd PVN revealed that basal CRH mRNA levels were increased by GDX in PF and C but not E males. We did not see a similar effect of GDX in our previous study (Lan, Yamashita et al. 2006). It is possible that differences in methodology between the two studies contributed to the different findings. We sectioned PVN regions at 14 μm and 30 μm in the two studies, respectively. Thus more CRH neurons might be included and analysed in the present study. There are previous reports showing that GDX increases the number of CRH-ir neurons in the hypothalamus (Almeida, Hassan et al. 1992; Bingaman, Magnuson et al. 1994) as well as restraint-induced Fos-ir, CRH hnRNA, and AVP hnRNA expression in the mpd PVN (Viau, Lee et al. 2003). On the other hand, Yukananov and colleagues counted the number of silver grains per cell and found no changes in basal CRH mRNA in the mpd PVN following GDX (Yukhananov and Handa 1997). Similarly, it was shown that GDX had no effect on ADX-induced increases in CRH mRNA

levels in the PVN in brains collected at 60 min after the onset of a 30 min restraint stress (Viau, Chu et al. 1999) or under basal conditions (Viau, Soriano et al. 2001). These studies together with our data suggest that the experimental conditions, methods of analysis and endpoint measured may all influence the results observed.

Importantly, changes in neuropeptide mRNA expression in the PVN-projecting neurons can also influence mpd PVN neuroscretory neurons. In areas upstream to the PVN, we found that neither prenatal treatment nor surgical condition influenced basal CRH mRNA levels in the oval nucleus of the aBNST. However, intact E had marginally lower CRH mRNA levels compared to intact C males in the fusiform nucleus of the aBNST. Furthermore, following high testosterone replacement, E males had significantly higher CRH mRNA in the CeA than C males. It has been shown that CRH mRNA levels in the fusiform nucleus of the aBNST are reduced following GDX, indicating that testosterone exerts a stimulatory effect on CRH mRNA expression in this region (Viau, Soriano et al. 2001). On the other hand, CRH mRNA levels in the CeA vary negatively with testosterone, but only in the presence of CORT (Viau, Soriano et al. 2001). Thus lower CRH mRNA levels in the fusiform nucleus of the aBNST and higher CRH mRNA levels in the CeA in E rats might reflect a reduced testosterone effect on CRH mRNA expression in these regions. Our finding that basal CRH mRNA levels were increased by GDX in PF and C males in the mpd PVN demonstrates the inhibitory role of testosterone on CRH mRNA expression, most likely due to its effects on neurons upstream from the PVN. That basal CRH expression in E males did not change

following GDX suggests a reduced capacity for testosterone to regulate CRH mRNA expression in the central CRH pathway compared to PF and C males.

In contrast, testosterone appeared to have enhanced effects on central AVP pathways in E and/or PF compared to C males. Consistent with previous findings of androgen-dependent influences on AVP mRNA in the pBNST and MeA (Miller, DeVries et al. 1992; De Vries, Wang et al. 1994; Viau, Soriano et al. 2001), we found that AVP mRNA expression in the pBNST and MeA were totally abolished by GDX. Importantly, there were no significant effects of prenatal or surgical treatment on basal AVP mRNA levels in the mpd PVN, consistent with the finding that GDX had no effect on basal AVP mRNA levels in this region (Viau, Soriano et al. 2001). However, like CRH, testosterone likely exerts its effects on the AVP upstream from the mpd PVN, although in this case the effects are CORT-independent. That is, Viau et al. reported glucocorticoid-independent stimulatory effects of testosterone on CRH and AVP mRNA expression within the anterior fusiform and posterior BNST, respectively, and on AVP expression within the MeA (Viau, Soriano et al. 2001). In the present study, we found that both E and PF males showed higher AVP mRNA levels in the pBNST following high testosterone replacement compared to either intact or low testosterone replacement conditions, respectively. Furthermore, E had higher AVP mRNA levels under intact conditions in the MeA compared to C males. In addition, inspection of Figure 11 C suggests a greater effect of low testosterone replacement on E compared to C males, although the differences were not statistically significant. Together, these findings

suggest enhanced AVP responsiveness to testosterone upstream from the PVN in E and/or PF compared to control animals. Importantly, although higher pBNST and MeA AVP mRNA levels in E males may suggest an enhanced inhibitory effect on PVN neurons, we also found decreased AR mRNA levels in the MPN and the principal nucleus of the pBNST (decribed below). If the reduced AR mRNA levels reflect a downregulation in AR, this may counteract the increased inhibitory effect of AVP from these PVN-projecting neurons.

4. Ethanol effects on AR mRNA expression within HPA regulating regions

As noted, recent mapping studies have demonstrated that ARs are not expressed by dorsal medial parvocellular PVN neurons that project to the median eminence, but are distributed in the ventral medial parvocellular PVN which projects to the brainstem and spinal cord (Simerly, Chang et al. 1990; Zhou, Blaustein et al. 1994; Williamson, Bingham et al. 2005; Bingham, Williamson et al. 2006; Williamson and Viau 2007). These findings suggest that androgenic influences on the HPA axis are not directly on the PVN, but rather on PVN projecting neurons. Candidate brain areas that mediate androgenic effects on the PVN are the amygdala, MPN, BNST and hippocampus, which contain high densities of AR. To date, there are no reports of prenatal ethanol effects on AR levels in the brain. However, it has been shown that prenatal stress upregulates both ARs and ERs in female offspring, and downregulates ARs in male offspring (Kaiser, Kruijver et al. 2003; Kaiser, Kruijver et al. 2003). Maternal ethanol consumption increases adrenal weights, basal CORT levels, and the

CORT response to stress in the pregnant dam (Weinberg and Bezio 1987), and alters adrenocortical development of the ethanol-exposed offspring (Weinberg, 1989). Thus, like prenatal stress, prenatal ethanol may alter AR expression and thus induce altered HPA response to testosterone.

We found that AR mRNA levels in the MPN and principal nucleus of pBNST did not change across surgical treatment in E and PF males. However, INT C males had higher AR mRNA levels in the MPN compared to C males in the other surgical treatment conditions, and higher AR mRNA levels in the principal nucleus of pBNST compared to C males in the GDX and GDX-H conditions. Importantly, we found that AR mRNA expression levels were decreased in INT E and PF males in the MPN and principal nucleus of pBNST compared to INT C males. Furthermore, in the MeA, AR mRNA levels were differentially altered by surgical treatment in E and PF but not C males. That is, INT E males had higher AR mRNA levels than GDX E males and INT PF males had higher AR mRNA levels than GDX-L PF males; by contrast C males did not differ in AR mRNA levels across surgical treatments.

Findings from previous studies suggest a unique biphasic regulatory pattern of AR mRNA changes following GDX that also depend on the time and tissue examined. Handa et al. (Handa, Kerr et al. 1996) reported that AR mRNA levels were increased in the MPOA and BNST 4 days after GDX, but decreased after 2 months. However, AR-ir was decreased in the MPOA and BNST after both short- and long-term GDX (Handa, Kerr et al. 1996). In addition, AR mRNA expression in the hippocampus was increased 7 weeks following GDX

(Burgess and Handa 1993). Kerr and colleagues found that hippocampal AR mRNA levels decreased following 4 days of GDX or AR antagonist measured by RT-PCR (Kerr, Allore et al. 1995). However, because AR binding as well as AR protein levels do not always parallel changes in mRNA levels (Kerr, Allore et al. 1995; Handa, Kerr et al. 1996), it remains to be determined whether changes in AR mRNA levels reflect changes in receptor levels and the sensitivity to androgens or possibily just changes in mRNA stability. Nevertheless, the steady state AR mRNA levels may reflect AR synthesis and thus the rate of synthesis may change with time following GDX to compensate for the receptor turnover rate (Handa, Kerr et al. 1996). If this is the case, the finding of AR downregultion in E males in the MPN and principal nucleus of pBNST, which both exert tonic inhibition to the neurosecretory neurons in the mpd PVN, supports our suggestion that E males are less sensitive to the inhibitory effects of testosterone than PF and C males. It is possible that AR downregulation represents a counteracting mechanism that attenuates the increased inhibitory effects of central AVP circuits on the mpd PVN, thus contributing to the HPA hyperresponsiveness observed in E males.

5. Ethanol effects on MR and GR expression in the dorsal hippocampus

As previously described, feedback deficits in E animals may result from decreased expression of MR and/or GR in the hippocampus. The hippocampus is a major negative feedback site of the HPA axis. MRs in the hippocampus are thought to mediate primarily

tonic inhibition of HPA activity (De Kloet, Vreugdenhil et al. 1998) and are important for glucocorticoid regulation of HPA activity during mild stressors (Pace and Spencer 2005); GRs are mainly responsible for feedback inhibition in response to stress-induced CORT elevations as well as tonic inhibition at the circadian peak (De Kloet, Vreugdenhil et al. 1998; Sapolsky, Romero et al. 2000).

Data from the Weinberg laboratory have shown that E animals exhibit deficits in the intermediate (Osborn, Kim et al. 1996) but not the fast (Hofmann, Glavas et al. 1999) negative feedback time domain. Furthermore, HPA responses to both MR and GR blockade were differentially altered in E and control females (Glavas, Yu et al. 2006), indicating a possible role for MR and GR in mediating the alterations in HPA feedback regulation observed in E animals. However, studies using binding assays found no differences in MR and GR densities or binding affinities among E, PF and C animals (Weinberg and Petersen 1991; Kim, Yu et al. 1999). Because hippocampal MR and GR mRNA levels appear to be regulated by glucocorticoids in a site-specific manner, such that ADX upregulates GR mRNA in subfields CA1-2 and DG, and MR mRNA only in subfields CA1-2, (Herman, Patel et al. 1989), we hypothesized that differential effects of prenatal ethanol exposure on MR and GR levels could be revealed through analysis of hippocampal subfields by *in situ* hybridization techniques. As well, compared to receptor binding assays that require removal of endogenous ligands (i.e. ADX), *in situ* hybridization allows the receptor mRNA levels to be detected under intact conditions. Indeed, recent data from Weinberg laboratory showed that ADX

induced a greater increase in MR mRNA levels in E females and GR mRNA levels in E males in the CA3 subfield, compared to their respective PF and/or C counterparts. Furthermore, CORT replacement following ADX was less effective in normalizing MR mRNA levels in E males in $CA1_a$ and DG, and there was a similar trend for E females in the CA3 (Glavas, Ellis et al. 2007). Together, these data suggest that E animals have altered sensitivity of both MR and GR to regulation by CORT, and that the MR/GR balance may be altered by prenatal ethanol exposure.

Sex differences in hippocampal CORT-binding and GR mRNA and protein levels suggest a role of gonadal hormone in glucocorticoid receptor expression (Turner and Weaver 1985; Ahima, Lawson et al. 1992; Bohn, Dean et al. 1994). The hippocampus contains high levels of AR, MR and GR, especially in CA1 pyramidal cells, which form the major signal output of the hippocampal trisynaptic circuit (Herman, Patel et al. 1989; Simerly, Chang et al. 1990; Kerr, Allore et al. 1995; Shughrue, Lane et al. 1997; Bingham, Williamson et al. 2006), indicating that AR may synergize or antagonize with other receptors in regulating hippocampal functions. In this study, we explored MR and GR mRNA in hippocampal subfields CA1-3 and the DG in relation to circulating testosterone levels. We found that, as expected, MR and GR mRNA levels were altered overall across surgical treatments. That is, GDX increased MR mRNA levels in CA1, CA2 and CA3, and GR mRNA levels in CA1. These results were consistent with findings showing that androgens exert a suppressive effect on the transcriptions of both receptors in some brain regions (Turner 1997). Handa's group

also found that DHT decreased GR mRNA levels in CA1, as well as prevented the increase in GR mRNA levels following ADX (Kerr, Beck et al. 1996). Since GR is an autologously regulated gene, they proposed that DHT binding to AR is substituiting for CORT feedback inhibition at the level of the hormone responsive element (HRE) of the GR gene. Hence DHT normalizes the GR upregulation that occurs with ADX, and downregulates GR when given to an intact animal.

We found no differences in MR mRNA among the three prenatal treatment groups. Overall, however, E and PF males had higher GR mRNA levels in CA1 compared to C males, and in the intact condition, E males had higher GR mRNA levels than C males in the CA3 and DG. Thus testosterone binding to AR was less effective in substituting for GR feedback inhibition in E and/or PF compared to C males. These data suggest that, as expected, testosterone had an inhibitory role on both GR and MR mRNA levels in the hippocampus across all prenatal groups. Importantly, however, prenatal ethanol exposure appears to differentially regulate basal hippocampal GR expression in adult males; an effect that may be, at least partially, nutritionally mediated, and that may reflect increased basal HPA tone.

Hippocampal MRs are important in terms of control of threshold or sensitivity of the stress response system, whereas GRs mediate steroid control of termination and recovery from stress (De Kloet, Vreugdenhil et al. 1998). We have previously shown that E males typically showed HPA hyperactivity in response to prolonged or repeated stressors (Weinberg 1992; Kim, Giberson et al. 1999). The findings that GR mRNA levels were

altered by prenatal ethanol exposure from this study may underly the impaired recovery in HPA hormones in E males.

6. Fetal programming of HPA functions by prenatal ethanol, prenatal stress, or prenatal undernutrition?

Fetal programming refers to the concept that adverse stimuli or insults (e.g. prenatal exposure to drugs or other toxic agents) during pregnancy may cause permanent alteration of fetal physiological and metabolic processes and lead to life-long, persisting dysfunction and susceptibility to diseases in adulthood (Bertram and Hanson 2002; Chadio, Kotsampasi et al. 2007; Kapoor, Petropoulos et al. 2007). The HPA axis is highly susceptible to programming during development. Early environmental experiences, including exposure to ethanol or glucocorticoids, and maternal undernutrition reprogram the HPA axis such that HPA tone is increased throughout life (Bertram and Hanson 2002; Matthews 2002; Zhang, Sliwowska et al. 2005; Iqbal, Brien et al. 2006; Chadio, Kotsampasi et al. 2007; Kapoor, Petropoulos et al. 2007).

Prenatal ethanol exposure, prenatal undernutrition and prenatal stress all lead to increased fetal exposure to glucocorticoids. Importantly, in the case of maternal ethanol consumption, there are both direct and indirect effects on the fetus. Ethanol crosses the placenta and directly activates the fetal HPA axis, resulting in increased maternal adrenal

weights, basal CORT levels, CORT responses to stress (Weinberg and Bezio 1987). Both protein and food restrictions during pregnancy result in lower placental 11-hydrozy-steroid dehydrogenase type 2 activity, the enzyme that converts physiological glucocorticoids to inactive 11-keto produces, thus resulting in overexposure of the fetus to maternal glucocorticoids (Langley-Evans, Phillips et al. 1994; Lesage, Blondeau et al. 2001). Furthermore, because the pregnant female and fetus constitute an interrelated functional unit, alterations in maternal HPA activity have significant implications for fetal HPA development. Maternal CORT crosses the placenta (Eguchi 1969), resulting in suppression of endogenous fetal HPA activity. Ethanol-induced activation of the maternal HPA axis occurs as early as day 11 of pregnancy, persists throughout gestation, may increase as gestation progresses, and occurs even with low concentrations of ethanol in the diet (Weinberg and Gallo 1982; Weinberg and Bezio 1987). Together these direct and indirect effects of ethanol can have permanent organizational effects on neural structures that regulate HPA activity throughout life (Levine and Mullins 1966). Furthermore, ethanol administration may affect nutrient intake directly, or cause secondary malnutrition due to its deleterious effects at almost every level of the gastrointestinal tract, as well as its direct toxic effects on the digestive glands, liver and pancreas (Weinberg 1984; Weinberg 1985; Lieber 2005). Thus, both maternal glucocorticoids and undernutrition may be involved in the HPA programming that results from prenatal ethanol exposure. However, studies have shown that maternal glucocorticoid levels (Lee and Rivier 1992; Slone and Redei 2002) do not have a major impact on the

increased CORT activity of the offspring. ADX of the pregnant dam has no effect on the increased CORT responses to restraint stress in E offspring (Slone and Redei 2002). Further, CORT treatment of ADX dams does not mimic the effect of prenatal ethanol on offspring HPA activity (Lee and Rivier 1992). These findings suggest that increased maternal CORT levels are not the primary mediator of increased stress responsiveness in E offspring. Given the complex nature of ethanol effects, which include direct effects of ethanol itself, mild undernutrition and increased maternal CORT, it is likely that the effect of ethanol on the offspring HPA axis result from a balance among these three influences.

Pair-feeding is a necessary and standard control for the nutritional effects of ethanol intake. PF dams are fed a reduced ration, equivalent to that consumed by their E partners, such that both calorie and protein intake are somewhat reduced in E and PF compared to control dams (Weinberg 1985). As such, the pair-feeding procedure can only control for the reduced food intake of the E dams, but can never control for the ethanol-induced changes in nutrient absorption and utilization. Furthermore, PF dams experience mild stress due to the hunger that accompanies consuming less than they would eat *ad libitum.* Thus, there is a mild prenatal stress component superimposed on the nutritional aspect of reduced food intake in the pair-feeding paradigm. Prenatal stress affects the hormonal and behavioural development of offspring (Koehl, Darnaudery et al. 1999; Kofman 2002; Matthews 2002; Seckl 2004), particularly with respect to the development of the HPA axis. We suggest that different mechanisms may underlie HPA dysregulation in E and PF males, with some effects in PF

offspring due to mild prenatal stress, which in itself could program the HPA axis and affect outcome (Hofmann, Simms et al. 2002; Glavas, Ellis et al. 2007).

In this study, we found specific effects of ethanol on body and thymus weights, CORT and LH responses, CRH expression in the PVN, the fusiform nucleus of the aBNST and CeA, AVP expression in the MeA and GR expression in the hippocampus. On the other hand, some effects of ethanol, including adrenal sensitivity to testosterone, AVP expression in the pBNST, and AR expression in the MPN and pBNST, may be, at least partially, nutritionally mediated, or mediated by the mild prenatal stress induced by pair-feeding. Importantly, our previous studies suggest that although E and PF animals may show similar alterations in HPA and/or HPG activity, it is possible that different mechanisms underly the changes seen in these two groups.

In the previous study, we showed that E males exhibit a blunted AVP mRNA response to restraint stress (Lan, Yamashita et al. 2006). Consistent with this, recent data has found that E males showed a higher CRH mRNA response and a lower AVP mRNA response following ADX compared to C males (Glavas, Ellis et al. 2007). Similarly, Lee and colleagues found that E males had lower AVP hnRNA levels compared to control males (Lee, Schmidt et al. 2000). Together with data from the present study, these findings suggest that E males exhibit an altered set-point of HPA activity, i.e. a shift toward greater CRH mediation of HPA regulation. On the other hand, the balance between CRH and AVP- mediated effects appears to be different in PF compared to E males. That is, although both E and PF males

showed a greater response to testosterone in terms of AVP responses in the BNST, PF males did not show the blunted CRH responses to testosterone shown by E males. In a previous study, we found that CORT replacement following ADX failed to normalize the ADX-induced increase in AVP mRNA levels in PF as it did in E males (Glavas, Ellis et al. 2007). Together, these data support the possibility that mechanisms underlying HPA dysregulation may differ in E and PF animals rather than occurring along a continuum of effects on the same pathway.

7. Working model of the mechanisms underlying the effect of testosterone in mediating prenatal ethanol effects on the neurosecretory neurons in the mpd PVN

a. Simplified model of the limbic-forebrain projections to the mpd PVN

A schematic diagram of the circuits that regulates the neurosecretory neurons in the mpd PVN is shown in Figure 19. This circuit has been described in detail in Chapter I. Briefly, ARs are not localized in mpd PVN, indicating that androgens act upstream from the PVN to regulate HPA output. Candidate brain areas that mediate androgenic effects on the PVN are the MPN, BNST, amygdala, and hippocampus, which contain high densities of AR (Simerly, Chang et al. 1990; Lisciotto and Morrell 1994; Zhou, Blaustein et al. 1994; Kerr, Allore et al. 1995; Shughrue, Lane et al. 1997; Romeo, Diedrich et al. 2000; Bingham, Williamson et al. 2006; Williamson and Viau 2007).

The PVN is directly innervated by GABAergic neurons from multiple brain regions, including the BNST and MPOA (Cullinan, Herman et al. 1993; Roland and Sawchenko 1993; Cullinan, Helmreich et al. 1996; Herman, Figueiredo et al. 2003). The CRH-projecting neurons in the aBNST and AVP-projecting neurons in the pBNST (Moga and Saper 1994; Champagne, Beaulieu et al. 1998) exert stimulatory and inhibitory influences, respectively, on CRH and AVP mRNA expression in the mpd PVN, (Herman, Cullinan et al. 1994). There are no direct projections from the amygdala to the mpd PVN. The influence of the amygdala on the HPA system is excitatory and involves a CRH-communicating pathway from the CeA through the fusiform nucleus of the aBNST (aBNST fu), and an AVP-communicating pathway from the MeA through the pBNST to the mpd PVN. It should be noted, however, CRH neurons in the CeA also have rich connections with brainstem structures [e.g. nucleus of the solitary tract (NTS), parabrachial nucleus (PBN)] that exert stimulatory innervation to the PVN (Schwaber, Kapp et al. 1982; van der Kooy, Koda et al. 1984). The CeA and MeA also contain large populations of GABAergic neurons which project to the predominantly GABAergic BNST neurons, and thus activate the PVN by disinhibition (Canteras, Simerly et al. 1992; Prewitt and Herman 1998; Dong, Petrovich et al. 2001a).

The inhibitory feedback effect of the hippocampus on HPA function is relayed via the subiculum, which contains glutamate neurons that stimulate the inhibitory interneurons in the MPOA or BNST (Herman and Cullinan 1997; Herman, Figueiredo et al. 2003).

b. Effects of testosterone on neuropeptide expression within the limbic forebrain circuits that project to the mpd PVN

A series of studies by the Viau laboratory have demonstrated glucocorticoid-independent effects of testosterone on neuropeptide expression in the limbic-forebrain projections to mpd PVN. As shown in Figure 20, AVP mRNA expression in the pBNST and MeA, and CRH mRNA expression in the aBNST, depend exclusively on stimulatory effects of testosterone. However, CRH mRNA expression in the CeA is inhibited by testosterone in adrenal-intact, but not in ADX rats suggesting that this effect is CORT-dependent (Viau, Chu et al. 1999; Viau, Soriano et al. 2001; Viau 2002). Androgens exert a suppressive effect on the transcriptions of both MR and GR in several brain regions, including the hippocampus (Kerr, Beck et al. 1996; Turner 1997). Thus the overall effect of testosterone on the CRH and AVP neurons in the mpd PVN is inhibitory (Almeida, Hassan et al. 1992; Bingaman, Magnuson et al. 1994; Handa, Nunley et al. 1994; Viau, Lee et al. 2003).

c. Effects of prenatal ethanol exposure on neuropeptide and AR expression in response to testosterone

In this study, we found that HPA activity in E males showed a number of alterations that relate specifically to testosterone status. As shown in Figure 21, E males showed lower CRH mRNA levels in the fusiform nucleus of the aBNST compared to C males under intact conditions, and higher CRH mRNA levels in the CeA compared to C males under high

testosterone replacement conditions, indicating a reduced capacity of testosterone to regulate CRH in these regions. However, AVP mRNA expression in the pBNST and MeA showed enhanced responses to testosterone in E and/or PF males. Furthermore, while testosterone had an inhibitory role in both MR and GR mRNA levels in the hippocampus across all prenatal groups, prenatal ethanol exposure appeared to differentially regulate basal hippocampal GR expression, with E males showing GR upregulation compared to PF and/or C males. In addition, AR mRNA levels in the MPN and the principal nucleus of pBNST were lower in E and PF compared to C males under intact conditions. This may counteract the increased inhibitory AVP signals from pBNST and MeA, and thus contribute to the HPA hyperresponsiveness seen in E and/or PF males.

We propose that although basal HPA hormone levels and CRH/AVP mRNA expression in the mpd PVN appear unchanged compared to control males, the upstream CRH and AVP pathways that regulate the neurosecretory neurons in the mpd PVN are altered in E males, which might set the stage for the HPA hyperresponsiveness that is observed. The enhanced capacity of testosterone to regulate the central AVP inhibitory pathways may be a compensatory mechanism that accounts for the unchanged basal HPA activity in E males. However, in the face of challenge by stressors, this mechanism may be insufficient to maintain normal HPA regulation.

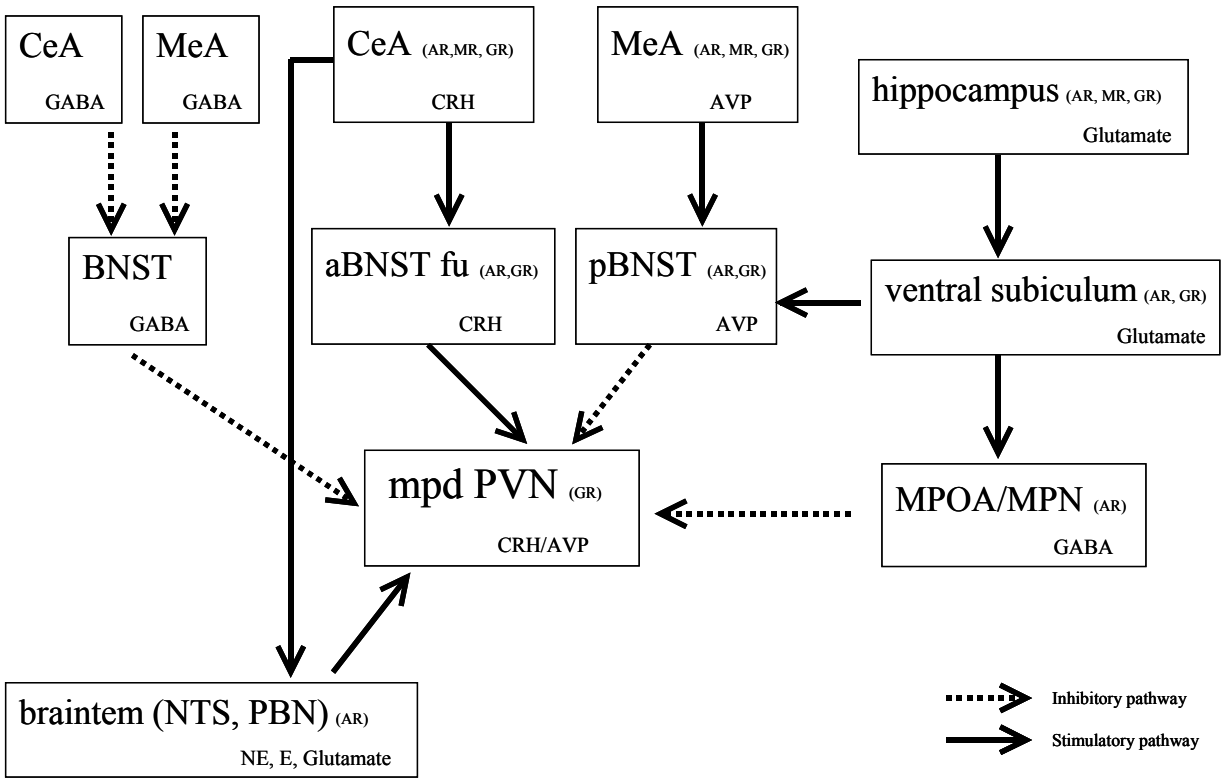


Figure 19 Simplified diagram of limbic-forebrain projections to the mpd PVN in normal animals (see text for details, pp. 128)

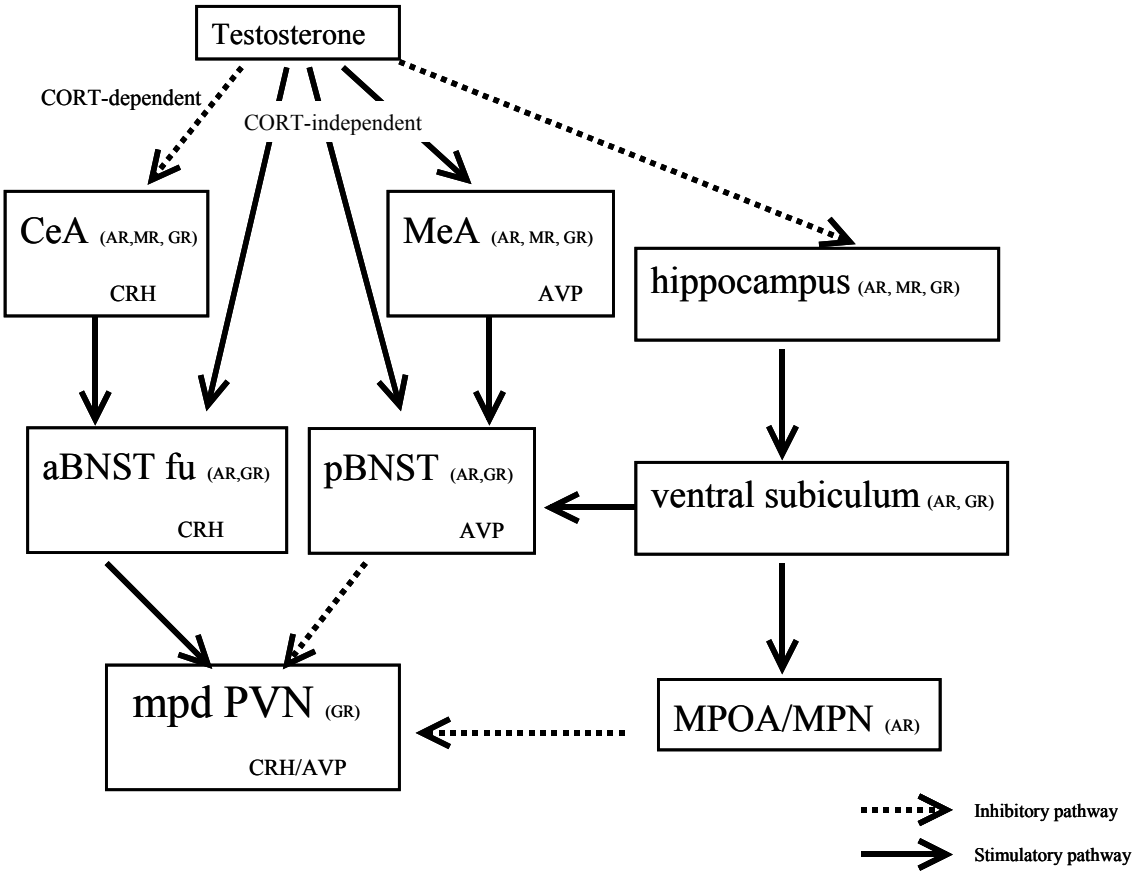


Figure 20 Effects of testosterone on neuropeptide expression within the limbic forebrain circuits that project to the mpd PVN in normal animals (see text for details, pp.130)

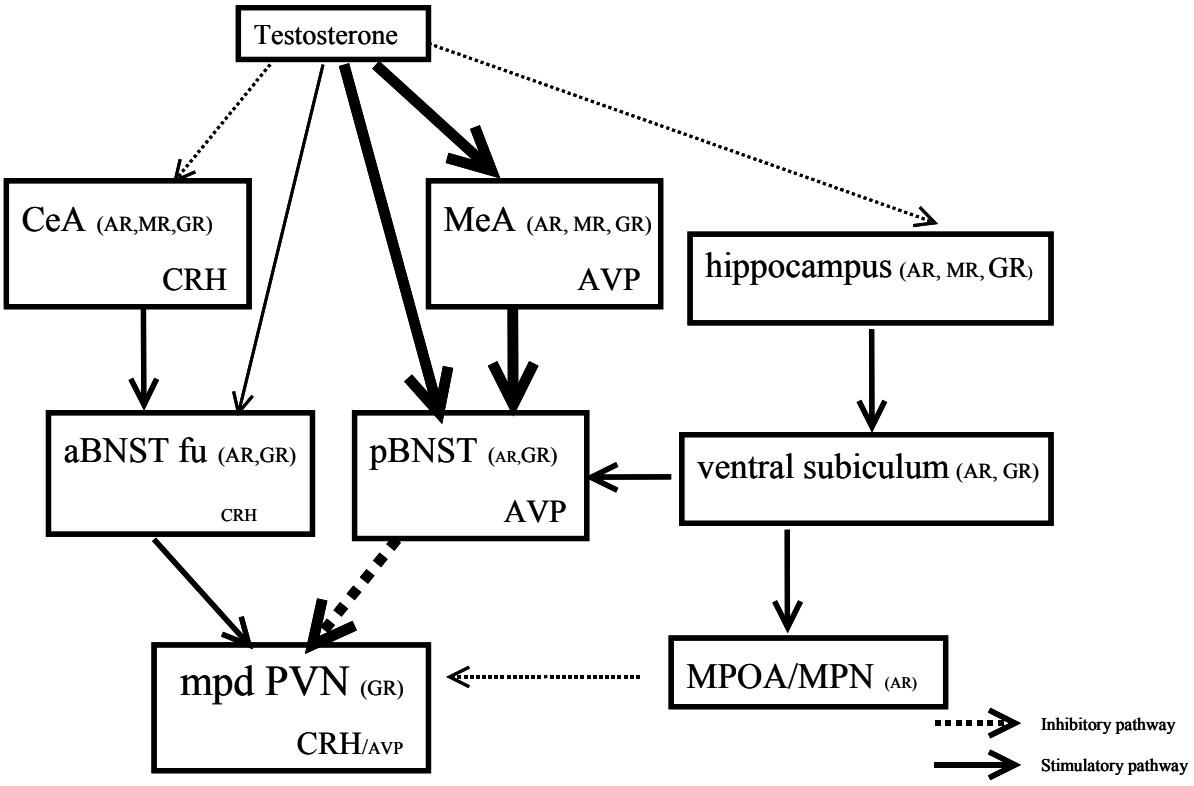


Figure 21 Working model to illustrate possible effects of prenatal ethanol exposure on neuropeptide and AR expression in response to testosterone (see text for details, pp. 130)

CHAPTER V: SUMMARY AND FUTURE DIRECTIONS

A. Summary

The main objective of this thesis was to investigate the influence of gonadal steroids, particularly testosterone, on HPA responsiveness in E compared to control males. In particular, we investigated the possibility that prenatal ethanol-induced changes in HPG regulation and/or HPA-HPG interactions play a role in the alterations in HPA activity observed in E males compared to controls. Our working hypothesis is that the alterations in HPA activity and regulation observed in E males compared to their controls counterparts are mediated, at least in part, by ethanol-induced changes in HPG effects on HPA regulation.

In the first study, we compared the effects of GDX, i.e., removal of testicular androgens, on basal and stress-related HPA activity as well as HPG regulation of adult E, PF and C male offspring. We tested the specific hypothesis that a decreased testicular influence could contribute to the HPA hyperresponsiveness typically observed in E males. We found intact E rats showed increased ACTH but blunted testosterone and LH responses to restraint stress, and no stress-induced elevation in AVP mRNA levels compared to those observed in PF and/or control rats. GDX significantly increased ACTH responses to stress in control but not E and PF males; eliminated differences among groups in plasma ACTH and AVP mRNA levels; and altered LH and gonadotropin-releasing hormone responses in E males. Findings from the first study indicated that central regulation of both the HPA and HPG axes are altered by prenatal ethanol exposure, with normal testicular influences on HPA function

markedly reduced in E animals. A decreased sensitivity to inhibitory effects of androgens could contribute to the HPA hyperresponsiveness typically observed in E males.

In the second study, we tested the hypothesis that the differential alterations in HPA activity observed in E males compared to their control counterparts are mediated, at least in part, by ethanol-induced changes in HPA sensitivity to testosterone. To this end, we explored dose-related effects of testosterone on HPA regulation and responsiveness. Our findings support and extend those of our previous study in providing strong evidence that regulation of both the HPA and HPG axes is altered by prenatal ethanol exposure, and that E males show altered sensitivity to the inhibitory effects of testosterone. HPA activity in E males showed a number of alterations that relate specifically to testosterone status. Sensitivity of both the thymus and adrenal to circulating testosterone was reduced in E rats. Furthermore, prenatal ethanol altered the responsiveness of central CRH and AVP pathways to testosterone. Basal CRH mRNA levels in the mpd PVN were increased by GDX in PF and C but not E males. INT E also had lower CRH mRNA levels in the fusiform nucleus of the aBNST than INT C males, but higher CRH mRNA levels under high testosterone replacement conditions in the CeA. Together, these findings suggest reduced capacity of testosterone to regulate the central CRH pathways in E males. In contrast, testosterone appeared to have greater effects on central AVP pathways in E and/or PF compared to C males. Both E and PF males showed higher AVP mRNA levels in the pBNST following high testosterone replacement compared to either intact or low testosterone replacement conditions, respectively, and E males had

higher AVP mRNA levels under intact conditions in the MeA compared to C males. Furthermore, while testosterone had an inhibitory role in both MR and GR mRNA levels in the hippocampus across all prenatal groups, prenatal ethanol exposure appeared to differentially regulate basal hippocampal GR expression, with E males showing GR upregulation compared to PF and/or C males. HPG regulation was also altered by prenatal ethanol exposure. Testosterone had less of an inhibitory effect on stress-induced LH increases in E than in PF and C males. In addition, AR mRNA levels in the MPN and the principal nucleus of pBNST were lower in E and PF compared to C males under intact conditions. If decreased AR mRNA levels in key brain areas reflects downregulation of AR levels, this may counteract the increased inhibitory AVP signals upstream from the paraventricular nucleus, and thus contribute to the HPA hyperresponsiveness observed in E males.

Together, these data suggest that altered HPA activity in E males represents a complex balance between reduced effects of testosterone on central CRH pathways and increased effects of testosterone on central AVP pathways, with some effects possibly mediated by the nutrional effects of ethanol.

B. Clinical Implications

Children born to alcoholic mothers show behavioral deficits, including hyperactivity, attentional deficits, impaired habituation and cognitive and perceptual problems (Shaywitz,

Cohen et al. 1980). Children with FASD also exhibit lack of inhibition, impulsivity and poor sensitivity to social cues (Streissguth, Clarren et al. 1985; Streissguth, Barr et al. 1986; Streissguth, Aase et al. 1991), which may occur in the absence of intellectual impairment. In fact, it has been suggested that offspring activity level may be a more sensitive indicator of alcohol's teratogenicity than physical features (Shaywitz, Cohen et al. 1980; Landesman-Dwyer 1982). Attentional deficits may reflect the child's inability to withhold a response (Streissguth, Barr et al. 1986). Previous data from the Weinberg laboratory, using an animal model, have shown that HPA hyperactivity and deficits in 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. HPA hyperactivity has also been reported in children with FASD (Spohr, Willms et al. 1993; Streissguth, Barr et al. 1994; Mattson, Schoenfeld et al. 2001; Bookstein, Sampson et al. 2002; Baer, Sampson et al. 2003) . Data from this thesis and previous studies (Sliwowska, Lan et al. 2008, in press; Lan, Yamashita et al. submitted) show that testosterone and ovarian steroids mediate prenatal ethanol effects on HPA regulation in male and female rats, respectively. To date, however, few clinical studies have focused on possible sexual dimorphism in either the behavioral problems observed or 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, Willms et al. 1993). Further work in this area is needed.

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 (e.g. 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 the Weinberg laboratory (Osborn, Kim et al. 1996; Gabriel, Yu et al. 2000; Glavas, Hofmann et al. 2001; Gabriel, Yu et al. 2006; Glavas, Ellis et al. 2007) and others (Redei, Halasz et al. 1993; Lee, Schmidt et al. 2000) showed that the balance between HPA drive and feedback is differentially altered in E males and females. An imbalance would impair the ability to maintain homeostasis in E animals and could progressively create a condition of neuroendocrine dysregulation and impaired behavioral adaptation. These changes have implications for the development of secondary disabilities in children with FAS. The findings also suggest that early treatment before puberty or different therapeutic approaches for each gender in adulthood should be taken into consideration.

C. Future Directions

This thesis study explored the effects of testosterone in mediating prentatal ethanol effects on HPA and HPG regulation in males. Our experimental questions were: 1) Do E males differ from controls in adrenal and gonadal hormone levels under intact conditions,

following GDX or following GDX with low or high testosterone replacement? 2) Do differences between E and controls with different circulating testosterone levels occur under both basal and stress conditions? 3) Do central measures of HPA and HPG activity under different circulating testosterone levels differ in E compared to controls? And 4) Are activities of central testosterone-sensitive pathway that regulate CRH and AVP neurosecretory neurons altered in E males compared to controls? The data suggest that adrenal and gonadal hormone responses and central regulation of both the HPA and HPG axes are altered by prenatal ethanol exposure. Part of the central testosterone-sensitive pathway that regulates CRH and AVP neurosecretory neurons was also altered by prenatal ethanol exposure. We concluded that balance between reduced effects of testosterone on central CRH pathways and increased effects of testosterone on central AVP, as well as downregulation of AR expression in key brain areas could contribute to the HPA hyperresponsiveness typically observed in E males.

For further understanding of how testosterone mediates prenatal ethanol effect on HPA function in male rats, several steps could be taken. The time points we chose for the present study appear to be optimal for measuring the stress levels of peripheral hormone, but were not optimal for revealing possible differences in central secretagogues gene expression, since changes in mRNA levels require a longer time frame (Kovacs and Sawchenko 1996; Akana and Dallman 1997; Viau, Chu et al. 1999). Therefore, we only measured basal mRNA levels for central secretogogues and receptors in the second study. Measurement of Fos

responses in the PVN, as well as hnRNA levels of CRH and AVP, which indicate neuronal activation as well as transcriptional drive to CRH- and AVP-expressing parvocellular neurons, is warranted to determine if testosterone plays a role in stress-induced changes in transcripts of central secretagogues in E compared to their counterparts.

We showed that the reduced capacity of testosterone to regulate HPA activity in E males might be due, at least in part, to downregulation of AR in the MPN and the principal nucleus of the BNST. Since testosterone can be aromatized to estrogen and act on ER, or reduced to DHT and act on AR, future experiments including DHT replacement, and DHT + estradiol replacement groups, would be helpful to understand whether AR or ER accounts for the reduced sensitivity of E males to circulating testosterone. Furthermore, there are areas other than MPN, BNST and MeA that contain AR and ER-β and may directly or indirectly influence CRH and AVP neurosecretory neurons in the PVN, such as anteroventral periventricular nucleus, lateral septum, ventral subiculum, and regions in the brainstem including the periaqueductal gray and catecholaminergic neurons in the ventral medulla (Williamson and Viau 2007). Exploring AR and ER-β levels in these areas will provide a clearer picture for us to understand the mechanisms underlying the effects of testosterone in mediating prenatal ethanol exposure on HPA regulations in male rats.

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