THE EFFECTS OF PRENATAL ETHANOL EXPOSURE ON 
HYPOTHALAMIC-PITUITARY-ADRENAL STRESS RESPONSE 
IN SPRAGUE-DAWLEY RATS

by

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ABSTRACT

Rats prenatally exposed to ethanol (E) exhibit hypothalamic-pituitary-adrenal (HPA) hyperresponsiveness to stressors. The objective of this thesis was to compare responses of E and control animals to acute and repeated stress and to determine how hormonal hyperresponsiveness is mediated by alterations in central regulation of the HPA axis. While multiple mechanisms may play a role in mediating HPA hyperresponsiveness, this thesis focused on the possible roles of increased drive from higher cortical inputs to the hypothalamus and increased anterior pituitary responsiveness to secretagogues. Previous studies have shown that following acute stress, E animals show hormonal hyperresponsiveness and alterations in central regulation primarily at the level of the PVN. In contrast, repeated stress may result in habituation as indicated by an attenuation of hormonal responses to the stressor; however, alterations in central regulation following repeated stress have not been examined in E animals.

Female and male offspring from E, pair-fed (PF) and control (C) dams were tested in adulthood. Animals were terminated after 1 d or 10 d of daily 1 hr restraint stress, either prior to (basal, non-stress condition), immediately after, or 3 hr after restraint. We measured plasma levels of adrenocorticotrophin (ACTH) and corticosterone (CORT), medial parvocellular paraventricular corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) mRNA levels, CRH receptor (CRH-R1) mRNA levels in the PVN and anterior pituitary, and proopiomelanocortin (POMC) mRNA levels in the anterior pituitary.

RestRAINT stress increased plasma ACTH and CORT above basal levels in all prenatal groups, and the response on d 10 was attenuated compared to d 1, indicating hormonal habituation to the stressor. Overall, females but not males exhibited increased CRH
mRNA over basal levels following 1 d of restraint, with a return to basal levels following 10 d of restraint. However, CRH mRNA levels were not differentially altered in either E females or males compared to their respective controls following restraint. In contrast, E females exhibited increased AVP mRNA levels compared to PF and C females following both 1 d and 10 d of restraint, and E and PF males showed greater AVP mRNA levels compared to C males overall. Furthermore, examination of the CRH:AVP ratio indicated that E females had a lower CRH:AVP ratio compared to C females following 10 d of restraint, and overall, E and PF males had a lower CRH:AVP ratio compared to C males. E males also exhibited a lower CRH:AVP ratio compared to C males under basal conditions. These data indicate a general shift towards increased AVP mRNA levels following repeated compared to acute restraint stress in E compared to control animals.

Following exposure to acute stress, both females and males in all prenatal treatment groups showed upregulated CRH-R₁ mRNA levels in the PVN, and the response on d 10 was attenuated compared to d 1, again suggesting habituation to the stressor. Examination of the 1d:10d ratio of CRH-R₁ mRNA expression in the PVN revealed that, following stress, E females exhibited a higher 1d:10d ratio of CRH-R₁ mRNA expression compared to PF and C females, and E and PF males also showed a greater 1d:10d ratio of CRH-R₁ mRNA expression compared to C males. In contrast, no differences were seen in CRH-R₁ mRNA in the anterior pituitary among E, PF and C females or males in any of the experimental conditions. It is possible that increased CRH-R₁ mRNA could play a role in mediating HPA hyperresponsiveness in E animals, but further studies are needed to confirm this.
Measurement of POMC mRNA indicated that females in all prenatal groups had higher POMC mRNA levels following exposure to 1 d of restraint and under basal conditions on d 10 compared to basal conditions on d 1. However, this was due primarily to increased responsiveness in PF and C females, as supported by the additional finding that overall, E females had lower POMC mRNA levels compared to PF and C females. For males, overall, POMC mRNA levels were greater under basal and stress conditions on d 10 compared to basal conditions on d 1. For E males, analyses revealed lower basal POMC mRNA levels on d 1 and d 10 compared to PF and C males. Additionally, exposure to both 1 d and 10 d of restraint stress increased POMC mRNA levels over d 1 basal conditions in E but not PF and C males, and E males also had greater POMC mRNA expression compared to C males following 10 d of restraint.

In conclusion, these findings demonstrate that prenatal ethanol exposure results in altered central regulation of the HPA axis following exposure to stressors. In the paradigm utilized here, E animals do show habituation to a mild repeated stress in terms of plasma hormone levels. However, E, PF and C animals exhibit differential alterations in central regulation. Differences among E, PF and C animals following exposure to both acute and repeated stress do not appear to be mediated by CRH mRNA in the PVN or CRH-R₁ mRNA in the anterior pituitary, but at least following repeated stress may in part be due to a greater shift toward AVP regulation and/or increased levels of CRH-R₁ mRNA in the PVN. For E males but not females, it appears that an increase in pituitary POMC mRNA levels following stress may also play a role in HPA hyperresponsiveness. Together, these data suggest that increased HPA drive and/or increased pituitary responsiveness to secretagogues may be differentially involved in mediating HPA hyperresponsiveness in females and males.
prenatally exposed to ethanol. In addition, these results further suggest that differences between females and males in response to stress involve the interaction of the HPA and HPG axes.
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LIST OF ABBREVIATIONS

ACTH – adrenocorticotrophin
ADX – adrenalectomy
ANOVA – analysis of variance
ARBD – alcohol-related birth defects
ARND – alcohol-related neurodevelopmental disorders
AVP – arginine vasopressin
AVP-R – arginine vasopressin receptor
BAL – blood alcohol level
°C – degrees Celsius
cDNA – complementary deoxyribonucleic acid
cRNA – complementary ribonucleic acid
cm – centimeters
cAMP – adenosine 3’’5’’-cyclic monophosphate
C – prenatal control diet group
CNS – central nervous system
CORT – corticosterone
CRH – corticotrophin releasing hormone
CRH-R – corticotrophin releasing hormone receptor
d – day
DEX – dexamethasone-21-phosphate
DTT – dithiothreitol
E – prenatal ethanol diet group
EDTA – ethylenediaminetetraacetic acid
FAS – fetal alcohol syndrome
g – grams
G – gestation day
GABA – γ-aminobutyric acid
GR – glucocorticoid receptor
GDX - gonadectomy
HPA – hypothalamic-pituitary-adrenal
HPG – hypothalamic-pituitary-gonadal
hnRNA – heteronuclear ribonucleic acid
hr – hour
L – lactation day
kg – kilogram
min – minutes
MR – mineralocorticoid receptor
mRNA – messenger ribonucleic acid
NAD – nicotine adenine dinucleotide
NADH – nicotine adenine dinucleotide in reduced form
NE – norepinephrine
PBS – phosphate buffer solution
PF – prenatal pair-fed diet group
PN – postnatal day
POMC – proopiomelanocortin

xiii
PVN – paraventricular nucleus

RIA – radioimmunoassay

ROD – relative optical density

s - second

SEM – standard error of the mean

SSC – standard saline citrate
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A. Fetal Alcohol Syndrome

Women chronically consuming alcohol during pregnancy may give birth to children with a condition called Fetal Alcohol Syndrome (FAS). FAS can be diagnosed by three major criteria: a characteristic facial dysmorphism including: a smooth philtrum, short palpebral fissures, epicanthal folds and thinned upper vermilion (Jones & Smith, 1973; Jones et al., 1973); growth deficits including decreased head circumference, birth weight and height (Jones & Smith, 1973); and alterations in the central nervous system (CNS), including microcephaly, decreased brain weight and delayed or altered intellectual development, and behavioural deficits such as hyperactivity, problems in social perception, poor impulse control and problems in memory, attention or judgment (Clarren & Smith, 1978; Aase, 1994).

FAS represents the extreme end of a spectrum of alcohol-related effects. If the full diagnostic criteria of FAS are not met, then alcohol-related effects are termed partial FAS, which includes children with varying degrees of growth, intellectual and behavioural deficits. Children who do not have all the characteristics of FAS or partial FAS, but display physical abnormalities induced by prenatal alcohol exposure are referred to as having alcohol-related birth defects (ARBD). Children who display behavioural or functional abnormalities are described as displaying alcohol-related neurodevelopmental disorders (ARND) (Stratton et al., 1996).

Alcohol affects the development of the fetus and may produce long-term consequences that compromise an individual’s ability to adapt to his/her environment. The effects range from growth retardation and physical abnormalities to altered cognitive and
behavioural function. Prenatal alcohol exposure may result in cardiac, renal and genital malformations (Jones & Smith, 1973; Clarren & Smith, 1978), immune dysfunction (Johnson et al., 1981), joint anomalies such as clinodactyly and camptodactyly (Jones et al., 1973; Aase, 1994), and decreased basal ganglia size, which has been implicated in spatial memory deficits (Mattson et al., 1994). Furthermore, growth deficits, such as decreased height, weight and head circumference may persist into adolescence (Streissguth et al., 1991; Streissguth, 1994; Day et al., 2002). In addition, maladaptive behaviours such as poor judgment, poor social adaptation, and attention and social problems have also been documented in adolescents and adults (Streissguth et al., 1991; Streissguth, 1994; Coles et al., 1997). These behavioural and social problems can lead to secondary disabilities that affect the ability of children to adapt to or function in their environment. Adults with FAS or partial FAS have a high rate of mental illnesses such as alcohol or drug dependence, depression, psychotic disorders and personality disorders that include being avoidant, antisocial and dependent (Famy et al., 1998). Thus, the effects of prenatal alcohol exposure are not limited just to childhood, but persist into adulthood.

Other long term effects of alcohol that can compromise an individual’s ability to function in adulthood involve changes in neuroendocrine function. Neuroendocrine systems that are altered by prenatal alcohol exposure include the hypothalamic-pituitary-thyroid axis which regulates metabolism, the hypothalamic-pituitary-gonadal axis which regulates sexual maturation, differentiation and behaviour, and the hypothalamic-pituitary-adrenal axis which is involved in mediating the response to stress as well as numerous physiological functions (Weinberg et al., 1986; Gabriel et al., 1998).
1. Teratogenic Effects of Alcohol

The teratogenic effects of alcohol on the offspring are widespread and depend partly on variables such as the amount, timing and length of exposure to alcohol during pregnancy (Randall, 1987). The critical period of development of an organ system, called organogenesis, is the time when it undergoes crucial steps in maturation and thus may be more vulnerable to the negative effects of any agent that may cause birth defects. The first trimester in humans is when the development of facial and skull bones occurs, whereas the second or third trimester is more often associated with growth retardation and neurological defects. A review of clinical and statistical studies of women consuming alcohol during critical periods in pregnancy by Coles (1994) suggests that alcohol exposure during the first trimester would result in disruptions in organogenesis and musculoskeletal anomalies, whereas exposure during the second and third trimester would result in growth retardation and central nervous system defects such as intellectual and behavioural deficits. Thus, alcohol exposure during the first trimester can result in the characteristic facial dysmorphology of FAS, whereas exposure during the second and third trimester is more likely to result in growth, intellectual and behavioural deficits because growth and brain development occur more rapidly during those stages of gestation. If maternal alcohol consumption occurs throughout pregnancy, the fetus may develop full FAS because of exposure to alcohol during critical periods of development for facial characteristic, growth patterns and CNS function.

In addition to timing, the amount and length of alcohol exposure in utero will affect the offspring’s development. A link between the minimum threshold for alcohol’s effects and the damage it does to the fetus has not been well established, which is in part due
to the widespread effects that alcohol has on the development of the fetus, even at moderate doses. In general, there is an increasing dose-response relationship in terms of amount of alcohol consumed and the range of damage to the developing fetus (Jacobson & Jacobson, 1994). However, even low levels of alcohol during pregnancy have adverse effects on the mental development of the fetus, whereas higher levels of alcohol exposure are necessary to produce deficits in gross motor coordination (Jacobson et al., 1993). Furthermore, animal studies have shown that binge drinking is more harmful to the fetus than steady rates of alcohol consumption, as peak blood alcohol concentrations will be higher (Pierce & West, 1986).

Other factors that may interact and contribute to the impact of alcohol exposure in utero include rate of alcohol metabolism, maternal nutritional status, and the use of other substances such as cocaine, narcotics, marijuana and nicotine (Schenker et al., 1990).

B. Animal Models of FAS

1. Benefits and Limitations of Animal Models

The benefits of using animal models to study in utero alcohol exposure are 1) the control of confounding factors, such as malnutrition, poor environment, and disease; 2) the ability to control dose, pattern and duration of the exposure; 3) the ability to examine the involvement of variables, such as maternal nurturing and environment, and genetic factors, and 4) the ability to elucidate the mechanisms involved in alcohol teratogenicity (Becker et al., 1994; 1996). However, limitations to using animal models include: small body and brain size, which make certain techniques difficult to implement (Becker et al., 1996). In addition, behaviours such as social judgment, language development or math skills obviously cannot
be modeled satisfactorily, thus making it difficult to use animal models to study the impact of prenatal alcohol exposure on affected children’s social behaviour and higher cognitive functioning. Also, the difference in metabolism and sensitivity to alcohol in particular species of animals may vary from those in humans.

2. Effects of Prenatal Ethanol Exposure in Rodent Models

Growth deficits that characterize FAS in children have been shown in rats prenatally exposed to ethanol (Diaz & Samson, 1980), whereas craniofacial dysmorphology has been shown in mice prenatally exposed to ethanol (Sulik et al., 1981). In addition, rodent models of prenatal alcohol exposure have been developed that display some of the other physical alterations observed in children with FAS. For example, rats prenatally exposed to ethanol show changes in organ growth and cellular development, in particular, the heart and kidney (Gallo & Weinberg, 1986), and increased fetal resorptions, while mice exhibit limb deficits including adactyly, ectodactyly and syndactyly and congenital malformations such as skeletal, neurological, urogenital and cardiovascular abnormalities (Randall et al., 1977; Randall & Taylor, 1979), and both rats and mice exhibit decreased brain growth (Randall et al., 1977; Pierce & West, 1986). Ethanol-induced physiological abnormalities such as hormonal hyperresponsiveness to stressors and immune challenges (Weinberg et al., 1996; Kim et al., 1999c), as well as neurotransmitter deficits (Druse et al., 1991; Rudeen & Weinberg, 1993; Kim & Druse, 1996) have also been observed in animal models. Additionally, rats prenatally exposed to ethanol have shown a range of behavioural deficits similar to those in children prenatally exposed to alcohol, for example, an inability to use environmental cues (Weinberg, 1992b); altered behavioural responses to aversive behavioural tasks (Osborn et al., 1998b); behavioural hyperactivity and hyperresponsiveness
such as increased open-field activity (Becker et al., 1996), and deficits in response inhibition as shown by increased levels of activity or exploration in running wheels and in nose-poke and head-dip tests (Driscoll et al., 1990). Furthermore, rodents prenatally exposed to ethanol exhibit learning deficits, performing more efficiently on tasks that reward a slower rate of response (Martin et al., 1977), and showing altered spatial learning and memory on the Morris water maze task (Kim et al., 1997; Gabriel et al., 2002). These data indicate that findings between humans and animals exposed to alcohol in utero are fairly congruent (Driscoll et al., 1990). Overall, animal models have helped further the understanding of alcohol's teratogenic effects on the fetus and the long-term effect on the behavioural and intellectual functioning of the developing child. Thus, animal models provide a method to study the mechanisms underlying these alterations, and cognitive and behavioural responses altered by the teratogenicity of alcohol.

3. Ethanol Administration

To study the effects of prenatal ethanol exposure, a number of different methods to administer ethanol that are currently utilized include injection, inhalation, gastric intubation, and drinking water or liquid diet. The advantages of administering ethanol via injection and gastric intubation are the ability to administer controlled doses of ethanol and to achieve high blood alcohol levels (BALs). However, these methods require extensive handling, and/or invasive techniques that result in a fair amount of stress on the pregnant dam and can lead to prenatal stress effects in the offspring. Prenatal stress in itself has been shown to produce hyperresponsiveness of the HPA axis (Suchecki & Neto, 1991). Administering ethanol via drinking water is non invasive, but the aversive taste of ethanol results in reduced consumption that leads to lower BALs and reduced water and food intake (Weiner, 1980).
Administering ethanol via alcohol vapors is non invasive, the amount and pattern of ethanol delivery can be highly controlled in order to achieve and maintain high BALs, and may eliminate some of the confounding factors of undernutrition (Lee et al., 2000b). However, humans consume ethanol by drinking, and thus administering ethanol via alcohol vapors is not representative of the way humans consume ethanol. The liquid diet method of administering is non invasive, provides the required nutrients to maintain adequate nutritional status and results in high BALs (Weinberg, 1985; Lieber & De Carli, 1989). However, several concerns have been raised about the liquid diet method such as; 1) variability in diet composition between shipments (e.g. change in size of the milk protein casein); 2) presence of flavorings and preservatives that may have toxicological effects, and 3) nutritional adequacy of these formula for pregnant rodents (Weiner, 1980), and 4) the reduced food intake of rats consuming liquid ethanol diets. There are advantages and disadvantages to each of the methods of alcohol administration. However, for our purposes, we use the liquid diet method because it is the least invasive, similar to the method humans consume ethanol, provides adequate nutrition and results in high BALs.

In rats, the consumption of ethanol via liquid diet results in reduced food intake compared to consumption of an ethanol-free diet (Weinberg, 1985). To control for the effects of undernutrition caused by the displacement of nutrients due to ethanol's high caloric content, a nutritional control group [pair-fed (PF)] in which nutritional intake is matched with that of the prenatal ethanol group, is included in models using the liquid diet to administer ethanol. Additionally, ethanol can also impair the digestion and absorption of nutrients which can lead to secondary malnutrition effects independent of adequate nutrition (Weinberg, 1984). Unfortunately, there is no control for secondary malnutrition. Thus, while ethanol is
the main teratogen causing FAS (Weinberg, 1985) nutritional effects of ethanol can also affect offspring development, and pair-fed controls should always be included. However, pair-feeding in itself is also an experimental treatment. For example, pair-feeding can result in a shift in CORT circadian rhythm, altered body and organ weights, and altered behaviour of both the maternal female and offspring (Gallo & Weinberg, 1981; Weinberg & Gallo, 1982). Because pair-feeding in itself is an experimental treatment, it is important to include an ad libitum fed control (C) group that has access to a diet that is nutritionally equivalent to that of animals consuming the liquid diets. The advantages of providing controls ad libitum access to the liquid control diet are being able to isocalorically match all nutrients to the diet consumed by alcohol and pair-fed animals. The disadvantage is that the animals may overeat liquid diets provided ad libitum, at least initially, and to gain weight at a faster rate than animals consuming a pelleted control diet (Weinberg, 1984). One can also use a pelleted diet that has been developed to match the daily intake of nutrients and calories to that of animals consuming the liquid diets. However, the physical state of the diet can affect digestion and absorption of nutrients, thus this procedure is not completely unconfounded. Using laboratory chow for the ad libitum fed control group does not provide a perfect match in terms of all nutrients, but does provide a nutritionally sound diet that allows one to compare data to that of most standard laboratory reared animals.

C. Stress, Stressor and the Stress Response

According to Selye (1973), stress is defined as a nonspecific response of the body to adverse stimuli, characterized by an enlarged adrenal cortex, shrinkage of the thymus, spleen and lymph nodes, and ulcers in the stomach and upper gut. Selye saw the concept of stress as a nonspecific physiological response to environmental stimuli, whereas Mason
(1971; 1975) defined stress in terms of both physiological and psychological stimuli, the response to these stimuli, and stimulus-response interactions. More recently, the concept of stress has shifted in definition to that of a state of threatened homeostasis (Chrousos et al., 1988). For the purpose of this thesis, stress is defined as an alteration in an organism's homeostasis, and stressors as the stimuli that induce this alteration. Adaptive responses include the physiological and behavioural responses that the organism activates in an attempt to restore homeostasis.

In order for an organism to survive, preservation of the internal milieu or homeostasis requires continuous adaptation to stressors (Aguilera, 1994). The organism's response to stress varies depending on the type of stressor, which can be broadly classified as physical (e.g., electric shock) or psychological (e.g., public presentation). It also depends on the duration (acute versus chronic), and intensity of the stressors, and the organism's prior exposure to the stressor, health, genetic makeup and early life experiences (Johnson et al., 1992; Aguilera, 1994). The acute stress response is beneficial to an organism because it prepares the body to meet impending stressors by mobilizing energy reserves, increasing cardiovascular and respiratory tone, suppressing anabolic processes, heightening awareness, sharpening cognition and focusing attention (Munck et al., 1984; Chrousos & Gold, 1992; Johnson et al., 1992). On the other hand, prolonged periods of stress can be detrimental to the organism's survival by suppressing reproductive and immune functions, causing weight loss, fatigue, hypertension, atherosclerosis and gastrointestinal ulcers (Stratakis & Chrousos, 1995). In addition to causing physiological changes, a dysregulation of the stress response has been implicated in psychiatric disorders such as depression, panic disorder and anorexia nervosa (Johnson et al., 1992).
D. Hypothalamic-Pituitary-Adrenal Axis

The HPA axis involves a hormonal cascade in response to a stressor or to circadian stimuli. In general, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) neurons of the parvocellular cells in the paraventricular nucleus (PVN) of the hypothalamus release their contents into the median eminence of the hypophyseal portal system. The release of CRH and AVP stimulates the synthesis and secretion of POMC gene-derived peptides such as adrenocorticotropic hormone (ACTH) from the anterior pituitary. Subsequently, the systemic circulation transports ACTH to act upon the adrenal cortex to stimulate the release of glucocorticoids such as cortisol in humans, and corticosterone (CORT) in rats. The HPA axis is regulated at multiple levels and sites within the brain, such as the hippocampus, hypothalamus, pituitary and frontal cortex via glucocorticoid (GR) and mineralocorticoid (MR) receptors. Thus, glucocorticoids released from the adrenal gland feedback at multiple CNS levels to regulate the HPA axis in response to stressful stimuli. In addition, glucocorticoids have a regulatory role on basal activity of the HPA axis by exerting negative feedback at various CNS components of the stress system (Stratakis & Chrousos, 1995) via MR (Herman et al., 1989) and an MR/GR interaction in the hippocampus (Herman & Spencer, 1998) and possibly via regulation of CRH receptors (CRH-R) in the PVN and pituitary (Luo et al., 1995; Zhou et al., 1996).

1. Corticotropin Releasing Hormone (CRH)

CRH, a 41 amino acid peptide (Vale et al., 1981) is found in many different regions of the brain including the parvocellular division of the PVN of the hypothalamus, brainstem, Barrington's nucleus, dorsal raphe and olfactory bulb (Imaki et al., 1991; Champagne et al., 1998), which suggests that this peptide is involved in many diverse
functions. For example, CRH has been associated with integrating autonomic and behavioural functions to stress within an organism (Smagin et al., 2001), including anxiety-like behaviours (De Souza, 1995), depression (Arborelius et al., 1999), and fear-related responses (Makino et al., 1999). However, CRH appears most abundantly in corticotroph cells in the pituitary and is the major regulator of ACTH release (Vale et al., 1981). Basal CRH levels are under the tonic control of glucocorticoids (Viau et al., 1999; Viau, 2002), as well as stimulatory and inhibitory influences from afferents such as the catecholaminergic neurons of the brainstem, afferent from the hippocampus, amygdala and circumventricular organs, ascending sensory pathways and other hypothalamic inputs to the PVN (Herman & Cullinan, 1997; Aguilera, 1998). CRH neurons of the PVN project to the median eminence where they terminate on the capillaries of the hypothalamo-hypophyseal portal vessels that reach the anterior pituitary. CRH stimulates the release of ACTH by interacting with CRH receptors (CRH-R) (Aguilera, 1998).

2. CRH Receptor (CRH-R)

CRH-Rs have a very wide distribution in the brain and have been localized in the hippocampus, cerebral and cerebellar cortex, olfactory pathway, amygdala and brainstem (Potter et al., 1994; Wong et al., 1994; Eliner et al., 1996; Primus et al., 1997). With the discovery of CRH-R subtypes, studies have shown specific localization of these receptors in the brain and pituitary. In rats and mice, CRH-R₁, the main receptor involved in the regulation of the stress response (Rivest et al., 1995; Smagin & Dunn, 2000), is found in the cerebral cortex, cerebrum, PVN of the hypothalamus and intermediate and anterior pituitary (Primus et al., 1997; Van Pett et al., 2000; Dautzenberg et al., 2001). CRH-R₁ is a seven transmembrane G protein receptor coupled to adenylate cyclase that exerts its effects via
adenosine 3', 5'-cyclic monophosphate (cAMP) and protein kinase A signaling mechanisms (Labrie et al., 1982; Aguilera et al., 1983; Battaglia et al., 1987; Chen et al., 1993; Kuryshev et al., 1996; Pozzoli et al., 1996). In the brain, CRH can upregulate its own activity and PVN CRH-R1 activity via an ultrashort positive feedback loop in order to maintain HPA responsiveness during stress (Ono et al., 1985; Rivest et al., 1995; Imaki et al., 1996; Mansi et al., 1996; Champagne et al., 1998; Jezova et al., 1999; Drolet & Rivest, 2001; Imaki et al., 2001). Basal CRH-R1 levels are under the inhibitory influence of glucocorticoids in the PVN and the pituitary (Luo et al., 1995; Zhou et al., 1996). During stress, however, AVP and CRH both modulate CRH-R1 mRNA levels with an additive down-regulatory or up-regulatory effect, whereas glucocorticoids play a minor role in CRH-R1 regulation (Hauger & Aguilera, 1993; Luo et al., 1995; Pozzoli et al., 1996).

3. Arginine Vasopressin (AVP)

AVP, a decapeptide, is produced in the hypothalamic paraventricular and supraoptic nuclei (Antoni, 1993). It plays a major role in maintaining fluid balance via neural connections with the posterior pituitary and is involved in regulating ACTH secretion from the anterior pituitary (Antoni, 1993; Aguilera, 1994). Presently, two major cell types have been identified in the hypothalamic PVN that produce AVP. AVP from magnocellular cells is transported to the posterior pituitary via neural connections and released into the peripheral circulation to maintain fluid balance, whereas AVP of parvocellular origin is secreted into the external zone of the median eminence to effect release of ACTH from the anterior pituitary (Antoni, 1993). Thus, AVP release from magnocellular cells responds primarily to osmotic changes, whereas AVP secretion from parvocellular cells responds predominantly to stress (Aguilera et al., 1994). However, the possibility that magnocellular cells contribute to ACTH
responsiveness during stress cannot be completely ruled out (Antoni, 1993). Approximately 50% of CRH axons projecting to the external zone of the median eminence contain AVP; these are separated into subpopulations of CRH - AVP containing neurons and CRH - AVP deficient neurons (Wolfson et al., 1985; Antoni, 1993; Walker et al., 2001). Basal AVP is under glucocorticoid and sex steroid control. For example, in adrenalectomized (ADX) and gonadectomized (GDX) males, AVP mRNA increase was blocked, but restored following testosterone replacement (Viau et al., 1999; 2001; Viau, 2002).

4. AVP Receptor (AVP-R)

Three major subtypes of AVP membrane receptors have been identified. The V2 subtype is found in the kidney, the V1a subtype is found predominantly in smooth muscle and liver and the V1b subtype is found mainly in the pituitary (Sugimoto et al., 1994; Lolait et al., 1995; Aguilera & Rabadán-Diehl, 2000). The V1b-R, the main regulator of ACTH release, is coupled to calcium phospholipid dependent signaling systems (Sugimoto et al., 1994; Lolait et al., 1995) and is under the regulation of glucocorticoids (Rabadán-Diehl et al., 1997).

5. Proopiomelanocortin (POMC)

Following the release of CRH and AVP from the hypothalamus, POMC, the precursor to ACTH, is synthesized. CRH stimulates POMC mRNA synthesis via cAMP pathways, whereas AVP stimulates POMC synthesis by promoting transcript processing after transcription via cAMP (Aguilera, 1994). POMC is synthesized mainly in the intermediate and anterior lobes of the pituitary and is also a precursor for β-endorphin, and α-, β- and γ-melanocyte-stimulating hormone (Habib et al., 2001). In corticotroph cells, POMC is cleaved
into ACTH and β-endorphin and secreted from the anterior pituitary (Habib et al., 2001). Basal POMC levels in the anterior lobe are regulated tonically by glucocorticoids and are independent of CRH (Eberwine & Roberts, 1984; Rabadan-Diehl et al., 1996; Aguilera, 1998). However, CRH increases transcription and AVP increases transcript processing of POMC following stress (Aguilera, 1998).

6. Adrenocorticotropicin (ACTH)

As noted, CRH stimulates POMC gene transcription, which eventually results in increased processed cytoplasmic POMC mRNA and enhanced ACTH stores, whereas AVP may play a greater role in ACTH turnover and release (Antoni, 1993). While AVP from the parvocellular cells of the PVN alone is a weak stimulator of ACTH release from the anterior pituitary during stress, it does potentiate the effect of CRH on ACTH release, possibly by increasing CRH stimulated cAMP production in the anterior pituitary (de Goeij et al., 1991; Aguilera, 1994; Aguilera et al., 1994). Furthermore, the release of ACTH from the anterior pituitary is under inhibitory regulation of glucocorticoids under both basal and stress conditions (Dallman & Jones, 1973; Aguilera, 1994).

Under some conditions, there may be a lack of correlation between CRH-R and ACTH responsiveness. For example, following either repeated immobilization or repeated ip hypertonic saline injection there was a decrease in CRH-R number despite an increase in pituitary ACTH response (Aguilera, 1994). In contrast, rats subjected to either repeated stress paradigm showed elevated V1b-R mRNA levels in the pituitary as well as increased ACTH responsiveness (Aguilera, 1994). These studies show that CRH-R and AVP-R regulation of ACTH responsiveness may involve different pathways, at least following either repeated immobilization or repeated ip hypertonic saline injection. Unfortunately, current studies on
how repeated restraint stress alters CRH-R, AVP-R and ACTH responsiveness, and the interactions among these, are limited. Restraint is primarily a psychological as opposed to a physical stressor, and causes no pain or injury; however, both immobilization and ip hypertonic saline injection are primarily physical stressors that cause physical discomfort. In summary, ACTH regulation primarily involves differential secretion of CRH and AVP, glucocorticoid sensitivity during feedback and CRH-R and AVP-R in the pituitary.

7. Corticosterone (CORT)

ACTH released from the anterior pituitary travels through the systemic circulation to affect the release of glucocorticoids, or CORT, from the adrenal cortex. CORT then feeds back at multiple sites in the brain, such as the hippocampus, pituitary and hypothalamus to regulate the HPA axis via MR and GR. MRs bind CORT with high affinity which suggests that they are involved mainly in regulating basal levels of CORT, whereas GRs bind CORT with a lower affinity and are occupied predominantly during high levels of circulating CORT, such as during the circadian peak or stress (DeKloet et al., 1998). The occupancy of GRs during high levels of circulating CORT suggests that GRs may be more involved in regulating HPA activation (DeKloet et al., 1998). Furthermore, MR/GR interactions under basal (Herman & Spencer, 1998) and stress (Paskitti et al., 2000; Gesing et al., 2001) conditions have also been reported. Thus, GRs and MRs mediate feedback under both basal and stress conditions. A possible mechanism whereby CORT may inhibit the HPA axis has been studied at the mRNA level as well as by direct measurement of peptide hormones. ADX results in increased POMC mRNA in the anterior pituitary (Eberwine & Roberts, 1984) and CRH mRNA in the PVN (Jingami et al., 1985), both of which can be reversed by glucocorticoids. Increased CORT levels have also been shown to decrease pituitary CRH-R
binding in vivo (Hauger et al., 1987). Additionally, CRH, POMC and CRH-R1 gene expression were reduced by dexamethasone (DEX) treatment (Zhou et al., 1996). Thus, these data suggest that possible mechanisms mediating the inhibitory effect of CORT are at the level of POMC, CRH and CRH-R1 mRNA synthesis and CRH-R binding.

E. HPA Axis and Stress

1. Response to Acute Stress

As previously mentioned, the organism's response to stress depends on the type of stressor, and its duration and intensity. Additionally, the response to stress depends on the endpoint measured. Various acute stressors that activate the HPA axis can result in increased levels of ACTH and CORT. Increased levels of ACTH and/or CORT following immobilization – which involves restricting limb movements (Hauger et al., 1988; 1990; Culman et al., 1991; de Goeij et al., 1991; Tizabi & Aquilera, 1992; Makino et al., 1995; Jezova et al., 1999), restraint – which involves restricting physical movement (Herman, 1995; Ma et al., 1999; Garcia et al., 2000), ether exposure (Ono et al., 1985; Hashimoto et al., 1988; Kovacs & Sawchenko, 1996) and acute exposure to alcohol (Zhou et al., 2000) have been shown. Similarly, various acute stressors can result in central changes in gene expression as shown by increased CRH and/or AVP mRNA following immobilization (Imaki et al., 1992; Makino et al., 1995b; Aubry et al., 1999; Jezova et al., 1999), restraint (Harbuz & Lightman, 1989; Imaki et al., 1996; Kiss et al., 1996; Ma et al., 1997; Ma & Lightman, 1998; Pinnock & Herbert, 2001), forced swim (Harbuz & Lightman, 1989) and icv injection of CRH – which mimics the effects of a stressor (Imaki et al., 1996). Also, CRH-R1 mRNA in the PVN have been shown to increase following immobilization (Luo et al., 1994; Nappi & Rivest, 1995), acute restraint (Imaki et al., 1996; 2001), LPS injection (Rivest et al., 1995;
Drolet & Rivest, 2001) and CRH injection (Mansi et al., 1996). In contrast, CRH-R₁ mRNA in the anterior pituitary appears to decrease following immobilization (Makino et al., 1995a), a CORT injection (Ochedalski et al., 1998) and CRH injection (Hauger & Aguilera, 1993). Thus, many types of acute stressors can result in increased levels of plasma ACTH and CORT, and of CRH, AVP and CRH-R₁ mRNA in the PVN, and in decreased CRH-R₁ mRNA in the anterior pituitary.

2. Response to Chronic Stress

In contrast, various chronic stressors such as repeated restraint and immobilization stress result in normal or slightly decreased levels of plasma ACTH and CORT, which suggests hormonal habituation (Hashimoto et al., 1988; Culman et al., 1991; Ma et al., 1999). Central alterations may also reflect habituation; for example, normal or slightly decreased levels of CRH mRNA have been observed following chronic immobilization and restraint stress (Lightman & Harbuz, 1993; Ma et al., 1999). In contrast, hypertonic saline injection (Ma & Aquilera, 1999) or immobilization stress that involves taping down the limbs of the animal and placing it dorsal surface up (Makino et al., 1995b) are paradigms where sensitization to repeated stress occurs. Sensitization typically results in increased plasma ACTH and CRH mRNA levels. In contrast to hormone and CRH levels, AVP appears to increase in all repeated stress models studied. For example, increased AVP mRNA has been observed following both repeated restraint (Herman, 1995; Ma et al., 1997; 1999) and immobilization (Makino et al., 1995b). An increase in AVP protein levels has also been shown in the external zone of the median eminence following chronic immobilization or psychosocial stress (de Goeij et al., 1992a, 1992b; Bartanusz et al., 1993). These data suggest a shift towards increased AVP production as an adaptive mechanism following
various chronic stressors (Aguilera & Rabadán-Diehl, 2000) in order to maintain HPA responsiveness. Additionally, chronic immobilization has been shown to decrease CRH-R₁ mRNA in the anterior pituitary (Makino et al., 1995a), and to result in lower CRH-R₁ mRNA levels in the PVN compared to that seen in response to an acute stressor (Makino et al., 1995a; Bonaz & Rivest, 1998). However, MR and GR peptide pools and gene expression do not appear to be affected by a chronic intermittent stress regimen that included such stressors as restraint, cold exposure and forced swim (Herman et al., 1999). Thus, decreased levels of plasma ACTH and CORT, as well as decreased PVN CRH and pituitary CRH-R₁ mRNA levels and increased PVN AVP mRNA expression may underlie the adaptive response to repeated or chronic stress.

**F. Effects of Prenatal Ethanol Exposure on the HPA Stress Response**

As previously mentioned, prenatal ethanol exposure has adverse effects on the HPA axis. This could have an impact on an organism’s health because the HPA hormones mediate the response to stress, interact with the immune system (Bateman et al., 1989), and maintain and modulate a wide range of physiological, cellular and metabolic processes. For example, glucocorticoids maintain cardiovascular function, blood pressure, blood glucose and liver glycogen levels and muscle work capacity (Munck & Guyre, 1986). During stress, glucocorticoids have a protective effect by suppressing inflammation (Munck & Guyre, 1986). Prenatal alcohol exposure can result in HPA hyperresponsiveness to stressors, characterized by increased HPA hormone levels and/or delayed recovery back to basal levels following stress. A recent study has shown that maternal drinking at conception and during pregnancy was associated with elevated poststress cortisol levels in infants (Jacobson et al., 1999). In addition, a case study reported pseudo-Cushing’s syndrome in an infant exposed to
alcohol via breast milk (Binkiewicz et al., 1978). This study suggests that alcohol also can have stimulatory effects on the HPA axis of the newborn.

Studies in rats have shown altered HPA axis function in the pregnant female who is consuming ethanol. Increased maternal adrenal weights, basal plasma CORT levels, and adrenocortical responses to stress have been found in pregnant female rats consuming ethanol (Weinberg & Bezio, 1987; Weinberg, 1989). These effects are specific to ethanol and occur regardless of whether diets are nutritionally supplemented. Similarly, HPA hyperresponsiveness is found in offspring prenatally exposed to ethanol, with effects specific to ethanol and not nutritional factors. In general, E, PF and C rats do not differ in basal or non-stress levels of CRH, ACTH and CORT (Taylor et al., 1981; 1982; 1983; Nelson et al., 1986; Weinberg, 1988; 1992a; Lee et al., 1990; 2000a; Kim et al., 1999a). In addition, Lee et al., (2000a) has shown no differences in PVN mRNA levels of the immediate early genes (IEGs) c-fos and NGFI-B, and unchanged resting median eminence content of AVP. However, from weaning age on, E rats display increased plasma ACTH levels following a variety of stressors including footshock, restraint, cold or CRH infusion (Nelson et al., 1986; Lee et al., 1990; 2000a; Weinberg et al., 1996; Ogilvie & Rivier, 1997; Kim et al., 1999a; Osborn et al., 2000), and increased β-endorphin following restraint (Weinberg et al., 1996). Additionally, E rats display increased plasma CORT responses to various physiological and psychological stressors such as footshock, cardiac puncture, noise and shake, restraint, forced swim, ether and cold (Taylor et al., 1982; 1983; Nelson et al., 1986; Weinberg, 1988; 1992a; Weinberg et al., 1996; Kim et al., 1999a), and to challenges with drugs such as ethanol and morphine (Taylor et al., 1981; 1983; Nelson et al., 1986). In addition, following exposure to footshock, E animals display significantly enhanced c-fos and NGFI-B mRNA and CRH
hnRNA levels, but not AVP hnRNA, compared to C animals (Lee et al., 2000a). Furthermore, E rats demonstrate deficits in negative feedback or recovery from stress. For example, E rats show prolonged CORT, ACTH and β-endorphin elevations during and following restraint stress (Weinberg, 1988; 1992a; Weinberg et al., 1996), and less attenuation in CORT responses when allowed to perform consummatory behaviours during novel cage stress as compared to control animals (Weinberg, 1988). Additionally, E rats appear to display deficits in the ability to use or respond to environmental cues, as they were less discriminating in their CORT responses to predictable versus unpredictable restraint stress compared to controls (Weinberg, 1992b). Finally, while HPA hyperresponsiveness and/or deficits in negative feedback have been shown in both female and male rats prenatally exposed to ethanol, ethanol appears to differentially affect female and male offspring depending on the nature and intensity of the stressor, and the time course, and hormonal endpoint measured (Weinberg, 1988; Halasz et al., 1993; Weinberg et al., 1996).

G. Mechanisms of Prenatal Ethanol Effects on HPA Hyperresponsiveness

At present, the mechanisms underlying HPA hyperresponsiveness in prenatally ethanol exposed rats are unknown. One or more of the following mechanisms may explain hyperresponsiveness: 1) increased stimulatory input from within the hypothalamus, limbic structures or higher cortical areas to the hypothalamus, as suggested by increased CRH and AVP synthesis; 2) similar stimulation from above the PVN but increased pituitary responsiveness to secretagogues; 3) altered neurotransmitter regulation of the HPA axis, and 4) deficits in negative feedback regulation of the axis.

First, there may be increased HPA drive of E animals. Increased basal hypothalamic CRH mRNA in 60 day old E and PF males exposed to ethanol during the last
two weeks of gestation (Redei et al., 1993) and in the PVN of 21 day old pups exposed to ethanol vapors during the second week of gestation (Lee et al., 1990), as well as decreased hypothalamic CRH content in 21 day old ethanol-exposed pups (Redei et al., 1989) have been demonstrated. However, prenatal ethanol exposure did not alter basal hypothalamic CRH levels (Angelogianni & Gianoulakis, 1989; Lee et al., 2000a) but decreased basal median eminence CRH levels in E and PF rats compared to control rats (Lee & Rivier, 1994). Furthermore, c-fos and NGFI-B mRNA levels do not differ between E and C animals under basal conditions (Lee et al., 2000a). However, following exposure to footshock E animals display increased c-fos and NGFI-B mRNA and CRH hnRNA levels compared to C animals (Lee et al., 2000a). Inconsistencies have been reported regarding basal levels, possibly due to different methods and timing of alcohol administration and the ages at which the animals were tested. Additionally, these effects may also be mediated by nutritional factors considering that these effects were also observed in PF rats.

Second, there may be increased pituitary responsiveness to secretagogues in E animals. Increased basal POMC mRNA levels have been reported in the anterior pituitary of E males (Redei et al., 1993). Concurrently, increased basal POMC mRNA levels may also suggest increased HPA drive as suggested by increased basal hypothalamic CRH mRNA in 60 day old E and PF males (Redei et al., 1993). Although CRH or VP administrations did not produce differential anterior pituitary ACTH release under basal conditions (Taylor et al., 1988; Lee et al., 1990; Lee & Rivier, 1992), an increased ACTH response to CRH in DEX suppressed E animals has been shown, which suggests increased pituitary responsiveness to secretagogues (Osborn et al., 2000). In contrast, Lee et al., (2000a) found no difference in plasma ACTH levels between E and C animals following icv CRH injection. These
differences in results may be attributable to the fact that Osborn et al., (2000) used DEX to block endogenous HPA activity prior to CRH infusion. DEX is a synthetic glucocorticoid that acts primarily at the pituitary and does not readily cross the blood brain barrier. It is possible that increased pituitary responsiveness to CRH may only be observed when endogenous HPA activity is blocked. Alternatively, it is possible that differential pituitary responsiveness to DEX occurs in E compared to control animals.

Third, altered neurotransmitter regulation and activity of the pituitary β-endorphin system may be involved in mediating HPA hyperresponsiveness in E animals. The effect of opioids, such as β-endorphin, on the HPA axis has been controversial. An in vitro study suggested that opioid peptides inhibit secretion of CRH from the hypothalamus. Administration of an opioid antagonist significantly increased basal CRH secretion demonstrating a tonic inhibitory effect of endogenous opioids on CRH secretion (Tsagarakis et al., 1990). However, another study suggested that the β-endorphin system may increase CRH biosynthesis and release of ACTH via CRH-dependent pathways (Wang et al., 1996). As the β-endorphin system is known to be altered in E animals, as they show prolonged β-endorphin elevations following restraint stress (Weinberg et al., 1996), stimulatory effects of β-endorphin on CRH could play a role in hyperresponsiveness; thus further research is warranted. Norepinephrine (NE) content in the cortex and hypothalamus was shown to be lower in E rats compared to controls following restraint stress (Rudeen & Weinberg, 1993). Lower NE content in E rats may be indicative of increased NE turnover, which suggests that prenatal ethanol effects on NE regulation of CRH secretion may be involved in HPA hyperresponsiveness. The GABA system also appears to be altered in E rats, as demonstrated by altered responsiveness to the anxiolytic effects of benzodiazepines (Osborn et al., 1998a).
Additionally, E animals show alterations in behaviours and physiological activity consistent with increased serotonin receptor mediated functions (Hofmann et al., 2002) and decreased serotonin levels and reuptake sites (Druse et al., 1991; Kim & Druse, 1996). As serotonin plays a role in increasing HPA activity, an increase in serotonin receptor mediated functions could increase HPA activity.

Fourth, there is evidence for deficits in negative feedback control of the HPA axis in prenatal ethanol exposed rats following DEX suppression. Both E females and males showed increased ACTH and CORT responses to acute ether stress 3 hr following DEX injection, suggesting a deficit in the intermediate time domain (Osborn et al., 1996). However, CORT injection resulted in similarly blunted ACTH responses in E, PF and C rats in response to either swim or ether stress, suggesting that the fast feedback time domain may not be altered (Hofmann et al., 1999). Additionally, decreased glucocorticoid receptor densities at HPA feedback sites in the brain do not appear to underlie HPA hyperresponsiveness. Exposure to chronic intermittent stress produced down regulation of hippocampus MRs and GRs in females and males in all prenatal groups (Kim et al., 1999b).

**H. Thesis Objective**

Prenatal ethanol exposure results in HPA hyperresponsiveness to stressors. The HPA axis is involved in influencing the immune system and the nervous system, both of which are important systems essential for survival of the organism. Thus, HPA hyperresponsiveness can result in severe physiological and behavioural consequences that can compromise an organism's health and survival. The mechanisms underlying HPA hyperresponsiveness are beginning to be elucidated. However, further studies are needed. While multiple mechanisms may play a role in mediating HPA hyperresponsiveness, I will
focus on the possible roles of increased drive from higher cortical inputs and/or the hypothalamus and increased pituitary responsiveness to secretagogues. While hormonal hyperresponsiveness in prenatal ethanol exposed rats is typically not apparent under basal conditions, central changes in basal HPA regulation have recently been observed (Glavas et al., 2001). Furthermore, HPA hyperresponsiveness and changes in central regulation may be further unmasked if the system is perturbed by stress. Presently, many studies involving prenatal ethanol exposed rats have examined HPA responsiveness to acute stress but not repeated stress. Following acute stress, E animals show hyperresponsiveness and central alterations. In addition, E animals do not show the same level of hormonal habituation to repeated stressors as do controls. However, we have not examined central changes in E animals to repeated stress. By comparing acute versus repeated stress, a window on central changes underlying HPA hyperresponsiveness in E animals may be revealed.

The objective of this thesis was to compare responses of E and control animals to acute and repeated stress and to determine how hormonal hyperresponsiveness is mediated by alterations in central regulation of the HPA axis. We tested the following hypotheses: 1) that HPA hyperresponsiveness in E animals following acute restraint stress is associated with increased levels of plasma ACTH and CORT, and of CRH, AVP and CRH-R₁ mRNA expression in the PVN, as well as increased POMC and CRH-R₁ mRNA expression in the anterior pituitary compared to PF and C animals; and 2) that following repeated restraint stress E animals will show increased levels of plasma ACTH and CORT, increased PVN CRH and CRH-R₁ mRNA, as well as a greater shift to PVN AVP mRNA expression and increased POMC and CRH-R₁ mRNA expression in the anterior pituitary compared to PF and C animals.
Female and male offspring from E, PF and C dams were tested in adulthood. Animals were terminated at d 1 or d 10 after daily 1 hr restraint stress, either prior to (basal, non-stress conditions), immediately after, or 3 hr after restraint. We measured plasma ACTH and CORT levels, and CRH, AVP and CRH-R₁ mRNA levels in the PVN, and POMC and CRH-R₁ mRNA levels in the anterior pituitary.
CHAPTER 2: METHODS AND MATERIALS

A. Animals and Mating

Adult Sprague-Dawley female (225-250 g) and male (250-275 g) rats were ordered from the Animal Care Center, University of British Columbia, Vancouver, B.C., Canada. Male and female animals were pair-housed by sex for a one to two week adaptation period with ad libitum access to water and standard laboratory chow (Jamison’s Pet Food Distributors Ltd., Delta, B.C., Canada). Males were placed singly in stainless steel hanging cages (25 x 18 x 18 cm), with mesh front and flooring. Females were randomly assigned to a male and cage papers were checked daily for the presence of a vaginal plug. The presence of a vaginal plug indicated day 1 of gestation (G1). Colony rooms were temperature controlled (21°C) and maintained on a 12 hr light-dark cycle with lights on from 0600 hr to 1800 hr. All animal use procedures were in accordance with the National Institutes of Health guidelines and were approved by the University of British Columbia Animal Care Committee.

B. Diets and Feeding

On G1, females were singly housed in polycarbonate cages (24 x 16 x 46 cm) and randomly assigned to one of three treatment groups: 1) Ethanol (E) females (n = 13) had ad libitum access to a liquid ethanol diet containing 36 % ethanol-derived calories (Bio-Serv Inc., Frenchtown, N.J., USA), 2) Pair-fed (PF) females (n = 13) had access to a liquid diet, with maltose-dextrin isocalorically substituted for ethanol. The amount provided was matched to the amount consumed by an E female (g/kg body weight) on the same day of gestation, and 3) Control (C) females (n = 13) had ad libitum access to standard laboratory chow and water. All animals were provided with fresh diet between 1400 hr and 1600 hr.
daily at the peak of the CORT diurnal rhythm. This feeding schedule was designed to prevent a shift in the CORT circadian rhythm, which may occur in animals on a restricted diet, such as the PF dams (Gallo & Weinberg, 1981). Experimental diets were removed and weighed daily to determine the amount consumed by each pregnant dam. These liquid diets were developed previously by our lab to ensure adequate nutrition to pregnant dams, regardless of ethanol intake (Weinberg, 1985). E and PF dams were provided with fresh liquid diet daily until G22 when they were then provided ad libitum access to standard laboratory chow and water. Pregnant females were handled only on G1, G7, G14 and G21 for cage changing and weighing. Birth of pups was designated postnatal day 1 (PN1). Pups were culled to a litter size of 10 (5 females: 5 males). If necessary, cross fostering of pups occurred with pups from litters born on the same day, in the same treatment group, to maintain a litter size of 10. Dams and pups were weighed on PN1, PN8, PN15 and PN21. Pups were weaned on PN21 and group housed by litter and sex. Animals were tested at 90 to 120 days of age.

C. Blood Alcohol Level Measurements

To determine maximal BALs, BALs were measured 1 hr after lights off on G11. These BALs should reflect close to peak levels as major eating bouts occur immediately after lights off. Blood was collected via tail vein sampling, which involved removal of the tip of the tail with a razor blade. Blood was collected into heparinized microcapillary tubes, centrifuged at 2400X for 20 min at 4°C. Plasma was removed and stored in 0.6 ml polypropylene microcentrifuge tubes at -20°C until time of assay. BALs were measured using a Sigma-Aldrich Canada Ltd. Ethanol Assay kit (Oakville, O.N., Canada, Cat # Sigma 333-A). The reaction is based on the conversion of ethanol by alcohol dehydrogenase to acetaldehyde (ethanol + NAD → acetaldehyde + NADH). The conversion of NAD to NADH
results in a change in UV absorbance that is detected by a spectrometer. The minimum amount of detectable ethanol is 10 mg/dl and the curve is linear to 150 mg/dl.

**D. Testing and Blood Sampling**

Animals were singly housed one week prior to testing and tested 1 hr after lights on in order to examine changes in plasma hormone levels and mRNA levels during the circadian trough. Animals were assigned to one of the following testing conditions: 1) basal dl (terminated immediately upon removal from home cage); 2) Id restraint-1hr (1 d of a 1 hr restraint stress and terminated immediately following restraint); 3) Id restraint-4hr (1 d of a 1 hr restraint stress and terminated 4 hr after stress onset); 4) basal d10 (9 d of a 1 hr restraint stress and terminated on d 10 immediately upon removal from home cage); 5) 10d restraint-1hr (10 d of a 1 hr restraint stress, and terminated immediately following restraint on d 10); and 6) 10d restraint-4hr (10 d of a 1 hr restraint stress and terminated 4 hr after stress onset on d 10). Restraint stress consisted of confinement in a polyvinyl chloride tube with plastic caps taped securely on each end. The front cap was ventilated with four openings (1 cm in diameter, 2 cm apart) and the end cap had a 1.5 cm opening for the tail. Tubes for males were 20 cm x 7.5 cm (length x inner diameter) and tubes for females were 20 cm x 5.5 cm (length x inner diameter). Restraint is primarily a psychological as opposed to a physical stressor, and causes no pain or injury. Animals were weighed on d 0, d 4 and d 9 of testing. Animals were terminated by decapitation in order to obtain ACTH and CORT. Trunk blood was collected on ice in 12 x 75 mm polystyrene tubes containing a 3.75 % ethylenediaminetetraacetic acid (EDTA) disodium salt/aprotinin (1000 KIU) solution, and centrifuged at 3500X g for 10 min at 4°C. EDTA was used to prevent coagulation of blood, and aprotinin, a protease inhibitor, was used to prevent ACTH denaturation. Plasma was
stored in 0.6 ml polypropylene microcentrifuge tubes at -70°C until time of assay. Brains and pituitaries were removed and immediately frozen in powdered dry ice and stored at -70°C until time of in situ hybridization. Adrenals were removed, cleaned and weighed immediately. The study was carried out over twenty three days with each group (E, PF and C) and each sex represented on every test day.

E. Radioimmunoassays

1. Plasma CORT Levels

Total CORT (bound and free) was measured via radioimmunoassay (RIA) in plasma extracted in 95 % ETOH using our adaptation (Weinberg & Bezio, 1987) of the method of Kaneko et al. (1981). Antiserum was obtained from Immunocorp (Montreal, P.Q., Canada); tracer [1, 2, 6, 7]-H CORT from Mandel Scientific (Guelph, O.N., Canada); and unlabeled CORT for standards from Sigma Chemical Co. (St. Louis, M.O., USA). Dextran treated charcoal (Fisher Scientific Ltd., Nepean, O.N., Canada) was used to absorb and precipitate free steroids after incubation. Samples were counted in Scintisafe Econo 2 scintillation fluid (Fisher Scientific Ltd., Nepean, O.N., Canada). The minimum detectable amount of CORT was 0.25 ug/dl. Intra- and interassay coefficients of variation were 1.55 % and 4.26 %, respectively.

2. Plasma ACTH Levels

Plasma ACTH was measured using a modification of the DIASORIN ACTH RIA kit (Diasorin Inc., Stillwater, M.N., USA, Cat # 24130). All reagents were halved and 50 ul of each sample were used to assay plasma ACTH. Samples were counted using a gamma
counter. The minimum detectable amount of ACTH was 20 pg/ml and the mid-range intra- and interassay coefficients of variation were 3.9% and 6.5%, respectively.

F. In Situ Hybridization

1. Brain Preparation

Frozen brains were sectioned using a cryostat in the coronal plane (14 um thickness) at the hypothalamic PVN (-1.8 from bregma; coordinates from Paxinos & Watson, 1997) (Figure 1); frozen pituitaries were also sectioned in the coronal plane (14 um thickness) (Figure 2). Sections were thaw mounted onto poly-L-lysine (Sigma-Aldrich Canada Ltd., Oakville, O.N., Canada) and gelatin coated slides (Fisher Scientific Ltd., Nepean, O.N., Canada) and stored at -70°C until processing.

2. Probes

Hybridization histochemical localization was performed using purified antisense oligonucleotide probes labeled on the 3' end using terminal deoxynucleotidyl transferase (New England Biolabs Ltd., Mississauga, O.N., Canada) and [35S]-deoxyadenosine 5'-triphosphate (Amersham Biosciences Inc., Piscataway, N.J., USA) for CRH and AVP in the PVN and POMC in the pituitary, and antisense cRNA probes labeled with [35S]-deoxyuridine 5'-triphosphate (Amersham Biosciences Inc., Piscataway, N.J., USA) for CRH-R1 in the PVN and pituitary.

CRH oligonucleotide probes were synthesized according to the sequence by Jingami et al. (1985) and Young et al. (1986b); and AVP and POMC oligonucleotide probes were synthesized according to the sequence by Ivell and Richter (1984) and Young et al.
radiolabeled antisense and sense cRNA copies were synthesized from full-length rat (1.3 kb Pst I fragment) cDNA, generously donated by Dr. V. Viau, subcloned into a pBluescript SK transcription vector (Stratagene, San Diego, C.A., USA) encoding CRH-R mRNA according to the sequence by Perrin et al., (1993). The pBluescript SK transcription vector containing rat CRH-R cDNA was linearized with Bam H1 for antisense cRNA copies or Hind III for sense cRNA copies. Linearization of the plasmid was confirmed with a 1.2 % agarose gel with ethidium bromide (10mg/ml) in 1 X tris-acetate-EDTA (TAE) buffer and a 1 Kb DNA ladder (Invitrogen Canada Inc., Burlington, O.N., Canada, Cat #15615-016) in 6 X loading buffer (50 % glycerol, 1 X TAE, 1 % saturated Bromophenol Blue, 1 % xylene cyanol suspension) and photographed under UV illumination. Radioactive cRNA copies were synthesized by incubation of transcription buffer containing 200mM Tris-HCL (pH7.9), 30mM MgCl₂, 10mM spermidine, 50mM NaCl (Promega Riboprobe in vitro transcription system, Madison, W.I., USA), linearized plasmid in nuclease-free water (0.483 ug/ul), 0.1 M DTT, 2.5mM ATP/GTP/CTP, [³⁵S]-UTP, 1U RNasin (Promega, Madison, W.I., U.S.A.), and T7 (for antisense CRH-R cRNA copies) or T3 (for sense CRH-R cRNA copies) for 60 min at 37°C. Transcription was stopped by the addition of tRNA (25ug/ml) and RQ1 DNase, incubated for 30 min at 37°C and then placed directly on ice. Probes were purified on G-50 Sephadex columns (Roche RNA Quick Spin Columns, Indianapolis, I.N., USA, Cat # 274015).
Probe counts for CRH, AVP and POMC were $3.85 \times 10^5$ cpm/100 ul, $3.79 \times 10^5$ cpm/100 ul and $2.71 \times 10^5$ cpm/100 ul, respectively. Probe counts for CRH-R\textsubscript{1} were $2.4 \times 10^6$ cpm/100 ul for the PVN and $1.82 \times 10^6$ cpm/100 ul for the pituitary.

3. Hybridization

In situ hybridization procedures were adapted according to Zoeller (1993; 1994). Sections hybridized with CRH, AVP and POMC oligonucleotide probes were thawed at room temperature and fixed in 4 % formaldehyde in phosphate buffer for 60 min, washed in 1 X PBS (phosphate buffer solution), acetylated in 0.25 % acetic anhydride in 0.1 M triethanolamine hydrochloride, washed in 2 X SSC (standard saline citrate) and dehydrated in graded concentrations of ethanol (50, 75, 95 and 100 %), delipidated in chloroform and followed by a 100 % ethanol wash. A 50 % hybridization buffer containing dextran, Superpure formamide, 20 X SSC, 50X Denhardt’s, tRNA (25 mg/ml), 1 M NaPO4 buffer (pH 7.4), 1 % 1 M dithiothreitol (DTT) and probe was applied to slides (100 ul per slide = 25 ul/section), which were then cover slipped and incubated overnight at 37°C. Sections hybridized with CRH-R\textsubscript{1} cRNA probes were also fixed in 4 % formaldehyde in phosphate buffer for 60 min, washed in 1 X PBS, digested in Proteinase K (0.1 ug/ml) for 9 min at 37°C, acetylated in 0.25 % acetic anhydride in 0.1 M triethanolamine hydrochloride, washed in 2 X SSC and dehydrated in graded concentrations of ethanol (50, 75, 95 and 100 %), delipidated in chloroform and followed by a 100 % ethanol wash. Slides were saturated with 100 ul (25 ul/section) of a 75 % hybridization buffer and labeled probe, which were then cover slipped and incubated overnight at 55°C.

Following hybridization, coverslips for sections hybridized with CRH, AVP and POMC oligonucleotide probes were removed in 2 X SSC, tissue sections were washed in a
series of stringent saline solutions (2 X SSC at room temperature, 2 X SSC/DTT, 1 X SSC, 1 X SSC/50 % formamide all at 40°C, 1 X SSC, 0.5 X SSC at room temperature) and dehydrated in 70 % ethanol. Coverslips for sections hybridized with CRH-R₁ cRNA probes were removed in 2 X SSC and digested with an RNase A solution (50 mg/500 ml buffer for 60 min at 37°C) to remove remaining RNA probe bound non-specifically. Tissue sections were washed in a series of stringent saline solutions containing DTT (10 mM) (2 X SSC, 1 X SSC, 0.5 X SSC all at room temperature, 0.1 X SSC at 60°C, 0.1 X SSC at room temperature) and dehydrated in a series of graded ethanol solutions containing DTT (10 mM) (50, 75, 95 and 100 %).

Air-dried slides were exposed to Kodak Biomax MR Film (Eastman Kodak Co., Rochester, N.Y., USA). Exposure times were 5 days for CRH, 5 hr for AVP, 18 hr for POMC, 10 days for CRH-R₁ in the brain and 4 days for CRH-R₁ in the pituitary.

Slides with [³⁵S]-deoxyadenosine 5'-triphosphate labeled CRH and [³⁵S]-deoxyadenosine 5'-triphosphate AVP probes were coated with Kodak NTB2 liquid autoradiographic emulsion of a 1:1 (with RO·H₂O) dilution, dried and stored in light tight boxes with desiccant at 4°C for 37 d and 49 hr, respectively. Emulsion coated slides were developed with Kodak D-19 developer at 14°C and fixed with Kodak Polymax T fixer at 14°C. Slides were coverslipped and mounted with permamount.

4. Controls

To determine the optimal concentration of proteinase K buffer for the cRNA probe pre-hybridization washes, concentrations of 0.0, 0.1, 1.0 and 5.0 ug/ml of proteinase K were made and tested in a pilot in situ hybridization. cRNA probes were hybridized to the PVN and pituitary and exposed for 10 days for CRH-R₁ in the brain and 4 days for CRH-R₁.
in the pituitary for each concentration of proteinase K buffer. The optimal concentration was determined to be 0.1 ug/ml for 9 min at 37°C.

To confirm specificity of the probe, single-stranded antisense cRNA probes were hybridized to the PVN, pituitary, amygdala, dorsal hippocampus and arcuate nucleus to detect specific hybridization to complementary mRNA. Single-stranded sense cRNA probes were also hybridized to the PVN, pituitary, amygdala, dorsal hippocampus and arcuate nucleus as a negative hybridization control.

To confirm spatial distribution pattern, structures known to express and to not express target mRNA molecules were also hybridized with sense and antisense cRNA probes, and designated as positive (expressing) and negative (non-expressing) controls. For CRH-R1 mRNA in the PVN, the central medial amygdala, dorsal hippocampus (CA1 region) and pituitary were used as positive controls. For POMC, the arcuate nucleus was used as the positive control and the PVN as the negative control.

To confirm optimal exposure times for autoradiographic films and emulsions, additional slides hybridized to [35S]-labeled probes were exposed before and after developing at the optimal exposure time to determine whether autoradiographic films were under developed or saturated. Slides were also developed along with C-14 standards in order to establish the saturation threshold for autoradiographic films. Adrenalectomy slides were also included to establish the upper threshold of the AVP signal for autoradiographic emulsions.

5. Autoradiographic Analyses

Semiquantitative densitometric analysis was performed using SCION image (National Institutes of Health, Bethesda, M.D.) for PC imaging software. CRH, AVP and
CRH-R₁ mRNA levels for the PVN and, POMC and CRH-R₁ for the anterior pituitary were examined using autoradiographic film. The dorsomedial region of the parvocellular PVN for CRH and AVP mRNA levels were further examined using autoradiographic emulsions.

The SCION image system digitized the continuous range of image gray shades into 256 discrete gray levels. The levels obtained were converted to relative optical densities (ROD) using the formula: \( \text{ROD} = \log_{10} \left( \frac{256}{256 \text{-gray levels}} \right) \). The ROD are reported as mean density; the average gray values within the selection, which is the sum of the gray values of all the pixels in the selection divided by the number of pixels. The dorsomedial region of the parvocellular PVN was determined by comparing adjacent sections of \([^{35}\text{S}]\)-labeled CRH and AVP hybridized sections and outlined, thus forming a fixed window. The ROD within the dorsomedial parvocellular PVN was measured and the background was assessed by measuring the ROD when the fixed window was placed over an adjoining area of the brain where no specific CRH or AVP hybridization was detected. The optical density was determined by subtracting the ROD of the background from the ROD of the dorsomedial parvocellular PVN. The optical density of the PVN of each subject was measured for 2 to 3 sections from the anterior, middle and posterior regions of the PVN that were similar in shape to the fixed window. The greatest densities of autoradiographic grains were within the non-saturated portion of the linear calibration curve. Autoradiographic films for \([^{35}\text{S}]\)-labeled CRH-R₁ in the PVN and anterior pituitary and POMC in the anterior pituitary were analyzed similarly.

**G. Statistical Analyses**

All data were analyzed by appropriate analyses of variance (ANOVA) for factors of prenatal treatment (E, PF and C) and experimental condition. Significant main and
interaction effects were further analyzed by Newman-Keuls post hoc analysis. Values were expressed as mean ± standard error of the mean (SEM).
Figure 1  Diagrammatic Representation of the PVN in Rats

Diagram of the rat PVN (upper panel) and a photomicrograph of the rat PVN (lower panel). Light gray-coloured area represents the lateral magnocellular area of the PVN (upper panel). Dark gray-coloured area represents the medial parvocellular area of the PVN (upper panel). Photomicrograph of a representative coronal section of the PVN of a C female rat exposed to an acute 60 min restraint stress showing CRH-R₁ mRNA levels.
Figure 2  Diagrammatic Representation of the Anterior Pituitary in Rats

Diagram of the rat pituitary (upper panel) and a photomicrograph of the rat pituitary (lower panel). Light gray-coloured area represents the posterior pituitary (upper panel). Black-coloured area represents the intermediate pituitary (upper panel). Dark gray-coloured area represents the anterior pituitary (upper panel). Photomicrograph of a representative coronal section of the pituitary of a C male rat exposed to an acute 60 min restraint stress showing POMC mRNA levels.
CHAPTER 3: RESULTS

A. Developmental Data

1. Ethanol Intake and Blood Alcohol Levels

Ethanol intake by pregnant female dams averaged 9.51±0.63, 12.80±0.37 and 12.57±0.44 g/kg body weight/day for week 1, 2 and 3 of gestation, respectively. These intake levels resulted in blood alcohol levels (BALs) of 136-144 mg/dl.

2. Maternal Body Weights and Gestation Length

A repeated measures ANOVA on maternal weight gain during gestation (G) (Table 1) revealed significant effects of group \[F(2,28) = 23.85, p<0.001\] and day \[F(3,84) = 624.37, p<0.001\], as well as a group by day interaction \[F(6,70) = 32.78, p<0.001\]. E and PF dams weighed less than C dams on G14 and G21 (p's<0.005), and E dams also weighed less than PF dams on G21 (p<0.05). Analysis of maternal body weight gain during lactation (L) (Table 2) revealed significant effects of day \[F(3,84) = 100.45, p<0.0001\] and a group by day interaction \[F(6,45) = 10.30, p<0.0001\]. Body weights increased in all groups on L7 compared to L1 (p's<0.05). C dams continued to increase in weight on L14 (p<0.05) and weighed the same on L1 as on L21. E and PF dams weighed less on L21 compared to L7 and L14 (p's<0.05) but did not differ from C dams.

An overall ANOVA indicated main effects of prenatal treatment for gestation length \[F(2,27) = 18.20, p<0.0005\] (Table 3). Post hoc analyses revealed a longer gestation length for E dams compared to PF and C dams (p's<0.0005).
3. Pup Data

Analysis of number of live or stillborn offspring (Table 3) revealed main effects of prenatal treatment for number of live births \( [F(2,27) = 5.78, p<0.05] \); there were no differences for number of stillborn. Post hoc analyses revealed fewer live births for E compared to PF and C pups \( (p's<0.05) \).

A repeated measures ANOVA on pup weight gain (Figure 3a and 3b) revealed significant effects of group \( [F(2,58) = 5.88, p<0.005] \), day \( [F(3,174) = 5178.06, p<0.0001] \) and a group by day interaction \( [F(6,137) = 5.07, p<0.0005] \); there were no effects of sex. Post hoc analyses indicated E pups weighed less than C pups on PN21 \( (p<0.05) \) but there were no difference in pup weight gain between groups on PN1, PN8 and PN15. All groups showed an increase in pup weight on PN8, PN15 and PN21 \( (p's<0.0001) \).

B. Experimental Data

1. Body Weight at the Time of Testing

A repeated measures ANOVA on body weight at the time of testing revealed significant effects of day in females \( [F(2,136) = 16.53, p<0.0001] \) (Figure 4a). Across prenatal groups, females weighed less on d 4 compared to d 0 and d 9 of testing \( (p's<0.01) \). There were no differences in weight throughout testing among E, PF and C females. Analysis of body weights for males revealed significant effects of prenatal treatment \( [F(2,69) = 5.28, p<0.01] \) and day \( [F(2,138) = 6.65, p<0.005] \) (Figure 4b). E males weighed less than PF and C males at the start and throughout testing \( (p's<0.05) \). Similar to females, males in all groups weighed less on d 4 compared to d 0 and d 9 of testing \( (p's<0.05) \).
2. Adrenal Weight at the Time of Testing

An overall ANOVA (sex x prenatal treatment x experimental condition) indicated that females showed significantly higher adrenal:body weight ratios compared to males \( [F(1,138) = 605.64, p<0.0001; \text{data not shown}] \). Data were then further analyzed separately for females and males. For females, analysis of adrenal:body weight ratios revealed a main effect of prenatal treatment \( [F(2,69) = 3.28, p<0.05] \) (Table 4a). E females exhibited a higher adrenal:body weight ratio compared to PF females \( (p<0.05) \) but not C females. Analysis of adrenal:body weight ratios for males revealed a significant effect of prenatal treatment \( [F(2,69) = 3.71, p<0.05] \) and experimental condition \( [F(3,69) = 4.27, p<0.01] \) (Table 4b). E males showed a higher adrenal:body weight ratio compared to C males \( (p<0.05) \) and all groups exhibited a lower adrenal:body weight ratio following 1 d of restraint compared to the ratio for both basal and stress levels on d 10 \( (p's<0.05; \text{data not shown}) \). Further analyses revealed a higher adrenal:body weight ratio in E males compared to PF and C males following 10 d of restraint \( (p's<0.05) \).

3. Plasma CORT

Due to the sexual dimorphism of the HPA axis (Kitay, 1961; 1963), hormone data were analyzed separately for females and males.

An overall ANOVA (prenatal treatment x experimental condition) revealed a significant effect of experimental condition for both females \( [F(5,88) = 57.49, p<0.0001] \) (Figure 5a) and males \( [F(5,89) = 72.27, p<0.0001] \) (Figure 5b). Plasma CORT levels were higher following 1 d of restraint compared to 10 d of restraint and both basal conditions for both females \( (p's<0.05) \) and males \( (p's<0.05) \). There were no other differences among E, PF and C females or males.
4. Plasma ACTH

An overall ANOVA revealed significant effects of experimental condition for both females \([F(5,94) = 35.55, p<0.0001]\) (Figure 6a) and males \([F(5,90) = 62.27, p<0.0001]\) (Figure 6b). Post hoc analyses indicated that both females and males had higher plasma ACTH levels following 1 d of restraint compared to 10 d of restraint and both basal conditions \((p's<0.0005)\). Furthermore, E females had higher basal plasma ACTH levels compared to PF and C females on d 1 \((p's<0.005)\). Additionally, E females showed a trend towards higher plasma ACTH levels following 10 d of restraint compared PF and C females \((p's<0.10)\). For males, in contrast, E males had lower plasma ACTH levels following 10 d of restraint compared to PF and C males \((p's<0.05)\).

5. CRH mRNA

To be consistent with hormone data, data on mRNA levels were also analyzed separately for females and males.

Overall ANOVAs indicated that females (Figure 9a) but not males (Figure 9b) exhibited higher CRH mRNA over basal levels following 1 d of restraint \([F(3,66) = 3.64, p<0.05]\), with a return to basal levels following 10 d of restraint. However, for both females and males CRH mRNA levels were not differentially altered in E compared to PF and C animals following restraint.

6. AVP mRNA

An overall ANOVA indicated main effects of prenatal treatment \([F(2,67) = 4.65, p<0.05]\), experimental condition \([F(3,67) = 6.27, p<0.001]\) and a prenatal treatment by experimental condition interaction for females \([F(6,67) = 2.54, p<0.05]\) (Figure 12a).
Overall, E females exhibited higher levels of AVP mRNA compared to PF and C females (p's<0.05). E females had higher levels of AVP mRNA following 1 d of restraint compared to both basal conditions, (p’s<0.05) and had higher AVP mRNA levels following both 1 d and 10 d of restraint compared to PF and C females (p’s<0.005). Additionally, E females exhibited higher AVP mRNA compared to C females following 10 d of restraint compared to basal levels on d 10 (p’s<0.05). Similar to females, an overall ANOVA revealed a main effect of prenatal treatment for males [F(2,65) = 4.31, p<0.05] (Figure 12b). Overall, E and PF males showed higher AVP mRNA levels compared to C males (p’s<0.05). E males also showed a trend towards higher AVP mRNA levels under basal conditions on d 1 (p<0.10), and higher AVP mRNA levels following 10 d of restraint compared to C males (p<0.05).

7. CRH:AVP Ratio

Examination of the CRH:AVP ratio indicated that E females had a lower CRH:AVP ratio compared to PF and C females following 10 d of restraint (p’s<0.05) (Figure 13a). Additionally, E but not PF or C females had a lower CRH:AVP ratio following 10 d of restraint compared to basal levels on d 10 (p’s<0.01). For males, an overall ANOVA revealed a main effect of prenatal treatment [F(2,66) = 7.90, p<0.001] (Figure 13b). Overall, E and PF males had a lower CRH:AVP ratio compared to C males (p’s<0.05). E males also showed a lower CRH:AVP ratio compared to C males under basal conditions on d 1 (p<0.05). Additionally, E and PF males showed lower basal and stress CRH:AVP ratios on d 10 compared to C males (p’s<0.05).
8. CRH-R₁ mRNA in the PVN and Anterior Pituitary

An overall ANOVA for CRH-R₁ mRNA levels in the PVN of the hypothalamus indicated experimental condition effects for both females [F(3,70) = 92.78, p<0.0001] (Figure 16a) and males [F(3,68) = 189.03, p<0.0001] (Figure 16b). Basal CRH-R₁ mRNA levels on d 1 and d 10 were undetectable in E, PF and F females and males. Further analyses revealed that 1 d of restraint resulted in higher CRH-R₁ mRNA levels compared to 10 d of restraint and both basal conditions for females (p’s<0.0005) and males (p’s<0.0005). Further examination revealed a higher 1d:10d CRH-R₁ mRNA ratio in E females compared to PF and C females (p’s<0.05) (Figure 17). In addition, following 10 d of restraint E and PF males showed a trend towards higher CRH-R₁ mRNA levels compared to C males (p’s<0.10). Further analyses confirmed a higher 1d:10d CRH-R₁ mRNA ratio in E and PF males compared to controls (p’s<0.005) (Figure 17). There were no significant differences among E, PF and C females (Figure 20a) or males (Figure 20b) in anterior pituitary CRH-R₁ mRNA under any experimental condition.

9. POMC mRNA

For females, an overall ANOVA revealed a significant effect of prenatal treatment for POMC mRNA levels in the anterior pituitary [F(2,65) = 4.33, p<0.05] (Figure 23a). Overall, E females had lower POMC mRNA levels compared to PF and C females (p’s<0.05). Further analyses revealed higher POMC mRNA levels following 1 d of restraint and under basal conditions on d 10 compared to basal levels on d 1 for all prenatal groups (p’s<0.05). For males, an overall ANOVA indicated a significant effect of experimental condition [F(3,66) = 3.87, p<0.05] and a prenatal treatment by experimental condition interaction for males [F(6,66) = 2.40, p<0.05] (Figure 23b). Post hoc analyses revealed
higher basal and stress POMC mRNA levels on d 10 compared to basal levels on d 1 (p’s<0.05). Further, E males had lower basal POMC mRNA levels on d 1 and d 10 compared to PF and C males (p’s<0.05). Additionally, exposure to 1 d and 10 d of restraint stress (p’s<0.05) increased POMC mRNA levels over basal levels on d 1 in E males but not PF and C males. There was also a trend towards higher POMC mRNA levels following 10 d of restraint in E and PF compared to C males (p’s<0.10).
Table 1  Maternal body weights (g) (mean ± SEM) during gestation

Maternal body weights of E, PF and C Dams during gestation (G) (n = 13 per group). There was a significant main effect of group (p<0.001), day (p<0.001) and a group x day interaction (p<0.001) for E, PF and C dams during gestation. On G14 and G21, E and PF dams weighed significantly less than C dams (* p's<0.005). On G21, E dams weighed significantly less than PF dams (^ p<0.05).

<table>
<thead>
<tr>
<th>Diet</th>
<th>G1</th>
<th>G7</th>
<th>G14</th>
<th>G21</th>
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</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>267.3±3.8</td>
<td>260.5±4.0</td>
<td>285.3±4.5*</td>
<td>336.4±6.9*^</td>
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<tr>
<td>Pair-fed</td>
<td>270.1±4.5</td>
<td>267.3±3.9</td>
<td>294.4±3.5*</td>
<td>358.5±4.5*</td>
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<tr>
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<td>286.2±2.4</td>
<td>325.4±3.8</td>
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Table 2  Maternal body weights (g) (mean ± SEM) during lactation

Maternal body weights of E, PF and C Dams during lactation (L) (n = 10-13 per group). There was a significant main effect of day (p<0.0001) and a group x day interaction (p<0.0001). On L7, all E, PF and C dams increased in weight compared to L1 (* p’s<0.05). On L14, C dams increased in weight compared to L1 and L21 (^ p’s<0.05). E and PF dams weighed less than on L21 compared to L7 and L14 but did not differ from C dams (~ p’s<0.05).

<table>
<thead>
<tr>
<th>Diet</th>
<th>L1</th>
<th>L7</th>
<th>L14</th>
<th>L21</th>
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<td>305.8±4.9</td>
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<td>335.5±4.6</td>
<td>315.5±4.3~</td>
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<tr>
<td>Control</td>
<td>316.4±4.2</td>
<td>327.0±3.1*</td>
<td>335.0±5.1^</td>
<td>308.8±2.2</td>
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Table 3  Gestation data of E, PF and C Dams

Gestation length (mean ± SEM), number of live-born pups (mean ± SEM) and number of stillborn pups (mean ± SEM) of E, PF and C Dams (n = 10-13 per group). Gestation length of E dams were significantly greater compared to both PF and C dams (* p’s<0.0005). The number of live-born pups of E dams were significantly less than both PF and C dams (^ p’s<0.05). However, there were no significant differences among E, PF and C dams in the number of stillborn pups.

<table>
<thead>
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<th>Prenatal Treatment</th>
<th>gestation length</th>
<th>live-born</th>
<th>stillborn</th>
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<tr>
<td>Pair-fed</td>
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<td>0.2±0.1</td>
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<tr>
<td>Control</td>
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Table 4  Adrenal:body weight ratios (x 10^5) (mean ± SEM) at time of testing

Adrenal:body weight ratios of E, PF and C female (upper panel) and male (lower panel) rats under basal conditions on d 1 and following 10 d of restraint (n = 5-7 per condition). There was a significant main effect of prenatal treatment for both females and males (p’s<0.05), and for males, a significant main effect of experimental condition (p<0.01). Overall, E females had a greater adrenal:body weight ratio compared to PF females (p<0.05). Overall, E males had a greater adrenal:body weight ratio compared to C males (p<0.05). Following 10 d of restraint, E males had a greater adrenal:body weight ratio compared to both PF and C males (* p’s<0.05).

a. female

<table>
<thead>
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<th>Prenatal treatment</th>
<th>Basal d1</th>
<th>10d restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad wt</td>
<td>Bdy wt</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.68±0.24</td>
<td>274.3±12.7</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.35±0.53</td>
<td>293.2±16.0</td>
</tr>
<tr>
<td>Control</td>
<td>6.93±0.35</td>
<td>291.5±8.1</td>
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</table>

b. male

<table>
<thead>
<tr>
<th>Prenatal treatment</th>
<th>Basal d1</th>
<th>10d restraint</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ad wt</td>
<td>Bdy wt</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.22±0.27</td>
<td>491.3±20.3</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>5.82±0.39</td>
<td>545.3±10.5</td>
</tr>
<tr>
<td>Control</td>
<td>5.30±0.26</td>
<td>537.2±19.0</td>
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</tbody>
</table>

Ad wt = Adrenal weight; Bdy wt = Body weight; Ad/Bdy = Adrenal:body weight ratio
Postnatal (PN) body weights of E, PF and C female (left panel) and male (right panel) pups on PN1, PN8, PN15 and PN21 (n = 10-13 litters per group). There was a significant main effect of group (p<0.005), day (p<0.0001) and a group x day interaction (p>0.0005) for both female and male pups in all prenatal treatment conditions. On PN21, both E female and male pups weighed less than C female and male pups, respectively (* p's<0.05). Both female and male pups in all prenatal treatment groups increased in weight from PN1 through to PN21 (^ p's<0.0001).
Body weights of E, PF and C female (left panel) and male (right panel) rats at time of testing (n = 5-7 per condition). There was a significant main effect of day in both females and males (p's<0.005), and in males, a significant main effect of prenatal treatment (p<0.01). On d 4 of testing, both females and males in all prenatal treatment conditions had lower body weights compared to day 0, with a recovery in body weights by d 9 of testing (* p's<0.05). Overall, E males weighed less than both PF and C males (p’s<0.05).
Figure 5  Plasma CORT levels (ug/dl) (mean ± SEM)

Plasma CORT levels of E, PF and C females (upper panel) and males (lower panel) under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, both females and males had greater plasma CORT levels following 1 d of restraint compared to 10 d of restraint, and greater plasma CORT levels following 10 d of restraint compared to both basal conditions (* p’s<0.05). However, there were no significant differences among E, PF and C females or males in any experimental condition. Table contains plasma CORT values for E, PF and C females (left columns) and males (right columns) under 1d and 10d restraint conditions at 4 hr. No significant differences among E, PF and C females or males.

Plasma CORT levels (ug/dl) (mean ± SEM) at 4 hr after restraint onset

<table>
<thead>
<tr>
<th>Prenatal treatment</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1d restraint</td>
<td>10d restraint</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.24±0.13</td>
<td>2.31±0.77</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>1.09±0.05</td>
<td>1.68±0.28</td>
</tr>
<tr>
<td>Control</td>
<td>6.12±2.34</td>
<td>6.51±2.50</td>
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</table>
Plasma ACTH levels of E, PF and C females (upper panel) and males (lower panel) under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, both females and males had greater plasma ACTH levels following 1 d of restraint compared to 10 d of restraint, and greater plasma ACTH levels following 10 d of restraint compared to both basal conditions (* p’s<0.0005). E females had greater plasma ACTH levels under basal conditions on d 1 (^ p’s<0.005) and following 10 d of restraint (# p’s<0.10) compared to both PF and C females. E males had lower plasma ACTH levels following 10 d of restraint compared to PF and C males (+ p’s<0.05). Table contains plasma ACTH values for E, PF and C females (left columns) and males (right columns) under 1d or 10d restraint at 4 hr. No significant differences among E, PF or C females of males.

Plasma ACTH levels (pg/ml) (mean ± SEM) at 4 hr after restraint onset

<table>
<thead>
<tr>
<th>Prenatal treatment</th>
<th>Female 1d restraint</th>
<th>Female 10d restraint</th>
<th>Male 1d restraint</th>
<th>Male 10d restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>45.29±3.49</td>
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<tr>
<td>Pair-fed</td>
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<td>50.87±5.41</td>
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<tr>
<td>Control</td>
<td>50.03±6.40</td>
<td>49.47±1.97</td>
<td>50.02±4.25</td>
<td>45.87±2.91</td>
</tr>
</tbody>
</table>
Figure 7  Dark-field photomicrographs of CRH mRNA levels in the dorsomedial PVN

Representative dark-field photomicrographs of CRH mRNA levels of E, PF and C females in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 8  Dark-field photomicrographs of CRH mRNA levels in the dorsomedial PVN

Representative dark-field photomicrographs of CRH mRNA levels of E, PF and C males in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 9  CRH mRNA levels in the dorsomedial PVN

CRH mRNA levels of E, PF and C females (upper panel) and males (lower panel) in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, females had greater CRH mRNA levels following 1 d of restraint compared to basal conditions on d 1 (* p<0.05). However, there were no significant differences among E, PF and C females within any experimental condition. There were also no differences among E, PF and C males in any experimental condition.
Figure 10  Dark-field photomicrographs of AVP mRNA levels in the dorsomedial PVN

Representative dark-field photomicrographs of AVP mRNA levels of E, PF and C females in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 11  Dark-field photomicrographs of AVP mRNA levels in the dorsomedial PVN

Representative dark-field photomicrographs of AVP mRNA levels of E, PF and C males in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Pair-fed</th>
<th>Control</th>
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</thead>
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<tr>
<td>Basal d1</td>
<td></td>
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<td>1d restraint</td>
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<td>Basal d10</td>
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<tr>
<td>10d restraint</td>
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</table>
AVP mRNA levels of E, PF and C females (upper panel) and males (lower panel) in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, E females had greater AVP mRNA levels compared to both PF and C females (p's<0.05). E females had greater AVP mRNA levels following 1 d of restraint compared to both basal conditions (* p's<0.05), and following both 1 d and 10 d of restraint compared to PF and C females (^ p's<0.005). E females had greater AVP mRNA levels compared to C females following 10 d of restraint compared to basal conditions on d 10 (+ p's<0.05). Overall, E and PF males had greater AVP mRNA levels compared to C males (p's<0.05). E males had greater AVP mRNA levels compared to C males under basal conditions on d 1 (# p<0.10) and following 10 d of restraint (~ p<0.05).
Figure 13  CRH:AVP ratio in the dorsomedial PVN

CRH:AVP ratio of E, PF and C females (upper panel) and males (lower panel) in the dorsomedial PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). E females had a lower CRH:AVP ratio compared to PF and C females following 10 d of restraint (* p’s<0.05), and a lower CRH:AVP ratio compared to basal conditions on d 10 (^ p’s<0.01). Overall, E and PF males had a lower CRH:AVP ratio compared to C males (p’s<0.05). E males had a lower CRH:AVP ratio compared to C males under basal conditions on d 1 (+ p<0.05). Both E and PF males had a lower CRH:AVP ratio compared to C males under basal conditions on d 10 and following 10 d of restraint (# p’s<0.05).
Figure 14  Photomicrographs of CRH-R₁ mRNA levels in the PVN

Representative photomicrographs of CRH-R₁ mRNA levels of E, PF and C females in the PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 15  Photomicrographs of CRH-R₁ mRNA levels in the PVN

Representative photomicrographs of CRH-R₁ mRNA levels of E, PF and C males in the PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
CRH-R₁ mRNA levels of E, PF and C females (upper panel) and males (lower panel) in the PVN under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, both females and males had greater CRH-R₁ mRNA levels following 1 d of restraint compared to 10 d of restraint, and greater CRH-R₁ mRNA levels following 10 d of restraint compared to both basal conditions (* p’s<0.0005). Basal CRH-R₁ mRNA levels on d 1 and d 10 were undetectable for E, PF and C females and males. E and PF males had greater CRH-R₁ mRNA levels compared to C males following 10 d of restraint (^ p<0.10).
Figure 17  1d:10d ratio of CRH-R1 mRNA levels in the PVN

1d:10d ratio of CRH-R1 mRNA levels of E, PF and C females and males in the PVN following 1 d or 10 d of restraint (n = 5-7 per condition). E females had a higher 1d:10d ratio of CRH-R1 mRNA levels compared to PF and C females (* p’s<0.05). Both E and PF males had a higher 1d:10d ratio of CRH-R1 mRNA levels compared to C males (^ p’s<0.005).
Figure 18  Photomicrographs of CRH-R₁ mRNA levels in the anterior pituitary

Representative photomicrographs of CRH-R₁ mRNA levels of E, PF and C females in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Representative photomicrographs of CRH-R\textsubscript{1} mRNA levels of E, PF and C males in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 20  CRH-R₁ mRNA levels in the anterior pituitary

CRH-R₁ mRNA levels of E, PF and C females (upper panel) and males (lower panel) in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). There were no significant differences among E, PF and C females or males in any experimental conditions.
Figure 21 Photomicrographs of POMC mRNA levels in the anterior pituitary

Representative photomicrographs of POMC mRNA levels of E, PF and C females in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 22  Photomicrographs of POMC mRNA levels in the anterior pituitary

Representative photomicrographs of POMC mRNA levels of E, PF and C males in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint.
Figure 23  POMC mRNA levels in the anterior pituitary

POMC mRNA levels of E, PF and C females (upper panel) and males (lower panel) in the anterior pituitary under basal conditions on d 1 or d 10, or following 1 d or 10 d of restraint (n = 5-7 per condition). Overall, females had greater POMC mRNA levels following 1 d of restraint and under basal conditions on d 10 compared to basal conditions on d 1 (* p's<0.05). However, overall, E females had lower POMC mRNA compared to PF and C females (p's<0.05). Overall, males had greater POMC mRNA levels under basal and stress conditions on d 10 compared to basal conditions on d 1 (^ p's<0.05). In addition, E males had lower POMC mRNA levels compared to PF and C males under basal conditions on d 1 and d 10 (+ p's<0.05). However, following both 1 d and 10 d of restraint POMC mRNA levels increased in E but not PF and C males, compared to d 1 basal conditions (# p's<0.05), and E and PF males also had greater POMC mRNA levels compared to C males following 10 d of restraint (~ p<0.10).
CHAPTER 4: DISCUSSION

The objective of this thesis was to compare responses of E and control animals to acute and repeated restraint stress, and to determine how hormonal hyperresponsiveness is mediated by alterations in central regulation of the HPA axis.

The present study extends previous work in our laboratory (Weinberg et al., 1996) and others (Lee et al., 2000a; 1990; Redei et al., 1993) examining the long term adverse effects of prenatal ethanol exposure on HPA responsiveness and on central changes underlying the increased response to stress observed in these animals. These findings demonstrate that prenatal ethanol exposure results in altered central regulation of the HPA axis following exposure to stressors. In the paradigm utilized here, E animals do show habituation to a mild repeated stress in terms of plasma hormone levels. However, E, PF and C animals exhibit differential alterations in central regulation. Differences among E, PF and C animals following exposure to both acute and repeated stress do not appear to be mediated by CRH mRNA in the PVN or CRH-R₁ mRNA in the anterior pituitary, but at least following repeated stress may in part be due to a greater shift toward AVP regulation and/or increased levels of CRH-R₁ mRNA in the PVN. For males but not females, it appears that an increase in pituitary POMC mRNA levels may also play a role in hyperresponsiveness. Together, these data suggest that increased HPA drive and/or increased pituitary responsiveness to secretagogues may be differentially involved in mediating HPA hyperresponsiveness in females and males prenatally exposed to ethanol. In addition, these results further suggest that differences between females and males in response to stress involve the interaction of the HPA and HPG axes.
A. Effects of Ethanol on Pregnancy Outcome

In the present study, maternal ethanol intake was consistently high throughout gestation, ranging from 9.51 to 12.57 g/kg body weight over the 3 weeks of gestation. This range of ethanol intake resulted in BALs of approximately 136-144 mg/dl. E and PF dams were similar in body weights through most of gestation and lactation, indicating that the lower maternal weight gain in E dams was in part mediated by ethanol-induced nutritional effects. However, direct effects of ethanol were also observed. E dams had longer gestation lengths and smaller litters than both PF and C dams. In addition, E pups had lower body weights compared to C pups at weaning. These data demonstrating adverse effects of maternal ethanol consumption on maternal weights and pregnancy outcome are consistent with previous data from our laboratory (Weinberg et al., 1995; Weinberg, 1985).

B. Effects of Acute and Repeated Restraint Stress

However, repeated restraint stress resulted in lower body weights in females and males in all prenatal treatment groups half way through testing, with a recovery by the final day of testing. The initial decrease in weight and recovery by the final day of testing indicate that this repeated stress paradigm was severe enough to cause a change in body weight, but mild enough for habituation to occur. During testing, E males had larger adrenals compared to both PF and C males following exposure to repeated stress. E males also had lower body weights compared to both PF and C males during testing, which indicates that the adverse effects of prenatal ethanol exposure on growth can persist into adulthood.

As expected, plasma ACTH and CORT levels were increased following exposure to acute stress in females and males across all prenatal groups, with attenuation following exposure to repeated stress. In addition, following exposure to acute and repeated stress,
plasma ACTH and CORT levels measured 4 hr after stress induction did not differ from basal levels (data not shown). This indicates that all animals habituated hormonally to the repeated stress and that this paradigm is mild enough for habituation to occur. However, direct effects of ethanol were observed. E females exhibited elevated plasma ACTH levels following exposure to repeated stress compared to both PF and C females, which suggests that E females show deficient or delayed habituation. These results are consistent with previous data from our laboratory, where E females subjected to 10 d of repeated restraint stress continued to show significant elevations of ACTH, whereas PF and C females did not differ from basal levels (Weinberg et al., 1996). In contrast, in E males, glucocorticoid negative feedback appears to be deficient as exhibited by lower plasma ACTH levels following exposure to repeated stress compared to PF and C males. It is unclear why E males exhibited lower plasma ACTH levels following repeated stress, thus further studies are warranted. It is becoming increasingly clear that E females and males respond differentially to stress, and these differences may be revealed depending on the nature and intensity of the stressor and the hormonal endpoint measured (Weinberg, 1992a).

E females exhibited elevated basal plasma ACTH levels, suggesting increased HPA drive. We did not observe central alterations in E females under basal conditions. However, a previous study in our lab (Glavas et al., unpublished) found that E females show elevated basal CRH mRNA levels suggesting increased HPA drive. Furthermore, Lee & Rivier (1994) have shown decreased basal CRH levels in the median eminence in E and PF rats compared to control rats suggesting increased peptide release or a smaller readily releasable pool of CRH. The lower CRH concentration may be due to an alcohol-induced translational efficiency of CRH mRNA and/or enhanced CRH protein degradation (Lee &
Thus, further investigation of the mechanism leading to decreased CRH levels in the median eminence is warranted. Furthermore, the possibility of a deficit in inhibitory regulation of glucocorticoids on basal plasma ACTH levels cannot be ruled out because basal plasma ACTH levels are regulated by glucocorticoids (Dallman & Jones, 1973; Aguilera, 1994). In contrast to the present study, previous studies have found no difference in basal plasma ACTH levels among E, PF and C rats regardless of whether method of alcohol administration involved exposure to ethanol vapors (Lee et al., 1990; 2000a) or exposure to liquid diet (Kim et al., 1999a). Further studies are needed to determine why differences in basal ACTH levels occur among studies. Although the results from the present study differ from previous findings, the difference in plasma ACTH response provides further support for the suggestion that HPA drive is increased in E animals.

In this study, a 1 hr restraint stress was chosen to detect optimal changes in mRNA levels following exposure to stress. This time point may not have been optimal to show ACTH and/or CORT hyperresponsiveness in E animals. For example, our laboratory has shown that E animals exhibit increased and prolonged elevations of plasma ACTH and CORT following exposure to repeated restraint stress when sampled during recovery (Weinberg et al., 1996).

C. Effects of Acute Stress in Central Regulation of the HPA Axis

There is general agreement that CRH mRNA is elevated in the PVN following exposure to acute stress (Ma et al., 1997a; 1997b; Ma & Lightman, 1998), which was confirmed in the present study for females. Females in all prenatal treatment groups exhibited higher CRH mRNA levels following exposure to a 1 hr restraint. In contrast, there were no increases in CRH mRNA levels in males in any prenatal treatment group. It is possible that
the 4 hr time point may not have been optimal for detecting changes in CRH mRNA levels in males for our restraint paradigm. This study was not designed to examine temporal changes in CRH mRNA levels but to examine CRH mRNA levels at one time point following exposure to stress. A detectable change in CRH mRNA following exposure to restraint stress has a window of 3 to 6 hr (Ma et al., 1997a; 1997b; Ma & Lightman, 1998). Future studies will examine a time course of changes in CRH heteronuclear (hn) RNA in both females and males in response to restraint.

It is also possible that males did not show any changes in CRH mRNA due to technical factors. In situ hybridization may not be a sensitive enough technique to detect small increases in large basal CRH mRNA pools (Kovacs & Sawchenko, 1996). Another possibility relates to increased mRNA stability and translational efficacy. Pools of mRNA could remain stable but biosynthesis to replenish released peptides could become more efficient; thus, detectable changes in mRNA are not always concomitant with changes in transcriptional activity following exposure to stress (Whitnall, 1993). Changes in CRH mRNA levels may not provide information regarding the temporal relationship between transcriptional activation and the stimulatory event. However, changes in CRH mRNA pools can provide information regarding habituation to stimulation (Herman et al., 1992).

According to the present results, E animals may not show hyperresponsiveness at the level of CRH mRNA, at least in response to acute 1 hr restraint stress. However, changes in CRH in E animals may be at the level of their primary transcripts. It is known that there is a temporal lag between stress induction and detectable changes in CRH mRNA pools (Dent et al., 2000). To detect immediate changes in activity of PVN CRH cell bodies in response to a stressor, immediate early genes (IEG) such as c-fos and nerve growth factor I-B (NGFI-B)
are used as markers to indicate activation of neurons in response to stress (Kovacs & Sawchenko, 1996). E animals exposed to footshock exhibited increased IEG mRNA levels related to transcription of genes coding for CRH and increased CRH hnRNA, a primary transcript, compared to C animals, suggesting increased activity of PVN CRH as a potential mechanism through which prenatal ethanol exposure may alter the HPA axis (Lee et al., 2000a). Furthermore, despite robust increases in primary CRH transcripts, upregulation of CRH mRNA levels are not always detected. This may also be due to technical factors, such as the inability to detect small increases in large basal mRNA pools, or alterations that occur in regulating secretion, axonal transport, transcription, and post-transcriptional processing events (Kovacs & Sawchenko, 1996).

On the other hand, whether changes in parvocellular AVP mRNA levels occur following exposure to acute stress is complex. No changes were detected in AVP mRNA 4 or 6 hr after a single restraint (Ma & Lightman, 1998; Pinnock & Herbert, 2001), whereas, another study reported that a single episode of restraint increased both AVP hnRNA and mRNA in the parvocellular PVN measured 90 min following restraint induction (Herman, 1995). These studies indicate that detection of AVP mRNA at different time points provide different results, possibly due to the short half-life of AVP mRNA levels. Control rats exhibited large decrements in the number of AVP mRNA producing neurons over 90 and 120 min post-stress time points (Herman, 1995). We found no increase in AVP mRNA 4 hr following exposure to acute stress in males in any prenatal treatment group; however, E females exhibited increased AVP mRNA levels compared to both PF and C females. These results are in agreement with the study of Lee et al. (2000a) who found a marginal increase in AVP hnRNA in response to footshock in E compared to C females. Although, E males in the
The present study showed no increase in AVP mRNA levels following exposure to acute stress, they did however exhibit increased basal AVP mRNA levels. One possibility explaining increased basal AVP mRNA levels in E males is altered gonadal influences on basal function due to the interaction of the HPG and HPA axes. Testosterone is involved in regulating AVP expression in the PVN under basal and stress conditions (Viau & Meaney, 1996) via androgen receptors in the amygdala in males (Viau et al., 2001). ADX was shown to increase AVP mRNA levels in male rats, which were attenuated by GDX and reversed by testosterone replacement (Viau et al., 2001). Furthermore, GDX increases hypothalamic CRH immunoreactivity by removing an androgen inhibitory tone on the HPA axis (Bingamen et al., 1994), again suggesting that gonadal steroids influence basal regulation of the HPA axis.

The finding that prenatal ethanol exposure resulted in increased AVP gene expression in females following exposure to acute stress, whereas males exhibited increased AVP gene expression under basal conditions suggests that increased HPA drive may play a role in HPA hyperresponsiveness in E animals. These data also support previous findings that ethanol differentially affects female and male offspring depending on the nature and intensity of the stressor, and the time course, and endpoint measured (Halasz et al., 1993; Weinberg, 1988; Weinberg et al., 1996).

Previous studies have shown that CRH-R₁ mRNA levels are undetectable in the PVN under basal conditions (Mansi et al., 1996; Rivest et al., 1995; Imaki et al., 2001; 1996). However, following exposure to acute restraint stress, CRH-R₁ and CRH mRNA in the PVN are typically upregulated (Imaki et al., 2001; 1996). The upregulation of CRH-R₁ mRNA appears to represent an adaptive response to stress (Mansi et al., 1996). It has been suggested that upregulation of PVN CRH-R₁ may be a mechanism which the HPA axis employs to
remain responsive to stress (Ono et al., 1985; Rivest et al., 1995; Imaki et al., 1996; 2001; Mansi et al., 1996; Champagne et al., 1998; Jezova et al., 1999; Drolet & Rivest, 2001;). As expected, we found undetectable basal CRH-R\textsubscript{1} mRNA levels and upregulation of CRH-R\textsubscript{1} mRNA in the PVN in both females and males in all prenatal treatment groups following exposure to acute stress. The existence of an ultrashort positive feedback loop in the control of CRH during stress has been suggested by Ono et al. (1985). Tract-tracing studies, histochemical mapping, retrograde transport of wheat-germ-conjugated gold particles and immunohistochemical studies provide anatomical support for a positive feedback loop at the level of the PVN (Swanson et al., 1987; Champagne et al., 1998; Drolet & Rivest, 2001). Whether the present study provides support for the ultrashort feedback loop remains to be elucidated, considering that the PVN can be activated via multiple extrahypothalamic stressor specific pathways. For example, following hemorrhage and/or hypotension, catecholaminergic pathways can activate the HPA axis, while behavioural and cardiovascular responses to stress activate the HPA axis via the central medial and cortical amygdaloid nuclei (Palkovits et al., 1998; Herman & Cullinan, 1997). The PVN receives direct cortical inputs from the amygdala via the lateral hypothalamus ventral amygdalofugal and stria terminalis pathway (Palkovits et al., 1998). One study found that exposure to a psychological stressor, where non-footshocked rats are surrounded by footshocked rats, resulted in elevated CRH mRNA levels and CRH content in the central amgydala. Furthermore, the psychological stressor resulted in a small but significant increase in CRH-R\textsubscript{1} mRNA in the PVN with no changes in CRH mRNA levels or content (Makino et al., 1999). Thus, elevated levels of CRH-R\textsubscript{1} gene expression in the PVN in both E females and males may also be due to increased HPA drive from extrahypothalamic structures such as the amygdala.
In contrast, CRH-R₁ mRNA in the anterior pituitary was not altered by stress in females or males in any prenatal treatment group. Studies have shown that in response to acute immobilization (Makino et al., 1995b) and CRH injection (Hauger & Aguilera, 1993) CRH-R₁ mRNA in the anterior pituitary decreases. One possibility is that our restraint stress paradigm was not severe enough to result in a decrease in CRH-R₁ mRNA levels. For example, immobilization is a fairly severe stressor that often involves taping down the limbs of the animal and placing them dorsal surface up (Makino et al., 1995b). On the other hand, restraint stress is primarily a psychological stressor that does not involve physical pain or injury. Another possibility relates to the temporal relationship between stress induction and changes in mRNA pools. The temporal lag of 3 to 6 hr between stress induction and the changes seen in mRNA pools do not reflect immediate changes observed in the response to stress. Changes in CRH-R₁ mRNA may represent the replenishing of releasable peptide stores in order to prepare for exposure to subsequent or prolonged challenges. As an immediate response to stress, an adaptive response may be at the level of receptor internalization and desensitization.

The present study showed that females in all prenatal treatment groups exhibited elevated POMC mRNA levels following exposure to acute stress, which is consistent with a previous study that reported increased POMC mRNA in the anterior pituitary following exposure to an acute 1 hr restraint stress (Harbuz & Lightman, 1989). E males also exhibited elevated POMC mRNA levels following exposure to acute stress, which suggests increased release of ACTH and β-endorphin. These data are consistent with previous data from our laboratory indicating elevated levels of both ACTH and β-endorphin in E males following exposure to an acute 1 hr stress (Weinberg et al., 1996). Together with data indicating that
the immediate release of ACTH and β-endorphin in response to stress may not correlate with increased POMC mRNA levels measured 3 to 6 hr after stress induction; these findings suggest that an adaptive response in E males may be to increase the amount of readily releasable stores of pituitary POMC-derived peptides. Furthermore, increased POMC mRNA levels in E males following acute stress suggests increased HPA drive and/or increased pituitary responsiveness to secretagogues. E males also exhibited lower basal POMC mRNA levels compared to both PF and C males. Disagreement exists with regard to basal pituitary activity in E animals, as investigators have reported both decreased (Halasz et al., 1993) and increased (Redei et al., 1993) basal POMC mRNA levels. Both of the previous studies employed the same method of alcohol administration during pregnancy and measured POMC mRNA levels via RNA extraction method. Thus, more studies are needed to resolve these differences.

D. Effects of Repeated Stress in Central Regulation of the HPA Axis

Following exposure to repeated stress, CRH mRNA levels were not elevated in either females or males across all prenatal treatment groups, which is consistent with previous results regarding repeated restraint stress (Ma & Lightman, 1998; Pinnock & Herbert, 2001), suggesting habituation over time. In contrast, there is general agreement that repeated restraint stress results in upregulation of parvocellular AVP mRNA in the PVN (Makino et al., 1995a; Pinnock & Herbert, 2001; Ma & Lightman, 1998; Ma et al., 1999). Our data showed that following exposure to repeated restraint stress, both E females and males exhibited increased AVP mRNA levels compared to C females and males, respectively, which is in agreement with increased hnAVP levels in response to footshock in E animals (Lee et al., 2000a). It is not clear why we did not find upregulation in AVP mRNA.
in our control animals. It is possible that the 4 hr time point may not have been optimal for detecting changes in mRNA levels or that there may have been an increase in mRNA stability and translational efficacy.

It has also been shown that repeated stress increases the proportion of CRH parvocellular neurons containing AVP from 50 % to 90 %, while the number of CRH parvocellular neurons remains unchanged (Bartanusz et al., 1993). In addition, repeated stress also increased AVP stores and colocalization of AVP with CRH in the median eminence (de Goeij et al., 1991; 1992a; 1992b). Furthermore, AVP secretion from the CRH terminals of the external zone of the median eminence increased in response to repeated stress (de Goeij et al., 1992b; Bartanusz et al., 1993). These data indicate that repeated stress results in a shift towards increased AVP transcription, translation, storage and secretion in order to maintain HPA responsiveness. We found a greater shift towards increased AVP mRNA levels in E compared to both PF and C females following exposure to repeated stress, while E males overall had higher AVP than C males and also exhibited increased basal AVP mRNA levels. Our data suggests that hyperresponsiveness found in E animals may in part be mediated by increased levels of AVP mRNA or a greater shift toward AVP mediation of HPA activity compared to C animals, and thus support the hypothesis that increased HPA drive in E animals may play a role in HPA hyperresponsiveness. However, whether changes in mRNA pools are directly related to peptide synthesis is still unknown. Presently, there are limited studies that examine whether induction of mRNA invariably leads to increases in peptide. A study using immunoautoradiography and in situ hybridization on adjacent sections, has reported increases in CRH peptide expression paralleled by changes in CRH mRNA levels in the PVN, suggesting that CRH biosynthesis is driven by the amount of
available mRNA; thus, mRNA levels may be an accurate predictor of CRH peptide levels (Herman & Morrison, 1996). However, these studies were done in ADX animals. Thus it is possible that AVP mRNA levels is in fact an accurate predictor of AVP peptide expression; however, it remains to be elucidated whether this is true following exposure to repeated restraint stress and whether the same relationship applies to AVP gene expression and biosynthesis. A shift toward increased AVP production following exposure to repeated stress has been suggested to maintain HPA responsiveness. It is important for the HPA axis to continually respond to stressors because a lack of response can lead to various physiological changes that are important to an organism’s health. As previously mentioned, glucocorticoids are important in maintaining cardiovascular function, blood pressure, blood glucose and liver glycogen levels, and muscle work capacity (Munck & Guyre, 1986). Furthermore, a dysregulation of the stress response has been implicated in psychiatric disorders such as depression, panic disorder and anorexia nervosa (Johnson et al., 1992), all of which can compromise an organism’s ability to function successfully in their environment.

Following exposure to repeated stress, both females and males in all prenatal treatment groups exhibited attenuated PVN CRH-R₁ mRNA levels compared to levels following 1 d of restraint stress, suggesting habituation to the repeated stress. In addition, effects of ethanol exposure were also observed. E females and both E and PF males exhibited higher levels of CRH-R₁ mRNA following exposure to repeated restraint stress compared to their PF and/or C counterparts. The increase in PVN CRH-R₁ mRNA in both E females and males compared to their respective controls suggests increased drive from the hypothalamus and/or extrahypothalamic structures in order to increase stress-induced CRH expression and subsequently to amplify the stress response. Thus, increased CRH-R₁ mRNA levels at the
PVN may in part mediate HPA hyperresponsiveness in E animals, although the increase in CRH-R1 gene expression in E males following stress may be mediated by ethanol-induced nutritional effects. As previously mentioned, the increase in CRH-R1 mRNA levels may not be sufficient to provide support for an ultrashort feedback loop but it does suggest that a resulting increase in CRH mRNA production may be centrally mediated. That the increase in CRH-R1 mRNA levels was not paralleled by detectable increases in CRH levels may be due to technical factors such as the inability to detect small changes in large pools of mRNA, and the fact that this study was not designed to examine the temporal changes in mRNA levels. It is possible that due to the short half-life of mRNA, a portion of the mRNA pool was degraded or translated into cellular biosynthetic processes, resulting in decreased mRNA levels which would subsequently be replenished by new CRH mRNA production; thus suggesting increased turnover of CRH mRNA. Another possibility is increased stability and translational efficacy of CRH mRNA.

In contrast, CRH-R1 mRNA in the anterior pituitary was not altered by repeated stress in either females or males in any prenatal treatment groups. A study has shown that in response to repeated immobilization CRH-R1 mRNA in the anterior pituitary decreases (Makino et al., 1995b). As mentioned previously, one possibility is that our repeated stress paradigm was not severe enough to result in a decrease in anterior pituitary CRH-R1 gene expression. Restraint stress is primarily a psychological stressor that does not involve physical pain or injury, whereas immobilization involves physical discomfort. Another possibility is that changes seen in mRNA pools do not reflect immediate changes observed in the response to stress. As an immediate response to stress, an adaptive response may be at the level of receptor internalization and desensitization. For example, following 10 d of repeated
immobilization anterior pituitary CRH-R binding, measured immediately following the final stress period, was down regulated (Hauger et al., 1988; 1990).

The present study showed that both females and males in all prenatal treatment groups exhibited elevated POMC mRNA levels following exposure to repeated stress. E males also exhibited elevated POMC mRNA levels following exposure to repeated stress, which suggests increased release of ACTH and β-endorphin. These results are consistent with previous findings where exposure to 6 d of alcohol vapors resulted in increased anterior pituitary POMC mRNA levels in adult rats (Rivier & Lee, 2001). Results from this study are consistent with previous data from our laboratory; both ACTH and β-endorphin levels remained elevated in E compared to PF and C males following exposure to 10 d of daily repeated stress (Weinberg et al., 1996). As discussed previously, the immediate release of ACTH and β-endorphin in response to stress may not necessarily correlate with increased POMC mRNA levels measured 3 to 6 hr after stress induction. Thus, increased POMC mRNA levels following exposure to repeated stress suggests that an adaptive response in E males is to increase the amount of readily releasable stores of pituitary POMC-derived peptides.

Furthermore, changes in POMC mRNA were not paralleled by changes in plasma ACTH. Levels of POMC mRNA were increased following exposure to repeated stress, whereas ACTH levels were decreased. These findings are consistent with a study where rats pretreated for 6 d to alcohol vapors exhibited increased POMC mRNA levels and decreased ACTH release in response to intragastric injection of alcohol and/or shocks (Rivier & Lee, 2001). This latter study suggested that small changes in the large stores of pituitary POMC mRNA did not affect ACTH release (Rivier & Lee, 2001). Another possibility is that changes
in POMC mRNA levels do not result in or affect the immediate release of ACTH, and that changes in POMC mRNA may replenish depleted ACTH peptide pools to prepare for a subsequent or prolonged challenge.

In the present study, certain changes in central regulation were observed in both E and PF animals. For example, both E and PF males exhibited lower plasma ACTH levels and increased CRH-R₁ mRNA levels in the PVN and POMC mRNA in the anterior pituitary compared to C males following exposure to repeated stress. Previous studies have shown that pair-feeding, in addition to providing as an essential nutritional control, is in itself an experimental treatment. Pair-feeding can produce shifts in the CORT circadian rhythm as well as alter body and organ weights and behaviour of both the maternal female and offspring (Gallo & Weinberg, 1981; Weinberg & Gallo, 1982). In addition, PF offspring display deficits in fast feedback regulation of the HPA axis (Hofmann et al., 1999). Furthermore, PF dams may experience increased stress due to their restricted feeding schedule. E females have ad libitum access to their liquid diets, but consume less than if their diets did not contain ethanol. Thus, PF dams receive an amount less than what they would consume ad libitum. Although both E and PF treatment groups are receiving the same number of calories, PF dams experience deprivation and possible stress, whereas E dams may not. This may induce changes in PF animals different from those observed in E and C animals. For example, PF dams demonstrate prolonged corticoid levels following stress and decreased corticosterone binding globulin binding capacity on G21 compared to both E and C dams (Weinberg & Gallo, 1982; Weinberg & Bezio, 1987). Although the inclusion of a PF group in animal models of prenatal ethanol exposure is necessary to isolate the teratogenic effects of ethanol from the effects of primary malnutrition in the offspring, the results of this
study provide further evidence that secondary malnutrition effects due to ethanol exposure in utero do occur. Thus, the results of the present study further demonstrate that pair-feeding is an experimental treatment, but also a necessary nutritional control.

The sex differences characterized in this study may be due to the well known interaction of the HPA and HPG axes under both basal and stress conditions. For example, E females but not males showed elevated basal plasma ACTH levels and prolonged ACTH levels compared to PF and C females following exposure to repeated stress. On the other hand, E males but not females showed elevated basal AVP mRNA levels and elevated POMC mRNA levels following exposure to stress compared to PF and C males. Female rats appear to be most sensitive to stress during proestrus and it appears that estrogen has a facilitatory effect on ACTH and CORT release, whereas progesterone appears to be responsible for inhibiting the facilitatory effects of estrogen (Viau & Meaney, 1991). In addition, higher CRH-R$_1$ mRNA levels in the PVN on the morning of proestrus have been shown in cycling female rats following immobilization stress (Nappi & Rivest, 1995). In males, the inhibitory effect on AVP mRNA induction in GDX and ADX animals was reversed with testosterone replacement; thus suggesting that testosterone has a direct permissive role in mediating AVP transcription (Viau et al., 1999). In addition, following exposure to restraint stress ACTH responses are inhibited by both CORT and testosterone implants in the medial préoptic area of the brain, which has been suggested as a site whereby CORT and testosterone interact to affect the release of ACTH (Viau & Meaney, 1996). Furthermore, the results of this study extend previous work demonstrating that HPA hyperresponsiveness in E females and males appear differentially (Weinberg, 1988; 1992a; Halasz et al., 1993; Redei et al., 1993; Osborn et al., 1996; Weinberg et al., 1996), and that
differential effects in females and males are revealed depending on the nature and intensity of the stressor, the time course and the hormonal endpoint measured (Weinberg et al., 1996).

In summary, the data from the present study indicate that prenatal ethanol exposure results in altered central regulation of the HPA axis in response to acute and repeated stress. The results indicate that hormonally, E animals are not different in our controls in showing habituation to repeated restraint stress. However, the mechanisms involved in the response to acute stress and in habituation to repeated stress appear to be differentially regulated in E females and males. Both E females and males showed hormonal habituation as indicated by attenuated levels of ACTH and CORT following exposure to repeated stress, which supports previous findings from our laboratory (Weinberg et al., 1996). Furthermore, E females but not males exhibited attenuated, yet slightly elevated plasma ACTH levels compared to PF and C females following exposure to repeated stress, suggesting deficient and delayed habituation, which again supports previous data from our laboratory (Weinberg et al., 1996). Results from this study found elevated basal plasma ACTH levels in E females but not males, suggesting increased HPA drive and possibly deficits in inhibitory regulation of basal plasma ACTH levels via glucocorticoids. Although these results differ from previous findings (Lee et al., 1990; Kim et al., 1999a), the difference in basal ACTH levels provides further support for the hypothesis that E animals exhibit increased HPA drive. E females also exhibited increased levels of AVP and PVN CRH-R1 mRNA following exposure to acute and repeated stress. This suggests that increased drive from the hypothalamus or extrahypothalamic areas to the PVN may play a role in mediating HPA hyperresponsiveness. E males exhibited increased levels of AVP, PVN CRH-R1 and POMC mRNA following exposure to acute and repeated stress. Similar to E females, this
also suggests that increased drive from the hypothalamus or extrahypothalamic areas to the PVN and increased pituitary responsiveness to secretagogues may play a role in HPA hyperresponsiveness. Furthermore, an increase in POMC mRNA levels following exposure to repeated stress without alterations in anterior pituitary CRH-R₁ mRNA levels suggests that deficits may also be at the level of CRH-R₁ binding.

Together, these data suggest that increased HPA drive from higher cortical areas, or within the hypothalamus, to the PVN, and increased pituitary responsiveness to secretagogues are involved in mediating HPA hyperresponsiveness in rats prenatally exposed to ethanol. In addition, these results further suggest that differences in females and males in response to stress involve the interaction of the HPA and HPG axes.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The objective of this thesis was to compare responses of E and control animals to acute and repeated restraint stress, and to determine how hormonal hyperresponsiveness is mediated by alterations in central regulation of the HPA axis.

The present findings demonstrate that E animals do show habituation to a mild repeated stress in terms of plasma hormone levels. However, E, PF and C animals exhibit differential alterations in central regulation. Differences among E, PF and C animals following exposure to both acute and repeated stress do not appear to be mediated by CRH mRNA in the PVN or CRH-R1 mRNA in the anterior pituitary, but at least following repeated stress may in part be due to a greater shift toward AVP regulation and/or increased levels of CRH-R1 mRNA in the PVN. For E males but not females, it appears that an increase in pituitary POMC mRNA levels following stress may also play a role in HPA hyperresponsiveness.

Both E females and males showed hormonal habituation as indicated by attenuated levels of ACTH and CORT following exposure to repeated stress, which supports previous findings from our laboratory (Weinberg et al., 1996). Furthermore, E females but not males exhibited attenuated, yet slightly elevated plasma ACTH levels compared to PF and C females following exposure to repeated stress, suggesting deficient and delayed habituation, which again supports previous data from our laboratory (Weinberg et al., 1996). Results from this study found elevated basal plasma ACTH levels in E females but not males, suggesting increased HPA drive and possibly deficits in inhibitory regulation of basal plasma ACTH levels via glucocorticoids. Although these results differ from previous findings (Lee
et al., 1990; Kim et al., 1999a), the difference in basal ACTH levels provides further support for the hypothesis that E animals exhibit increased HPA drive.

The finding that neither E females nor males exhibited elevated CRH mRNA levels suggests that CRH gene expression may not play a role in HPA hyperresponsiveness in our repeated stress paradigm, possibly due to the increased stability of CRH mRNA and translational efficacy. However, both E females and males exhibited increased AVP levels and a lower CRH:AVP ratio, suggesting a shift towards increased AVP production. Elevated levels of AVP mRNA levels suggests that increased HPA drive may play a role in HPA hyperresponsiveness in E animals.

Both E females and males exhibited increased levels of PVN CRH-R1 mRNA following exposure to restraint stress. This suggests that increased drive from the hypothalamus or extrahypothalamic areas to the PVN may play a role in mediating HPA hyperresponsiveness. E males also exhibited increased levels of anterior pituitary POMC mRNA following exposure to restraint stress suggesting that increased drive from the hypothalamus or extrahypothalamic areas to the PVN and increased pituitary responsiveness to secretagogues may play a role in HPA hyperresponsiveness. Furthermore, an increase in POMC mRNA levels following exposure to repeated stress without alterations in anterior pituitary CRH-R1 mRNA levels suggests that deficits may also be at the level of CRH-R1 binding.

Together, these data suggest that increased HPA drive from higher cortical areas, or within the hypothalamus, to the PVN, and increased pituitary responsiveness to secretagogues are involved in mediating HPA hyperresponsiveness in rats prenatally exposed
to ethanol. In addition, these results further suggest that differences in females and males in response to stress involve the interaction of the HPA and HPG axes.

Presently, there are few studies that have examined the mechanisms involved in the adaptive response to stress following prenatal ethanol exposure. The present study has lead to many questions and provides direction for future research.

Although both E females and males did not exhibit changes in CRH mRNA levels, there are subsequent events following that may be affected by prenatal ethanol exposure. For example, these results suggest that CRH peptide pools and release should be examined. Prenatal ethanol exposure has shown unaltered basal hypothalamic CRH levels (Angelogianni & Gianoulakis, 1989; Lee et al., 2000) but decreased basal median eminence CRH levels in E and PF rats compared to control rats (Lee & Rivier, 1994). On the other hand, unchanged resting median eminence content of CRH has also been found (Lee et al., 2000a). Disagreement exists with regard to CRH resting and release levels, thus further research is warranted.

Both E females and males exhibit increased AVP mRNA levels. It remains to be investigated whether increased AVP mRNA levels translates into increased AVP biosynthesis. Although resting hnAVP levels in alcohol-treated rats were not different from controls, lower AVP peptide levels were detected in the median eminence in alcohol-treated rats exposed to an acute ethanol challenge (Lee et al., 2001), suggesting a discrepancy between steady state PVN levels and peptide release. It is possible that increased mRNA levels may lead to increased peptide synthesis but not necessarily release. Thus, these results suggest that the number of CRH parvocellular neurons containing AVP, and AVP stores and colocalization with CRH in the median eminence should also be examined using
immunoautoradiography in order to elucidate the relationship between hypothalamic changes and biosynthesis. For the same reasons, CRH-R binding in the anterior pituitary and ACTH peptide pools should also be examined. The present study and others (Rivier & Lee, 2001) found that changes in POMC mRNA levels were not paralleled by changes in plasma ACTH, which again suggests a discrepancy between mRNA levels and protein release.

Considering that E animals exhibit increased AVP mRNA levels following restraint stress, it is possible that V1b-R may also be altered in prenatal ethanol exposed rats. For example, following 14 d of repeated stress, AVP-R binding was increased in the anterior pituitary in rats (Aguilera et al., 1994); thus further research is warranted to determine whether alterations in V1b-R also play a role in HPA hyperresponsiveness in E animals.

It has been suggested that hnRNA and IEGs such as c-fos are used as markers to indicate activation of neurons in response to stress (Kovacs & Sawchenko, 1996). The present study showed no changes in CRH and CRH-R1 mRNA levels, which may suggest increased mRNA stability or increased translational efficacy. Another possibility is that the methods used were not sensitive enough to detect small differences in large reserve pools of mRNA. Under basal conditions, hnRNA pools are fairly low but show a robust signal in response to challenges; thus making it a more sensitive marker for indicating activation of neurons compared to mRNA (Hoffman & Lyo, 2002). In response to a challenge, hnRNA synthesis is increased followed by increased mRNA levels and eventually biosynthesis in order to replenish depleted peptide pools (Hoffman & Lyo, 2002). Thus, further research examining changes in IEGs as well as hnRNA levels may indicate whether or not E animals show hyperresponsiveness at the level of unprocessed mRNA pools.
Previous studies typically have found no differences among E, PF and C animals under basal conditions (Taylor et al., 1981; 1982; 1983; Nelson et al., 1986; Weinberg, 1988; 1992a; Lee et al., 1990; 2000a; Kim et al., 1999a). However, the present study found elevated basal plasma ACTH levels in E females, and elevated basal AVP and POMC mRNA levels in E males. In addition, previous work in our lab has shown that sham operated E females also showed increased basal CRH mRNA levels (Glavas et al., unpublished). Together, these data suggest that increased basal ACTH and CRH levels may, at least in part, be due to increased HPA drive. However, a deficit in inhibitory regulation of basal ACTH and CRH levels by glucocorticoids at the hypothalamus and pituitary can not be completely ruled out. Corticosteroid receptor densities at HPA feedback sites such as the hypothalamus and anterior pituitary were unaltered in E compared to PF and C animals under basal conditions (Kim et al., 1999b). However, the cytosolic binding assay that was used did not allow for regional distinctions within the region of interest because it required homogenization of the whole structure. Thus, measures of GR and MR proteins and their respective mRNA levels using immunohistochemistry and in situ hybridization, respectively, would be a more sensitive measure.

In addition, previous studies have shown marked sex differences in prenatal ethanol effects in response to stress and on HPA regulation at different levels of the axis (Weinberg, 1988; 1992a; Halasz et al., 1993; Redei et al., 1993; Osborn et al., 1996; Weinberg et al., 1996). The results of the present study extend this previous work demonstrating differential HPA responsiveness in E females and males compared to their control counterparts. These data suggest that gonadal hormones play a role in mediating prenatal ethanol effects on HPA activity. In support of this, studies have found altered HPG
function in E females and males. For example, suppressed pre- and postnatal testosterone surges in E fetuses/neonates (McGivern et al., 1993; 1998) have been reported. E females show delayed puberty onset (McGivern & Yellon, 1992) and premature reproductive aging as suggested by earlier onset of acyclicity (McGivern et al., 1995). Thus, further research examining the influence of estrogen and progesterone cyclicity and testosterone on the HPA axis following prenatal ethanol exposure may provide important insight on how gonadal hormones interact with HPA hormones.

A. Clinical Implications

Cognitive deficits and behavioural abnormalities have been shown in both clinical populations and animal models of prenatal ethanol exposure. Maladaptive behaviours such as hyperactivity, distractability, impaired judgement and poor attention span (Streissguth et al., 1980; 1991), as well as slower central information processing (Jacobson, 1998), are consequences in individuals diagnosed with FAS that may compromise their ability to adapt to their environment. It has been suggested that HPA hyperresponsiveness following prenatal ethanol exposure may underlie some of the behavioural abnormalities (Kim et al., 1997) and immune deficits (Johnson et al., 1981; Giberson & Weinberg, 1995; Giberson et al., 1997) observed in both humans and animals.

HPA hyperresponsiveness, characterized by increased HPA hormone levels and/or delayed recovery back to basal levels following stress, has been shown in both humans and animals. A recent study found that maternal drinking at conception and during pregnancy was associated with higher poststress cortisol levels in infants (Jacobson et al., 1999). Many animal studies have also shown that prenatal ethanol exposure produces HPA hyperresponsiveness (Taylor et al., 1982; Angelogianni & Gianoulakis, 1989; Lee et al.,
The long-term adverse consequences of HPA hyperresponsiveness include gastrointestinal ulceration, immunosuppression, weight loss, fatigue, myopathy, steroid diabetes, hypertension, psychogenic dwarfism, reproductive dysfunction and neuronal death (Sapolsky, 1992; Stratakis & Chrousos, 1995). In addition, HPA hyperresponsiveness, reflecting dysregulation of the HPA axis, has been implicated in psychiatric disorders such as depression, panic disorder, obsessive-compulsive disorder and anorexia nervosa (Chrousos & Gold, 1992; Johnson et al., 1992). Given that HPA hyperresponsiveness has adverse physiological and behavioural consequences which could compromise the health and survival of the organism; it is unfortunate that there is limited clinical information on how prenatal alcohol exposure affects the HPA axis of the developing child indicating the importance of this issue for further study.

As previously mentioned, the constancy and preservation of homeostasis requires continuous habituation to stressors in order for an organism to survive, and the inability to successfully adapt to prolonged periods of stress can be detrimental to an organism’s survival. Deficits in habituation to repeated stimuli presentation have been documented in human neonates prenatally exposed to alcohol suggesting that deficits in habituation to repeated stimuli may be involved in impaired cognitive and intellectual functioning in adulthood (Streissguth et al., 1983). Furthermore, the present findings confirm that rats prenatally exposed to ethanol also do not successfully habituate to prolonged periods of stress in adulthood.

A hallmark characteristic of FAS is impaired growth and organs, which suggest that direct effects of ethanol on cellular processes may, at least in part, be at the level of
protein synthesis (Schenker et al., 1990). The present study also suggests that changes in mRNA levels may mediate the adaptive response to stress. These data suggest that examination of the mechanisms involved in the teratogenic effects of alcohol warrants investigation at the level of molecular mechanisms. Unfortunately, studies investigating the molecular mechanisms involved in prenatal ethanol exposure have been limited. However, caution must be used in extrapolating data obtained from rats to humans, given the large phylogenetic gap between the two species. Nevertheless, as previously discussed, basic research using animal models have been instrumental in the study of prenatal ethanol exposure.

B. Conclusions

In conclusion, the results of this thesis demonstrate that prenatal ethanol exposure has long-term effects on HPA responsiveness to stressors. Our data suggest that central alterations in HPA hyperresponsiveness occur differentially in E females and males, and may be due to increased HPA drive and/or increased pituitary responsiveness to secretagogues.

Although the effects of prenatal ethanol exposure are entirely preventable, it remains prevalent in society. Thus, it is important to examine the mechanisms involved in order to develop preventative measures that may assist in ameliorating some of the physiological deficits that become apparent when prenatal ethanol exposed offspring are exposed to challenges. Furthermore, a better understanding of the effects of prenatal ethanol exposure and its underlying mechanisms may also lead to pharmacological or behavioural treatments for affected individuals.
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