THE EFFECTS OF PRENATAL ETHANOL EXPOSURE
ON ANTERIOR PITUITARY SENSITIVITY AND ELEVATED PLUS MAZE
BEHAVIOUR FOLLOWING CRH STIMULATION

by

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B.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Anatomy)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July 1997

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ABSTRACT

Previous studies have shown that following prenatal ethanol exposure, rats exhibit hormonal hyperresponsiveness of the hypothalamic-pituitary-adrenal (HPA) axis and behavioural hyperresponsiveness to stressful stimuli. The studies conducted in this thesis investigated the effects of prenatal ethanol exposure on HPA axis function and on behaviour in the elevated plus maze. Male and female Sprague-Dawley rats from prenatal ethanol (E), pair-fed (PF) and ad lib-fed control (C) treatment groups were tested in adulthood in all studies. Two major studies were conducted.

The first study investigated the hypothesis that the HPA hyperresponsiveness observed in animals prenatally exposed to ethanol is due at least in part to an increased responsiveness of the anterior pituitary to CRH stimulation. Following dexamethasone suppression, CRH-induced plasma ACTH and CORT were compared between animals from E, PF and C treatment groups. Higher doses of CRH resulted in higher and more prolonged ACTH and CORT responses in both males and females. Overall, females demonstrated greater increases in plasma ACTH levels in response to exogenous CRH compared to males. Importantly, E females but not E males showed increased plasma ACTH responses to CRH as compared to their PF and C counterparts during the trough but not during the peak of the HPA circadian rhythm. The results of this study suggest that E animals exhibit increased anterior pituitary responsiveness to CRH stimulation at least during the trough of the HPA circadian rhythm. Furthermore, females may be more vulnerable to the effects of prenatal ethanol exposure compared to males.
The second study investigated the hypothesis that behavioural hyperresponsiveness observed in animals prenatally exposed to ethanol is mediated by an increased sensitivity of the central nervous system to CRH. The anxiogenic effects of low or high doses of CRH administered centrally with or without D-Phe-CRH (a CRH antagonist) on elevated plus maze behaviour were compared between animals from E, PF and C treatment groups. Intracerebroventricular administration of CRH increased fear-related behaviour as indicated by decreased time on and entries into open arms, and increased time on closed arms; and decreased exploration and motor activity as indicated by decreased entries into closed arms, total entries and total rears. D-Phe-CRH reversed the effects of CRH on some but not all of the behavioural measures. Overall, females required higher doses of CRH to elicit behavioural effects which are the same levels as seen in males, suggesting that females are less sensitive to the behavioural effects of CRH than males. Importantly, E males showed increased fear-related behaviour and decreased exploration and motor activity in the elevated plus maze compared to PF and C males. Furthermore, following administration of CRH, E animals showed increased fear-related behaviour and decreased exploration and motor activity compared to PF and C animals. The results of this study suggest that E animals exhibit an increased responsiveness to the behavioural effects of centrally administered CRH.
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ACKNOWLEDGMENTS

I would like to extend my sincere gratitude to my supervisor, Dr. J. Weinberg, for her encouragement and support throughout this project. I would also like to thank the members of my advisory committee, Dr. J. T. Emerman, Dr. C. McIntosh and Dr. W. Vogl, for their valuable suggestions and advice.

I would also like to thank the following people for their technical as well as emotional support: Wayne Yu, Linda Herbert, Glenn Edin, Kwon Kim, Lawrence Chan, Wendy Simms, Jill Osborn, Kara Gabriel, Pam Giberson, Kathy Keiver, Candace Hoffman and Maria Glavas. Thank you for all your encouragement, for sharing your scientific and worldly knowledge, and most of all, for helping me keep my sanity.

Lastly, I would like to thank my family and friends, especially Kin, Myra, Anne, Melissa, Glenn, Stephen, Mark and Lawrence, whose emotional support were invaluable.

This work was supported by a grant from the National Institutes of Health.
CHAPTER 1: GENERAL INTRODUCTION

I. Fetal Alcohol Syndrome (FAS)

It has long been known from anecdotal evidence that the consumption of alcohol during pregnancy is harmful to the fetus, but it was not until the late sixties and early seventies when Jones and Smith (1973), and Lemoine and colleagues (1968) independently documented the harmful effects on the offspring of alcohol consumption during pregnancy. “Fetal Alcohol Syndrome” or FAS has since been used to describe the cluster of malformations found in these offspring.

A. Diagnosis

The major diagnostic criteria for FAS include: (1) confirmed maternal alcohol exposure, (2) pre and/or postnatal growth deficiency, (3) craniofacial anomalies and (4) central nervous system dysfunction (Stratton et al, 1996). Growth deficiencies in the diagnosis of FAS include characteristics such as decreased height, weight and brain size (Streissguth et al., 1980). Craniofacial anomalies include small head circumference, short palpebral fissures, hypoplastic philtrum, thin vermilion border of the upper lip and a flat midface (Streissguth et al, 1980 & 1985). Central nervous system dysfunction includes mental retardation, and neuromotor and behavioural disorders (Hill, 1989). If some, but not all of the above criteria are met, the term “Fetal Alcohol Effects” (FAE) has been suggested (Sokol & Clarren, 1989). More recently, more specific terms have been proposed, the term Alcohol-Related Birth Defects (ARBD) was suggested when congenital anomalies involving cardiac, skeletal, renal, ocular and auditory systems following prenatal ethanol exposure are
observed. The term “Alcohol-Related Neurodevelopmental Disorder” (ARND) was also suggested when central nervous system neurodevelopmental abnormalities and/or behavioural or cognitive abnormalities are observed (Sokol & Clarren, 1989; Stratton et al, 1996).

B. Behavioural and Cognitive Deficits

Infants with FAS may demonstrate decreased ability to habituate to environmental cues, decreased suckling, increased cerebral excitation, severe tremors, irritability with decreased vigorous body activity, insecurity, sleeping disorders, decreased alertness and failure to thrive (Hill & Tennyson, 1980; Pierog et al, 1977; Streissguth et al., 1980). In addition, infants with FAS may also demonstrate symptoms which are similar to alcohol withdrawal in adults, such as irritability followed by tremors, spontaneous seizures, hypertonia, abdominal distention and increased respiratory rate (Pierog et al, 1977).

Older children diagnosed with FAS/FAE typically demonstrate below average IQ (Conry, 1990; Streissguth et al, 1989). Furthermore, these children may demonstrate attention deficits, poor social adaptation and problems with comprehension which persist through adolescence and adulthood. These behavioural deficits may persist; a follow-up study on 61 adolescents and adults with FAS, by Streissguth et al (1991) showed that due to behavioural, social and cognitive disabilities, none of these people were independent in terms of either housing or income.
C. Incidence and Epidemiology

Different reports have indicated different incidences of FAS and/or ARBD depending on the population groups being examined. Philipson (1988) reported an incidence of 1.9 per 1000 live births worldwide. Olegard et al. (1979) reported an incidence of 3.3 per 1000 live births in Sweden. Dehaene et al. (1991) reported an incidence of 1.2 per 1000 live births in France. Studies done in the United States reported incidences ranging from 0.6 to 3.1 per 1000 live births in Cleveland and Boston respectively (Sokol et al, 1980; Ouellette et al, 1977); and incidences of 2.9 among Native Americans, 0.6 among African Americans, 0.09 among Caucasians, 0.08 among Hispanics and 0.03 among Asians per 1000 live births (Chavez et al, 1988). Studies done in Native Indian populations in Canada report incidences ranging from 25.0 to 189.0 per 1000 live births in British Columbia and Northwestern Canada, respectively (Asante, Nelms-Matzke, 1985; Robinson et al, 1987).

As expected the reported prevalence of FAS and/or ARBD varies depending on the drinking habits of the population being studied and the training of the clinician identifying the disorder; nevertheless, the findings are relevant enough to warrant attention from the scientific community. In addition, because of various social pressures, the complete prevention of maternal drinking has not been possible; therefore, the mechanisms underlying FAS and/or ARBD need to be investigated to aid proper intervention or treatment.
D. Animal Models

Studies in humans have limitations. A number of variables such as socioeconomic status, health, diet and level of alcohol intake may confound the conclusions which can be drawn from human studies. Animal models were therefore developed.

A number of different methods have been utilized to administer alcohol prenatally. Alcohol has been administered to the pregnant dam via gastric intubation or injection. It has also been administered by placing ethanol in the drinking water or a liquid diet (Weinberg, 1984; Weiner, 1980). The advantage of the gastric intubation and injection methods is that the dose of ethanol received by the pregnant female is controlled by the experimenter; however, these methods require a great deal of handling and are invasive, resulting in a fair amount of stress on the animal. Placing ethanol in drinking water is the simplest method of administration; however, most rodents find the taste of ethanol aversive, and thus they will not voluntarily consume very concentrated solutions of ethanol. This method therefore poses problems in achieving a high blood alcohol concentration in pregnant animals, which will result in teratogenic effects of alcohol on the offspring, as seen in human FAS. Animal studies have demonstrated that high levels of alcohol consumption as seen in binge drinking produces a more pronounced effect of alcohol teratogenesis, as compared to the intake of the same dose of alcohol over a longer interval of time (West et al, 1989).

For the purposes of this thesis, administration of ethanol via a liquid diet was used. This method of administration is less stressful to the pregnant female. Furthermore, the liquid diet is the only available source of food for the rodents; thus, this method of ethanol administration results in a consistent intake of high doses of ethanol resulting in consistently elevated blood alcohol levels (Lieber & DeCarli, 1982).
Research using animal models of prenatal ethanol exposure have demonstrated growth deficiencies (Gallo & Weinberg, 1986; Diaz & Samson, 1980), craniofacial anomalies (Sulik, 1983), and neuromotor and behavioural disorders (Weinberg & Gallo, 1981a; Bond & DiGiusto, 1978; Riley, 1990) similar to those seen in human FAS. Furthermore, animal studies have provided a deeper understanding of other physiological effects of prenatal ethanol exposure which are difficult to assess definitively in human case studies.

II. Neuroendocrinology of the Stress Response

A. Stress

Stress is a physiological response within an organism, elicited by "evocative agents" called stressors which threaten "balance, equilibrium or harmony", or homeostasis (Selye, 1950). Both biological and psychological stimuli can elicit a stress response. The body attempts to correct the effects of a disturbance by activating the sympathomedullary adrenal and the HPA axes, the end products of which are medullary catecholamines and glucocorticoids, respectively (Kopin, 1995). These hormones serve as major endocrine regulators of carbohydrate and lipid metabolism, cardiovascular tone, muscle function, immunocompetence and behaviour. The net metabolic effect of catecholamines and glucocorticoids is catabolic or antianabolic, which results in the provision of glucose or energy for the body to cope with the disturbance resulting from stressors (Berne and Levy, 1993). This dissertation will primarily focus on the HPA response to stressors.
B. Hypothalamic-Pituitary-Adrenal (HPA) Axis

Activation of the HPA axis begins with neuronal input to the parvocellular cells of the paraventricular nuclei in the hypothalamus. Following a psychological or physiological stressor, higher brain centers such as the prefrontal cortex and the hippocampus stimulate the hypothalamus to release corticotropin-releasing hormone (CRH) and vasopressin which are transported via the hypothalamo-hypophyseal portal system to the corticotropes of the anterior pituitary where CRH binds to its receptors. CRH then acts via cyclic AMP mediated processes to stimulate the release and increase the rate of synthesis of adrenocorticotropin (ACTH), \( \beta \)-endorphin (\( \beta \)-EP) and other pro-opiomelanocortin-derived peptides. ACTH, when released into the systemic circulation, binds to its receptors in the adrenal cortex, and similarly via cyclic AMP mediated processes, stimulate the release and increase the rate of synthesis of corticosterone (CORT) in rodents, and cortisol in humans (Jones & Gillham, 1988).

C. Feedback Inhibition or Negative Feedback

Prolonged elevation of HPA hormones may have detrimental effects on the organism; therefore, a mechanism exists which signals the termination of further release of HPA hormones. The HPA axis operates with a negative feedback system, such that increasing levels of hormones result in the inhibition of their own secretion or inhibition of secretion of their secretagogues. Similar to the HPA stimulatory cascade, feedback inhibition is a receptor mediated process.
In general, feedback inhibition can be categorized into ultra-short-loop negative feedback, short-loop feedback and long-loop negative feedback. Ultra-short-loop feedback involves the inhibition of secretion and/or biosynthesis of hormones by their own secretion. For example, increasing levels of CRH inhibit further CRH secretion from the hypothalamus (Calogero et al., 1988), and, increasing levels of ACTH inhibit further ACTH secretion from the pituitary (Motta et al, 1965; Vernikos-Danellis & Trigg, 1967). Short-loop negative feedback involves the inhibition of the secretion and/or biosynthesis of hormones by inhibition of their direct secretagogues. Thus, increasing levels of CORT inhibit ACTH secretion (Stark et al, 1968), and increasing levels of ACTH inhibit CRH secretion (Suda et al, 1986; 1987). Long-loop feedback involves the inhibition of the secretion and/or biosynthesis of hormones by inhibition of the secretagogues further up in the cascade of hormone release. Thus, increasing levels of CORT inhibit CRH secretion (Plotsky & Vale, 1984; Suda et al, 1984).

Of these modes of feedback inhibition, CORT feedback has been the most extensively studied. CORT receptors have not only been localized in the anterior pituitary and the hypothalamus, where short and long-loop CORT feedback inhibition occur, respectively; they have also been found in other brain regions, suggesting that other sites of CORT feedback exist. Two types of CORT receptors - mineralocorticoid (type I) and glucocorticoid (type II) receptors have been identified. Mineralocorticoid receptors which are high affinity CORT binding receptors have been found in the hippocampus and the lateral septum. Glucocorticoid receptors which are low affinity CORT binding receptors have been found in the lateral septum, the dentate gyrus, the nucleus of tractus solitarius and the central amygdala (Dallman et al, 1987). Mineralocorticoid receptors, being high affinity binding
receptors, are essentially fully occupied by CORT at all times. In contrast, glucocorticoid receptors, being low affinity binding receptors, are only half maximally occupied during the peak (PM) and after stress during the trough (AM) of the HPA circadian rhythm. Their role may therefore be more important in feedback inhibition following stressor-induced increases in CRH and/or ACTH secretion. The presence of unbound receptors permits further CORT binding, and thus allows for feedback regulation (Reul and De Kloet, 1985).

CORT feedback on ACTH and CRH secretion has been categorized according to the time domains in which feedback occurs. Fast, intermediate and slow feedback time domains have been implicated. Fast feedback occurs within seconds to minutes following an increase in CORT levels. It occurs while plasma CORT is increasing, and it is sensitive to the rate of increase. It acts primarily by inhibiting ACTH and CRH release. Intermediate feedback occurs 2-10 hours following the initiation of a relatively short period of increase in CORT levels. It acts primarily by inhibiting ACTH release, and CRH synthesis and release. Slow feedback occurs during a prolonged pathological increase of CORT of 12 or more hours. It acts by inhibiting ACTH release and synthesis by inhibiting POMC mRNA synthesis, as well as CRH synthesis and release (Keller-Wood & Dallman, 1984).

D. Facilitation or Positive Feedback

The HPA axis also aids in the adaptability of organisms to multiple stressors by exhibiting positive feedback or facilitation. That is, prior increase in levels of HPA hormones may result in facilitation of the hormonal response to a subsequent novel stressor. Facilitation involves an increased responsiveness or a lack of the expected decreased responsiveness of the HPA hormones. Facilitation may occur following a novel stressor
while the organism is under a chronic stressor. It may also occur following a novel stressor presented within 24 hours following an acute stressor (Aguilera, 1994; Dallman et al, 1991; Odio & Brodish, 1990; Young & Akil, 1988). The mechanisms involved in facilitation are still unclear.

E. Circadian Rhythmicity

The HPA axis exhibits a circadian rhythm, which emerges 21 days following birth, and is fully established by 28 days following birth (Jones & Gillham, 1988). Circadian rhythms in CRH, ACTH and CORT levels have been reported in both rats and humans (David-Nelson & Brodish, 1969; Moldow & Fischman, 1984; Szafarczyk, et al, 1980). The peak activity of HPA hormones generally occur near the onset of daily activity, which in rodents and other nocturnal animals occur in the late afternoon or early evening (PM), while, the trough activity occurs in the morning (AM), approximately 12 hours following peak activity (Keller-Wood & Dallman, 1984). The circadian drive from higher brain centers send neural signals to the suprachiasmatic nuclei and paraventricular nuclei of the hypothalamus (Dallman et al, 1987). Studies show that morning (trough) ACTH levels can be achieved even when the connections of the pituitary to the brain are lesioned. However, evening (peak) ACTH levels cannot be achieved unless the connections of the pituitary to the brain are intact, suggesting that there is a daily drive from the brain to the HPA axis that is functioning only during the time of peak basal activity (Keller-Wood & Dallman, 1984).
III. CRH and the Central Nervous System

A. Localization of CRH

CRH was first isolated in its crude form by Saffran and Schally in 1955. However, its chemical identity was not known until 1981 when Vale and colleagues identified its 41-amino acid sequence from extracts of sheep hypothalamus. Since then, the amino acid sequence of CRH in humans, rats, pigs, goats and cows have also been determined; CRH in all of these organisms has a similar primary sequence (Rivier and Plotsky, 1986).

As previously discussed, CRH plays an important role in the HPA axis. It is released from the hypothalamus, and is the primary secretagogue of ACTH, β-endorphins and other POMC-related peptides. Interestingly however, CRH and its receptor have been localized not only in the median eminence and the anterior pituitary, but also in numerous other brain regions such as the frontal, parietal and temporal lobes of the cerebral cortex, the hippocampus, olfactory bulbs, amygdala, septal nuclei, caudate putamen, nucleus accumbens, thalamus, superior colliculus, periaqueductal gray, tegmental nucleus and trigeminal tract (Aguilera et al., 1990; De Souza, 1986; De Souza et al, 1985). In addition, electrophysiological studies have shown that CRH exerts predominantly excitatory actions in a variety of brain areas including the locus ceruleus, hippocampus and cerebral cortex (Nemeroff, 1988).

B. Physiological Effects of CRH

CRH administered centrally (intracerebroventricularly) has been demonstrated to activate the sympathetic nervous system, increasing norepinephrine and epinephrine levels in
rats and dogs; CRH antagonists have been shown to reverse these elevations (Brown & Fisher, 1983; Brown et al, 1985). CRH administered centrally has also been demonstrated to increase adrenal sympathetic nerve activity; this increase was absent when CRH was administered peripherally (intravenously), suggesting that this excitatory action is not mediated by the effects of CRH on pituitary-adrenal activity (Kurosawa et al, 1986). In addition, CRH administered centrally has been shown to produce changes in EEG activity suggestive of increased arousal (Ehlers et al, 1983).

Other physiological effects of CRH administered centrally include inhibition of luteinizing hormone (LH), growth hormone, and testosterone secretion, but not follicle-stimulating hormone, thyroid-stimulating hormone and prolactin secretion. Furthermore, CRH administered centrally has also been shown to have widespread effects on gastrointestinal function, causing a decrease in gastric acid secretion, gastric emptying and small bowel transit, and an increase in large bowel transit (Dunn and Berridge, 1990).
C. Behavioural Effects of CRH

The behavioural effects of CRH are dependent on both the dose of CRH administered and the testing conditions. In familiar environments (environments to which animals were previously habituated) CRH administered centrally has been shown to increase locomotor activity and rearing in rats (Koob et al, 1993; Sutton et al, 1982). In novel environments such as the open field, low doses of CRH (0.01 μg) administered centrally have been shown to increase locomotor activity and rearing (Sutton et al, 1982; Veldhuis and De Wied, 1984); while high doses of CRH (1-2 μg) have been shown to decrease locomotor activity and rearing (Britton et al, 1982; 1984; Sutton et al, 1982).

In both familiar and novel environments, low doses of CRH (0.1 μg) administered centrally have been shown to increase ingestive behaviour (Gosnell et al, 1983); while high doses of CRH (greater than 1.0 μg) have been shown to decrease ingestive behaviour (Britton et al, 1982; Levine et al; 1983; Morley & Levine, 1982). In both familiar and novel environments, CRH administered centrally has been shown to increase grooming (Britton et al, 1982; Dunn et al, 1987; Morley & Levine, 1982; Sherman & Kalin, 1987) and stress-induced freezing (Sherman & Kalin, 1988); and to decrease pentobarbital-induced sleeping time (Shibasaki et al, 1991), frequency of sleeping (Sherman & Kalin, 1986 & 1987), and exploratory behaviour in the multi-compartment chamber test (Spadaro et al, 1990).

In general, the interpretation of the central effects of CRH has been that of increased behavioural arousal and anxiety which are typically seen in stress. In anxiety tests such as the elevated plus maze, CRH has been shown to decrease time spent on the open arms suggesting increased anxiety levels (File et al, 1988). Together, previous studies demonstrate the
important role of CRH in integrating hormonal, visceral and behavioural responses for organisms to cope with stress.

D. Central vs. Peripheral Nervous System Effects of CRH

It is important to note that CRH administered centrally has also been shown to activate the HPA axis (Ono et al, 1984; Veldhuis & De Wied, 1984). However, most of the physiological as well as behavioural effects previously mentioned have been shown to exist in the absence of peripheral CRH effects (i.e. pituitary-adrenal activity). Among the effects of CRH just cited, only the gastrointestinal effects of CRH have been shown to be completely blocked by adrenalectomy, implicating the involvement of glucocorticoids or catecholamines released from the adrenals in this particular response. In general, the relative independence of the effects of centrally administered CRH from those of pituitary-adrenal activity has been experimentally shown by administering CRH peripherally (subcutaneously or intravenously), a route which does not allow it to cross the blood-brain barrier, and then demonstrating an absence of the effects normally seen when CRH is administered centrally. The independence of the effects of centrally administered CRH from peripherally administered CRH has also been shown by procedures such as hypophysectomy, adrenalectomy or dexamethasone (a glucocorticoid analogue) administration, both of which inhibit pituitary-adrenal activity; following these procedures, the effects of centrally administered CRH have persisted.

Furthermore, the specificity of the central actions of CRH have been demonstrated via central administration of CRH competitive antagonists such as α-CRH and D-Phe-CRH, which do not cross the blood-brain barrier. Central administration of these antagonists such
as α-CRH and D-Phe-CRH has been demonstrated to block the behavioural effects normally elicited by CRH. For example, the increased locomotor activity induced by CRH in photocell cages is attenuated by administration of α-CRH or D-Phe-CRH (Menzaghi et al, 1994). Behaviour believed to be modulated by CRH such as the decrease in time spent on open arms in the plus maze following exposure to social defeat stress, has also been shown to be antagonized by central administration of α-CRH (Heinrichs, et al, 1992).

Similar to CRH in the HPA axis, CRH in extrahypothalamic sites have also been demonstrated to have circadian rhythmicity; however, unlike the rhythmicity of hypothalamic CRH, extrahypothalamic CRH is in general not affected by CORT levels (Owens et al, 1990), which further suggests an independence in functioning of centrally active CRH from peripherally active CRH.

IV. Prenatal Ethanol Exposure and Stress Responsiveness

A. Prenatal Ethanol Exposure and the HPA Axis

Prenatal ethanol exposure alters virtually every aspect of the endocrine system of the offspring, affecting growth, thyroid, gonadal and adrenocortical hormone levels (see Weinberg, 1993). These effects are caused by either direct effects on the endocrine glands or central effects on the brain and/or hypothalamic-pituitary functioning. Studies have shown reduction of growth hormone levels during the preweaning period in animals exposed to ethanol during the last week of gestation (Thadani and Schandberg, 1979); reduction in thyroid hormone levels in ethanol-exposed sheep fetuses (Rose et al, 1981), and two- or three-week old rats prenatally exposed to ethanol (Kornguth et al., 1979; Portoles et al,
1988); reduction in brain dihydrotestosterone (Kakihana et al, 1980) and plasma testosterone levels (Kelce et al, 1989) in rat pups at birth; potentiation of plasma prolactin levels from weaning to adulthood; and reduction in plasma LH levels from weaning to 35 days of age (Esquifino et al, 1986). This dissertation will focus on the effects of prenatal ethanol exposure on the HPA axis.

Few human studies have investigated the effects of prenatal ethanol exposure on the HPA axis. A case study of four children with FAS indicated that plasma Cortisol levels were within normal limits (Root et al., 1975). However, some human studies have shown that prenatal ethanol exposure can alter HPA functioning. Binkiewicz et al. (1978) reported a pseudo-Cushing’s syndrome in an infant exposed to ethanol via breast milk. Recently, Jacobson et al (1993), reported that infants with a history of prenatal alcohol exposure demonstrated elevated salivary cortisol following a routine blood draw, which is an acute stressor. Furthermore, animals studies have clearly shown that prenatal ethanol exposure, similar to prenatal stress (Meaney et al, 1993), alters the HPA axis of the offspring.

At birth, prenatal ethanol exposed rats have been shown to have increased brain and plasma CORT levels (Kakihana et al, 1980; Taylor et al, 1982b; Weinberg, 1989). During the pre-weaning age (the first 3 weeks of life), prenatal ethanol exposed rats have been shown to have reduced adrenocortical response to stressors such as ether, novel environments, saline injection, cold stress, alcohol and morphine (Kakihana et al, 1980; Taylor et al, 1982b, Weinberg 1989). However, this decreased adrenocortical responsiveness does not last.

Following weaning, prenatal ethanol exposed animals have been shown to be hyperresponsive to stressors. In response to stressors such as cardiac puncture, 30 min noise/shake, or, continuous or intermittent footshock stress, animals prenatally exposed to
ethanol show higher plasma CORT levels compared to pair-fed and ad-lib fed control animals (Nelson et al, 1984 & 1986; Taylor et al, 1982a). In addition, prenatal ethanol exposed animals demonstrate prolonged elevation and/or delayed recovery to basal levels. Following 1 min intermittent footshock stress, animals prenatally exposed to ethanol demonstrate prolonged decline to basal of plasma ACTH levels compared to control animals (Taylor et al, 1986). At 120 min of a 240 min restraint stress, CORT levels show a significant decrease in pair-fed and control animals; however, prenatal ethanol exposed animals do not show a significant decrease from peak levels throughout the 240 min restraint stress (Weinberg, 1992). Prenatal ethanol exposed animals also demonstrate a delayed habituation to environmental cues of HPA hormones. Females prenatally exposed to ethanol show reduced habituation of CORT levels to a one-hour restraint stress, as well as deficits in pituitary-adrenal response inhibition in a consummatory task compared to pair-fed and ad-lib fed control females (Weinberg, 1988).

Data from different studies suggest that females may be more vulnerable than males to the effects of ethanol on HPA responsiveness. Studies by Taylor et al (1982a; 1986; 1988) and Kelly et al (1991) have demonstrated HPA hyperresponsiveness following prenatal ethanol exposure primarily in females. However, work in our laboratory has shown that under appropriate conditions, HPA hyperresponsiveness following prenatal ethanol exposure may also be demonstrated in males. We have shown that males prenatally exposed to ethanol may show a greater, more prolonged CORT elevation over a 4-hour restraint stress period compared to control males, whereas females prenatally exposed to ethanol did not differ significantly from control females under these conditions (Weinberg, 1992). Furthermore, in a study investigating feedback inhibition following a stress response, it has been shown that
during the trough of the HPA circadian rhythm, females and not males prenatally exposed to ethanol demonstrate higher stressor-induced CORT levels following dexamethasone suppression compared to pair-fed and control females. However, during the peak of the HPA circadian rhythm, both females and males prenatally exposed to ethanol demonstrate higher stress-induced CORT levels following dexamethasone suppression compared to pair-fed and control females and males (Osborn et al, 1996). Thus, the parameters of the test situation, such as the nature of the stressor and the time of day testing is conducted, play an important role in determining whether differential effects of prenatal ethanol exposure are observed in males and in females.

Although it is known that HPA hyperresponsiveness can have detrimental effects on the health and even survival of the organism, the mechanisms underlying this hyperresponsiveness in organisms prenatally exposed to ethanol is still unclear. It is therefore important to investigate this phenomenon further, in order to ultimately find possible means of alleviating its harmful effects on persons prenatally exposed to alcohol.

B. Prenatal Ethanol Exposure and Stress-related Behaviour

As previously mentioned, clinical studies have shown that children prenatally exposed to ethanol are hyperactive, uninhibited, impulsive and have attention deficits that may reflect an inability to inhibit responses (Streissguth et al, 1985 & 1986; Streissguth, 1986). These behavioural deficits are particularly seen in challenging or stressful situations (Streissguth et al, 1986).

Animal studies have also shown that animals prenatally exposed to ethanol demonstrate hyperactivity, attention deficits, lack of inhibition, gait abnormalities hearing
abnormalities and reduced habituation (Driscoll et al, 1990). Rats prenatally exposed to ethanol demonstrate increased ambulation in open-field testing (Bond and Di Giusto, 1977). They also demonstrate heightened startle reactivity in the absence of morphological, body weight or physical developmental aberrations (Anandam et al, 1980). In addition, they demonstrate a response inhibition in a passive avoidance task (Barron and Riley, 1990). In general, behavioural studies suggest that prenatal ethanol exposure result in behavioural hyperresponsiveness in the offspring; however, the mechanisms underlying this hyperresponsiveness is still unclear. It is important to investigate this phenomenon further in order to find both psychological and pharmacological means of treatment and intervention.

V. Thesis Objective and Hypotheses

Prenatal ethanol exposure results in hormonal hyperresponsiveness of the HPA axis, and behavioural hyperresponsiveness in the adult offspring. The objective of this dissertation is to investigate these hormonal and behavioural aberrations with the following working hypotheses:

Hypothesis 1: The hypothalamic-pituitary-adrenal hyperresponsiveness observed in animals prenatally exposed to ethanol is due in part to an increased responsiveness of the anterior pituitary to CRH stimulation.

Hypothesis 2: The behavioural hyperresponsiveness observed in animals prenatally exposed to ethanol is mediated by an increased sensitivity of the central nervous system to...
CHAPTER 2: GENERAL METHODS

I. Breeding and Feeding

Sprague-Dawley rats (Canadian Breeding Farms, St. Constant, Quebec) were bred, and their offspring used as subjects for each study. Rats used for breeding were housed under conditions of constant temperature (21 °C), lighting (lights on from 600 to 1800 hr) and handling. They were group housed for 1-2 wk following transport into the colony room before breeding started. Males were then singly housed in stainless steel mesh hanging cages (24 x 30 x 18 cm), and were maintained on standard laboratory chow (Ralston Purina of Canada, Woodstock, Ontario) and water. Females were placed singly with males and cage papers were checked daily for vaginal plugs. Day 1 of gestation was considered the day the plug was found. All animal use procedures were in accordance with NIH guidelines and were approved by the University of British Columbia Animal Care Committee.

On day 1 of gestation, females were re-housed in polycarbonate cages (24 x 16 x 46 cm) and randomly assigned to 1 of 3 groups: 1) Ethanol (E) which received liquid ethanol diet (36% ethanol-derived calories), ad libitum, 2) Pair-fed (PF) which received liquid control diet (maltose-dextrin isocalorically substituted for ethanol) with each female receiving the same amount of diet consumed by a female in the ethanol group matched per kg body weight on the same day of gestation; or 3) Control (C) which received laboratory chow and water, ad libitum.
II. **Diets**

The liquid diets were previously developed in our laboratory to provide adequate nutrition to pregnant females regardless of ethanol intake (Weinberg, 1985) and were prepared by Bio-Serv, Inc., Frenchtown, New Jersey (Table 1). This method of feeding has been demonstrated to be reliable in obtaining high blood alcohol levels and result in physical dependence and tolerance. Protein provided 25% and ethanol provided 36% of total calories. Maltose-dextrin was isocalorically substituted for ethanol in the liquid control diet.

The diets were made fresh every 3 days and refrigerated until feeding. The diet was offered in glass bottles with ball point drinking tubes to prevent spillage and evaporation. Fresh diet was placed on the cages daily just prior to lights off (1700 h) to avoid a shift in the maternal CORT circadian rhythm. It has been demonstrated that if animals receive a restricted amount of food (such as that received by the PF group), circadian rhythms will re-entrain to the feeding time thus shifting the CORT rhythm (Gallo & Weinberg, 1981). Bottles from the previous day were removed and weighed at this time to determine the amount of diet consumed. Experimental diets were continued until gestation day 22 when they were replaced by laboratory chow and water *ad libitum*, in order to minimize the adverse effects of ethanol on maternal lactation.

Females were undisturbed except for weighing and cage cleaning on days 1, 7, 14, and 21 of gestation. At birth, designated day 1 of lactation dams and pups were weighed and all litters culled to 10 (5 males and 5 females). Dams and pups were weighed and cages cleaned on days 1, 8, 15, and 22 of lactation. On day 22 pups were weaned, ear marked and group housed by sex and by litter. They remained group housed until testing. Both female and male offspring were used in all experiments. Animals were numbered within each
experiment and studies were run so that the investigators did not have knowledge of the prenatal treatment. No more than 1 male and 1 female from each litter were assigned to any adult experimental treatment condition.

III. Blood Sampling

Testing was conducted at consistent times within the circadian rhythm for each experiment. The testing colony room was closed off for at least 8 hours prior to testing to prevent elevation of hormone levels due to disturbance. All blood sampling was done in the lab immediately adjacent to the colony room.

For the studies in which blood samples were taken, blood was collected using a cold tuberculin syringe connected to indwelling jugular cannulae. Syringes used for collecting blood were coated with EDTA and aprotinin to prevent coagulation and denaturation. Blood samples for ACTH determination were collected and stored in plastic tubes because ACTH binds to glass. Blood was centrifuged at 3500g for 10 minutes at 4°C, and plasma was transferred to plastic Eppendorf tubes using plastic pipettes and stored at -70°C until assayed.

IV. Surgeries

A. Jugular Cannulation

Animals were implanted with indwelling jugular cannulae under halothane anesthesia 36-48 hr prior to testing. It has been demonstrated that catecholamines and other hormones return to basal levels by 24 hours after implantation (Wixson et al, 1987). The surgical and sampling procedures were in accordance with Rivier et al (1982). Cannulae were cold
sterilized with the Clindox-S system and implanted under semi-sterile conditions. The incision area on the rat was cleaned with 70% ethanol prior to surgery.

The indwelling cannula consisted of PE50 tubing with a beveled silastic tip. Cannulae were inserted into the left internal jugular vein and secured in place with 3 sutures. The free end of the cannulae were then tunneled subcutaneously, then exteriorized dorsally between the scapulae. The tip of the free end of the cannula was folded over and capped with PE 20 tubing until the testing day. On the testing day, a sampling cannula consisting of PE50 tubing with a blunted 22G x 1 1/2" needle at one end was inserted into the free exterior end of the indwelling catheter. A Luer LOK PRN adapter attached to a needle hub for injection and sampling was attached to the other end of the sampling cannulae.
B. **Intracerebroventricular (ICV) Cannulation**

ICV cannulae were implanted into the right lateral ventricle of the subjects. Surgery was done under a combination of ketamine (70-80 mg/kg body weight Ketaset, Ayerst Laboratories, Montreal) and xylazine (7-9 mg/kg body weight Rompun, Miles Canada Inc., Ontario) anaesthesia. The lateral ventricles were located using a stereotaxic instrument, using the following coordinates: AP=-0.8 mm, ML=-1.0 mm and DV=-4.1 mm relative to bregma, with the incisor bar set at 5.0 mm (Paxinos & Watson, 1986) (Figure 1). A small hole was drilled into the skull and the guide cannula lowered into place. Four small stainless steel screws were implanted around the cannula, and dental acrylic was applied over the screws and the lower part of the cannula to hold it in place. The skin was sutured and a dummy cannula was inserted into the guide cannula. A 1% betadine solution was applied to the surface of the sutured skin to prevent infection. Infusion of substances into the ventricle was accomplished with an infusion pump (Harvard Apparatus Syringe Infusion Pump 22, Southatick, Massachusetts) at a controlled rate of 5 μl/min. After animals were tested, cannulae placements were verified by infusion of toluene blue dye into the cannulae. The brains were then extracted and sectioned coronally. Only animals demonstrating dye in both lateral ventricles were included in the data analysis.

V. **Solutions Administered**

CRF administered intravenously (IV) was dissolved in PBS with 1% bovine serum albumin. CRF administered ICV was dissolved in sterile water. CRF solutions were not vortexed but were mixed by gentle inversion of the tubes to prevent denaturation.
Tubing used to connect the infusion pump to ICV cannulae were coated with bovine serum albumin using 1% BSA in PBS at least 24 hr prior to testing day, to prevent binding of CRF to the cannulae during infusion.

VI. Testing Room

The testing room had controlled temperature and lighting similar to the colony room. The room was kept as quiet as possible and a white noise of 40 dB was used to mask extraneous sounds.

VII. Behavioural Test: Elevated Plus (+) Maze

The +-maze was designed according to the specifications of Pellow et al (1985). It was constructed of black Plexiglass attached to a wooden base for support, and consisted of two open arms (50 x 10 cm) and two closed arms (50 x 10 x 40 cm) such that the two closed arms were opposite each other, and the two open arms were opposite each other. The maze was elevated 50 cm off the floor by four wooden legs (Figure 2).

Animals were pair-housed 1 week prior to testing in the elevated +-maze to increase baseline activity. Behaviour in the +-maze was videotaped. Each videotape was scored by two independent individuals and a mean of the two scores was used for data analyses. The following measures were taken: time on and entries into open arms and closed arms, total number of entries and total number of rears.
VIII. Assays

A. Plasma Corticosterone Levels

Total CORT (bound plus free) was measured by radioimmunoassay (RIA) in plasma extracted in absolute ethanol (1:10 v/v), using an adaptation (Weinberg & Bezio, 1987) of the method of Kaneko et al., (1981). Antiserum was obtained from Immunocorp, Montreal, PQ. Tracer, [1,2,6,7-3H]-CORT, was obtained from Dupont, New England Nuclear, Mississauga, Ontario; unlabelled CORT for standards was obtained from Sigma, St. Louis, Missouri. Dextran coated charcoal was used to absorb and precipitate free steroids after incubation. Samples were counted in Formula 989, (Dupont, New England Nuclear, Mississauga, Ontario). The intra and interassay coefficients of variation were 3% and 3.9%, respectively.

B. Plasma ACTH Levels

Plasma ACTH was assayed using a modification of the Incstar equilibrium RIA (Incstar Inc., Stillwater, Minnesota) with all reagent volumes halved and 50 µl rather than 100 µl plasma per sample used. The antiserum cross reacts 100 % with Porcine ACTH1-39 and Human ACTH1-24 but shows less than 0.01% crossreactivity with α-melanocyte-stimulating hormone, β-endorphin, β-lipotropin, leucine enkaphalin, methionine enkephalin, bombesin, calcitonin, parathyroid hormone, FSH, vasopressin, oxytocin, and substance-P (Orth, 1979). The midrange intra and interassay coefficients of variation were 3.9% and 6.5%, respectively.
IX. Statistical Analyses

All data were analyzed by appropriate analyses of variance (ANOVA) for factors of sex, prenatal treatment (E, PF and C) and adult experimental treatment. Post hoc analyses were done using the Newman-Keuls method.
Table 1. Liquid Rat Diet

<table>
<thead>
<tr>
<th></th>
<th>Pair-fed Diet (kcal/l)</th>
<th>Ethanol Diet (kcal/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROTEIN</td>
<td>258</td>
<td>258</td>
</tr>
<tr>
<td>FAT</td>
<td>255</td>
<td>255</td>
</tr>
<tr>
<td>CARBOHYDRATE</td>
<td>486</td>
<td>118</td>
</tr>
<tr>
<td>ETHANOL</td>
<td>0</td>
<td>368</td>
</tr>
<tr>
<td>TOTAL</td>
<td>999</td>
<td>999</td>
</tr>
</tbody>
</table>

1 Theoretical parameters of diet as specified by Bio-Serv. The actual values have a variability of less than or equal to 10% of the theoretical values due to analytical variability, sampling variability, and moisture levels.
Figure 1. Location of Intracerebroventricular Cannula

1 Coronal section of rat brain, adapted from Paxinos & Watson (1986). The ICV cannulae were lowered into the right lateral ventricle (LV).
Figure 2. Elevated Plus Maze
CHAPTER 3: PRENATAL ETHANOLO EXPOSURE AND THE RESPONSIVENESS OF THE ANTERIOR PITUITARY TO CORTICOTROPIN-RELEASING HORMONE

I. Introduction

Rodents prenatally exposed to ethanol show increased HPA responsiveness to stressors as previously discussed (see Chapter 1). The mechanisms underlying this hyperresponsiveness is still unclear. Possible mechanisms may involve either stimulatory or inhibitory components of the cascade of HPA hormones involved during a stress response. In particular, the observed hyperresponsiveness may be due to a deficit in feedback inhibition of HPA activity and/or an increased sensitivity of the anterior pituitary and/or adrenals to secretagogues. Previous work from our laboratory investigating inhibitory components of the HPA axis showed that E animals demonstrate deficits in feedback inhibition following dexamethasone (a glucocorticoid analogue) suppression compared to C animals. This deficit was shown in E females but not E males during the trough, and in both E males and E females during the peak of the HPA circadian rhythm (Osborn et al, 1996). Work in our laboratory and in that of others investigating stimulatory components of HPA activity showed that the adrenal glands do not exhibit an altered sensitivity to ACTH stimulation following prenatal ethanol exposure at least when tested at the trough of the HPA circadian rhythm (Lee & Rivier, 1994; Taylor et al, 1982a), suggesting that the hyperresponsiveness of the HPA axis seen in E offspring may be mediated at the level of the anterior pituitary or higher. A previous study demonstrated that the anterior pituitary of adult offspring does not appear to exhibit altered sensitivity to CRH stimulation following prenatal ethanol exposure.
However, that study did not examine a full dose-response curve; the study examined only one dose of CRH (7 μg/kg body weight), at one time point (1 hr) following CRH administration. The present study was designed to investigate the effects of prenatal ethanol exposure on the sensitivity of the anterior pituitary to CRH stimulation during both the trough and peak of the HPA circadian rhythm.

II. Objectives and Experimental Design

The objective of this study was to test the hypothesis that the HPA hyperresponsiveness observed in animals prenatally exposed to ethanol is due, at least in part, to an increased responsiveness of the anterior pituitary to CRH stimulation. To test this hypothesis, anterior pituitary responsiveness of E, PF and C animals to CRH administered peripherally was compared following dexamethasone suppression. Dexamethasone, is an analogue of CORT which preferentially binds to glucocorticoid receptors. It binds to the hypothalamus and the anterior pituitary to inhibit CRH and ACTH secretion, respectively. The purpose of dexamethasone for the experiments in this study is to suppress endogenous release of CRH, in order more effectively to observe the effects of CRH administered exogenously. Responsiveness was assessed by measuring plasma ACTH levels following CRH infusion.

Three major experiments were conducted. In Experiment 1, control animals were tested to investigate the dose-response relationship between CRH and pituitary ACTH release. Based on the data gathered from Experiment 1, optimal low and high doses of CRH for Experiments 2 and 3 were determined. In Experiments 2 and 3, animals from E, PF and
C groups were tested to compare the responsiveness of their anterior pituitary to CRH simulation during the trough (AM) and the peak (PM) of the circadian rhythm of the HPA axis, respectively.

III. General Methods

Subjects were the adult male and female offspring, 90 - 120 days of age, from the C treatment group, for Experiment 1; and from the E, PF and C treatment groups, for Experiments 2 and 3, bred as described in Chapter 2. The offspring were group housed by litter and by sex, until one week before testing, when animals were singly housed. At 36 to 48 hours prior to testing, internal jugular cannulae were implanted as described in Chapter 2. On the day of testing, animals were removed one at a time from the colony room and transferred to an adjacent room. This was done with minimal noise and disturbance to the rest of the animals in the colony room to prevent activation of the HPA axis of animals not currently being tested. In the adjacent room, a sampling cannula was attached to the exteriorized end of the subject’s jugular cannula. The subjects were then injected with dexamethasone intraperitoneally (i.p.) and immediately placed singly into a holding bucket (30 x 20 cm) where they remained throughout testing.

Specific doses of hormones and sampling times are described in each experiment. For all three experiments, dexamethasone was administered three hours prior to infusion of CRH or saline. Immediately prior to infusion of CRH or saline (vehicle control), a basal blood sample (0 min) was drawn using cold syringe coated with EDTA and aprotinin. CRH or saline was then immediately infused and blood samples collected at specific intervals following infusion. The volume of the blood samples taken was approximately 0.4 ml for all
time points except for the last time point (at 25 min for Experiment 1 and at 30 min for Experiments 2 and 3) when 1.0 ml of blood was taken.

Blood samples taken at each time point were analyzed for determination of plasma ACTH. Plasma CORT levels of blood samples from the last time point (30 min), from Experiments 2 and 3 from were also determined. Hormone levels were determined using RIA procedures as outlined in Chapter 2.

IV. EXPERIMENTS

A. Experiment 1: Pituitary ACTH responsiveness to CRH following dexamethasone suppression.

A.1. Specific Objectives and Experimental Design

The specific objectives of this experiment were to confirm the suppressive actions of dexamethasone on HPA activity and to determine the doses of CRH which would elicit an ACTH response from the anterior pituitary. To confirm the actions of dexamethasone, the pituitary ACTH response to CRH stimulation was tested in the presence and the absence of dexamethasone. The doses of CRH tested were: 0, 5, 15 and 20 μg/kg body weight for both males and females. The doses of dexamethasone used were derived from a previous study by Osborn et al (1996): 0 (saline) and 15 μg/100 g body weight for males or 30 μg/100g body weight for females.

For each treatment condition a sample size of n=2 to 4 was used, which was sufficient to provide an indication of pituitary ACTH responsiveness to the doses of exogenous CRH.
administered. The treatment conditions and their resulting ACTH levels are summarized in Tables 2 and 3.

A.2. Results

Plasma ACTH levels indicated that DEX effectively suppressed the hypothalamic-pituitary-adrenal axis in both males and females. CRH (5, 10 or 20 μg/ kg body weight) elicited an increase in plasma ACTH levels at 5 and 15 min following CRH infusion as compared to basal levels. At 25 min following CRH infusion plasma ACTH levels showed a pattern of decrease toward basal levels. Using these data, the doses of CRH chosen for Experiment 2 were: 0, 2, 10 and 20 μg/kg body weight; the doses chosen for Experiment 3 were: 0, 2 and 10 μg/kg body weight.

B. Experiment 2: Prenatal ethanol exposure and pituitary ACTH responsiveness to CRH at the trough (AM) of the hypothalamic-pituitary-adrenal circadian rhythm.

B.1. Specific Objective and Experimental Design

The specific objective of Experiment 2 was to compare anterior pituitary sensitivity to CRH stimulation in E, PF and C animals following dexamethasone suppression during the trough (AM) of the HPA circadian rhythm. Dexamethasone was administered (i.p.) three hours prior to CRH or saline infusion. The doses of dexamethasone used for this experiment were: 15 μg/100g body weight for males and 30 μg/100g body weight for females. Note that since the suppressive action of dexamethasone was confirmed in Experiment 1, CRH stimulation was only tested in the presence of dexamethasone; it was not necessary to test the effects of CRH
stimulation in the absence of dexamethasone. The doses of CRH used for this experiment were: 0 (saline), 2, 10 and 20 μg/kg body weight, as determined from Experiment 1. Blood samples were taken immediately prior to CRH or saline infusion (0 min), and at 5, 15 and 30 minutes following CRH or saline infusion.

B.2. Results

B.2.1. Developmental Results

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 11.6 ± 1.2, 13.8 ± 0.9 and 12.4 ± 4.4 g/kg body weight for wk 1, 2 and 3 of gestation, respectively.

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group (p<0.001) and days (p<0.001), as well as a group by day interaction (p<0.05). Post-hoc tests revealed that E and PF females weighed significantly less than C females on gestation day 7 (p’s<0.001), day 14 (p’s<0.01), and day 21 (p’s<0.01) (Table 4). Analysis of maternal weights during lactation revealed a significant main effect of days (p<0.001), as well as a group by day interaction (p<0.001). E and PF females weighed significantly less than C females on lactation day 1 (p’s<0.05). By lactation day 8, there were no significant differences among groups (Table 5).

There were no significant differences among groups for litter size or number of stillborn. Analysis of body weights for pups indicated a significant main effect of group (p<0.001) and a group by day interaction (p<0.001). Post hoc tests indicate that E and PF pups
weighed less than C pups on days 1, 8 and 15 (p’s<0.01) following birth. On day 22 following birth E pups weighed less than C pups (p<0.01) (Table 6).

E, PF and C animals did not differ in body weights at the time of testing in adulthood.

B.2.2. Experimental Results

**Plasma ACTH.** An overall ANOVA (sex x adult experimental treatment [CRH/saline] x prenatal treatment [E/PF/C] x time) indicated that females showed significantly higher plasma ACTH levels compared to males (p<0.05) (Figure 3). Data were then further analysed separately for males and females. For both males and females, there were significant adult experimental treatment by time interactions (p’s<0.001). That is, at 5, 15 and 30 min after CRH infusion, males and females infused with CRH (all doses) had significantly higher plasma ACTH levels compared to males and females infused with saline, respectively (p’s<0.05).

Further analyses of adult experimental treatment x prenatal treatment x dose interactions at each sampling time revealed different patterns of response for males (Figure 4) and females (Figures 5). For males, post hoc analyses indicated that prior to infusion (0 min) of CRH or saline, there were no significant differences in basal plasma ACTH levels among E, PF and C males. At 5 min following infusion of 2 μg/kg body weight CRH, and at 15 min following infusion of 10 μg/kg body weight CRH, PF males showed higher plasma ACTH levels compared to their E and C counterparts (p's<0.01), respectively. There were no significant differences in plasma ACTH levels among E, PF and C males at 30 min following infusion of CRH (all doses) (Figure 4).

For females as for males, prior to infusion (0 min) of CRH or saline, there were no significant differences in basal plasma ACTH levels among E, PF and C females. In contrast to
males however, at all time points (5, 15 and 30 min) following infusion of 2 µg/kg body weight CRH, E females showed higher plasma ACTH levels compared to their PF and C counterparts (p's<0.05, except at 30 min, E<C, p<0.07). Similarly, following infusion of 20 µg/kg body weight of CRH, E females showed significantly higher plasma ACTH levels compared to their PF counterparts at 30 min post-infusion (p<0.05), and trends toward higher plasma ACTH levels compared to their C counterparts at 15 (p<0.07) and 30 min (p<0.11) post-infusion, respectively (Figure 5).

**Plasma CORT.** Plasma CORT levels were determined from blood samples taken from 30 min following CRH or saline infusion. As expected, overall, females had significantly higher plasma CORT levels compared to males (p<0.001). There were significant effects of adult experimental treatment for both males and females (p's<0.001) (Figure 6A). Males infused with CRH (all doses) showed higher plasma CORT levels compared to males infused with saline (p's <0.001, except for males infused with 2 µg/kg body weight of CRH, p<0.08). In addition, there was a significant dose-response relationship; males infused with 10 or 20 µg/kg body weight CRH showed higher plasma CORT levels compared to males infused with 2 µg/kg body weight CRH (p<0.01 and p<0.001 respectively).

Similarly, females infused with CRH (all doses) showed significantly higher plasma CORT levels compared to females infused with saline (p's<0.05), and a significant dose-response relationship was observed. Plasma CORT levels were significantly higher following infusion of 10 µg/kg body weight CRH compared to 2 µg/kg body weight CRH (p's<0.05); and were higher following infusion of 20 µg/kg body weight CRH compared to 10 µg/kg body...
weight CRH (p<0.05). A significant overall effect of prenatal treatment was also observed (p<0.05). Overall, E females showed higher plasma CORT levels compared to C females (p<0.05); however, post hoc comparisons only reached significance at the 20 µg/kg body weight CRH dose (p<0.05).

C. Experiment 3: Prenatal ethanol exposure and pituitary ACTH responsiveness to CRH at the peak (PM) of the hypothalamic-pituitary-adrenalc circadian rhythm.

C.1. Specific Objective and Experimental Design

The specific objective of Experiment 3 was to compare anterior pituitary sensitivity to CRH stimulation in E, PF and C animals following dexamethasone suppression during the peak of the HPA circadian rhythm (PM). Dexamethasone was administered (i.p.) three hours prior to CRH or saline infusion. The doses of dexamethasone used for this experiment were: 15 µg/100g body weight for males and 30 µg/100g body weight for females. As in Experiment 2, CRH stimulation was only tested in the presence of dexamethasone. The doses of CRH used for this experiment were: 0 (saline), 2 and 10 µg/kg body weight, as determined from Experiment 1. Blood samples were taken immediately prior to CRH or saline infusion (0 min), and at 5, 15 and 30 minutes following CRH or saline infusion.
C.2. Results

C.2.1. Developmental Results

Ethanol intake of pregnant females was consistently high throughout gestation, averaging $10.4 \pm 1.3$, $12.0 \pm 1.0$ and $11.3 \pm 0.9$ g/kg body weight for wk 1, 2 and 3 of gestation, respectively.

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group ($p<0.001$) and days ($p<0.001$), as well as a group by day interaction ($p<0.01$). Post-hoc tests revealed that E and PF females weighed significantly less than C females on gestation day 7 ($p'<0.001$), day 14 ($p'<0.001$), and day 21 ($p'<0.05$) (Table 7). Analysis of maternal weights during lactation revealed a significant main effect of days ($p<0.001$). E and PF females weighed significantly less than C females on lactation day 1 ($p'<0.05$). By lactation day 8, there were no significant differences among groups (Table 8).

There were no significant differences among groups for litter size or number of stillborn. Analysis of body weights for pups indicated a significant main effect of group ($p<0.001$) and a group by day interaction ($p<0.001$). Post hoc tests indicate that E and PF pups weighed less than C pups on days 1, 8, 15 and 22 ($p'<0.01$) following birth (Table 9).

E, PF and C animals did not differ in body weights at the time of testing in adulthood.

C.2.2. Experimental Results

**Plasma ACTH.** An overall ANOVA (sex x adult experimental treatment [CRH/saline] x prenatal treatment [E/PF/C] x time) indicated that females showed significantly higher plasma ACTH levels compared to males ($p<0.05$) (Figure 7). Data were further analysed separately for males and females. For both males and females, there were significant adult experimental
treatment by time interactions (p’s<0.001). That is, at 5, 15 and 30 min after infusion of CRH or saline, males and females infused with CRH (all doses) had significantly higher plasma ACTH levels compared to males and females infused with saline, respectively (p’s<0.001).

Further analyses of adult experimental treatment x prenatal treatment x dose interactions at each sampling time revealed that for both males (Figure 8) and females (Figure 9), there were no significant differences in plasma ACTH levels among animals in E, PF and C groups across all doses at all time points except at 30 min following saline infusion, when E males showed significantly lower plasma ACTH levels compared to C males (p<0.05).

**Plasma CORT.** Plasma CORT levels were determined from blood samples taken from 30 min following CRH or saline infusion. As expected, overall, females had higher plasma CORT levels compared to males (p<0.001). Furthermore, there were significant effects of adult experimental treatment for both males and females (p’s<0.001) (Figure 6B). Males infused with 10 μg/kg body weight CRH showed higher plasma CORT levels compared to males infused with saline or 2 μg/kg body weight CRH (p’s<0.001); and, females infused with 2 or 10 μg/kg body weight of CRH showed higher plasma CORT levels compared to females infused with saline (p<0.05 and p<0.001, respectively). In addition, there was an overall effect of prenatal treatment for males (p<0.01) as well as trends toward prenatal treatment by adult experimental treatment interactions for both males (p<0.07) and females (p<0.10). Specifically, E and PF males infused with 10 μg/kg body weight CRH showed lower plasma CORT levels compared to their C counterparts (p’s<0.001), whereas PF females
infused with 10 μg/kg body weight of CRH showed significantly higher plasma CORT levels compared to their E and C counterparts (p’s<0.05) (Figure 6B).

V. DISCUSSION

The results of the present study demonstrate a dose-response relationship between exogenously administered CRH and plasma ACTH and CORT. Higher doses of CRH resulted in higher and more prolonged ACTH and CORT responses in both males and females during both the trough and the peak of the HPA circadian rhythm. The data also demonstrate increased plasma ACTH levels in response to exogenous CRH in females as compared to males during both the trough and the peak of the HPA circadian rhythm, reflecting the known sexual dimorphism in pituitary-adrenal activity. Importantly, effects of prenatal ethanol exposure were also observed. During the trough of the HPA circadian rhythm, E females demonstrated increased plasma ACTH and CORT levels in response to exogenous CRH compared to PF and C females. There were no differences in plasma ACTH and CORT levels in the absence of exogenous CRH. In contrast, during the peak of the HPA circadian rhythm, E, PF and C animals did not differ significantly in plasma ACTH levels in response to exogenous CRH, whereas plasma CORT levels were decreased in E and PF males compared to C males following CRH infusion (10 μg/kg body weight). Furthermore, in the absence of exogenous CRH (i.e. infusion of vehicle), E males demonstrated decreased plasma ACTH levels compared to C males at the peak of the HPA circadian rhythm.

In experiment 1, ACTH levels observed in response to increasing doses of exogenous CRH without dexamethasone suppression were consistent with those observed in previous
studies (Lee & Rivier, 1993; Wynn et al, 1988). Furthermore, in all of the experiments, ACTH levels in response to exogenous CRH were lower following dexamethasone suppression than in conditions without dexamethasone, as expected. All doses of CRH resulted in elevated ACTH, with a peak seen between 5 and 15 min following CRH infusion. Higher doses of CRH resulted in more prolonged elevations in ACTH levels.

The present study utilized dexamethasone to inhibit the release of endogenous CRH. Previous studies have shown that E animals (aged 14 -22 days) exhibit increased hypothalamic CRH content following ether or cold stress (Angelogianni and Gianoulakis, 1989), as well as increased CRH mRNA levels in the parvocellular division of the paraventricular nucleus following mild, inescapable electroshocks (Lee et al, 1990) compared to control animals. It is not known whether the increased levels of hypothalamic CRH and CRH mRNA are exhibited between 90-120 days of age, which is when testing was carried out for the present study, or, whether the amount of CRH endogenously released in E animals following exposure to stressors is higher than in C animals. However, if hypothalamic CRH is in fact increased in E compared to C animals following exposure to a stressor, and assuming that hypothalamic CRH and CRH mRNA reflect the amount of peptide released during a stress response, then endogenous CRH levels may interact with the effects of exogenously administered CRH on ACTH release. For this reason, dexamethasone, a glucocorticoid analogue, was utilized in the present study to inhibit the release of endogenous CRH. Dexamethasone acts on the anterior pituitary and the hypothalamus to inhibit synthesis and release of CRH (Carnes et al, 1987; De Kloet et al, 1975; Jingami et al, 1985). Experiment 1 clearly demonstrated the inhibitory effects of dexamethasone on the HPA axis.
Dexamethasone-treated animals showed decreased basal plasma ACTH levels compared to saline-treated animals.

The present study showed an increased responsiveness of the anterior pituitary of E females to CRH stimulation during the trough but not during the peak of the HPA circadian rhythm. Previous studies have demonstrated a marked difference in the intrinsic basal hormone levels and stress responsiveness between the trough and the peak of the HPA circadian rhythm. Normally, peak HPA activity occurs prior to the onset of awakening, which occurs during the early morning in humans, and during the late afternoon in rats (around lights out); whereas the trough occurs during the late afternoon/early evening in humans, and during the early morning in rats (around lights on). Endocrinological studies have shown that there is a central neural drive from the brain to pituitary ACTH secretion that is “on” during the peak but not during the trough of the HPA circadian rhythm (Keller-Wood & Dallman, 1984). During the trough, there is evidence that pituitary ACTH secretion is autonomous in rats; that is, it does not require input from the brain. This is supported by data showing that circulating ACTH levels in the morning (trough) are similar in female rats with lesions in the medial basal hypothalamus to those in normal intact rats. Furthermore, circulating ACTH levels do not increase in the late afternoon (peak) in lesioned females, unlike levels in intact rats (Kaneko et al, 1980). Although in the present study dexamethasone clearly suppressed HPA activity in all animals, it is still possible that the central neural drive present during the peak of the HPA circadian rhythm played a role in the differences observed between the peak and the trough responses.

Studies on positive feedback or facilitation of HPA activity may also help to explain the difference between the results obtained at the trough (experiment 2) and peak (experiment
3) of the HPA circadian rhythm. As previously discussed (see Chapter 1), facilitation may occur following a novel stressor while the organism is being exposed to a chronic stressor, or, following a novel stressor presented within 24 hours of an acute stressor. The present study demonstrates a diurnal variation in ACTH secretion similar to that seen in stress-induced facilitation. In particular, previous studies on stress-induced facilitation (Akana et al, 1992; Dallman, 1992) have demonstrated that following cyanoketone treatment (an adrenal enzyme inhibitor which blocks the acute CORT response to stress), facilitation of ACTH secretion following a 30 min restraint stress administered within 12 hours of another 30-min restraint stress, was exhibited only during the trough and not during the peak of HPA activity. Akana and colleagues (1992) suggested that this diurnal variation was a result of "a neural site that lies before the final hypothalamic neuroendocrine neurons"; that is, it is controlled by higher brain centers. The mechanisms underlying facilitation are still unclear. However, it is believed that facilitation occurs when there is either an increase in CRH released by the hypothalamus as a result of stimulation from a neural site proximal to the CRH neurons in the paraventricular nuclei of the hypothalamus (Akana et al, 1992) and/or an increase in ACTH secreted by the anterior pituitary for a given quantity of CRH (Dallman, 1993). It is possible that, similar to stress-induced facilitation, the hyperresponsiveness of the HPA axis in E animals following stress is due to increased responsiveness of the anterior pituitary for a given quantity of CRH, as suggested by the results of the present study. Furthermore, similar to stress-induced facilitation, this hyperresponsiveness may be exhibited only during the trough and not during the peak of the HPA circadian rhythm.

Alternatively, it is possible that the increased plasma ACTH response to exogenous CRH shown by E animals in the present study is a result of a deficit in feedback inhibition
following dexamethasone suppression rather than an increased responsiveness of the anterior pituitary to CRH. Previous work in our laboratory examined feedback inhibition of HPA activity by measuring plasma CORT and ACTH in dexamethasone-blocked animals following stress (Osborn et al, 1996). The data demonstrated that E females but not E males exhibit increased plasma CORT levels compared to PF and C animals during the trough of HPA activity, while both E females and E males show increased plasma CORT levels and E females also exhibit increased plasma ACTH levels compared to PF and C animals during the peak of HPA activity. Thus, more robust deficits in feedback inhibition were seen in E animals during the peak as compared to the trough of the HPA circadian rhythm. In contrast, the results of the present study showed more robust effects of prenatal ethanol exposure during the trough as compared to the peak of HPA activity. It is important to note that the present study utilized exogenous CRH to stimulate the anterior pituitary, while the previous study utilized exposure to a stressor and thus endogenous CRH to stimulate the anterior pituitary. The differences in the results of these two studies suggest that the increased ACTH responsiveness exhibited by E females in the present study probably does not involve mechanisms related to feedback inhibition.

In addition, data from the present study indicated decreased plasma ACTH levels in E compared to C males at 30 min following infusion of saline (i.e. 0 μg/kg body weight of CRH) (Figure 8), and decreased plasma CORT levels in E compared to C males at 30 min following infusion of CRH (10 μg/kg body weight) (Figure 6). These decreased ACTH and CORT levels may have been a result of increased feedback inhibition in E compared to C males. During the peak of HPA activity, basal CORT levels are higher than at the trough,
and are at levels which start occupying the type II glucocorticoid receptors which are believed to be primarily responsible for negative feedback (see Chapter 1). As previously discussed, E animals typically exhibit increased CORT levels following exposure to a stressor compared to C animals. In the current study, animals experienced a mild stressor, an intraperitoneal injection for dexamethasone administration, 3 hours prior to infusion of CRH/saline. This injection undoubtedly elicited an increase in CORT levels. Although CORT levels would no longer be elevated by 3 hours following injection, the 3 hour interval is within the intermediate feedback time domain. Therefore, the combination of increased basal HPA activity during the peak of the rhythm and the prior stress of intraperitoneal injection for administration of dexamethasone which may have increased CORT levels in E compared to C animals, may have played a role in an overall increased feedback inhibition in E males, thereby resulting in the decreased ACTH and CORT levels at time of testing.

Previous studies have demonstrated that although pair-feeding provides an essential nutritional control group, pair-feeding is itself a type of experimental treatment. The present study indicated some differential effects in pair-fed animals. Increased plasma ACTH responsiveness to CRH stimulation in PF males during the trough of HPA activity, as well as increased plasma CORT levels in PF females during the peak of HPA activity were observed, suggesting that nutritional effects and/or the stress associated with pair-feeding may also play a role in the hyperresponsiveness of the HPA axis. The present data further demonstrate the long term effects of pair-feeding and highlight the importance of including an ad libitum fed control group in prenatal alcohol studies.

Overall, females in the present study demonstrated increased ACTH responsiveness to CRH stimulation compared to males. This demonstrated sex difference is consistent with
previous endocrinological studies on rats which showed increased ACTH responsiveness to stress in females compared to males (Chisari et al, 1995; McCormick et al, 1995; Shanks et al, 1994). Interestingly, a recent study on CRH stimulation following dexamethasone suppression in humans also demonstrated increased HPA activity in females compared to males (Heuser et al, 1994). Previously, it has been shown that gonadal hormones play a mediating role in the demonstrated sexual dimorphism of the HPA axis. In particular, estradiol increases HPA activity, whereas testosterone decreases HPA activity. This is supported by data showing that following gonadectomy in females, adrenal weight decreased, while estradiol replacement increased pituitary ACTH content and adrenal weight. Furthermore, following gonadectomy in males, pituitary ACTH content and adrenal weight increased, while testosterone replacement prevented both these changes (Kitay, 1963).

In addition to the expected sexual dimorphism in responsiveness of the HPA axis, the data from the present study showed more robust alterations in anterior pituitary responsiveness in E females than in E males, suggesting that females may be more vulnerable to the effects of prenatal ethanol exposure compared to males. This is consistent with previous studies on stress responsiveness following prenatal ethanol exposure. Studies by Taylor et al (1982a; 1986; 1988) and Kelly et al (1991) and colleagues have demonstrated HPA hyperresponsiveness following prenatal ethanol exposure primarily in females. Previous studies conducted in our laboratory have also demonstrated that following prenatal ethanol exposure, females often exhibit more robust increases in HPA activity compared to males. For example, in a study examining adrenocortical responsiveness to a novel test cage, both males and females demonstrated a reduction in CORT response to novelty in the presence of the opportunity to perform a consummatory task; however, females and not
males prenatally exposed to ethanol showed less attenuation of their CORT response to novelty compared to pair-fed and control animals (Weinberg, 1988). In another study examining adrenocortical responsiveness to ether stress and cardiac puncture, handled females prenatally exposed to ethanol showed higher CORT levels in response to the stressors compared to males in the same treatment condition (Weinberg & Gallo, 1982). However, previous work in our laboratory has shown that specific testing parameters such as the time course and hormonal endpoint measured, and the type of stressors used affect whether E males and/or females demonstrate HPA hyperresponsiveness. Therefore, it is possible that under different testing conditions, E males may still exhibit an increased anterior pituitary responsiveness.

In conclusion, the present study supports the hypothesis that the increased HPA responsiveness to stressors seen in females prenatally exposed to ethanol may be due, at least in part, to an increased anterior pituitary sensitivity to CRH stimulation. This altered anterior pituitary responsiveness appears to occur during the trough and not during the peak of HPA activity. Interestingly, the majority of the studies demonstrating hyperresponsiveness of the HPA axis to stressors in E animals have been conducted during the trough of the HPA circadian rhythm. The present study also suggests that there is a sex difference in anterior pituitary responsiveness to CRH stimulation following prenatal ethanol exposure. Further studies need to be conducted to ascertain the molecular mechanisms involved in CRH-induced ACTH secretion in E animals in order to further the results of this study.
Table 2. Plasma ACTH levels (mean ± SEM) in males, following CRH infusion.

A. ACTH levels in saline-treated animals (no dexamethasone)

<table>
<thead>
<tr>
<th>CRH (µg/kg body weight)</th>
<th>Time post-CRH infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min (basal)</td>
</tr>
<tr>
<td>0</td>
<td>175.5 ± 83.7</td>
</tr>
<tr>
<td>5</td>
<td>170.1 ± 46.6</td>
</tr>
<tr>
<td>10</td>
<td>238.5 ± 34.9</td>
</tr>
<tr>
<td>20</td>
<td>140.6 ± 84.1</td>
</tr>
</tbody>
</table>

B. ACTH levels in dexamethasone-treated animals

<table>
<thead>
<tr>
<th>CRH (µg/kg body weight)</th>
<th>Time post-CRH infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min (basal)</td>
</tr>
<tr>
<td>0</td>
<td>49.2 ± 3.5</td>
</tr>
<tr>
<td>5</td>
<td>81.5 ± 28.2</td>
</tr>
<tr>
<td>10</td>
<td>45.9 ± 2.7</td>
</tr>
<tr>
<td>20</td>
<td>44.1 ± 3.9</td>
</tr>
</tbody>
</table>
Table 3. Plasma ACTH levels (mean ± SEM) in females, following CRH infusion.

### A. ACTH levels in saline-treated animals (no dexamethasone)

<table>
<thead>
<tr>
<th>CRH (µg/kg body weight)</th>
<th>Time post-CRH infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min (basal)</td>
</tr>
<tr>
<td>0</td>
<td>205.3 ±44.0</td>
</tr>
<tr>
<td>5</td>
<td>229.2 ±46.0</td>
</tr>
<tr>
<td>10</td>
<td>217.9 ±8.5</td>
</tr>
<tr>
<td>20</td>
<td>331.8 ±175.9</td>
</tr>
</tbody>
</table>

### B. ACTH levels in dexamethasone-treated animals

<table>
<thead>
<tr>
<th>CRH (µg/kg body weight)</th>
<th>Time post-CRH infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min (basal)</td>
</tr>
<tr>
<td>0</td>
<td>50.9 ±4.8</td>
</tr>
<tr>
<td>5</td>
<td>85.5 ±35.4</td>
</tr>
<tr>
<td>10</td>
<td>43.7 ±1.2</td>
</tr>
<tr>
<td>20</td>
<td>70.9 ±3.3</td>
</tr>
</tbody>
</table>
Table 4.

Maternal weights (g) (mean ± SEM) during gestation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>274.5 + 2.9</td>
<td>279.8 + 3.0*</td>
<td>320.4 + 4.7*</td>
<td>391.6 + 8.4*</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>271.2 + 3.5</td>
<td>274.4 + 3.7*</td>
<td>311.1 + 5.4*</td>
<td>381.3 + 11.2*</td>
</tr>
<tr>
<td>Control</td>
<td>284.2 + 7.3</td>
<td>317.7 + 7.5</td>
<td>363.6 + 11.4</td>
<td>442.0 + 4.0</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 = day 8 < day 15 < day 22, p’s<0.05.
- For days 7, 14 and 21: (*)E = PF < C, p’s< 0.01.
Table 5.

Maternal weights (g) (mean ± SEM) during lactation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>321.9 + 5.6*</td>
<td>366.8 + 9.1</td>
<td>392.0 + 6.4</td>
<td>376.9 + 4.8</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>323.7 + 8.0*</td>
<td>348.6 + 7.5</td>
<td>379.3 + 7.5</td>
<td>366.2 + 8.6</td>
</tr>
<tr>
<td>Control</td>
<td>348.3 + 6.2</td>
<td>370.2 + 5.6</td>
<td>386.7 + 6.0</td>
<td>378.5 + 4.1</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 < day 8 = day 15 = day 22, p's<0.05.
- For day 1: (*) E=PF<C, p’s<0.05.
Table 6.
Pup weights (g) (mean ± SEM).

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.0*</td>
<td>5.6*</td>
<td>14.7*</td>
<td>13.1*</td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 0.1</td>
<td>± 0.6</td>
<td>± 0.6</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.2*</td>
<td>5.8*</td>
<td>16.5*</td>
<td>15.9*</td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 0.2</td>
<td>± 0.4</td>
<td>± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>7.2</td>
<td>6.7</td>
<td>20.5</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 0.1</td>
<td>± 0.8</td>
<td>± 0.6</td>
</tr>
</tbody>
</table>

- Main effect of days, $p<0.001$: day 1 < day 8 < day 15 < day 22, $p's<0.05$.
- For days 1, 8 and 15: (*) E=PF < C, $p's<0.01$.
- For day 22: (**) E<C, $p's<0.01$.
Table 7.
Maternal weights (g) (mean ± SEM) during gestation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>262.7 + 2.6</td>
<td>267.6 + 3.8*</td>
<td>293.3 + 3.5*</td>
<td>361.6 + 4.3*</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>267.3 + 3.5</td>
<td>265.4 + 3.3*</td>
<td>287.8 + 3.7*</td>
<td>348.5 + 9.2*</td>
</tr>
<tr>
<td>Control</td>
<td>269.7 + 4.4</td>
<td>294.3 + 4.7</td>
<td>325.1 + 4.7</td>
<td>406.0 + 14.3</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 = day 8 < day 15 < day 22, p’s<0.05.
- For days 7, 14 and 21: (*)E = PF < C, p’s< 0.05.
Table 8.

Maternal weights (g) (mean ± SEM) during lactation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>299.3 + 4.6*</td>
<td>335.3 + 3.6</td>
<td>354.8 + 5.1</td>
<td>333.0 + 4.9</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>304.5 + 5.5*</td>
<td>334.2 + 6.4</td>
<td>349.3 + 4.3</td>
<td>337.6 + 5.4</td>
</tr>
<tr>
<td>Control</td>
<td>316.4 + 5.3</td>
<td>355.6 + 5.3</td>
<td>365.8 + 5.0</td>
<td>346.1 + 4.9</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 < day 8 = day 15 = day 22, p’s<0.05.
- For day 1: (*) E=PF<C, p’s<0.05.
Table 9.

Pup weights (g) (mean ± SEM).

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.8 *</td>
<td>± 0.2</td>
<td>15.4 *</td>
<td>+ 0.5</td>
</tr>
<tr>
<td></td>
<td>5.4 *</td>
<td>+ 0.1</td>
<td>14.2 *</td>
<td>+ 0.5</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.0 *</td>
<td>+ 0.2</td>
<td>15.8 *</td>
<td>+ 0.5</td>
</tr>
<tr>
<td></td>
<td>5.5 *</td>
<td>+ 0.2</td>
<td>15.1 *</td>
<td>+ 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>6.5</td>
<td>± 0.2</td>
<td>18.0</td>
<td>+ 0.5</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>+ 0.2</td>
<td>17.2</td>
<td>+ 0.5</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 < day 8 < day 15 < day 22, p’s<0.05.
- For days 1, 8, 15 and 22: (*) E=PF<C, p’s<0.01.
Figure 3. ACTH Levels (Experiment 2 - Trough): Adult Experimental Treatment Effect Across Sampling Times

• Main effect of sex, p<0.05: males < females, p<0.05.
• Main effect of adult experimental treatment, p<0.001: 0 < 2 = 10 = 20 μg CRH, p’<<0.05.
• Adult experimental treatment by time interaction, p<0.001: for both males and females, at 5, 15 and 30 min post-infusion, (*)0 < 2 = 10 = 20 μg CRH, p’<<0.05.
ACTH LEVELS
Experiment 2 (trough)

- ○ 0 ug CRF  ■ 2 ug CRF  ● 10 ug CRF  ▽ 20 ug CRF

### Males

Plasma ACTH (pg/ml)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Females

Plasma ACTH (pg/ml)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ug CRF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ug CRF</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4. ACTH Levels (Experiment 2 - Trough): Adult Experimental Treatment by Prenatal Treatment Interaction Across Sampling Times in Males

- At 5 min post-infusion, adult experimental treatment by prenatal treatment interaction, p<0.05: for males infused with 2 μg CRH, (*)PF > E, p<0.01.
- At 15 min post-infusion, adult experimental treatment by prenatal treatment interaction, p<0.05: for males infused with 10 μg CRH, (*)PF > C, p<0.01.
ACTH LEVELS - Males

Experiment 2 (trough)

<table>
<thead>
<tr>
<th>CRH (ug/kg body wt)</th>
<th>0</th>
<th>2</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td></td>
<td></td>
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<tr>
<td>Pairfed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
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</tr>
</tbody>
</table>

Plasma ACTH (pg/ml)

- 0 min
- 5 min
- 15 min
- 30 min

CRH (ug/kg body wt)
Figure 5. ACTH Levels (Experiment 2 - Trough): Adult Experimental Treatment by Prenatal Treatment Interaction Across Sampling Times in Females

- At 5 min post-infusion, for females infused with 2 μg CRH, (*)E > PF = C, p’s <0.05.
- At 15 min post-infusion, for females infused with 2 μg CRH, (*)E > PF = C, p’s <0.001; for females infused with 20 μg CRH, (*)E > C , p <0.05.
- At 30 min post-infusion, for females infused with 2 μg CRH, (*)E > PF = C, p’s <0.05; for females infused with 20 μg CRH, (*)E > PF = C, p’s <0.05.
ACTH LEVELS - Females
Experiment 2 (trough)

CRH (ug/kg body wt)

Plasma ACTH (pg/ml)

0 min

5 min

15 min *

30 min *
Figure 6.  Corticosterone Levels (Experiments 2 - Trough and Experiment 3 - Peak): Adult Experimental Treatment by Prenatal Treatment Interaction in Males and Females

A. Experiment 2 - Trough
- Main effect of sex, p<0.001: males < females, p<0.001.
- Main effect of prenatal treatment, p<0.05: for females, E < PF = C, p<0.07 and p<0.05, respectively.
- Main effect of adult experimental treatment, p<0.001: for males, (#)0 < 2 < 10 = 20 μg CRH, p’s <0.01 (except 0 < 2 μg CRH, p<0.08); for females, (#)0 < 2 < 10 < 20 μg CRH, p’s <0.05.
- Females infused with 20 μg CRH, (*)E > C, p<0.05.

B. Experiment 3 - Peak
- Main effect of sex, p<0.001: males < females, p<0.001.
- Main effect of prenatal treatment, p<0.01: for males, E = PF < C, p’s<0.01
- Main effect of adult experimental treatment, p<0.001: for females, (#)0 < 2 = 10 μg CRH, p’s<0.05.
- Males infused with 10 μg CRH, (*)E = PF < C, p’s <0.001.
- Females infused with 10 μg CRH, (*) PF > E = C, p’s <0.05.
CORT LEVELS

- Ethanol
- Pairfed
- Control

A: Experiment 2 (trough)

B: Experiment 3 (peak)

Males

Females

Plasma CORT (ug/100 ml)

CRF (ug/kg body wt)
Figure 7. ACTH Levels (Experiment 3 - Peak): Adult Experimental Treatment Effect Across Sampling Times

- Main effect of sex, \( p<0.05 \): males < females, \( p<0.05 \).
- Main effect of adult experimental treatment, \( p<0.001 \): for both males and females: \( 0<2<10 \) \( \mu g \) CRH, \( p'<0.01 \).
- Adult experimental treatment by time interaction, \( p<0.001 \): for both males and females, at 5, 15 and 30 min post-infusion, (*)\( 0<2<10 \) \( \mu g \) CRH, \( p'<0.05 \).
ACTH LEVELS
Experiment 3 (peak)

Males

Females

Plasma ACTH (pg/ml)

Time (min)
Figure 8. ACTH Levels (Experiment 3 -Peak): Adult Experimental Treatment by Prenatal Treatment Interaction Across Sampling Times in Males

- At 30 min post-infusion, for males infused with saline, (*)E < C, p < 0.05.
ACTH LEVELS - Males
Experiment 3 (peak)

Plasma ACTH (pg/ml)

CRF (ug/kg body wt)
Figure 9. ACTH Levels (Experiment 3 - Peak): Adult Experimental Treatment by Prenatal Treatment Interaction Across Sampling Times in Females

- No significant prenatal treatment by adult experimental treatment effect.
ACTH LEVELS - Females
Experiment 3 (peak)

- **Ethanol**
- **Pairfed**
- **Control**

**Graphs:**
- **0 min**
- **5 min**
- **15 min**
- **30 min**

**X-axis:** CRF (ug/kg body wt)

**Y-axis:** Plasma ACTH (pg/ml)
CHAPTER 4: PARENATAL ETHANOL EXPOSURE AND BEHAVIOUR IN THE ELEVATED PLUS MAZE

I. Introduction

Rodents prenatally exposed to ethanol demonstrate cognitive and behavioural deficits similar to those seen in children exposed to alcohol in utero. These cognitive and behavioural deficits are seen particularly in challenging or stressful situations (Streissguth et al, 1986), and reflect hyperactivity, hyperresponsiveness and/or deficits in response inhibition. For example, increased open field activity (Bond, 1981 & 1986), increased wheel running (Martin et al, 1978), increased startle reactions (Anandam et al, 1980) and increased exploratory behaviour (Bond & DiGiusto, 1977; Riley and Meyer, 1984), as well as deficits in passive avoidance learning (Bond & DiGiusto, 1978; Gallo & Weinberg, 1982; Riley et al, 1979 & 1986), taste aversion learning (Riley et al, 1984), and nose poking behaviour (Riley et al, 1979) have been observed following prenatal ethanol exposure. In addition, prenatal ethanol exposure has also been shown to result in altered behavioural responses to stressors, including increased stress-induced analgesia, increased stress-induced ethanol consumption, and an inability to adapt to a stressful swimming paradigm.

The mechanisms underlying the altered behavioural responses, activity levels and performance following prenatal ethanol exposure are still unclear. Previous studies have demonstrated alterations in levels of and/or responsiveness to neurotransmitters and exogenous pharmacological agents following prenatal ethanol exposure, including deficits in whole brain levels of noradrenaline, dopamine and serotonin, as well as decreased synthesis and uptake of dopamine and serotonin (Druse, 1992). Furthermore, studies investigating specific brain
regions have shown increased hippocampal adrenaline and decreased septal dopamine concentrations under stressed conditions; and, increased septal serotonin, and 5-hydroxyindoleacetic acid concentrations under non-stressed conditions in female rats prenatally exposed to ethanol (Kelly, 1996). In addition, altered striatal dopamine concentrations in infant macaques have been demonstrated. However, the effects are complex, with increased dopamine levels observed when the mean maternal peak blood alcohol levels during gestation were below 249 mg/dl, and decreased dopamine levels when the mean maternal peak blood alcohol levels were 260-540 mg/dl (Clarren et al, 1990). Alterations in CNS neurotransmitter system, as well as in hormonal function (as discussed previously in this thesis) may play important roles in the behavioural problems associated with FAS.

This section of this thesis will focus on the role of CRH, a hormone with known neuroendocrine function as well as known neurotransmitter-like actions in the CNS, in mediating the behavioural alterations seen in rodents prenatally exposed to ethanol. CRH, when administered intracerebroventricularly (ICV), has been shown to elicit anxiety- or fear-related behaviours similar to those seen following exposure to stressors (Dunn and Berridge, 1990). Previous studies have successfully shown its anxiogenic effects using the elevated plus maze (Heinrichs et al, 1992; Menzaghi et al, 1994), an apparatus designed (Handely & Mithani, 1984) and validated as a test of anxiety for both rats and mice (Lister, 1987; Pellow et al, 1985; Pellow & File, 1986). Thus, the elevated plus maze is utilized in this study, to investigate possible alterations in behavioural sensitivity to CRH following prenatal ethanol exposure. CRH is of particular interest in studying FAS because it is known to affect directly both the behavioural and neuroendocrine systems of the body, suggesting that it plays a crucial role in integrating the organism’s response to stress by its actions in the CNS as well
as the pituitary gland. This is particularly important because it is in challenging or stressful situations that organisms prenatally exposed to ethanol show behavioural and hormonal hyperresponsiveness.

II. General Objective and Experimental Design

The objective of this study was to test the hypothesis that the behavioural hyperresponsiveness observed in rodents prenatally exposed to ethanol is mediated by an increased sensitivity of the brain to CRH. To test this hypothesis, we compared the sensitivity of E, PF and C male and female rats to the effects of centrally administered CRH on elevated plus maze behaviour.

Two major experiments were conducted. In Experiment 1, control animals were tested to determine the effects of increasing doses of CRH (o-CRH 138-135-15) on behaviour, and to determine the doses of D-Phe-CRH ([D-Phe^{12}, Nle^{21,38}, C^{a}MeLEU^{37}]r/h CRH (12-41)), a competitive antagonist of CRH, which would reverse the behavioural effects of CRH. In Experiment 2, animals from E, PF and C groups were tested on the + maze following low or high doses of CRH administered with or without D-Phe-CRH.

III. General Methods

Subjects were the adult male and female offspring, 60 - 90 days of age, from the C treatment group, for Experiment 1; and from E, PF and C treatment groups, for Experiment 2, bred as described in Chapter 2. One week prior to testing, subjects were pair-housed with same sex littermates and implanted with ICV cannulae in their right lateral ventricle using a
stereotaxic instrument, with procedures as outlined in Chapter 2. On the day of testing, animals were removed from their home cage and immediately infused with vehicle, CRH or D-Phe-CRH, or both CRH and D-Phe-CRH. Animals were then singly housed in a holding cage until testing in the plus maze 30 min following infusion.

The animals were placed on the plus maze for 5 min and their behaviour videotaped. The variable measured for Experiment 1 was time on open arms. The variables measured for Experiment 2 were: time on and entries into open and closed arms, total entries and total rears.

All experimental manipulations occurred during the light phase at approximately the same time each experimental day (700 to 1200 hr) to control for circadian variations in behavioural and hormonal responsiveness.

IV. Experiments

A. Experiment 1: The anxiogenic effects of CRH on elevated plus maze behaviour.

A.1. Specific Objective and Experimental Design

The specific objective of this experiment was to determine an effective low dose and high dose of CRH which would elicit behavioural effects, and an effective dose of D-Phe-CRH which would reverse the effects of CRH, in control males and females. The following doses of CRH were tested: 0 (vehicle), 0.1, 0.5, 0.75, 1.0, 1.5 and 2.0 μg/rat. The following doses of D-Phe-CRH were tested: 0 (vehicle), 1.0, 2.0, 3.0 and 5.0 μg/rat.

The experiment was conducted in two phases. The first phase determined the effective doses of CRH which elicited behavioural effects on the + maze. Results for this phase are shown in Table 10A and B. The second phase determined the effective doses of D-Phe-CRH
to antagonize the effects of CRH. Results for this phase are shown in Table 11A and B. For each treatment condition, a sample size of n=3 to 5 was used, which was sufficient to provide an indication of behavioural responsiveness to the doses of CRH and D-Phe-CRH administered.

A.2. Results

Consistent with previous studies investigating the anxiogenic effects of CRH, animals infused with CRH showed a decrease in time spent on open arms compared to animals infused with vehicle.

Effective low and high doses of CRH for each sex were established. The data from this experiment suggested that the effective low dose of CRH for males was 0.75 μg/rat (Table 10A); while the effective low dose of CRH for females was 1.0 μg/rat (Table 10B). At doses of CRH lower than these, the animals did not show a decrease in time spent on the open arms. The effective high dose of CRH for males was 1.5 μg/rat (Table 10A); the effective high dose for females was 2.0 μg/rat (Table 10B). At doses of CRH higher than these, the animals appeared ill from the side effects of CRH such as diarrhea and markedly decreased movement. The effective doses of D-Phe-CRH which reversed the effects of CRH were 1.0 μg/rat for males (Table 11A) and 2.0 μg/rat for females (Table 11B). These doses of D-Phe-CRH were effective in reversing the effects of both the high and low doses of CRH for each sex.
B. Experiment 2: Prenatal ethanol exposure and the anxiogenic effects of CRH on elevated plus maze behaviour.

B.1. Specific Objective and Experimental Design

The specific objective of this experiment was to compare the anxiogenic effects of CRH in E, PF and C animals on elevated plus maze behaviour. E, PF and C males (n=9-11) were assigned to one of the following adult experimental treatment groups (Table 12A): 0 (vehicle), 0.75 µg CRH (low dose), 0.75 µg CRH + 1.0 µg D-Phe-CRH, 1.5 µg CRH (high dose), and 1.5 µg CRH + 1.0 µg D-Phe-CRH. Similarly, E, PF and C females (n=8-10) were assigned to one of the following adult experimental treatment groups (Table 12B): 0 (vehicle), 1.0 µg CRH (low dose), 1.0 µg CRH + 2.0 µg D-Phe-CRH, 2.0 µg CRH (high dose), and 2.0 µg CRH + 2.0 µg D-Phe-CRH. These doses were determined based on the data obtained from Experiment 1.

B.2. Results

B.2.1. Developmental Results

Due to the number of subjects required for this experiment, and the need to run the experiments while the subjects were 60 - 90 days of age, it was necessary to conduct two consecutive and overlapping experiments. Thus, two separate breedings were undertaken and two sets of developmental data are presented here.

Breeding 1. Ethanol intake of pregnant females was consistently high throughout gestation, averaging 9.5 ± 1.0, 11.5 ± 1.3 and 11.2 ± 0.8 g/kg body weight for wk 1, 2 and 3 of gestation, respectively.
A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group (p<0.001) and days (p<0.001), as well as a group by day interaction (p<0.01). Post-hoc tests revealed that E and PF females weighed significantly less than C females on gestation day 7 (p’s<0.001), day 14 (p’s<0.001), and day 21 (p’s<0.01) (Table 13). Analysis of maternal weights during lactation revealed a significant main effect of days (p<0.001); body weight increased in all females during lactation. However, there were no significant differences among E, PF and C females during lactation (Table 14).

There were no significant differences among groups for litter size or number of stillborn pups. Analysis of body weights for pups indicated a significant main effect of days (p<0.001). Post hoc tests indicate that PF pups weighed less than C pups on day 1 (p<0.01) following birth. The difference between E and C pups did not reach significance. By day 8 following birth, there were no significant differences among groups (Table 15).

**Breeding 2.** Ethanol intake of pregnant females was consistently high throughout gestation, averaging 11.0 ± 1.9, 11.2 ± 1.2 and 10.0 ± 0.7 g/kg body weight for wk 1, 2 and 3 of gestation, respectively.

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of group (p<0.05) and days (p<0.001). Post-hoc tests revealed that E and PF females weighed significantly less than C females on gestation day 7 (p’s<0.01). PF females weighed significantly less than C females on day 14 of gestation (p<0.01) (Table 13). Analysis of maternal weights during lactation revealed a significant main effect of days (p<0.001), as well as a group by day interaction (p<0.001). E females weighed significantly less than PF females on lactation day 1 (p<0.01). By lactation day 8, there were no significant differences among groups (Table 14).
There were no significant differences among groups for litter size or number of stillborn pups. Analysis of body weights for pups indicated significant main effects of group (p<0.001), sex (p<0.01), days (p<0.001), as well as a group by day interaction (p<0.001). Post hoc tests indicate that male pups weighed more than female pups on days 15 and 22 following birth (p's<0.05). E and PF pups weighed less than C pups on days 1, 8, 15 and 22 (p's<0.01) following birth. In addition E pups weighed less than PF pups on days 1 and 8 (p's<0.05) following birth (Table 15).

B.2.2. Experimental Results

**Time on Open and Closed Arms.**

In females, there were no significant overall effects of prenatal treatment (E, PF or C) for time on open arms (Figure 10) or time on closed arms (Figure 11). However, there were significant overall effects of adult experimental treatment (CRH/D-Phe-CRH/vehicle) for both time on open and time on closed arms (p's<0.05). Post hoc analysis showed that females infused with the low dose of CRH spent less time on open arms and more time on closed arms compared to females infused with vehicle (p's<0.05). Treatment with D-Phe-CRH reversed the effects of the low dose of CRH on both time on open and time on closed arms to levels not significantly different from those females infused with vehicle. Inspection of Figures 10 and 11 suggests that the main effects of the low dose of CRH were due primarily to its effects on E and PF females; however, there was no significant prenatal treatment by adult experimental treatment interaction.

In males, there was a trend towards an overall effect of prenatal treatment for time on open arms (p<0.10) (Figure 12), and a significant overall effect of prenatal treatment for time
on closed arms (p<0.05) (Figure 13). Overall, E males spent more time on open arms (p's<0.08 - trend only) compared to C males, and less time on closed arms (p's<0.05) compared to PF and C males. There were significant overall effects of adult experimental treatment on time on both open and closed arms (p'<0.01). Post hoc analyses showed that males infused with either the low or high dose of CRH spent less time on open arms (p's<0.05), and more time on closed arms (p<0.06 - trend only and p<0.05, respectively) compared to males infused with vehicle. Treatment with D-Phe-CRH reversed the effects of the low dose of CRH on both time on open and time on closed arms, and the effects of the high dose of CRH on time on closed arms to levels not significant different from those of males infused with vehicle. D-Phe-CRH did not reverse the effects of the high dose of CRH on time on open arms.

Entries Into Open and Closed Arms, Total Entries and Total Rears.

In females, there were no significant main effects of prenatal treatment on entries into open and closed arms, total entries or total rears. However, significant overall effects of adult experimental treatment were observed for open arm entries (p<0.001), closed arm entries (p<0.05), total entries (p<0.001) and total rears (p<0.001).

Effects of adult experimental treatment on open arm entries are shown in Figure 14. Overall, females infused with either the low or high doses of CRH showed a decrease in open arm entries compared to females infused with vehicle or D-Phe-CRH alone (p's<0.001). Further analysis of adult experimental treatment effects within each prenatal treatment condition indicated a differential sensitivity of E, PF and C groups to CRH and D-Phe-CRH. Treatment with D-Phe-CRH reversed the suppression only in C but not in E and PF females.
Effects of adult experimental treatment on closed arm entries are shown in Figure 15. Overall, females infused with either the low or high doses of CRH showed a decrease in closed arm entries compared to females infused with vehicle or D-Phe-CRH alone (p's<0.01). Post hoc analyses indicated that the low dose of CRH decreased closed arm entries in females in all prenatal treatment groups. Treatment with D-Phe-CRH did not reverse this suppression to the levels seen in females treated with vehicle or D-Phe-CRH alone. In contrast, closed arm entries were significantly decreased in E but not PF or C females infused with the high dose of CRH compared to their vehicle infused counterparts (p<0.05). Treatment with D-Phe-CRH reversed this suppression.

Effects of adult experimental treatment on total entries are shown in Figure 16. Overall, females infused with either the low or high doses of CRH showed a decrease in total entries compared to females infused with vehicle or D-Phe-CRH alone (p's<0.001). Treatment with D-Phe-CRH reversed this suppression only for E and C but not for PF females.

Effects of adult experimental treatment on total rears are shown in Figure 17. Overall, females infused with either the low or high doses of CRH showed a decrease in total rears compared to females infused with vehicle or D-Phe-CRH alone (p's<0.001); treatment with D-Phe-CRH did not reverse this decrease. In contrast, total rears were significantly decreased in E and C but not PF females infused with the high dose of CRH compared to their vehicle infused counterparts (p<0.05 and p<0.01, respectively). Treatment with D-Phe-CRH reversed these suppressions.

In males, there were no significant overall effects of prenatal treatment on entries into open and closed arms, total entries and total rears. However, significant overall effects of
adult experimental treatment were observed for open arm entries (p<0.001), closed arm entries (p<0.05), total entries (p<0.001) and total rears (p<0.001).

Effects of adult experimental treatment on open arm entries are shown in Figure 18. Overall males infused with either the low or high doses of CRH showed a decrease in open arm entries compared to males infused with vehicle or D-Phe-CRH alone (p's<0.05); treatment with D-Phe-CRH did not reverse the overall effects of either the low or high dose of CRH. In fact, D-Phe-CRH when infused alone decreased open arm entries compared to levels in males infused with vehicle (p<0.05).

Effects of adult experimental treatment on closed arm entries are shown in Figure 19. Overall, males infused with either the low or high doses of CRH showed a decrease in closed arm entries compared to males infused with vehicle (p's<0.05). Furthermore, males infused with the low dose of CRH showed a decrease in closed arm entries compared to males infused with D-Phe-CRH alone (p<0.05). Treatment with D-Phe-CRH reversed the effects of the low dose but not the high dose of CRH to levels not significantly different from males infused with vehicle.

Effect of adult experimental treatment on total entries are shown in Figure 24. Overall, males infused with either the low or high doses of CRH showed a decrease in total entries compared to males infused with vehicle or D-Phe-CRH alone (p's<0.05). Post hoc analyses indicated that total entries were significantly decreased in E and C but not PF males infused with the low dose of CRH compared to their vehicle infused counterparts (p<0.10 - trend only and p<0.05, respectively); treatment with D-Phe-CRH reversed the effects of the low dose of CRH in C but not E males. In fact, D-Phe-CRH when infused with the high dose
of CRH actually decreased total entries in E and C but not PF males compared to their vehicle-infused counterparts (p’s<0.05).

Effects of adult experimental treatment on total rears are shown in Figure 20. Overall, males infused with both the low and high doses of CRH showed a decrease in total rears compared to males infused with vehicle (p’s<0.05); treatment with D-Phe-CRH reversed the effects of the low dose but not the high dose of CRH to levels not significantly different from males infused with vehicle. In addition, D-Phe-CRH when infused alone decreased total rears compared to levels in males infused with vehicle (p<0.05).

V. DISCUSSION

Behaviour on the plus maze has been demonstrated to be sensitive to compounds with both anxiolytic and anxiogenic properties. For example, chlordiazepoxide and diazepam which are anxiolytics, have been shown to decrease fear-related behaviour, while yohimbine and pentylenetetrazole, which are anxiogenics, have been shown to increase fear-related behaviour on the plus maze (Pellow et al, 1985). Importantly, CRH, the anxiogenic agent used in this study, has been shown to increase fear-related behaviour; furthermore, D-Phe-CRH, the CRH antagonist used in this study, has been shown to decrease or reverse the fear-related behaviour elicited by CRH (Heinrichs et al, 1992; Menzaghi et al, 1994).

When the plus maze was designed by Handley and Mithani (1984), they based it on previous data by Montgomery (1958), which showed that elevated open alleys evoke an approach-avoidance conflict which was considerably stronger than that evoked by enclosed alleys. His interpretation of this behaviour was that exposure to novel stimuli can evoke both exploratory drive and fear drive, thus generating an approach-avoidance conflict.
Furthermore, the elevated open alleys evoke a greater fear drive, and therefore more avoidance behaviour, than enclosed alleys. A recent study has validated that it is the open space of the open arms, and not the novelty or the height that is the main anxiogenic stimulus in the elevated plus maze (Treit et al, 1993). Researchers using the elevated plus maze have thus tried to quantify behaviour by measuring both time on and entries into the open and closed arms. In general, decreased time on and entries into open arms, and increased time on closed arms have been used as indices of anxiety/fear, while increased closed arm entries, total entries and total rears have been used as indices of exploration and motor activity (Belzung, 1994; Lister, 1987; Pellow et al, 1985). Although most studies use this interpretation of these behavioural measures, different ways of quantifying each measure exist. Time on and entries into open or closed arms are measured either as absolute values or as percentages of total time on or total entries into the open and closed arms together. In addition, while most studies count open arm entries when the rodent has all four feet in an open arm, some studies include instances when the rodent has its two front feet in an open arm (Zimmerberg and Farley, 1993). For the purposes of this thesis, the absolute values were used in measuring both time on and entries into arms. Open arm entries in experiment 1 were initially counted using the strictest criterion when all four feet were in an open arm. In experiment 2 however, open arm entries were counted when at least two feet were in an open arm. The decision to expand the criterion for measuring open arm entries in experiment 2 was made following examination of the data from a much greater number of subjects tested. The role of two-feet open arm entries as an index of measurement in elevated plus maze behaviour was thoroughly investigated in experiment 2. Data showed that similar to four-feet open arm entries, two-feet open arm entries occurred more often in vehicle-treated groups.
than in CRH-treated groups; therefore, two-feet open arm entries were taken into account as part of measuring open arm entries. Taken together, information from previous studies and analysis of the current data suggest that decreased time on and entries into open arms, and increased time on closed arms are measures of fear-related behaviour; and increased entries into closed arms, total entries and total rears are measures of exploration and motor activity.

The results from this experiment demonstrate that E males behave differentially in the elevated plus maze compared to C males, exhibiting decreased fear-related behaviour, as indicated by E males spending more time on open arms and less time on closed arms than C males. The results from this experiment also demonstrate that following administration of CRH, rats exhibit increased fear-related behaviour as indicated by decreased time on and entries into open arms, and increased time on closed arms, and decreased exploration and motor activity as indicated by decreased entries into closed arms, total entries and total rears. D-Phe CRH reversed the effects of CRH on all measures involving time except for the effects of the high dose of CRH on time on open arms in males. D-Phe-CRH did not reverse the effects of CRH on all measures involving entries except for the effects of the low dose of CRH on closed arm entries in males. These data suggest that D-Phe-CRH was effective in antagonizing CRH effects in some but not all behavioural measures. D-Phe-CRH used in this study may not have been sufficient to antagonize CRH in certain treatment groups. Furthermore, D-Phe-CRH may have had partial CRH agonistic properties since administration of D-Phe-CRH alone resulted in decreased open arm entries and total rears in males compared to vehicle, which were effects similar to those elicited by CRH.

This study also demonstrates that E animals have an altered sensitivity to the central effects of CRH. Although there were no significant prenatal treatment by adult experimental
treatment interaction effects on any of the measures, comparison of effects within prenatal treatment conditions demonstrated that E animals exhibit an increased sensitivity to CRH. Following infusion with the high dose of CRH, E males showed a significant decrease in open arm entries whereas E females showed a significant decrease in closed arm entries compared to their vehicle-infused counterparts. CRH did not have similar differential effects on control animals. In addition, E males infused with D-Phe-CRH together with the low dose of CRH still demonstrated significant decreases in open arm entries and total entries compared to E males infused with vehicle. In contrast, infusion of D-Phe-CRH together with the low dose of CRH to C males resulted in the restoration of open arm entries and total entries to levels not significantly different from C males infused with vehicle. It appears that the antagonistic effects of D-Phe-CRH are more robust in reversing the effects CRH in C animals as compared to E animals. Taken together, these data suggest that although administration of CRH results in overall effects across prenatal treatment conditions, the comparisons within prenatal treatment conditions demonstrate that CRH may have differential effects on the behaviour of E animals.

Comparison of behavioural effects within prenatal treatment conditions also demonstrated that pair-feeding resulted in an altered sensitivity of the CNS to CRH. PF females infused with D-Phe-CRH together with CRH still showed a significant decrease on open arm entries and total entries compared to PF females infused with vehicle, whereas D-Phe-CRH resulted in the restoration of open arm entries and total entries in C females to levels not significantly different from those of vehicle-infused animals. In addition, the high dose of CRH decreased open arm entries in PF males compared to levels in their vehicle-infused counterparts; these effects were not observed in C males. These data suggest an
increase sensitivity of PF animals to CRH. In contrast, for some measures, including high dose CRH effects on total rears in PF females and low dose CRH effects on open arm entries in PF males, the data suggest a decreased sensitivity of PF animals to CRH. Previous studies have demonstrated that although pair-feeding provides an essential nutritional control group, pair-feeding is itself a type of experimental treatment (Lee & Rivier, 1994; Weinberg, 1985; 1989). A recent study investigating the effects of prenatal protein malnutrition on elevated plus maze behaviour demonstrated that prenatal protein malnutrition in female rats resulted in increased exploration of the open arms, suggestive of lower anxiety in these animals (Almeida et al, 1996). The present study did not show overall effects of pair-feeding on elevated plus maze behaviour, but did indicate altered sensitivity of PF animals to CRH. Although these results do not provide us with a consistent indication of how pair-feeding affects the sensitivity of the CNS to CRH, the present data further emphasize that pair-feeding may have significant long term effects on behaviour and highlight the importance of including an ad libitum fed control group in prenatal alcohol studies.

This study also demonstrates that there is a sex difference in response to CRH. Males are more sensitive to CRH than females as shown by the finding that higher doses of CRH were required in females to elicit behavioural effects to the same level as seen in males. While different doses of CRH were being tested in experiment 1, it was observed that doses which produced moderate behavioural effects in females, produced illness in males, resulting in sluggishness and diarrhea which are known side effects of pathologically high doses of CRH. In addition, the increased sensitivity of males to CRH is consistent with a recent study demonstrating sex differences in anxiety behaviour as measured by the elevated plus maze; males were demonstrated to be more anxious in the elevated plus maze test compared to
females (Zimmerberg and Farley, 1993). Interestingly, in the same study, female gonadal hormones were found to play a crucial role in the observed behavioural sex difference. When females were ovariectomized prepubertally, they demonstrated increased anxiety levels.

In conclusion, these data suggest that the insult of prenatal ethanol exposure affects behavioural responsiveness to centrally administered CRH increasing fear-related behaviour and decreasing exploratory behaviour when tested in the elevated plus maze. This differential responsiveness may be due to an altered sensitivity of the CNS to the neurobehavioural effects of CRH. It is also possible that this differential responsiveness may be due at least in part to E animals exhibiting increased endogenous release of extrahypothalamic CRH in response to the stress of a novel environment, thus potentiating the effects of exogenously administered CRH. Although there were no significant differences between vehicle infused E, PF and C animals, this possibility can not be excluded in view of the fact that there were overall effects of prenatal ethanol exposure on elevated plus maze behaviour, such as those seen in time on open arms and time on closed arms in males (Figures 12 and 13). Further studies need to be carried out to determine the mechanisms underlying this altered sensitivity to central administration of CRH in rats prenatally exposed to ethanol.
Table 10.

Time (mean ± SEM) spent on the open arms following administration of CRH.

A. Males

<table>
<thead>
<tr>
<th>CRH (µg)</th>
<th>Time in the Open Arm (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.5 ± 11.9</td>
</tr>
<tr>
<td>0.1</td>
<td>11.0 ± 11.0</td>
</tr>
<tr>
<td>0.5</td>
<td>17.2 ± 6.0</td>
</tr>
<tr>
<td>1.0</td>
<td>17.6 ± 10.3</td>
</tr>
<tr>
<td>2.0</td>
<td>13.8 ± 9.6</td>
</tr>
</tbody>
</table>

B. Females

<table>
<thead>
<tr>
<th>CRH (µg)</th>
<th>Time in the Open Arm (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41.8 ± 13.8</td>
</tr>
<tr>
<td>0.1</td>
<td>39.2 ± 7.7</td>
</tr>
<tr>
<td>0.5</td>
<td>41.1 ± 11.1</td>
</tr>
<tr>
<td>1.0</td>
<td>34.0 ± 14.3</td>
</tr>
<tr>
<td>1.5</td>
<td>19.5 ± 7.4</td>
</tr>
<tr>
<td>2.0</td>
<td>8.0 ± 8.0</td>
</tr>
</tbody>
</table>
Table 11.

Time (mean ± SEM) spent on the open arms following administration of CRH and D-Phe-CRH.

### A. Males

<table>
<thead>
<tr>
<th>CRH (µg)</th>
<th>D-Phe-CRH (µg)</th>
<th>Time in the Open Arm (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>47.1 ± 8.7</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>41.0 ± 9.8</td>
</tr>
<tr>
<td>0.75</td>
<td>0</td>
<td>34.0 ± 24.0</td>
</tr>
<tr>
<td>0.75</td>
<td>1.0</td>
<td>51.5 ± 12.0</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>11.0 ± 7.3</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>43.5 ± 14.5</td>
</tr>
<tr>
<td>1.5</td>
<td>5.0</td>
<td>132.3 ± 38.2</td>
</tr>
</tbody>
</table>

### B. Females

<table>
<thead>
<tr>
<th>CRH (µg)</th>
<th>D-Phe-CRH (µg)</th>
<th>Time in the Open Arm (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>52.0 ± 18.2</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>89.0 ± 31.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>20.8 ± 7.7</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>28.7 ± 15.7</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>60.9 ± 23.1</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>16.2 ± 8.3</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>12.5 ± 6.5</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>64.3 ± 33.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>124.0 ± 88.0</td>
</tr>
</tbody>
</table>
Table 12.

Treatment conditions

A. Males

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRH (µg/rat)</th>
<th>D-Phe-CRH (µg/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Phe-CRH</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>CRH (low dose)</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>CRH (low dose) + D-Phe-CRH</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>CRH (high dose)</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>CRH (high dose) + D-Phe-CRH</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

B. Females

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRH (µg/rat)</th>
<th>D-Phe-CRH (µg/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Phe-CRH</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>CRH (low dose)</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>CRH (low dose) + D-Phe-CRH</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>CRH (high dose)</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>CRH (high dose) + D-Phe-CRH</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Table 13.

Maternal weights (g) (mean ± SEM) during gestation.

A. Breeding 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>258.3 ± 3.2</td>
<td>253.1 ± 3.9*</td>
<td>277.4 ± 3.2*</td>
<td>323.3 ± 12.4*</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>260.0 ± 3.2</td>
<td>254.1 ± 2.5*</td>
<td>279.4 ± 3.3*</td>
<td>343.5 ± 5.3*</td>
</tr>
<tr>
<td>Control</td>
<td>264.7 ± 3.9</td>
<td>293.1 ± 3.2</td>
<td>315.4 ± 4.6</td>
<td>395.4 ± 9.8</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 = day 7 < day 14 < day 21, p's<0.05.
- For days 7, 14 and 21: (*)E = PF<C, p's<0.01.

B. Breeding 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>285.4 ± 3.4</td>
<td>288.3 ± 3.8*</td>
<td>316.5 ± 6.5</td>
<td>365.3 ± 10.9</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>280.8 ± 3.4</td>
<td>282.2 ± 3.1*</td>
<td>304.4 ± 5.2**</td>
<td>353.3 ± 14.0</td>
</tr>
<tr>
<td>Control</td>
<td>289.5 ± 3.8</td>
<td>309.7 ± 3.9</td>
<td>332.9 ± 5.8</td>
<td>402.2 ± 17.1</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 = day 7 < day 14 < day 21, p’s<0.05.
- For day 7: (*) E = PF < C, p’s<0.01.
- For day 14: (**) PF < C, p<0.01
Table 14.

Maternal weights (g) (mean ± SEM) during lactation.

A. Breeding 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>285.8 ± 3.8</td>
<td>331.1 ± 4.4</td>
<td>340.9 ± 2.5</td>
<td>321.4 ± 3.0</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>297.0 ± 4.4</td>
<td>332.0 ± 4.7</td>
<td>350.8 ± 5.0</td>
<td>331.6 ± 3.7</td>
</tr>
<tr>
<td>Control</td>
<td>310.2 ± 6.1</td>
<td>335.7 ± 6.5</td>
<td>350.2 ± 5.6</td>
<td>335.6 ± 5.2</td>
</tr>
</tbody>
</table>

• Main effect of days, p<0.001: day 1 < day 8 = day 15 = day 22, p’s<0.05.

B. Breeding 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>308.8 ± 6.5*</td>
<td>357.2 ± 6.6</td>
<td>371.3 ± 3.4</td>
<td>367.1 ± 3.9</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>325.6 ± 5.0</td>
<td>350.0 ± 4.1</td>
<td>377.5 ± 3.4</td>
<td>363.4 ± 3.9</td>
</tr>
<tr>
<td>Control</td>
<td>338.1 ± 3.5</td>
<td>357.8 ± 3.5</td>
<td>370.8 ± 7.2</td>
<td>356.2 ± 5.7</td>
</tr>
</tbody>
</table>

• Main effect of days, p<0.001: day 1 < day 8 = day 15 = day 22, p’s<0.05.
• For day 1, (*)E < PF, p<0.01.
Table 15.

Pup weights (g) (mean ± SEM).

A. Breeding 1

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>Day 1 Males</th>
<th>Females</th>
<th>Day 8 Males</th>
<th>Females</th>
<th>Day 15 Males</th>
<th>Females</th>
<th>Day 22 Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>6.1 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>15.9 ± 0.5</td>
<td>15.5 ± 0.5</td>
<td>31.8 ± 0.5</td>
<td>31.0 ± 0.5</td>
<td>46.9 ± 1.1</td>
<td>46.0 ± 0.9</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.0 * ± 0.2</td>
<td>5.7 ± 0.1</td>
<td>15.3 ± 1.1</td>
<td>15.9 ± 0.6</td>
<td>32.5 ± 0.8</td>
<td>31.5 ± 0.7</td>
<td>50.4 ± 1.4</td>
<td>48.6 ± 1.3</td>
</tr>
<tr>
<td>Control</td>
<td>6.5 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>17.0 ± 0.4</td>
<td>16.2 ± 0.3</td>
<td>33.0 ± 0.7</td>
<td>31.6 ± 0.7</td>
<td>45.2 ± 4.7</td>
<td>49.5 ± 0.9</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 < day 8 < day 15 < day 22, p's<0.05.
- For day 1, (*)PF < C, p<0.01.

B. Breeding 2

<table>
<thead>
<tr>
<th>Prenatal Treatment</th>
<th>Day 1 Males</th>
<th>Females</th>
<th>Day 8 Males</th>
<th>Females</th>
<th>Day 15 Males</th>
<th>Females</th>
<th>Day 22 Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>5.5 ** ± 0.2</td>
<td>5.2 ** ± 0.2</td>
<td>14.7 ** ± 0.4</td>
<td>13.6 ** ± 0.7</td>
<td>32.3 * ± 0.8</td>
<td>30.3 * ± 0.8</td>
<td>49.9 * ± 1.0</td>
<td>46.9 * ± 1.2</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.0 * ± 0.1</td>
<td>5.7 * ± 0.2</td>
<td>16.0 * ± 0.3</td>
<td>15.1 * ± 0.3</td>
<td>32.0 * ± 0.7</td>
<td>30.7 * ± 0.5</td>
<td>49.6 * ± 1.1</td>
<td>47.0 * ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>6.8 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>18.7 ± 0.5</td>
<td>17.7 ± 0.4</td>
<td>35.2 ± 0.6</td>
<td>33.6 ± 0.6</td>
<td>55.8 ± 1.2</td>
<td>53.1 ± 1.2</td>
</tr>
</tbody>
</table>

- Main effect of days, p<0.001: day 1 < day 8 < day 15 < day 22, p's<0.05.
- For days 1 and 8, (**)E < (*)PF < C, p's<0.05.
- For days 15 and 22, (*)E = PF < C, p's <0.01.
Figure 10. Time on Open Arms - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.05:
  low C < vehicle, p<0.05.
Figure 11.  Time on Closed Arms - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.05:
  low C > vehicle, p<0.05.
TIME ON CLOSED ARMS
Females

[Graph showing time on closed arms for females across different conditions: Ethanol, Pairfed, Control. The graph includes data for vehicle, D-Phe-CRH, low C, low C + D, high C, high C + D.]
Figure 12. Time on Open Arms - Males

- A trend towards a main effect of prenatal treatment, p<0.10: (E) > C, p<0.08.
- Main effect of adult experimental treatment, p<0.01:
  low C = high C = high C+D < vehicle, p's<0.05.
TIME ON OPEN ARMS
Males

- Ethanol
- Pairfed
- Control

![Graph showing time on open arms for males with group comparisons]
Figure 13. Time on Closed Arms - Males

- Main effect of prenatal treatment, p<0.05:
  \( (#) E < PF = C, p's < 0.05. \)
- Main effect of adult experimental treatment, p<0.01:
  low C = high C > vehicle, p<0.06 and p<0.05, respectively.
TIME ON CLOSED ARMS
Males

- Ethanol
- Pairfed
- Control

![Graph showing time on closed arms for males across different conditions with error bars for each group.](image-url)
Figure 14. Open Arm Entries - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001:
  low C = low C+D = high C = high C+D < vehicle = D-Phe-CRH, p's<0.001.
- Comparison within prenatal treatment group:
  For E females, (α)low C = low C+D = high C = high C+D < vehicle = D-Phe-CRH, p's<0.05.
  For PF females, (β)low C = low C+D = high C+D < vehicle = D-Phe-CRH, p's<0.05; and high C < vehicle, p<0.05.
  For C females, (k)low C = high C < vehicle = D-Phe-CRH, p's<0.05 for low C compared to vehicle and D-Phe-CRH, and p's<0.06 for high C compared to vehicle and D-Phe-CRH.
OPEN ARM ENTRIES
Females

- Ethanol
- Pairfed
- Control

Entries

vehicle  D-Phe-CRH  low C  low C + D  high C  high C + D
Figure 15. Closed Arm Entries - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001:
  low C = low C+D = high C = high C+D < vehicle = D-Phe-CRH, p’s <0.01
- Comparisons within prenatal treatment group:
  For E females, (α)high C < vehicle, p<0.05
CLOSED ARM ENTRIES
Females

- Ethanol
- Pairfed
- Control

Full Entries

vehicle  D-Phe-CRH  low C  low C + D  high C  high C + D
Figure 16.  Total Entries - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001:
  \[ \text{low C} = \text{low C+D} = \text{high C} = \text{high C+D} < \text{vehicle} = \text{D-Phe-CRH}, p's<0.001. \]
- Comparisons within prenatal treatment group:
  For E females, (α)\[ \text{low C} = \text{high C} < \text{vehicle}, p's<0.05. \]
  For PF females, (β)\[ \text{low C} = \text{low C+D} = \text{high C} = \text{high C+D} < \text{vehicle}, p's<0.05. \]
  For C females, (κ)\[ \text{low C} = \text{high C} < \text{vehicle}, p's<0.05. \]
TOTAL ENTRIES
Females

- Ethanol
- Pairfed
- Control

Full + Partial Entries

vehicle  D-Phe-CRH  low C  low C + D  high C  high C + D
Figure 17.  Total Rears - Females

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001:
  low C = low C+D = high C = high C+D < vehicle = D-Phe-CRH, p's<0.001.
- Comparisons within prenatal treatment group:
  For E females, (α)high C < vehicle, p<0.05
  For C females, (κ)high C < vehicle, p<0.01.
TOTAL REARS
Females

- Ethanol
- Pairfed
- Control

Graph showing the total rears for females across different conditions and treatments.
Figure 18. Open Arm Entries - Males

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001.
  low C = low C+D = high C = high C+D < vehicle, p's <0.05;
  D-Phe-CRH < vehicle, p<0.05; and, D-Phe-CRH > low C = low C+D = high C = high C+D, p's<0.05.
- Comparisons within prenatal treatment group:
  For E males, (a)low C = low C+D = high C = high C+D < vehicle, p's<0.05.
  For PF males, (b)high C = high C+D < vehicle, p<0.06 and p<0.01 respectively.
  For C males, (k)low C = high C+D < vehicle, p's<0.05.
OPEN ARM ENTRIES
Males

Ethanol
Pairfed
Control

Entries

vehicle  D-Phe-CRH  low C  low C + D  high C  high C + D
Figure 19.  Closed Arm Entries - Males

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.05:
  high C < vehicle, p<0.05;
  low C = high C+D < vehicle = D-Phe-CRH, p’s<0.05.
CLOSED ARM ENTRIES
Males

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Pairfed</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle D-Phe-CRH low C low C + D high C high C + D</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Full Entries

vehicle D-Phe-CRH low C low C + D high C high C + D
Figure 20. Total Entries - Males

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001:
  - low C = low C+D = high C = high C+D < vehicle = D-Phe-CRH, p’s <0.05
- Comparisons within prenatal treatment group:
  For E males, (α)low C = low C+D = high C+D < vehicle, p<0.10, p<0.05 and p<0.05, respectively.
  For C males, (κ)low C = high C+D < vehicle, p<0.05.
Figure 21.  Total Rears - Males

- No significant main effect of prenatal treatment.
- Main effect of adult experimental treatment, p<0.001
  low C = low C+D = high C = high C+D < vehicle, p's <0.05;
  D-Phe-CRH < vehicle, p<0.05.
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The studies on this thesis focused on the examination of: 1) a possible mechanism which may underlie the HPA hyperresponsiveness seen in animals prenatally exposed to ethanol; and 2) a possible mechanism which may underlie the behavioural hyperresponsiveness seen in animals prenatally exposed to ethanol. Specifically, the experiments in the first study (Chapter 3) investigated the possible alterations in sensitivity of the anterior pituitary to CRH stimulation following dexamethasone suppression in E animals; while the experiments in the second study (Chapter 4) investigated the possible alterations in sensitivity of the CNS to the anxiogenic effects of CRH as measured by the elevated plus maze in E animals.

The first study in this thesis compared CRH-induced plasma ACTH and CORT in E, PF and C animals. Higher doses of CRH resulted in higher and more prolonged ACTH and CORT responses. Overall, females showed higher ACTH and CORT responses. Importantly, data showed that following dexamethasone suppression, E females exhibited increased plasma ACTH responses to CRH compared to C females during the trough but not during the peak of HPA activity. Thus, the results of this study suggest that there is a sex difference in anterior pituitary responsiveness to CRH stimulation following prenatal ethanol exposure, demonstrating that females are more vulnerable to the effects of prenatal ethanol exposure than males. Furthermore, the results from this study also suggest that the HPA hyperresponsiveness to stressors seen in offspring prenatally exposed to ethanol may be mediated by alterations in the sensitivity of the anterior pituitary to CRH stimulation at least during the trough of the HPA circadian rhythm. To further the results of this study, cellular
mechanisms involved in the alteration of anterior pituitary sensitivity to CRH need to be investigated. CRH acts on the anterior pituitary via a cAMP-mediated second messenger pathway. Using standard adenylate cyclase assays, the in vitro production of cAMP by the anterior pituitary corticotropes in response to CRH stimulation may be investigated. Changes in CRH receptor levels in the anterior pituitary may also be evaluated using CRH receptor binding assays.

The second study in this thesis investigated fear-related behaviour, exploration and motor activity in the elevated plus maze in response to centrally administered CRH. Data showed that centrally administered CRH increased fear-related behaviour as indicated by decreased time on and entries into open arms, and increased time on closed arms; and decreased exploration and motor activity as indicated by decreased entries into closed arms, total entries and total rears. Overall, females required higher doses of CRH to elicit behavioural effects to the same levels as in males, suggesting that males are more sensitive to the effects of CRH on elevated plus maze behaviour. D-Phe-CRH reversed the effects of CRH in some but not all of the behavioural measures. Overall, E animals demonstrated decreased fear-related behaviour in the elevated plus maze. Furthermore, following administration of CRH, E animals showed increased fear-related behaviour and decreased exploration and motor activity. Thus, the results from this study support the hypothesis that the behavioural hyperresponsiveness to stressors seen in offspring prenatally exposed to ethanol may be mediated in part by increased sensitivity of the CNS to the anxiogenic effects of CRH. To further the results of this study, the cellular mechanisms involved in the alteration of CNS sensitivity to CRH need to be investigated. Similar to the recommended experiments in the first study of this thesis, adenylate cyclase activity in various regions of
the brain in response to CRH stimulation \textit{in vitro}, and CRH receptor levels in the CNS following prenatal ethanol exposure need to be ascertained.

Together, the results of the studies in this thesis highlight the crucial role of CRH in integrating both behavioural and hormonal systems of the body in response to stressful stimuli. Although the studies conducted in this thesis investigated the peripheral and central effects of CRH separately, the mechanisms underlying hormonal and behavioural hyperresponsiveness seen in animals prenatally exposed to ethanol may be closely interrelated. Specifically, the hormones of the HPA axis may play an important role in modulating behaviour. CRH has been demonstrated to increase hypothalamic CRH, which in turn increases plasma ACTH and CORT; that is, centrally administered CRH also plays a role in activating the HPA axis (Hotta et al, 1991; Ono et al, 1985). Furthermore, glucocorticoids have been demonstrated to affect learning, retention and exploratory behaviour by integrating neurotransmitter systems which directly affect behaviour (McEwen et al 1986). Interestingly, the HPA hormones have also been shown to affect the brain during development (McEwen et al, 1986). Ethanol when consumed by a pregnant dam, is a stressor which activates the HPA axis. The elevated maternal HPA hormones may in turn affect the development of the fetal brain. Thus, the increased levels of HPA hormones may also play a role in the increased behavioural responsiveness to stressors seen in E animals during development and adulthood.

Finally, the findings from these studies may have clinical implications. Children prenatally exposed to ethanol are hyperactive, uninhibited and impulsive in behaviour, and have attention deficits which may reflect an inability to inhibit responses (Streissguth et al, 1983; 1985; 1986). These behavioural deficits are particularly seen in stressful or
challenging situations (Streissguth, 1986). Maternal drinking during pregnancy has been shown to be associated with higher post-stress cortisol levels in infants (Jacobson et al., 1993). CRH, ACTH and glucocorticoids are known to modulate behaviour during stress (McEwen et al, 1986). Thus, it is possible that sustained increases in hormones of the HPA axis could play a role in mediating the increased hyperactivity and behavioural arousal that are observed in children prenatally exposed to ethanol.

As further studies are conducted on the molecular mechanisms involved in both hormonal and behavioural hyperresponsiveness following prenatal ethanol exposure, pharmacological intervention and treatment of altered hormonal and behavioural symptoms associated with FAS may be possible.

Even though FAS is one hundred percent preventable, alcohol is so much a part of our culture, and so aggressively marketed to those least able to resist that FAS is still prevalent. Active prevention and continuing research must therefore continue on all fronts to safeguard our children’s future and the future of our people.
REFERENCES


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