EFFECTS OF FOETAL ETHANOL EXPOSURE ON HYPOTHALAMIC-PITUITARY ADRENAL AXIS FUNCTION AND BEHAVIOUR ON THE ELEVATED PLUS MAZE

by

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This thesis investigated effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal (HPA) axis function and on behaviour on the elevated plus maze (+-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.

The hormonal studies investigated the hypothesis that a deficit in feedback inhibition of the HPA axis may underlie the hormonal hyperresponsiveness seen in E rats. The effects of dexamethasone (DEX) blockade on corticosterone (CORT) levels and adrenocorticotrophin (ACTH) levels were examined over a 36 hour (h) period. Following DEX, E males and females had significantly higher stress CORT levels and/or ACTH than PF and C animals, and showed differential responsiveness following DEX administration depending upon the time of day tested. At the trough of the circadian rhythm, E males did not differ from PF and C males, whereas E females had increased stress CORT levels compared to PF and C females. In contrast, at the peak of the circadian rhythm, E males showed increased stress CORT levels but not ACTH, whereas E females showed increased stress CORT and ACTH levels. These data support the hypothesis that E animals may exhibit deficits in HPA feedback inhibition.

Two other possible mechanisms for HPA hyperresponsiveness were investigated during the trough of the CORT circadian cycle. First, adrenal sensitivity to exogenous ACTH was examined. No significant differences were found among prenatal treatment
groups in adrenal sensitivity to ACTH. Second, corticotrophin releasing factor (CRF) mRNA expression in the hypothalamus was measured in DEX suppressed animals 1 h after exposure to ether vapor. E males showed a trend toward higher CRF mRNA levels and E females demonstrated significantly higher CRF mRNA levels than their respective controls. These data suggest that HPA hyperresponsiveness seen in E animals is not due to increased adrenal sensitivity to ACTH but may be due to increased synthesis of CRF.

The behavioural studies investigated the hypothesis that alterations in behaviour seen in E animals is in part mediated by alterations in the GABA-ergic system or increased sensitivity to central CRF. Both E males and females demonstrated behavioural hyperactivity and alterations in fear on the +-maze. In addition, the hyperactivity seen in E animals appeared to be reduced by prior exposure to the open field. Furthermore, E males and females demonstrated increased sensitivity to the effects of benzodiazepine on the +-maze compared with respective controls. Due to methodological issues, the studies on central CRF sensitivity were inconclusive. These data suggest that E animals may exhibit differential responses to aversive environments but the underlying dysfunction may be altered by prior experience to aversive stimuli. Further, the data suggest that prenatal ethanol exposure may have long lasting effects on the GABA-ergic system.
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LIST OF ABBREVIATIONS

ANOVA - analyses of variance

ARBD - alcohol related birth defects

AVP - arginine vasopressin

BAL - blood alcohol level

BDZ - benzodiazepine

β-EP - beta endorphin

bw - body weight

C - control

cAMP - adenosine 3', 5'-cyclic monophosphate

CBG - corticosterone binding globulin

cGMP - guanosine 3', 5'-monophosphate

CNS - central nervous system

CORT - corticosterone

CRF - corticotrophin releasing factor

d - day

DA - dopamine

DEX - dexamethasone

DTT - dithiothreitol

E - ethanol

EEG - electroencephalograph

ERE - oestrogen responsive elements
ETOH - ethanol
FAE - fetal alcohol effects
FAS - fetal alcohol syndrome
FSH - follicular stimulating hormone
GABA - gamma aminobutyric acid
GR - glucocorticoid receptor
h - hour
hCRF - alpha-helical corticotrophin releasing factor
HPA - hypothalamic-pituitary-adrenal
ICV - intracerebroventricular
IP - intraperitoneal
LC - locus coeruleus
mRNA - messenger RNA
NE - norepinephrine
OF - open field
OT - oxytocin
OVX - ovariectomy
PBS - phosphate-buffered saline
PEA - proenkephalin
PF - pair-fed
POMC - pro-opiomelanocorticotrophin
PVN - paraventricular nucleus
RIA - radioimmunoassay

SC - subcutaneously

SEM - standard error of the mean

SSC - 300 mM NaCl/30 mM sodium citrate

wk - week

VP - vasopressin

5-HT - serotonin

+-Maze - plusmaze

%Topen - (time in open arms / (time in open arms + time in closed arms)) x 100

%Tclosed - (time in closed arms / (time in open arms + time in closed arms)) x 100
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FOREWARD

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For all portions of these papers that are reported in this thesis, Jill Osborn was the major contributor involved in conducting the research, analysing the data and writing the papers.
A. FOETAL ALCOHOL SYNDROME

For more than 250 years, the effect of chronic maternal alcohol consumption on the developing foetus has been a topic of public concern (Warner & Rossett, 1975). However adverse effects of prenatal alcohol exposure were not clinically recognised until Lemoine and colleagues (1968), in France, and Jones and colleagues (1973), in Seattle, independently described a cluster of abnormalities in children whose mothers chronically consumed high doses of alcohol during pregnancy. Jones and Smith termed this cluster of symptoms the Foetal Alcohol Syndrome (FAS) (Jones & Smith, 1973).

No single test can positively identify FAS, making it difficult for clinicians to diagnose affected infants. The Foetal Alcohol Study Group of the Research Society on Alcoholism developed criteria to aid in the diagnosis of FAS as well as terminology to be used when discussing the syndrome (Sokol & Clarren, 1989). In accordance with the work of Jones and colleagues (1973), it has been suggested that the minimal diagnostic criteria for FAS include symptoms in each of three categories: prenatal and/or postnatal growth deficiencies, central nervous system (CNS) impairment, and a characteristic facial dysmorphology all associated with high maternal alcohol intake (Sokol & Clarren, 1989). Further, it has been recognised from both clinical experience and epidemiological findings that FAS is only the most extreme end of the spectrum of deficits resulting from
prenatal alcohol exposure and that not all infants exposed to alcohol in utero develop full FAS. The term possible Foetal Alcohol Effect(s) (FAE) has been proposed to indicate that alcohol is being considered as one of the possible causes of a patient's birth defects (Sokol & Clarren, 1989). However, the term FAE has frequently been used in the literature to indicate birth defects judged milder than full FAS. Sokol and Clarren (1989) have suggested that the term FAE may be ambiguous since the relationship of the defects to maternal alcohol consumption may not be fully documented. These authors have recommended the term alcohol-related birth defects (ARBD) for describing the more subtle but still disabling symptoms that can result from prenatal exposure to alcohol (Sokol & Clarren, 1989). Children with ARBD exhibit signs/symptoms from one or more of the three diagnostic categories described above and have anatomic or functional deficits that may be attributed to the impact of prenatal alcohol exposure (Sokol & Clarren, 1989).

A.1 EPIDEMIOLOGY

FAS has been recognised for many years as one of the three most commonly identifiable causes of mental retardation, ranking third behind Down syndrome and neural tube defects (Abel, 1984). Recently, however, Abel and Sokol (1987) have rated it as the leading cause of mental retardation in the Western world. Its reported prevalence varies significantly with the social drinking habits of the population under study and the training of the clinicians working in the area in identifying the disorder. FAS has been identified
in children from all ethnic groups and socio-economic classes. However, it appears that specific ethnic groups (Native American and African American) and individuals from lower socio-economic classes have higher rates of alcohol abuse and thus increased numbers of infants diagnosed with FAS.

The average number of children diagnosed with FAS in the general population ranges from 0.43 to 3.1 per 1000 live births (Abel, 1984). In some Native American communities, the incidence may be as high as 1 in 100 live births (May et al., 1983). In 23 communities in British Columbia and 14 communities in the Yukon Territories, the rate of FAS/FAE described in children below the age of 16 was 26 and 46 per 1000 respectively (Robinson et al., 1987). Although these findings appear significant, it is believed that they may, in fact, underestimate the actual numbers of affected individuals since the diagnosis relies heavily on trained clinicians to identify the cluster of symptoms, i.e. subtle but characteristic facial features, growth deficiencies, and the presence of CNS problems (Streissguth & LaDue, 1987). The recognition of FAS also relies on communication among members of an interdisciplinary health care team who have provided prenatal, perinatal and postnatal care and are aware of maternal drinking histories. Little and colleagues (1990) examined medical charts of 40 infants born to 38 alcohol abusers. Although there were positive prenatal drinking histories in the obstetricians' reports and the infants showed various signs of FAS, a diagnosis of FAS or ARBD was not mentioned in any of the 40 charts. Early diagnosis, early intervention and systematic follow-up are key components in the management of infants with FAS. Lack
of effective communication among professionals can contribute to failure in making an already difficult diagnosis or to making an incorrect diagnosis.

A.2 PHARMACOKINETICS OF ALCOHOL

Because alcohol readily crosses the placental barrier, the alcohol levels of the foetus and the mother are approximately equivalent when pregnant women drink (Waltman & Iniguez, 1972). The distribution of alcohol is almost uniform throughout the foetus and is proportional to the tissue water content. Therefore, alcohol content is particularly high in the amniotic fluid, placenta, liver, pancreas, kidney, lung, thymus, heart, and brain (Abel, 1980). Furthermore, the foetus' ability to metabolise alcohol is limited due to deficiency of hepatic alcohol dehydrogenase, the primary enzyme in the pathway for alcohol metabolism (Dow & Riopelle, 1987). Thus, the foetus relies on passive diffusion across the placenta and maternal elimination to reduce blood alcohol levels (BAL). In addition, alcohol elimination from the amniotic fluid is approximately twice as slow as that from maternal blood, resulting in high alcohol concentrations in the amniotic fluid even when alcohol in the maternal blood has been completely eliminated (Brien et al., 1983). Therefore the developing foetus is exposed to high levels of alcohol for longer periods of time than is the mother.
Interestingly, FAS does not occur consistently in all infants exposed to high levels of alcohol in utero. The full syndrome is seen in only about one-third of infants born to women with chronic alcoholism with the remaining two-thirds of infants showing symptoms ranging from severe disabilities to no apparent deficits, suggesting that factors other than alcohol ingestion alone are involved. Factors contributing to the pathogenesis of FAS include: genetic factors which may influence alcohol metabolism, maternal health, nutritional status, drinking pattern, parity, the timing of alcohol exposure, and the use or abuse of other substances (nicotine, caffeine, marijuana, cocaine, and narcotics) (Schenker et al., 1990).

The teratogenic effects of in utero exposure to alcohol are closely related to both the timing and level of exposure. The nature of the resulting birth defects reflects the stage of embryonic development when the toxicological insult occurred. Studies have suggested that first trimester exposure is associated with organ and musculoskeletal anomalies, whereas second and third trimester exposures are associated with growth, intellectual and behavioural deficits. Neonates born to women who reduced their alcohol consumption before the third trimester were similar to offspring of rare drinkers in growth parameters, but exhibited more congenital anomalies associated with FAS/ARBDS (Rosett et al., 1983). These findings are supported by animal experiments which demonstrated that alcohol exposure during organogenesis resulted in significant skeletal and visceral anomalies but not significant behavioural abnormalities or growth.
deficits (Sulik, 1983). Women who consumed large amounts of alcohol during the second and third trimester, after the twelfth week of gestation, had infants with fewer physical anomalies but more growth, mental, and behavioural abnormalities than those who ceased drinking before the twelfth week of gestation (Aronson & Olegard, 1987). Similarly, rodents exposed to alcohol during the period equivalent to the second and third trimester in humans (the period of most rapid brain development) exhibited significant cognitive and behavioural abnormalities (Meyer et al., 1990b).

The scientific community has yet to establish "safe" levels of alcohol consumption during pregnancy. Animal research has established that the teratogenic actions of alcohol are dose-dependent (Randall et al., 1977); however, literature on human consumption during pregnancy is less clear. Epidemiological studies have indicated a definite risk for the development of "alcohol related birth defects" in infants of women who had consumed greater than six drinks per day during pregnancy (Ernhart et al., 1989). Data from Streissguth and colleagues (1989 & 1990) suggest that "social" drinking (1 ounce of absolute alcohol per day) could result in intellectual deficits. Three drinks per day during pregnancy were associated with lower IQ's in children at four years of age (Streissguth et al., 1989); two drinks a day during pregnancy were associated with a seven point decrement in IQ at seven years of age (Streissguth et al., 1990).

Importantly, it appears that it is not the amount of alcohol consumed but rather the peak blood alcohol level reached that is the key factor in producing deficits (Pierce & West, 1986). Both animal (West et al., 1989) and human studies (Clarren, 1986; Clarren...
et al., 1990) have demonstrated that binge drinking at high levels may have more devastating effects on the developing foetus than intake of the same dose of alcohol over a longer interval of time. For example, a specific dose of alcohol given to rats, condensed over a few hours as compared to spread over a 24 hour period, resulted in higher blood alcohol concentrations and produced more severe microcephaly, and greater neuronal loss, behavioural hyperactivity and impaired spatial navigation (West et al., 1989). Therefore, when discussing a "safe" level of alcohol, it is probable there is no single dose-response relationship for ethanol teratogenesis but rather that each abnormal outcome in structure, function, morphology or growth has its own dose-response and gestational timing parameter (Clarren, 1986).

A.4 CLINICAL FEATURES OF FAS

As noted above, the minimal diagnostic criteria for FAS include prenatal and/or postnatal growth deficiencies, CNS abnormalities and a characteristic facial dysmorphology. However alcohol has been shown to have teratogenic effects on almost every system of the body (Schenker et al., 1990). The multiplicity of abnormalities that may be associated with FAS include not only congenital facial malformations and/or mental retardation but also a variety of organ, musculoskeletal, neurologic, and developmental differences. Each individual displays a variable combination and severity of symptoms so that, although commonalities exist clinically, each person with FAS is also unique.
Intrauterine growth retardation appears to be directly proportional to the degree of maternal alcohol intake, even with statistical adjustment for other contributing variables such as smoking, parity and gestational age (Streissguth et al., 1980). Children with FAS are usually below the third percentile in weight, height and head circumference. It has been shown that ingestion of one ounce of alcohol per day during the last trimester of pregnancy can result in a decrease in birth weight by 150 grams (Umbreit & Ostrow, 1980). However, if alcohol consumption is reduced during the last trimester, growth outcome improves (Rosett & Weiner., 1983). Unlike prenatal growth deficiency observed in offspring of smokers, postnatal "catch-up growth" does not usually occur in children with FAS. Whereas infants of smokers demonstrate significant "catch-up growth" and are the same size or just slightly smaller than infants of nonsmokers at 12 months of age, infants with FAS who are on average 700 grams smaller at birth were approximately 4000 grams smaller than infants of non-drinkers at 12 months. For most children, the growth deficiencies persist throughout adolescence into adulthood. Streissguth and colleagues (1991), in a follow-up of 61 adolescents and adults with FAS, demonstrated continued growth deficiencies in height and head circumference for both men and women; the effect of prenatal alcohol exposure on weight was more variable, with weights ranging from extremely thin to very heavy.

Alcohol exposure in utero can result in malformations in almost all systems of the body with varying levels of incidence. As previously noted, craniofacial dysmorphology is one of the most characteristic traits and is used in the clinical diagnosis of FAS. The dysmorphic characteristics include: midface hypoplasia, thin upper lip, a long flat
philtrum, low set ears, low anterior hairline, short palpebral fissures, epicanthal folds, upturned nose, ptosis, strabismus and microphthalmia. Cardiac malformations occur in 29-41% of infants with FAS (Sandor et al., 1981), the most common being atrial or ventricular septal defects. Genital and renal malformations occur in almost half of infants with FAS (Clarren & Smith, 1978). These include genital hypospadia, labial hypoplasia, aplastic, dysplastic or hypoplastic kidneys, ureteral duplications, megaloureter, hydrenephrosis, cystic diverticulae and vesicovaginal fistulae.

Microcephaly occurs in greater than 80% of infants with FAS (Clarren & Smith, 1978). Though generally prenatal in onset, it becomes more evident as the child matures, thus reflecting deficient brain growth. The most common brain anomalies are associated with failure or interruption of neuronal and glial migration and include cerebellar dysgenesis, cerebral nuclear dysgenesis, agenesis of the corpus callosum and neuroglial heterotopias (Clarren, 1986; Mattson et al., 1992). Neural tube defects including lumbosacral myelomeningocele and anencephaly occur at a higher rate in children with FAS than in the normal population (Freidman, 1982). Other clinical neurologic findings include alterations in cerebellar function (Hanson et al., 1978), generalised hypotonicity (Streissguth & LaDue, 1987), increased rates of cerebral palsy and hemiparesis or hemiplegia (Olegard et al., 1979), and an increased incidence of seizure disorders (Burd & Martosolf, 1989).

Furthermore, immune dysfunction has been recorded to occur at a higher incidence in children with FAS. Johnson and colleagues (1981) reported that patients
with FAS had increased rates of bacterial infections, decreased erythrocyte-antibody complement, rosette forming lymphocytes, and diminished mitogen-induced lymphocyte proliferative responses, suggesting immune system dysfunction. A number of malignancies have been reported to occur in children with FAS including rhabdomyosarcoma, Wilms-tumor, acute lymphocytic leukaemia, adrenal carcinoma, hepatoblastoma, neuroblastoma, and ganglioneuroblastoma (Zaunschirm & Muntean, 1984).

Orthopaedic anomalies are found in as many as half of the children diagnosed with FAS (Goldberg, 1987). Many of the abnormalities occur at rates higher than in the general population including congenital hip dislocation, limited supination or synostosis of the elbow, hypoplasia of the terminal phalanges, thoracic cage abnormalities, hypoplasia of the radial head, clinodactyly of the toes, camptodactyly of the fingers, delayed skeletal maturation and club foot (Smith et al., 1981; Spiegel et al., 1979). Other anomalies seen with FAS include spinal stenosis, abnormalities of the cervical spine, scoliosis, ligamentous laxity, flexion contractures of the elbow and polydactyly (Smith et al., 1981; Spiegel et al., 1979).

Upper airway obstruction due to physical anomalies has been reported in a number of infants with FAS (Usowicz et al., 1986). Respiratory complications associated with upper airway obstruction include obstructive apnea, respiratory arrest, chronic hypoxia, and pulmonary hypertension; these complications place these infants at increased risk for sudden infant death syndrome.
A.5 BEHAVIOURAL PROBLEMS.

Characteristics in early life of infants with FAS include irritability with decreased total body activity, decreased suckling, cerebral excitation, severe tremors, decreased ability to habituate, insecurity, sleeping disorders, decreased alertness and failure to thrive (Hill & Tennyson, 1980; Pierog et al., 1977; Streissguth et al., 1980). In severe cases, alcohol withdrawal symptoms may also occur. Withdrawal symptoms are similar to those of adults with chronic alcoholism: irritability followed by tremors, spontaneous seizures, hypertonia, abdominal distention, opisthotonos, hyperacusis and increased respiratory rate (Pierog et al., 1977). Moreover, other symptoms described in adults with chronic alcoholism, such as hyperactivity, tachycardia, tremors of the body, severe intention tremors and abnormal fears have also been reported in early life in children with FAS (Hill & Tennyson, 1980). These symptoms decrease over the first 18 months of life.

Children with FAS tend to be hyperactive, impulsive, emotionally labile, easily distractible and have higher rates of learning disabilities. Landesman-Dwyer and colleagues (1981) found that 3.5-4.5 year old children of moderate drinkers (0.45 oz per day) had decreased attention spans and were inattentive and fidgety. In addition, it has been reported that some children with FAS/ARBD also fulfil the diagnostic criteria of autism (Harris et al., 1995; Nanson, 1992). Many of the behavioural problems seen in children with FAS persist through adolescence and into adulthood, the most common being attention deficits, poor social adaptation, and problems with comprehension and abstraction (Streissguth et al., 1991). Furthermore, adolescents and adults with FAS have
been documented to have spatial memory deficits as well as social-behavioural problems such as lying and defiance (Streissguth et al., 1991). Streissguth and colleagues (1991) conducted a follow-up study of 61 adolescents and adults with FAS and found none were independent in terms of either housing or income, due primarily to lifelong behavioural, social and cognitive disabilities.

A.6 COGNITIVE IMPAIRMENT.

Mental retardation is one of the most devastating effects of alcohol exposure in utero. When tested as adolescents and adults, the average IQ of 82 persons diagnosed with FAS/FAE was 70, which is in the borderline to mildly retarded range (Streissguth et al., 1989). In an earlier study, Streissguth and colleagues (1978) reported that the IQs of 20 children with FAS varied between 16 and 105 with a mean of 65. Research by Conry (1990) supports these findings; evaluation of 19 school age children revealed IQ's ranging between 40 and 101. The number of physical malformations appears to be inversely related to intellectual outcome; the greater the number of anomalies, the lower the IQ (Streissguth et al., 1989). In addition, children with FAS have higher rates of speech and language problems (Autti-Ramo & Granstrom, 1991a; Autti-Ramo & Granstrom, 1991b; Greene et al., 1990), auditory disorders (Church & Gerkin, 1988), visual disorders (Stromland, 1990), and visual perceptual problems (Aronson, et al., 1985). Although some individuals with FAS have IQs within the normal range they still may exhibit learning disabilities, perceptual, behavioural and language disorders and therefore are unable to function at the same level as nonexposed children.
A.7 MOTOR DEFICITS.

Jones and colleagues (1973) reported poor performance on motor tests, with delayed motor development, in children (ranging from 3 to 57 months of age) with FAS. Even during the neonatal period, infants with FAS showed motor abnormalities such as increased body tremors, abnormally increased hand-to-mouth activity, decreased total body movements, and increased head to the left orientation (Landesman-Dwyer et al., 1978). Recently, Autti-Ramo and Granstrom (1991b) assessed 53 infants (ages 18-19 months) who were exposed to alcohol prenatally and found motor delay to be more common than cognitive delay. In addition, research has suggested that the neuromotor deficits associated with FAS are not influenced by the postnatal environment. Children who were placed in foster homes did not show improvement in motor testing when compared to matched pairs who remained in the care of their birth mothers (Kyllerman et al., 1985). Thus, the abnormalities and delays in motor development are thought to be prenatal in origin and resulting from in utero alcohol exposure.

Motor delay has been reported in infants with FAS at birth (Jones & Smith, 1973), eight months (Harris et al., 1993; Streissguth et al., 1989), twelve months (Golden et al., 1982), four years (Barr et al., 1990; Streissguth et al., 1989), six and seven years (Kyllerman et al., 1985; Streissguth et al., 1985), and six to eighteen years (Conry, 1990) of age. Infants demonstrated below normal scores on the Psychomotor Developmental Index of the Bayley Scales of Infant Development as well as weak grasp, tremulousness and poor eye-hand co-ordination (Jones et al., 1973; Jones & Smith, 1973; Streissguth et
al., 1989; Golden et al., 1982; Harris et al., 1993). Four year old children exposed to alcohol in utero scored significantly lower on the Wisconsin Fine Motor Steadiness Battery including time to complete the grooved peg board, errors on the grooved form boards, and latency to self correct (Streissguth et al., 1989). There appeared to be a dose response correlation between the amount of alcohol consumed by the mother and the motor performance scores of the offspring; increased alcohol consumption resulted in decreased motor performance scores. Autti-Ramo and Granstrom's (1991b) research supported these findings; evaluation of 80 children exposed to alcohol in utero was carried out 1-3 times during the first year of life. Exposure throughout pregnancy resulted in increased incidence and severity of developmental delays whereas reduction in maternal alcohol intake by the second trimester resulted in only slight abnormalities in motor development. In addition, Barr and colleagues (1990) reported that 4-year-old children exposed to alcohol in utero scored lower on finger tapping and tactual performance tests, and had lower subjective fine motor scores as rated by examiners blinded to their condition.

Six to seven year old children with FAS scored on average 1.5 standard deviations below the mean on the motor age examination (Johnson et al., 1951) and the modified Oseretsky Test (Kyllerman et al., 1985). Although the children could complete all test items, they lacked plasticity, economy and speed of performance. Interestingly six out of 21 children in this study also had noticeable tremors. Finally, Conry (1990) evaluated 19 Native American children with FAS between the ages of six and eighteen years, comparing them to gender and age matched controls in order to control for differences
due to cultural isolation. Children in the FAS group received significantly lower scores on sensory-motor tasks such as reaction time, non-dominant finger tapping, grip strength, and motor speed as assessed using the Detroit Test of Learning Aptitude as well as on the Beery Buktenika Test of Visual Motor Integration.

Motor development in infants with FAS corresponds more closely to their mental age than to their chronological age (Jones & Smith, 1973). It has been suggested that the deficits in gross motor performance seen in infancy dissipate with age, whereas fine motor deficits persist (Dehaene et al., 1984). However, a number of recent studies have demonstrated that some deficits in gross motor co-ordination persist. Barr et al., (1990) evaluated 449 4-year-old children exposed to various levels of alcohol during gestation, on a battery of gross motor tasks adapted from the gross motor scale developed at the Crippled Children's Division of the University of Oregon Medical School. The assessment consisted of 14 tasks to evaluate control of head, trunk, lower extremities and locomotion. Children exposed to alcohol in utero scored significantly lower than nonexposed children on the majority of tests, specifically those associated with balance. Again, most of the alcohol-related motor deficits were dose-related, reinforcing the concept of no known "safe" level of consumption.

**B. ANIMAL MODELS OF FAS**

To identify alcohol as a classic teratogenic agent, animal models have been developed. Human studies are often unable to control for or isolate specific alcohol
effects from environmental effects such as pattern and level of exposure, maternal nutritional status, health, age and ability to metabolise alcohol, parity, and the exposure to other substances of abuse (nicotine, caffeine, marijuana, cocaine, and narcotics). Furthermore, the mechanisms for induction of the abnormalities cannot be examined and the length of time required to evaluate late onset problems and developmental abnormalities is very long in humans. Animal models have allowed researchers to conduct controlled studies in which the specific effects of alcohol can be isolated.

B.1 RODENT MODEL

Rodent models have been highly utilised in foetal alcohol research due to the fact that rodents are small, easily handled, have a short gestation, are relatively inexpensive to purchase, house and feed (Keane & Leonard, 1989). In addition, rat strains have been bred in controlled environments which helps to control for early experience variability. The rat foetus has a similar metabolism and foetal development follows a sequence of stages similar to the human foetus but differs in the timing of stages with respect to birth. For example, in order to understand the impact of alcohol on brain development in rodent models or to compare the results from animal studies to humans, it is important to consider differences in brain development between the two species. For humans, the major brain growth spurt occurs during the third trimester of gestation and growth then continues for about two years postnatally (West, 1987). In contrast, the major brain growth spurt in the rat occurs during the first 10-14 days of postnatal life (this can be called the "third trimester equivalent") (West et al., 1989). Although alcohol can affect
the brain at any stage of development, it is probably the most vulnerable period is during
the brain growth spurt. Thus rodent models may use a prenatal exposure paradigm which
primarily affects neurogenesis and cell proliferation or may use a postnatal exposure
paradigm which covers the brain growth spurt and primarily affects neuronal migration
and differentiation and most of the gliogenesis and synaptogenesis (West, 1987). In the
present studies the effects of prenatal ethanol exposure on the hypothalamic-pituitary-
adrenal (HPA) axis were studied using a prenatal model, as the HPA axis develops and
begins to function during prenatal life in the rat.

B.2 ADMINISTRATION OF ALCOHOL AND NUTRITION

Chronic ingestion of alcohol has profound effects on nutritional status. Primary
malnutrition can result from displacement of other nutrients by alcohol because of
alcohol’s high energy content. Animals have been shown to decrease water and food
consumption when given alcohol (Weinberg, 1984) and human studies have
demonstrated that chronic consumption of high doses of alcohol results in weight loss
even though the calorie intake may be sufficient to maintain body weight (Lieber, 1991a;
Pirola & Lieber, 1972; Pirola & Lieber, 1976). Alcohol can also cause secondary
malnutrition as a result of maldigestion or malabsorption of nutrients from the
gastrointestinal tract, alteration of nutrient activation, utilisation and degradation, and
changes in metabolism (Lieber, 1991a; Lieber, 1991b; Lieber, 1988; Lieber, 1986;
Lieber, 1983; Weinberg, 1984). Furthermore, the transport of nutrients and oxygen
across the placenta may be disrupted by vascular changes in the placenta induced by
chronic alcohol intake (Gordon et al., 1982; Jones et al., 1981; Mukherjee & Hodgen, 1982; Pratt, 1980).

Malnutrition itself has been demonstrated to be teratogenic and nutritional state can affect alcohol metabolism (Weinberg, 1985). Although malnutrition may have teratogenic effects, it is not the primary cause of developmental abnormalities seen in FAS (Weinberg, 1985). The inclusion of pairfed groups (animals whose caloric intake has been matched to that of alcohol consuming animals) has demonstrated that although poor nutrient (especially low protein (Shorey & Erickson, 1982)) intake can act synergistically with alcohol, alcohol is the main teratogen causing FAS (Weinberg, 1985; Wiener, 1980; Wiener et al., 1981). However, it must be remembered that while pair feeding can control for primary malnutrition it cannot control for secondary malnutrition. In addition, although pair feeding provides an essential nutritional control group, pair feeding itself is an experimental treatment (Weinberg, 1984). For example, pair feeding can produce shifts in the circadian rhythm of a number of physiologic variables as well as alter body and organ weights and behaviour of both the maternal female and offspring (Gallo & Weinberg, 1981; Weinberg, 1989; Weinberg & Gallo, 1982).

Another factor which must be considered when feeding ethanol to rats is the method of administration. There are a number of different methods for administration of ethanol including: injection, intubation, placing ethanol in the drinking fluid, and ethanol in a liquid diet. The method of administration must suit the scientific question being studied. In the present studies, the route of administration had to be non-stressful as it has
been demonstrated that perinatal administration of glucocorticoids can result in alterations in cellular growth, neuronal myelination and differentiation, and interfere with normal biochemical, and physiologic processes (Weichsel, 1977). Injection and intubation allow for administration of a controlled dose of ethanol and high blood alcohol levels (BAL) to be reached; but both require a great deal of handling and involve a fair amount of stress. Placing ethanol in the drinking water is non-stressful but the taste of alcohol is aversive, resulting in the rodents not drinking concentrated solutions; therefore high BALs are not reached and rodents reduce their liquid and food intake (Wiener, 1980). Addition of ethanol through a liquid diet provides a non-stressful method of administration which allows for high BALs to be reached and adequate nutritional status to be maintained (Lieber & De Carli, 1989). Therefore, ethanol in a liquid diet was chosen as the method of administration in the present studies.

**B.3 EFFECTS OF ETHANOL EXPOSURE ON RODENTS**

Rodents exposed to ethanol (ETOH) *in utero* develop many abnormalities similar to those seen in children with FAS including: developmental delay, balance deficits, gait abnormalities and behavioural and cognitive deficits. Foetal ethanol exposed (E) rats demonstrate delayed incisor eruption, delays in the ability to elevate the head above the supporting surface and in the ability to elevate the pelvis using hindlimbs, as well as impaired balance and locomotor co-ordination (i.e. ability to traverse two parallel horizontal rods) (Meyer *et al.*, 1990a). E animals show deficits in balance tasks (i.e. they have more difficulty staying on a rotating drum and fall off inclined planes at more
gradual angles than unexposed animals) (Abel & Dintcheff, 1978). Furthermore, E animals demonstrate delays in development of certain postural reflexes including righting reflex (ability to return to all four feet after being placed on the dorsum); negative geotaxis (ability to rotate 180 degrees from a head down position on an inclined plane); and reflex suspension (ability to maintain a grip on a cross bar) (Norton et al., 1988).

E rats have also been shown to have gait abnormalities similar to those seen in humans exposed to alcohol in utero. Chronic maternal alcohol exposure (Hannigan & Riley, 1988), as well as exposure only during the period corresponding to the growth spurt of the cerebellum (Meyer et al., 1990b), and consumption of alcohol in a "binge-like" fashion (Bonthius & West, 1990; Goodlett et al., 1991), have all been demonstrated to lead to gait and co-ordination difficulties in E offspring. Animals exposed to ethanol prenatally and perinatally demonstrate impairments on several tests of balance and motor ability and have significantly lower whole brain and cerebellar weights when compared to control animals (Goodlett et al., 1991; Hannigan & Riley, 1988; Meyer et al., 1990b; Norton et al., 1988; Pierce & West, 1986; Sulik, 1983; West, 1987; West et al., 1989). Gait differences include shorter stride length, increased angle of placement of hindfeet and decreased symmetry in gait relative to controls (Meyer et al., 1990a; Hannigan & Riley, 1988). The histological evaluation of E animal brains has demonstrated alterations in layer 5 of the cortex and changes in total cortical thickness (Kotkoskie & Norton, 1989), changes in microvasculature in the cerebellum, hippocampus, and dentate gyrus (Kelly et al., 1990), and altered cerebellar morphology (Hannigan & Riley, 1988) suggesting that the motor deficits may be a result of altered brain development.
Furthermore, behavioural and cognitive deficits have been demonstrated in E rats (Abel, 1979). Behavioural deficits which reflect hyperactivity and hyperresponsiveness have also been seen in E animals. These include increased open field activity, (Bond, 1981; Bond, 1986), increased wheel running (Martin et al., 1978), increased startle reaction (Anandam et al., 1980) and increased exploratory behaviour (Bond & DiGiusto, 1977a; Riley & Meyer, 1984). In addition, E rats appear to have deficits in response inhibition; they demonstrate deficits in passive avoidance learning, (Bond & DiGiusto, 1977b; Bond & DiGiusto, 1978; Gallo & Weinberg, 1982; Riley et al., 1979a; Riley et al., 1986) taste aversion learning (Riley & Meyer., 1984), and reversal learning (Lochry & Riley, 1980) as well as alterations in nose poking behaviour (Riley et al., 1979b).

Neurotransmitters and neuropeptide systems have also been shown to be adversely affected by prenatal ETOH exposure and may, in part, play a role in behavioural and cognitive problems seen in FAS (Druse, 1992). Moderate to high doses of ETOH result in significant decreases in whole brain serotonin (5-HT), dopamine (DA), and norepinephrine (NE) levels, decreases in 5-HT and DA synthesis and reuptake, low turnover of NE, and variable change in glutamate, acetylcholine, histamine, and GABA levels (Druse, 1992). Further, E rodents also demonstrate alteration in physiological responses including decreased ability to thermoregulate following administration of ETOH, pentobarbital, diazepam, or morphine (Nelson et al., 1983b; Taylor et al., 1981; Taylor et al., 1987).
C. PSYCHONEUROENDOCRINOLOGY AND STRESS

The survival of an organism in an ever changing world requires the organism to be able to maintain its internal environment within narrow limits. The integration and coordination of multiple specialised cells allows the animal to maintain its existence and reproduce. To survive, the animal requires the capacity to adjust and to adapt to hostile conditions in the external environment, and to co-ordinate reproduction with those factors in the internal and external environments that are most conducive to survival of the offspring (Sapolsky, 1992). Selye (1936) defined stress as “an alteration in the body’s hormonal and neuronal secretions caused by the central nervous system in response to a perceived threat” and defined a stressor as “a change in an organism’s internal or external environment which is perceived by the organism as threatening.”

The term stress has a very broad usage in the scientific and popular literature. Stress has been used to describe a mental/emotional state, a perceived threat in the external environment, or as the physiologic changes which occur secondary to external threats. Selye’s definitions of stress and stressor mentioned above will be used in this dissertation. Selye’s work and that of Cannon serve as the foundation for stress research (Mason, 1975a; Mason, 1975b; Selye, 1973). Cannon considered stress as a disturbance in an organism’s homeostasis caused by a stressor. Stressors included events such as cold exposure, hypoxemia, hypoglycemia, hemorrhage and emotional stimuli. Cannon’s work emphasized the role of the autonomic nervous system in restoring homeostasis. The other pioneer in this field, Selye emphasized the role of the pituitary-adrenal system in
the stress response. He proposed that glucocorticoid release was a non-specific stress response to all stressors and was integral to the stress response. In addition, he suggested that acute stress allowed an organism to adapt to the environment while chronic stress exhausted this adaptive response and was potentially harmful to the organism's survival. Selye (1936) first described a stress syndrome produced by 'diverse noxious agents' such as surgical injury, spinal shock, exposure to cold, excessive or sublethal doses of a variety of drugs. He termed the acute phase the 'general alarm reaction' occurring over minutes or hours and the chronic phase the 'general adaptation syndrome', which is the organism's attempt to adapt to stimuli repeatedly presented over days. Overall, the perceived threat (internal or external) sets up a cascade of events that results in a physiologic reaction including endocrine and behavioural changes.

Over the past two decades the concept of absolute nonspecificity of bodily responses to all stressors as Selye (1973) described has been modified to reflect differences in the neuroendocrine response to various stressors (Kopin, 1995). The development of new techniques has allowed for more precise measurements of neurotransmitters and hormone responses resulting in the finding that there are different patterns of neuroendocrine responses to different stressors. For example, in a recent study by Kopin (1995) it was demonstrated that the catecholamine and andrenocorticotrophin (ACTH) responses varied with exposure to two different stressors, haemorrhage and formalin. Further, it was shown that exposure to the same stress but at different intensities, haemorrhage of 10% or 25% of blood volume and formalin concentration 1% or 4%, results in a different magnitude of neuroendocrine responses.
Furthermore, Cannon's and Selye's concept that the differences in the stress response seen among individuals may be due to genetic variations and that these variations can be modified by experience has stimulated a lot of research in the stress and chronic illness field. It is thought that maladaptive neuroendocrine responses to stressors may be involved in psychiatric, metabolic and/or autoimmune diseases as well as disturbances of growth and development (Stratakis & Chrousos, 1995).

C.1 HORMONES AND STRESS

Stress can affect the release of virtually every hormone in the body, and thus has widespread effects on many physiologic functions. The present discussion will be limited to the endocrinological effects of stress. The body responds to stressors by releasing a number of hormones: corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) from the hypothalamus, ACTH and β-endorphin (β-EP) from the anterior pituitary, and corticosterone (CORT) from the adrenal cortex (Asterita, 1985). In addition, prolactin, lutenizing hormone, and growth hormone secretion increase with acute stress, thyroid stimulating hormone secretion can be increased (Armario et al., 1984a) or decreased (Armario et al., 1984b) depending upon the stressor, and follicle stimulating hormone tends to be unaffected by stress (Armario et al., 1984c). Furthermore, epinephrine and NE are released from the adrenal medulla and NE from sympathetic nerves (Asterita, 1985). All these hormones alter the animal's internal environment in order to help increase the animal's ability to withstand stress by affecting
the cardiovascular, energy producing and immune systems (Asterita, 1985; Stratakis & Chrousos, 1995).

The control of the HPA axis is through various feedback loops which inhibit further release of hormones (Calogero et al., 1988). The median eminence (ME) can be viewed as an interface between hypothalamic/extrahypothalamic neuronal systems and the pituitary-adrenal system. Approximately 30 neurochemicals can be found in the ME, reinforcing the complexity of the control of the HPA axis (Jacobowitz, 1988). The HPA axis responds to stressors by releasing CRF and AVP. CRF and AVP release can be stimulated by NE, acetylcholine, enkephlins, angiotensin II, histamine, serotonin (5-HT), and β-EP (Calogero, 1995), and can be inhibited by γ-aminobutyric acid (GABA) (Calogero, 1995), α-melanocyte stimulating hormone, ACTH, and CORT (Asterita, 1985; Jacobowitz, 1988). CORT not only inhibits the release of CRF but also appears to decrease its synthesis (Uht et al., 1989).

ACTH is primarily controlled by CRF secretion from the hypothalamus into the hypothalamic-hypophysial portal system (Asterita, 1985; Goodman, 1988). However, AVP and oxytocin (OT) can modulate the effects of CRF which may be important in the mediation of the stress response (Gibbs, 1986). AVP stimulates the release of ACTH, potentiating the response of CRF (Gibbs, 1986; Gillies et al., 1982; Yates et al., 1971). OT also stimulates secretion of ACTH in rats (Antoni et al., 1983; Lutz-Bucher et al., 1982) but is inhibitory in primates (Antoni et al, 1983; Legros et al., 1982)). CORT
feedback is the main inhibitory stimulus controlling ACTH secretion (Jones & Gillham, 1988; Keller-Wood & Dallman, 1984; Wilkinson et al., 1981). Administration of high doses of dexamethasone (DEX), a synthetic glucocorticoid, to normal rats and adrenalectomized rats results in decreased CRF receptors in the rat pituitary in parallel with the decrease in ACTH secretion (Hauger et al., 1987; Hauger et al., 1989; Spinedi et al., 1991; Uht et al., 1989; Wynn et al., 1983). In addition, prolonged restraint stress (more than 12 hours) may also result in a reduction of CRF receptor numbers in the anterior pituitary (Hauger et al., 1988).

The final common pathway in the HPA system is the release of CORT by the adrenal cortex. ACTH released from the anterior pituitary reaches the adrenal cortex through the systemic circulation (Goodman, 1988). ACTH stimulates both the synthesis and secretion of CORT (Goodman, 1988; Jones & Gillham, 1988; Keller-Wood & Dallman, 1984). Once released, CORT acts to alter metabolism and utilisation of fats, proteins and carbohydrates, to control electrolyte balance, and to stimulate or suppress the immune system depending upon the length of exposure (Asterita, 1985; Goodman, 1988). In addition, CORT rigidly controls the activity of the HPA axis by feedback to multiple levels of the system further inhibiting the release of "stress hormones" (Keller-Wood & Dallman, 1984).

The control of the stress response through multiple feedback loops occurs in at least 3 different time domains and at least 3 different levels (Jones & Gillham, 1988; Keller-Wood & Dallman, 1984). In addition, the stress response varies with the intensity
of the stimulus, the length of exposure (chronic vs acute), and prior exposure to that specific stimulus or other stimuli (Keller-Wood & Dallman, 1984). Therefore, the physiological mechanisms controlling hormonal responses to stress, specifically the CORT negative feedback system, are not easily discerned.

CORT feedback inhibition of ACTH and CRF occurs within seconds (fast rate sensitive inhibition), over 2-10 hours (h) (intermediate inhibition), and over h to days (slow inhibition) (Jones & Gillham, 1988; Keller-Wood & Dallman, 1984). The fast feedback inhibition appears to be rate sensitive acting during the period of increasing plasma CORT concentration (Abe & Critchlow, 1977; Jones & Tiptaft, 1977; Kaneko et al., 1981). The rate of CORT rise required for inhibition varies between sexes, and is greater in females than males (Critchlow et al., 1963). The sex steroids appear to influence the HPA axis indirectly through inactivation of corticosteroids, through hepatic enzyme systems (Glenister & Yates, 1961; Kitay, 1961) or binding proteins (Sandberg & Slaunwhite, 1959; Slaunwhite et al., 1962) resulting in higher total CORT but proportionately lower free CORT concentration and higher clearance rates of CORT in female rats. Fast feedback inhibits release of ACTH and CRF but does not affect synthesis of ACTH and CRF (Keller-Wood & Dallman, 1984). In vitro studies have demonstrated that perfusion of rat pituitaries with DEX or CORT inhibits CRF-stimulated ACTH secretion without affecting ACTH content (Widmaire & Dallman, 1984; Widmaire & Dallman, 1983a; Widmaire & Dallman, 1983b). In addition, pretreatment of the pituitary with a protein synthesis inhibitor (cycloheximide) does not alter the response to feedback inhibition (Widmaire & Dallman, 1983b). Interestingly, only
steroids with both 21-hydroxyl and 11-β-hydroxyl groups cause fast feedback inhibition while 11-deoxycorticosterone and 11-deoxycortisol antagonise the fast feedback effects of CORT (Jones & Tiptaft, 1977).

Delayed feedback inhibition (intermediate and slow) acts independently of circulating CORT levels at the time of the stress and requires 45 min to 120 min to develop (Dallman & Yates, 1969). Delayed inhibition of ACTH and CRF appears to depend on the levels of CORT achieved (Dallman & Yates, 1969; Sayer & Sayer, 1947), the interval since the administration of the CORT (Dallman et al., 1987; Dallman & Jones, 1973; Dallman & Yates, 1969) and the total dose of the CORT administered (Jones & Tiptaft, 1977; Takebe et al., 1971). Intermediate inhibition can be detected at 30 min with the maximal effect occurring at 2-4 h (Dallman et al., 1987; Dallman & Yates, 1969; Kendall, 1971; Takebe et al., 1971) and an attenuation of the response at 6-12 h after administration of CORT (Dallman & Yates, 1969; Keller-Wood & Dallman, 1984). The latency and duration of the inhibition is dependent upon the total dose and duration of the CORT exposure (Abe & Critchlow, 1980); prolonged periods of inhibition occur with extremely high doses (4 mg CORT) or repeated exposure (Jones et al., 1974).

In addition, the receptors for delayed feedback appear to be different from those for fast feedback since either 21-hydroxyl or an 11-β-hydroxyl group are effective (Jones & Tiptaft, 1977). Intermediate inhibition results in decreased release of both ACTH and CRF and a decrease in CRF synthesis, whereas slow inhibition also decreases ACTH synthesis (Keller-Wood & Dallman, 1984). Within 2 h of exposure to CORT there is a
decrease in ACTH release (Abou-Samra et al., 1986), a decrease in pituitary sensitivity to CRF (Rochefort et al., 1959; Zatz & Reisine, 1985) and to K⁺ and Ca²⁺ stimulation (Arimura et al., 1969; Fleischer & Vale, 1968; Koch et al., 1974; Kraicer et al., 1973), inhibition of acetylcholine-stimulated CRF release (Edwardson & Bennett, 1974) and CRF synthesis (Hauger et al., 1987; Hauger et al., 1989), and decreased ACTH response to haemorrhage (Plotsky & Vale, 1984). Prolonged exposure to CORT for greater than 12 h results in inhibition of both ACTH release (Engeland et al., 1975) and ACTH synthesis (Schacter et al., 1982).

In addition to effects on the pituitary and hypothalamus, CORT also has inhibitory feedback effects on the brain. Two major classes of CORT receptors have been identified within the brain (Funder, 1986; Spencer et al., 1990). Type I receptors which are high affinity, low capacity receptors are located in large concentrations in the hippocampus and lateral septum, with little to none in the hypothalamus (Gerlach & McEwen, 1975; Stumpf & Sar, 1976; Waremboug, 1975). Type I receptors are 90% occupied during the trough of the normal circadian cycle and thus are thought to play a tonic role in circadian rhythms (De Kloet & Reul, 1987; Reul & De Kloet, 1985; Reul & De Kloet, 1986). Type II receptors, which are low affinity high capacity receptors, have a widespread distribution throughout the brain and are located in the highest density in the paraventricular nuclei of the hypothalamus, lateral septum, dentate gyrus, nucleus tractus solitarius and central amygdala (Agnati et al., 1985; Gustafsson et al., 1983; Waremboug, 1975). Type II receptors are 5-50% occupied during normal circadian rhythms and thus
thought to be involved with feedback regulation of stress-induced and nocturnal increases of CORT (De Kloet & Reul, 1987; Reul & De Kloet, 1985; Reul & De Kloet, 1986).

The complex system of feedback loops controlling the HPA axis gives rise to a malleable but stringent regulatory system. It allows for varied responses in proportion to the magnitude of the stimuli and habituation or sensitisation to repeated exposure to a stimulus or to varied stimuli (Keller-Wood & Dallman, 1984; Pitman et al., 1990). Its rigorous control over the HPA axis allows the animal to cope with environmental stressors (internal and external) and protects against over-stimulation and detrimental effects of continued exposure to "stress hormones".

C.2 CORTICOTROPHIN RELEASING FACTOR AND BEHAVIOUR

It has been demonstrated that CRF, ACTH, and CORT modulate behaviours during stress (McEwen et al., 1986). CRF neurons have been localised in a number of areas in the CNS other than the hypothalamus including limbic structures, cortex and in close association with the central autonomic system i.e. hypothalamus and locus coeruleus (LC) (Brown, 1986; De Souza & Insel, 1990; Sawchenko & Swason, 1990; Schiper et al., 1983). It has therefore been suggested that CRF's actions extend beyond that of HPA axis stimulation to involve simultaneous activation and co-ordination of metabolic (Brown et al., 1982a; Brown et al., 1982b), circulatory (Brown & Fisher, 1985; Brown & Fisher, 1986; Brown et al., 1986), and behavioural responses (Britton et al., 1982; Britton et al., 1981; Sutton et al., 1982) to stress. Furthermore, it is now
generally accepted that CRF fulfils the requisite criteria to be considered a neurotransmitter (Nemeroff, 1992).

Intracerebroventricular (ICV) administration of CRF can activate the HPA axis but can also inhibit luteinizing hormone (Ono et al., 1984; Ono et al., 1985; Rivier & Plotsky, 1986; Rivier & Vale, 1985; Taya & Sasamoto, 1989) and growth hormone release (Rivier & Plotsky, 1986; Rivier & Vale, 1985; Taya & Sasamoto, 1989). ICV-CRF appears to have no effects on follicle stimulating hormone, thyroid stimulating hormone or prolactin (Rivier & Vale, 1985). In addition, ICV-CRF can activate the sympathetic and parasympathetic nervous systems (Brown, 1986; Brown & Fisher, 1985; Brown et al., 1982a; Brown et al., 1982b, Brown et al., 1986) and has widespread effects on the gastrointestinal system (Dunn & Beridge, 1990; Tache et al., 1990) all of which are associated with response to or adaptation to stress. For example, CRF administration results in increased splanchnic nerve activity and increases in plasma NE and E levels (Kurosawa et al., 1986). These neurochemicals in turn cause a number of metabolic and physiologic changes (Brown et al., 1982a). ICV-CRF and ether stress both result in increases in catecholamine levels and are reversed by the administration of α-helical CRF (hCRF, a CRF antagonist) (Brown et al., 1986). In addition, CRF may be involved with parasympathetic stimulation, e.g. ICV injected CRF modifies the baroreflexic control of heart rate (Fisher, 1989). This is prevented by atropinemethylnitrate, a vagal nerve blocker, but not propanolol, a sympathetic nerve blocker. Electroencephalograph (EEG) activity and behavioural arousal are increased by ICV administration of CRF; low doses cause EEG activity characteristic of arousal and high doses also cause arousal followed
by seizure activity (Ehlers et al., 1983). Further, ICV-CRF can result in inhibition of gastric acid secretion and gastric emptying and stimulation of large bowel transit and fecal excretion (Tache et al., 1990).

Within normal physiologic range, behavioural responses to ICV-CRF but not peripheral doses of CRF resemble stress-induced behaviour or are frequently opposite to the behaviours seen with administration of anxiolytic agents (Dunn & Berridge, 1990; Koob & Britton, 1990). These include decreases in: feeding in both familiar and novel environments (Levine et al., 1983), sexual behaviour (Sirinathsinghji et al., 1983), entry into the open arms of the elevated plus maze (File et al., 1988; File, 1987), social interaction (Dunn & File, 1987), and in high doses, locomotion in an open field environment (Britton et al., 1981; Sutton et al., 1982). Further, CRF increases exploration in familiar surroundings and increases grooming behaviour (Dunn et al., 1987; Morley & Levine, 1982; Sutton et al., 1982; Veldhuis & De Wied, 1984), stress-induced analgesia (Dunn & Berridge, 1990), acoustic startle (Swerdlow et al., 1986; Swerdlow et al., 1989), shock-induced freezing (Sherman & Kalin, 1986), defensive withdrawal (Sherman & Kalin, 1986), and, at low doses, exploration in a novel environment (Britton et al., 1981; Sutton et al., 1982). At low doses, (0.0003 ug) ICV-CRF also impairs passive avoidance behaviour when administered either immediately following training or prior to retention testing (Veldhuis & De Weid, 1984). Many of these CRF-induced responses are attenuated by the administration of benzodiazepines (Dunn & Berridge, 1990; Koob & Britton, 1990). In addition, CRF antagonists (α-helical
CRF and CRF antisera) attenuate or reverse stress induced behaviours (Dunn & Berridge, 1990; Koob & Britton, 1990).

Thus, the function of CRF may extend beyond activation of the HPA axis to other physiological functions; further, CRF may be a primary mediator of the behavioural state of stress and behavioural responses to stress. Centrally, CRF appears to increase the "emotionality" of the animal, i.e. increase the sensitivity of the animal to stressful aspects of the environment. In addition, abnormally high levels of cerebrospinal fluid CRF and cortical CRF receptor density as well as a blunting of ACTH response to CRF have been demonstrated in a number of psychiatric illnesses (Nemeroff et al., 1988). Therefore, centrally, CRF may play a role in the abnormal behavioural responses seen in children with FAS including the hyperactivity, decreased attention span, lack of habituation, lack of inhibition, and increases in abnormal fears.

D. HPA AXIS IN FAS

It is recognised that pre and/or post natal environmental factors can have long lasting neuroendocrine and behavioural effects on the organism’s ability to cope with stress. Alcohol exposure during the prenatal and early postnatal periods constitutes an early insult to the organism which could alter the development of foetal endocrine function as well as foetal metabolic or physiological functions. These alterations could occur through direct effects of alcohol or indirect effects of alcohol-induced changes in maternal endocrine function. As previously mentioned, alcohol readily crosses the
placental barrier resulting in approximately equivalent foetal and maternal blood alcohol levels (Waltman & Iniguez, 1972). Thus, maternal alcohol consumption may result in a direct stimulation or suppression of foetal endocrine activity. Furthermore, altered maternal endocrine function disrupts the hormonal interactions between maternal and foetal systems, thereby disturbing the normal maternal foetal hormone balance (Anderson, 1981).

Although clinical studies have established that alcohol consumption markedly alters HPA function in adults who chronically abuse alcohol, few clinical studies have investigated HPA function in children prenatally exposed to alcohol. The few studies conducted on endocrine systems of children with FAS have found few significant differences but have focused mainly on growth retardation and have only examined basal hormones and thyroid and growth hormone challenge tests (Anderson, 1981). A case study of four children with foetal alcohol syndrome (FAS) indicated that plasma cortisol levels are within normal limits (Root et al., 1975). However, Binkiewicz and colleagues (1978) reported pseudo-Cushing’s syndrome in an infant exposed to alcohol via the breast milk, suggesting that alcohol can have stimulatory effects on the HPA axis of the newborn. Recently, Jacobson and colleagues (1993), reported that children with a history of prenatal alcohol exposure demonstrated elevated salivary cortisol following an acute stressor (i.e., a routine blood draw).
Animal studies provide evidence of altered HPA function of the maternal female, as well as altered HPA and β-EP system function in their offspring. Ethanol consumption in pregnant females results in increased maternal adrenal weights, basal CORT levels, adrenocortical responses to stress and corticoid stress increments without altering the binding capacity of plasma corticosterone binding globulin (CBG) (Weinberg, 1989, Weinberg & Bezio, 1987; Weinberg & Gallo, 1982). The ethanol-induced activation of the maternal HPA axis occurs as early as day 11 of pregnancy and persists throughout pregnancy, even at low concentrations of ethanol in the diet (5.5% w/v). In addition, with continued ethanol exposure, stimulatory effects of ethanol on maternal adrenal weights and basal CORT levels may persist through to parturition (Weinberg & Bezio, 1987). Furthermore, this HPA hyperresponsiveness appears to be independent of nutritional status and is specifically a result of ethanol exposure (Weinberg & Bezio, 1987). Females consuming diets with varied protein levels but consistent alcohol levels all demonstrated increased adrenal weights and adrenocortical hyperactivity. Together, these data suggested that ethanol consumption during pregnancy results in hypersecretion and hyperresponsiveness of the maternal HPA axis.

As hormones can freely pass through the placenta, ethanol-induced maternal hormone changes may have negative effects on the developing foetus. It has been suggested that the alterations seen in the E animal's HPA axis may be a result of ethanol acting as a maternal stressor elevating maternal CORT levels and indirectly elevating foetal levels. As mentioned previously, perinatal administration of glucocorticoid can
interfere with normal development (Lee et al., 1990; Weichsel, 1977). However, it has been demonstrated that offspring from dams challenged with stressors, ACTH, or adrenalectomy do not display the hyperresponsiveness seen in E animals (Weinberg et al., 1986). Therefore, it appears that ethanol may have direct as well as indirect actions on HPA axis development and function.

During the early neonatal period, E animals have elevated basal levels of brain, plasma, and adrenal CORT and decreased CBG binding capacity (Kakihana et al., 1980; Taylor et al., 1983; Weinberg et al., 1986, Weinberg, 1989), as well as elevated plasma and decreased pituitary β-EP levels (Angelogianni & Gianoulakis, 1989) compared to control animals. At 21 days of gestation, E animals are found to have greater relative adrenal weights but lower plasma CORT levels than pair-fed (PF) and control (C) animals. By 3-5 days of age, basal CORT levels in E animals return to normal. Furthermore, during the preweaning period (approximately the first three weeks of life), E animals exhibit suppressed or blunted HPA and β-EP responses to a wide variety of stressors including ether, novelty, saline injection, and cold stress, as well as to drugs such as ethanol and morphine (Angelogianni & Gianoulakis, 1989; Taylor et al., 1986a; Weinberg, 1989; Weinberg et al. 1986). Interestingly, this blunted stress response in E animals is a transient phenomenon and by day 15-21, the HPA response to stress appears to normalise.

In adulthood, basal CORT, ACTH, β-EP levels in E offspring do not differ from those in PF and C offspring (Taylor et al., 1982; Weinberg & Gallo, 1982). However,
adult E offspring are hyperresponsive to a variety of stressors including cardiac puncture (Taylor et al., 1982), restraint (Taylor et al., 1982; Weinberg, 1988; Weinberg, 1992a), noise and shaking (Taylor et al., 1982), novel environments (Weinberg, 1988), intermittent shock (Nelson et al., 1984; Nelson et al., 1986), ether (Angelogianni & Gianoulakis, 1989; Weinberg & Gallo, 1982) and cold (Angelogianni & Gianoulakis, 1989). E animals also demonstrate deficits in pituitary-adrenal response inhibition or recovery from stress. For example, E animals show prolonged CORT, ACTH, and β-EP elevations during and following restraint stress (Weinberg, 1988; Weinberg, 1992a) and also show smaller CORT decreases when allowed access to water in a novel environment (Weinberg, 1988) as compared to control animals. Similarly, E offspring show more prolonged ACTH elevations than control animals following 10 min footshock stress (Taylor et al., 1986b). Interestingly, HPA hyperresponsiveness and/or the deficits in response inhibition may be manifested differentially in males and females depending on the nature and intensity of the stressor, the time course measured, and the hormonal endpoint examined.

The ability to respond to environmental cues in stressful situations also appears to be deficient in E animals. Unlike PF and C animals, E animals do not respond differentially to predictable versus unpredictable restraint stress (Weinberg, unpublished). In addition, they demonstrate reduced corticosterone responses to a novel environment when allowed access to water, whereas PF and C demonstrate significant attenuation of the corticosterone response when allowed to drink (Weinberg, 1988). Furthermore, intrauterine exposure to ETOH also results in persistent effects on behavioural responses
to stressors including increased stress-induced analgesia (Nelson et al., 1985b), increased stress-induced ETOH consumption (Nelson et al., 1983a), and an inability to adapt to a stressful swimming paradigm (Taylor et al., 1983).

A possible mechanism underlying the HPA hyperresponsiveness seen in E offspring is a deficit in feedback inhibition of the HPA axis. As the hippocampus is a principal target site for glucocorticoid feedback in the brain (McEwen et al., 1986; Sapolsky et al., 1984), a recent study (Weinberg & Petersen, 1991) investigated the possibility that an ethanol-induced decrease in hippocampal glucocorticoid receptor concentration might, in part, mediate this altered HPA responsiveness. The data demonstrated that there were no significant differences in specific binding density or binding affinity for either Type I (mineralocorticoid) or Type II (glucocorticoid) receptors in the hippocampus of E animals compared to control animals, indicating that feedback deficits in E animals do not occur at the level of the hippocampal receptors, at least under basal or nonstressed conditions. In contrast, support for a deficit in feedback inhibition in E animals comes from the work of Nelson et al., (1985a) demonstrating that E animals appear to have an accelerated rebound of basal CORT levels following a high dose of the synthetic glucocorticoid, dexamethasone-21-phosphate (DEX). Clinically, the DEX suppression test has been used to evaluate HPA axis function in a number of psychiatric conditions and it appears that feedback inhibition of CORT is altered in a number of affective disorders (Nemeroff et al., 1988).
E. THESIS OBJECTIVES

The ability to respond appropriately to the environment is a basic mechanism for survival. Animals must respond to environmental stressors (external and internal) and be able to re-establish homeostasis once the stressor has been overcome. Hyperresponsiveness and/or deficits in the capacity to respond differentially to and recover from stress (like those seen in E offspring) can have detrimental affects on health and even survival. Prolonged exposure to glucocorticoids can result in alterations in metabolism and immunosuppression. The mechanism underlying hyperresponsiveness of the HPA and β-EP system as well as behavioural hyperactivity of E offspring are unknown at the present. The experiments undertaken in this thesis were done to examine two different hypotheses.

Hypothesis I: HPA axis hyperresponsiveness and/or delays in recovery from stressors that occur in E animals result from deficits in feedback inhibition of the HPA axis induced by prenatal ethanol exposure.

Hypothesis II: behavioural hyperresponsiveness observed in E animals is a result of elevated brain CRF levels and/or an increased CNS sensitivity to CRF which is induced by prenatal exposure to ethanol.
CHAPTER II: GENERAL METHODS

A. DIETS

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

B. BREEDING AND FEEDING

Sprague-Dawley rats (Canadian Breeding Farms, St. Constant, PQ) were housed under constant conditions (temperature, lighting, and handling). The males and females were group housed for 1-2 week (wk) prior to breeding to allow recovery from transport and adaptation to the colony room. 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. The colony room had controlled temperature (21 °C) and lighting, with lights on from 0600 to 1800 h.
Females were placed singly with males and cage papers were checked daily for vaginal plugs. Day 1 (d1) 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 d1 of gestation, females were rehoused 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), liquid control diet (maltose-dextrin isocalorically substituted for ethanol) with each animal pair-fed the amount consumed by a female in the ethanol group /kg body weight (bw)) on the same day of gestation or 3) Control (C), laboratory chow and water, ad libitum.

The diets were made fresh every 3 days and refrigerated until feeding. The diet was offered in a glass bottle 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 corticosterone (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 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 d22 when they were replaced by laboratory chow and water ad libitum, in order to minimize the adverse effects of ETOH on maternal lactation.
On d12-14 of gestation, a subset of females from each breeding were tested for blood alcohol levels (BAL). Blood samples (0.2-0.5 ml) were obtained from the tail of unanaesthetized females at 1900 h under red light and BALs determined by the Sigma Diagnostic Kit 332-UV (based on Bonnischsen & Theorell, 1951).

Females were undisturbed except for weighing and cage cleaning on d1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dams and pups were weighed and all litters culled to 10 (5 males and 5 females). If a litter had less than 5 males and 5 females at birth, pups from another litter born on the same day were cross-fostered to make up the required pup number. Dams and pups were weighed and cages cleaned on d1, 8, 15, and 22 of lactation. On d22 pups were weaned, ear marked and group housed by sex and by litter. They remained group housed until testing in adulthood (greater than 60 days of age).

In adulthood, male and female rats from prenatal E, PF, and C groups were selected for testing. Because pups within a litter are not independent subjects, the minimum number of litters bred were 10 per group (E, PF, C). In addition, to control for litter effects, no more than 1 female and 1 male per litter were tested at any 1 sampling time or in any 1 condition within an experiment. One week prior to testing, the animals were singly housed and moved from the breeding colony room to the testing colony room. Both female and male offspring were used in all experiments. Animals were given experiment numbers and studies were run so that the investigators did not have knowledge of the prenatal treatment.
C. BLOOD SAMPLING

All testing was conducted at consistent times within the circadian cycle. The testing colony room was closed off for at least 4 to 6 h prior to testing to prevent artificial elevation in hormone levels due to disturbance. All blood sampling was done in the lab immediately adjacent to the colony room.

Blood samples for the studies were collected by 1 of 3 methods: cardiac puncture under ether anaesthesia; decapitation; or via indwelling catheters. In the cardiac puncture technique, animals were lightly anaesthetised with diethyl ether or metofane (Janssen Pharmaceutica, Mississauga, ON, Canada) and blood samples drawn using heparinized syringes. The entire sampling procedure was completed within 2 minutes (min) of touching the animal’s cage, which is rapid enough to obtain a reliable measure of CORT, without any effect of disturbance or etherization (Davidson et al., 1968). ACTH levels could not be obtained with this procedure as they must be obtained within seconds of touching the animal’s cage. The blood was then placed in glass test tubes (10 x 75 mm), centrifuged at 2200g for 10 min at 4°C, and plasma was collected and stored at -20°C until assayed. The decapitation technique was used to collect samples for ACTH levels as they must be drawn within 10-15 sec of touching the animal's cage to obtain true measures of concentration. Trunk blood was collected on ice in plastic test tubes (12x75 mm) containing 7.5 mg EDTA and 1000 KIU aprotinin. The blood was centrifuged at
2200g for 10 min at 4°C and plasma transferred with plastic pipettes to plastic microcentrifuge tubes for storage at -70°C until assayed. The cannula technique was used in experiments requiring multiple samples over time and the administration of various exogenous substances. A modified indwelling jugular cannula was implanted under halothane anaesthesia at least 24 h before testing. It has been demonstrated that catecholamines and other hormones return to basal levels 24 h after implantation. (Wixson et al., 1987). (See section D for surgical procedure) Cold tuberculin syringes coated with EDTA and aprotinin were used for blood collection. The blood was centrifuged at 4°C at 2200 g for 10 min and plasma transferred with plastic pipettes to plastic microcentrifuge tubes for storage at -70°C until assayed.

D. SURGICAL PROCEDURES

D.1 MODIFIED INDWELLING JUGULAR CANNULAE

Animals were implanted with indwelling jugular cannulae under halothane anaesthesia 24-48 h prior to testing. The surgical and sampling procedure was in accordance with Rivier et al., (1982a). Cannulae were cold sterilised with the Clindox-S system and implanted under semi-sterile conditions. The incision area on the rat was cleaned with 95 % ETOH prior to surgery.
The cannula consisted of PE50 tubing with a bevelled silastic tip. Cannulae were inserted into the left internal jugular vein and secured in place with 4 sutures. The free end of the cannula was then tunneled subcutaneously to be 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. The sampling cannulae consisted of PE50 tubing with a 22G x 1 1/2" blunted needle at one end, which was inserted into the free exteriorized end of the indwelling cannula; a Luer LOK PRN adapter attached to a needle hub for injection and sampling was attached to the other end of the sampling cannulae.

D.2 INTRACEREBROVENTRICULAR (ICV) CANNULAE

ICV cannulae were implanted under ketamine/xylazine anaesthesia (0.8mg per kg bw of each ketamine and xylazine). Animals were placed in a stereotaxic apparatus and the skull exposed. Co-ordinates for the lateral ventricles (Paxinos & Watson, 1986) are approximately 0.8 mm posterior and 1 mm lateral to the bregma, and 4.5 mm ventral to the skull surface, with the incisor bar set at 3.3 mm. A hole was drilled into the skull and the guide cannula lowered into place. Four small stainless steel screws were inserted 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. Cicatrin antibiotic powder (Wellcome Burroughs Inc., Kirkland, PQ, Canada) was sprinkled over the suture site to help prevent infection. Infusion of substances into the ventricle was accomplished with an infusion pump (Harvard Apparatus Syringe Infusion Pump 22, Southatick, Mass, USA) which is capable of
delivering small volumes at a controlled rate. The infusion pump was connected via tubing to an internal cannula that was inserted into the guide cannula. After animals were tested, cannulae placements were verified by infusion of toluene blue dye into the cannula followed by corneal brain slicing. Only animals demonstrating dye in both lateral ventricles were included in the data analysis.

E. BEHAVIOURAL TESTS

E.1 ELEVATED PLUS MAZE (+-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). The maze was arranged so the two closed arms were opposite each other and the two open arms were opposite to each other. The maze was elevated 50 cm off the floor by four wooden legs.

The +-maze behaviour was videotaped. Each videotape was scored by two independent individuals and a mean of the two scores was used; intra and intertester coefficients of variation were 2.6% and 4.2% respectively. The time spent on the open arms, on the closed arms, and on the centre portion were recorded. The number of full entries (all four feet) onto open and closed arms, number of partial (one or two feet) onto the open arms and number of rears on the closed arms were recorded. In one experiment
ambulation (number of midline crosses), number of turns and number of rears on both the open and the closed arms were also determined.

E.2 OPEN FIELD (OF)

The OF was constructed of a wooden base with an arborite surface. It was 100 x 100 x 40 cm and was divided up into 16 squares. It was illuminated by two 60 watt bulbs suspended 75 cm above the surface. The OF behaviour was scored directly by a single investigator. Ambulation (all four feet crossing into a square) in the central 4 squares and the outer 12 squares and the number of rears were recorded.

F. ASSAYS

F.1 BLOOD ETHANOL LEVELS

Blood ethanol levels were determined by the enzymatic method for measuring ethanol at a wave length of 340 nm (Sigma Diagnostic Kit 332-UV) (revised from Bonnischsen & Theorell, 1951). The assay is structured upon the principle that alcohol dehydrogenase catalyses the oxidation of ethanol to acetaldehyde with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH resulting in a increased absorbency of 340 nm light (Lundquist, 1957). The consequent increase in absorbency is directly proportional to ethanol concentration in the sample.
F.2 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, ON; unlabelled CORT for standards was obtained from Sigma, St. Louis, MO. Dextran coated charcoal was used to absorb and precipitate free steroids after incubation. Samples were counted in Formula 989, (Dupont, New England Nuclear, Mississauga, ON). The intra and interassay coefficients of variation were 3% and 3.9%, respectively.

F.3 PLASMA ACTH LEVELS

Plasma ACTH was assayed by a modified procedure of the Incstar equilibrium RIA (Incstar Inc., Stillwater, Minnesota, USA) with all reagent volumes halved and 50 μl plasma per tube. The antiserum cross reacts 100% with Porcine ACTH 1-39 and Human ACTH 1-24 but shows less than 0.01% crossreactivity with α-melanocyte stimulating hormone, β-endorphin, β-lipotropin, leucine enkephalin, methionine enkephalin, bombesin, calcitonin, parathyroid hormone, follicular stimulating hormone (FSH), vasopressin, oxytocin, and substance-P (Orth, 1979). The midrange intra and interassay coefficients of variation were 3.9% and 6.5% respectively.
G. MEASUREMENTS OF mRNA

G.1 IN SITU HYBRIDIZATION

Brains were quickly collected on dry ice, wrapped in parafilm and aluminum foil, sealed in a plastic bag and stored at -70°C. Brains were transferred to the cryostat 1 h before slicing and warmed to -20°C. Brains were mounted onto the specimen holder with O.C.T. compound (Mile INC., Elkhart, IN, USA). Sections were taken until the paraventricular nucleus (PVN) of the hypothalamus was isolated using a 5% toluidine blue stain. Twelve coronal sections (12 μm) were taken through the PVN and mounted onto twice-gelatin coated slides. Slides were stored in a sealed container at -70°C.

Prehybridization treatments were performed according to Zoeller & Rudeen, (1992). Sections were briefly warmed to room temperature, immersed in 4% formaldehyde/phosphate-buffered saline (PBS) for 5 min, rinsed in PBS, and soaked for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% NaCl (pH 8.0). Slides for AVP and CRF mRNA were then rinsed in 2X SSC (300 mM NaCl/30 mM sodium citrate); slides for GR mRNA were rinsed in 1X SSC. Slides were then dehydrated by a graded series of ethanol baths, delipidated in chloroform, rehydrated in 95% ethanol and air dried. Hybridization buffer (50 μl) was applied to each slide;
slides were covered with a parafilm cover slip and incubated at 37°C for 20 h in humid chambers.

Hybridization buffer contained 50% formamide, 4X SSC, transfer RNA (250 µg/ml), sheared single stranded salmon sperm DNA (100µg/ml), 1X Denhardt’s solution (0.02% each of BSA, Ficoll, and polyvinylpyrrolidone), 10% (w/v) dextran sulfate (molecular weight 500,000), 100 mM dithiothreitol (DTT), and probe. After hybridization, the coverslips were floated off in 1X SSC and slides were washed 2 times in 2X SSC/50% formamide at 40°C for AVP and CRF mRNA slides and 52°C for GR mRNA slides. Following two 30 min washes in 1X SSC at room temperature AVP and CRF mRNA, slides were dipped in distilled water, equilibrated in 70% ethanol and air dried. GR mRNA slides were rinsed 2 times in 2X SSC at room temperature and then incubated in 2X SSC/1mM EDTA/50 µg/ml RNaseA at 37°C for 30 min. GR mRNA slides were rinsed 2 times in 2X SSC at room temperature, incubated for 5 min in 50% formamide/2X SSC, washed in a series of ethanol /0.1X SSC baths, dipped in deionized water, washed in 70% ethanol for 3 min and then air dried.

G.2 PROBES

AVP and CRF mRNA Probes

Oligonucleotides (Table 2) were synthesised on an Applied Biosystems Model 30B DNA synthesiser and purified by PCR columns according to manufacturer’s
instructions, purified by electrophoresis on an 8% polyacrylamide 8 M urea TBE gel, followed by electrophoresis into 1% LMP agarose, and phenol extracted. These purified oligomers exhibited a single band on a sequencing gel following $^{32}$P-labelling.

Purified oligonucleotides were 3'end labelled by incubating 50 units of terminal oligodeoxynucleotidyl transferase (Boehringer-Mannheim, Indianapolis, IN) in a solution containing 5 pmol oligomer and 50 pmol $^{35}$S-dATP (New England Nuclear) in the presence of 200 mM potassium cacodylate, 25 mM-Tris HCl, 0.25 mg/ml BSA, and 1.5 mM CoCl$_2$ (pH 6.6) for 15 min at 37°C. A phenol/chloroform extraction and ethanol precipitation was completed and the probe was stored at -20°C in 10mM Tris/1 mM EDTA, pH 8.0/50 mM DTT until used.

**GR mRNA Probe**

GR mRNA was measured using a cRNA probe. A 700-base-pair Pstl/EcoRI rat cRNA fragment corresponding to the 3' portion of the coding region was isolated from pRM9 (Miesfeld *et al.*, 1986). The probe was then subcloned into pBluescript (+) vectors as described in the transcription kit (Promega Biotec, Madison, Wisc., USA). The $^{33}$P-UTP labelled sense and antisense RNA probes were generated by specific polymerase (T7 or SP6) transcription after linearization with restriction enzymes PstI and EcoRI. cRNA probes are dissolved in RNAase-free water containing 0.1% SDS and stored at -80°C.
G.3 AUTORADIOGRAPHY AND SIGNAL QUANTITATION

Slides were held against film (Kodak Biomax MR) with $^{35}$S-standards (American Radiolabelled Chemical, Inc.) in cassettes for 1.5 h for AVP mRNA, 7 days for CRF mRNA, and 2 days 21 h for GR mRNA. Images were scanned on a Studio Scan II colour flatbed SCSI # 6 scanner using Fotolook SA 2.03. Microdensitometry was performed over the PVN using a Macintosh (Iifx)-based image analysis system (Image 1.42, Wayne Rasband, NIMH). Signal density was used as an index of mRNA levels; $^{35}$S-standards were used to control for film exposure. The hybridization signal was evaluated for each probe as follows. Using the $^{35}$S-standards, a calibration curve was calculated as a percent value. The average area of the PVN was determined and a triangle drawn. The area within the triangle was measured and remained constant between slides. An average was taken between the 2 consecutive sections for statistical analysis.
Table 1. Liquid Rat Diet

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.
Table 1.

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<th>CONTROL DIET (kcal/l)</th>
<th>ETHANOL DIET (kcal/l)</th>
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<tr>
<td>PROTEIN</td>
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<td>258</td>
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<tr>
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<td>255</td>
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<tr>
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<td>368</td>
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<td>TOTAL</td>
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Table 2. Synthetic Oligonucleotides
* Sequences are listed from 5’ to 3’ and are complementary to the base number or mRNA-coding region listed in the next column.
Table 2.

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CHAPTER III: HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS HYPERRESPONSIVENESS

A. EFFECTS OF PRENATAL ETHANOL EXPOSURE ON ACTH STIMULATION OF ADRENAL GLANDS

A.1 INTRODUCTION

One possible mechanism for the enhanced HPA response to stress seen in E offspring may be a greater sensitivity of the adrenal gland to ACTH. Previous studies (Lee & Rivier, 1994; Lee et al., 1990; Taylor et al., 1982) report some data suggesting that neither the adrenal nor the pituitary of E animals is hyperresposive to secretagogues. However, these studies did not systematically examine full dose-response curves for either ACTH or CRF and did not use both males and females. In the present study adrenal sensitivity to ACTH was examined in E, PF, and C male and female offspring at 90-110 days of age.

A.2 METHODS

Sprague-Dawley males (n = 25) and females (n = 53) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed according to Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were
obtained from the tail from 3 unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma Diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951). Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dam and pups were weighed and all litters culled to 10 (five males and five females). Dams and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 90-110 days of age.

One wk prior to testing, animals were singly housed and randomly assigned to postnatal treatment group. Testing order was counterbalanced across prenatal treatment, sex, and dose of ACTH (n = 9-10 for each of E, PF, and C, males and females for each dose of ACTH). Animals were implanted with indwelling jugular cannulae 24 to 48 h prior to testing. On the test day, the animals were removed from their home cage, injected with a dose of dexamethasone-21-phosphate (15 μg / 100 g body weight for males or 30 μg / 100 g body weight for females) (appropriate dose previously determined in a pilot study). Animals were quickly attached to the sampling cannulae and placed in the sampling bucket in the testing room. White noise (40 dB) was used to mask any extraneous room noises. The cannulae were flushed with 0.2 cc of saline to insure patency and left to hang freely over the edge of the bucket so as not to restrict movement. Three hours later, the cannulae were flushed with 0.05 cc of saline and a basal blood sample (0.2 cc) was drawn. Animals were then infused with ACTH (Cortrosyn, Organon, W. Orange, NJ) at doses of 0 (saline), 0.05, 0.1, 0.5, 1.0 units ACTH per rat in an injection volume of 0.3 cc. Another 0.3 cc saline (approximately the volume of the
sampling cannulae) was infused to insure complete administration of the ACTH. Blood samples were drawn (0.2 cc) at 1 h intervals for 4 h. All blood samples were centrifuged at 2200 g for 10 min at 4° C. Plasma was stored at -20° C until analysed. Fluid replacement following each sample consisted of physiologic saline of equivalent volumes. Animals were terminated at the end of the 4 h period. All testing was completed between 0700 and 1200 h.

A.3 STATISTICAL ANALYSES

All data were analysed by appropriate analyses of variance (ANOVA) for factors of prenatal treatment, sex, dose, and time of sample. Significant main and interaction effects were further analysed by Tukey's paired comparisons.

A.4 RESULTS

Developmental Data

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 9.7 ± 0.3, 11.2 ± 0.3, 10.7 ± 0.2 g/kg bw/day for wk 1, 2, and 3 of gestation, respectively. Blood alcohol levels were consistent with previous levels (Weinberg, 1985), averaging 120.1 ± 3.0 mg/dl.
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 x days interaction (p < 0.001). Post-hoc tests indicated that E and PF females weighed significantly less than C females on gestation d 7, 14, and 21 (p’s < 0.001). In addition, E females weighed significantly less than PF females on gestation d 22 (p’s < 0.05). Analysis of maternal weights during lactation similarly revealed a group x days interaction (p < 0.001); E and PF females weighed significantly less than C females on lactation d 1 and 8 (p’s < 0.01). E dams also weighed significantly less than C dams (p < 0.05) on lactation d 15; PF females showed a similar trend toward lower weight than C females on lactation d 15 (p < 0.10). By lactation d 22 there were no significant differences among groups.

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 group (p < 0.01) and a group x days interaction (p < 0.01). Post hoc tests indicated that E and PF pups weighed significantly less than C pups on d 1, 8, 15, and 22 of age (p’s < 0.01). There were no significant differences in offspring weight at the time of testing.

**Experimental Results**

There were no significant differences in CORT responses to ACTH among E, PF, and C animals at any dose or time tested (Figs. 1-5).
Overall, females had significantly higher CORT responses to ACTH than males (p < 0.0001) although patterns of responses were similar in males and females. In addition, for both males and females, there were significant dose x time interactions (p < 0.001). At 1 and 2 h, both males and females infused with all dosages of ACTH had significantly higher CORT levels than saline infused males and females (p's < 0.001 and p's < 0.05 respectively). In addition, at 2 h both males and females infused with 0.5 and 1.0 IU ACTH had significantly higher CORT levels than males and females infused with 0.05 IU ACTH (p's < 0.05 and p's < 0.01 respectively). Both males and females infused with 1.0 IU ACTH had higher CORT levels than animals infused with 0.1 and 0.5 IU of ACTH (p's < 0.001). Females infused with 0.5 IU ACTH also had significantly higher CORT levels than females infused with 0.1 IU ACTH (p < 0.05).

At 3 h post-infusion, both males and females infused with 1.0 IU ACTH had significantly higher CORT levels than animals infused with saline or any of the other dosages of ACTH (p's < 0.001). Females infused with 0.5 IU of ACTH had higher CORT levels than females infused with saline (p < 0.05); males infused with 0.5 IU of ACTH showed a similar trend toward higher CORT levels than males infused with saline (p < 0.06).

At 4 h post infusion, both males and females infused with 1.0 IU ACTH had CORT levels significantly higher than animals infused with saline or any other dosages of ACTH (p's < 0.001). For males, animals infused with 0.5 IU ACTH had significantly higher CORT levels than males infused with saline (p < 0.001) and showed a trend
towards higher CORT levels than males infused with 0.05 IU ACTH (p's < 0.07). Males infused with 0.1 IU ACTH also demonstrated a trend towards higher CORT levels than males infused with saline (p < 0.07). For females, animals infused with 0.1 and 0.5 IU ACTH had significantly higher CORT levels than saline infused females (p < 0.001). Females infused with 0.5 IU ACTH had higher CORT levels than females infused with 0.05 and 0.1 IU ACTH (p < 0.001 and p < 0.05 respectively).

A.5 DISCUSSION

The results from this experiment demonstrate that E animals are not hyperresponsive to exogenous ACTH compared to PF and C animals during the trough of the circadian cycle. Administration of ACTH resulted in a dose response relationship; that is, higher doses resulted in more prolonged CORT responses. The initial peak at 1 h was similar among all doses of ACTH; however at the later time points CORT levels following the highest doses of ACTH did not return to basal levels as did the CORT levels following lower doses. In addition, a sex difference in adrenal sensitivity was seen, females have higher CORT responses to exogenous ACTH than males at every dose. There were no significant differences in adrenal response to ACTH among E, PF and C animals. These data suggest that the insult of prenatal ethanol exposure does not affect adrenal sensitivity to ACTH at least when measured at the trough of the circadian cycle. Thus they suggest that the HPA hyperresponsiveness and/or delays in recovery from stressors that occur in E offspring are not due to an increased adrenal sensitivity to ACTH.
CORT levels in response to the increasing doses of exogenous ACTH in the present study were consistent with those observed in previous experiments (Keller-Wood et al., 1983c; Lake & Gann, 1972; Macchi & Hechter, 1954; Normand et al., 1980). All doses of ACTH resulted in elevated CORT, with a peak seen at 1 h and a decline thereafter. Higher doses of ACTH resulted in prolonged elevations in CORT levels. It has been shown that in vivo, normal adrenal glands of dogs (Keller-Wood et al., 1983a) and rats (Kaneko et al., 1981) respond to less than 10 pg/ml ACTH with an elevation in CORT levels. The maximal CORT secretion is seen in both dogs and rats at as little as 200 pg/ml ACTH (Coultron, 1973; Tam & Greer, 1982). In addition, Keller-Wood and colleagues (1983b) demonstrated that the elevation in CORT levels after administration of exogenous ACTH was proportional to the logarithm of ACTH concentration up to about 300 pg/ml and further increases in amounts of exogenous ACTH prolonged the duration of the maximum CORT secretory rate beyond the period when plasma ACTH levels were elevated. This suggests that another mechanism may be involved in the prolongation of the CORT response. It has been demonstrated that ACTH binding to adrenocortical cells results in a parallel production of adenosine 3' 5'-cyclic monophosphate (cAMP) (Buckley & Ramachandran 1982; Normand et al., 1980). The production of cAMP increases with increasing levels of exogenous ACTH. Although the exact mechanism for the prolongation of the CORT response has yet to be determined, it has been suggested that this prolongation may result from retention of ACTH by adrenal cells and/or the accumulation of a biosynthetic intermediate, such as cAMP, which is activated by ACTH (Macchi & Hechter, 1954).
The sex difference observed in adrenal response to exogenous ACTH is also consistent with that observed in previous studies (Kitay 1961; Skelton & Bernardis, 1966). Although the patterns of response were similar in males and females, females demonstrated higher CORT responses to exogenous ACTH than males. It has been demonstrated that female rats have heavier adrenal glands (Kitay, 1961; Skelton & Bernardis, 1966) and have greater CORT response to stress (Kitay, 1961; Weinberg 1992b) than males. The sex difference in adrenal cortex thickness and secretion has been shown to begin at about 40 days of age, is fully manifested at 55 days, and begins to decrease after 360 days of age (Sencar-Cupovic & Milkovic, 1976). Kitay (1961) demonstrated that administration of exogenous ACTH to male rats resulted in a peak plasma CORT level 30 min after infusion with a return to the resting levels by 120 min. In contrast, females reached a peak at 15 min after infusion, maintained the peak level for 30 min and then showed a decline towards basal levels; however females did not reach basal levels by 120 min. In addition, the concentration of CORT in the adrenal vein following anaesthesia, laparotomy and manipulation of abdominal viscera has been demonstrated to be 2.5 times higher in females than males (Kitay, 1961). Furthermore, the increase in adrenal sensitivity in females does not appear to be a direct effect of circulating sex hormones at the time of testing. Females gonadectomized prior to weaning still demonstrate greater stress CORT levels as adults than gonadectomized males (Skelton & Bernardis, 1966). Together these data suggest that adrenal glands of female rats are more sensitive to ACTH than are adrenal glands of male rats. This differential sensitivity does not appear to be completely dependent on gonadal function in adulthood.
Consistent with previous studies (Lee & Rivier, 1994; Lee et al., 1990; Taylor et al., 1982), the present study demonstrates that HPA hyperresponsiveness seen in E animals is not a result of differential adrenal sensitivity to ACTH at least when measured at the trough of the CORT circadian rhythm. It has been shown that the adrenal gland markedly changes its sensitivity to ACTH during the circadian cycle being maximally sensitive during lights-off and minimally sensitive during lights on (Kaneko et al., 1981). Therefore, it is possible that differential sensitivity to ACTH in E compared to PF and C animals might be observed at the peak of the circadian rhythm. In the present experiment, however, it appears that in the AM, during the trough of the circadian cycle, prenatal ethanol exposure exerts long-term effects on the hypothalamus and/or pituitary and not on the adrenal gland itself.
Figure 1. Corticosterone Levels in Male and Female Rats Following Infusion of Saline.

Points represent mean ± SEM.
There were no significant differences among E, PF, and C males (above) or females (below).
Figure 2. Corticosterone Levels in Male Rats Following Infusion of 0.05 and 0.1 units of ACTH.

Points represent mean ± SEM.
There were no significant differences among E, PF, and C males infused of 0.05 (above) and 0.1 (below) units of ACTH.
Figure 3. Corticosterone Levels in Male Rats Following Infusion of 0.5 and 1.0 units of ACTH.

Points represent mean ± SEM.
There were no significant differences among E, PF, and C males infused of 0.5 (above) and 1.0 (below) units of ACTH.
Figure 4. Corticosterone Levels in Female Rats Following Infusion of 0.05 and 0.1 units of ACTH.

Points represent mean ± SEM.

There were no significant differences among E, PF, and C females infused of 0.05 (above) and 0.1 (below) units of ACTH.
Figure 5. Corticosterone Levels in Female Rats Following Infusion of 0.5 and 1.0 units of ACTH.

Points represent mean ± SEM.

There were no significant differences among E, PF, and C females infused of 0.5 (above) and 1.0 (below) units of ACTH.
B. EFFECTS OF PRENATAL ETHANOL EXPOSURE ON HYPOTHALAMIC-PITUITARY-ADRENAL SENSITIVITY TO DEXAMETHASONE SUPPRESSION.

B.1 INTRODUCTION

The present study utilised dexamethasone (DEX) suppression to explore further the hypothesis that the HPA hyperresponsiveness and/or delays in recovery from stressors that occur in E offspring result from deficits in feedback inhibition of the HPA axis induced by prenatal ethanol exposure. Dexamethasone (DEX), a synthetic glucocorticoid, has been shown to inhibit ACTH release, and consequently leads to marked reduction in plasma CORT levels (Hauger et al., 1987; Hauger et al., 1989; Spinedi et al., 1991; Uht et al., 1989; Wynn et al., 1983). Binding studies have demonstrated that DEX preferentially binds to the pituitary suggesting that DEX suppression indicates pituitary sensitivity to feedback inhibition (De Kloet et al., 1975; Spencer et al., 1995). The effects of low dose DEX blockade on stress CORT levels to a mild and an intense stressor were examined at 3 and 6 h post-DEX injection in E, PF and C males and females.

B.2 METHODS

Sprague-Dawley males (n = 25) and females (n = 62) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed according to
Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were obtained from the tail from 3 unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma Diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951).

Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dams and pups were weighed and all litters culled to 10 (five males and five females). Dams and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 90-110 days of age.

One wk prior to testing, animals were singly housed and assigned to injection dose and testing time. Test order was counterbalanced across prenatal treatment, sex, and injection dose. Animals (n = 8 each from E, PF, and C, males and females) were assigned to 1 of 4 doses of DEX (0 [saline], 0.1, 0.5, or 1.0 µg/100 g bw) and to a testing time of 3 or 6 h post-injection. On the test day, between 0700 h and 0830 h, animals were given an intraperitoneal (IP) injection of approximately 3.0 cc containing the assigned dose of DEX and were then returned to their home cages until testing. Three or 6 h later animals were subjected to an IP injection with a 25G X 5/8" needle. Animals were then placed in a holding room for 15 min after which time blood samples were taken by cardiac puncture under ether anaesthesia. The blood was centrifuged at 4°C for 10 min at 2200 g and plasma was collected and stored at -20°C until CORT levels were...
measured using RIA. One week later the procedure was repeated using a more intense stressor, exposure to ether vapours for approximately 45 sec, and blood samples were obtained by decapitation for determination of CORT levels.

B.3 STATISTICAL ANALYSES

Data were analysed by appropriate analyses of variance (ANOVAs) for factors of group (E, PF, C), sex, dose of DEX, time of stressor, and stressor type. Significant effects were further analysed with Tukey's post-hoc tests. Because of the complexity of the four-way ANOVAs, the data were further analysed separately for males and females for each stressor using three-way ANOVAs for the factors of group, dose, and time, as well as separate 2 way ANOVAs at 3 and 6 h for the factors of group and dose.

B.4 RESULTS

Developmental Data

Ethanol intake of pregnant females was constantly high throughout gestation averaging $9.2 \pm 0.2$, $11.6 \pm 0.2$, $11.7 \pm 0.2$ g/kg bw/day 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 x days interaction ($p < 0.001$). Post-hoc tests indicated that E and PF females weighed significantly less than C females on gestation d 7, 14, and 21 ($p < 0.001$). In
addition, E females weighed significantly less than PF females on gestation d 7 and 14 (p’s < 0.05). Analysis of maternal weights during lactation similarly revealed a group x days interaction (p < 0.001). E females weighed significantly less than C females on lactation d 1 (p < 0.01). Furthermore, E females weighed significantly less than PF females on lactation d 8 and 15 (p’s < 0.05).

There were no significant differences among groups for litter size or number of stillborn pups. Analysis of body weights of pups indicated a significant main effect of group (p < 0.01) and a group x days interaction (p < 0.01). Post hoc tests indicated that E and PF pups weighed significantly less than C pups on d 1 and 8 (p’s < 0.05). In addition PF pups weighed significantly less than C pups on d 15 and 22 (p’s < 0.05). There were no significant differences in pup weight at the time of testing.

**Experimental Results**

Four-way ANOVAs (sex x group x dose x stressor) were carried out separately for 3 h and 6 h data. These ANOVAs revealed main effects of sex, dose and stressor at both the 3 and 6 h test times (p’s < 0.05). Females in all groups exhibited higher CORT levels than males (p < 0.01) reflecting the typical sex difference in HPA axis activity (Weinberg, 1988). Animals injected with 0.5 or 1.0 μg DEX/100 g bw had lower CORT levels than animals injected with 0 and 0.1 μg DEX/100 g bw (p < 0.05) (Figs. 6-9). In addition, animals injected with 0.5 μg DEX/100 g bw had higher CORT levels than those
injected with 1.0 μg DEX/100 g bw (p < 0.05). All animals showed higher CORT levels to ether vapours (Figs. 8 and 9) than to IP injection (p < 0.01) regardless of the injection dose of DEX (Figs 6 and 7)

The results of the 2-way ANOVAs (group x dose) for each type of stressor demonstrated some group differences. There were no significant group differences for either males or females among E, PF, and C animals at 3 or 6 h in response to the IP stressor (Figs. 6 and 7). However, males did demonstrate a group x dose interaction at 3 h but not at 6 h in response to the ether stressor, (p < 0.05) reflecting a different pattern of response for the 3 groups across doses of DEX. At 3 h, E and PF males showed a trend towards higher CORT levels than C males at doses of 0.1 and 0.5 μg/100 g bw (p's <0.05). In contrast, there was a marginally significant effect of group (p's < 0.06) for females subjected to the ether stressor at both 3 and 6 h. Post-hoc analysis demonstrated a trend for higher CORT levels in E compared to C females (p's < 0.1); PF females did not differ from either group. In addition, at 6 h a significant group x dose interaction was demonstrated (p < 0.05); E and PF females injected with 1.0 μg/100 g bw of DEX had higher CORT levels than C females injected with 1.0 μg/100 g bw of DEX following ether stress (p's <0.05) (Fig. 9).
B.5 DISCUSSION

The results from this study indicate that administration of DEX to block HPA activity suppressed CORT secretion in a dose dependent manner in all animals regardless of the prenatal treatment. Furthermore, as expected, males and females exhibited differential CORT responses to the same dose of DEX. In addition, the ability of DEX to suppress the CORT response was greater following IP injection as compared with ether. Further, the results demonstrate some differences in the suppressability of the CORT response to ether stress among animals in E, PF, and C groups. At 3 h post-injection, E males showed a trend towards higher CORT levels than C males at doses of 0.1 and 0.5 μg/100 g bw DEX and E females overall had higher CORT levels than C females. At 6 h post-injection, there were no significant differences among males but E females had higher CORT levels than C females at 1.0 μg/100 g bw DEX.

DEX has been shown to be a more potent blocker of the stress-induced ACTH release at the pituitary than CORT (De Kloet et al, 1975). In vivo and in vitro studies have demonstrated that DEX preferentially binds to the pituitary (De Kloet et al., 1975; Spencer et al., 1990). It has been shown that the hippocampus accumulates 3H-corticosterone to a greater extent than in the hypothalamus and anterior pituitary, while 3H-DEX accumulates to a greater extent in the anterior pituitary than in the hypothalamus and hippocampus (De Kloet et al., 1975). Administration of DEX (200 μg/day) for 7 days to adrenalectomized rats has been shown to reduce ACTH/β-lipotropin precursor mRNA
(19% of intact controls) but to have little effect on prepro-CRF mRNA (102% of intact controls) (Jingami et al., 1985). In addition, subcutaneous injection of DEX at doses of 1-50 μg/kg bw has been shown to produce a selective and time related decrease in available Type II receptors in the pituitary and to have no effect on Type II receptors in the hippocampus (Spencer et al., 1995). DEX in the drinking water has been demonstrated to activate pituitary Type II receptors at varying doses (0.3 μg/ml, 0.8 μg/ml, and 10.0 μg/ml), whereas hippocampus and hypothalamus Type II receptors are only activated at an exceedingly high dose of DEX (10 μg/ml) (Miller et al., 1992).

Furthermore, low dose DEX (1.5 μg/ml) in the drinking water overnight does not affect Type II binding in the hippocampus but 1.0 μg/ml of DEX in the drinking water for 1, 2, and 3 days does significantly reduce Type II activation (Spencer et al., 1990). Binding studies have also indicated that there is a differential time course of $^3$H-DEX and $^3$H-CORT binding in the hippocampus, hypothalamus and anterior pituitary (De Kloet et al., 1975). $^3$H-DEX is taken up more slowly and retained longer by the hippocampus than $^3$H-CORT; the hypothalamus takes up $^3$H-DEX and $^3$H-CORT equally but retains $^3$H-DEX for longer; and the anterior pituitary takes up $^3$H-DEX more rapidly and retains it longer than $^3$H-CORT. Thus, it appears that the ability of DEX to suppress stress-induced ACTH secretion may be a function of dose and time and a single low to moderate doses reflects predominately anterior pituitary suppression.

The results of the present study are consistent with previous studies in demonstrating that CORT responses increases as stressor intensity increases. Animals
exposed to ether, a more severe stressor, had higher CORT levels following both saline and DEX injection than animals exposed to IP injection, a mild stressor, following both saline and DEX injection. The HPA axis is very sensitive to mild stressors and may show a graded response to increasing intensities of a mild stressor, resulting in increased circulating ACTH and CORT. It has been demonstrated that with increases of volume of arterial haemorrhage there is an increase in plasma ACTH (Plotsky, 1987) and with increasing challenges of low intensity psychological stressors such as handling and novel environment (Armario et al., 1986a; Armario et al., 1986b) or increasing intensity of current-delivered footshock (Kant et al., 1983) there is an incremental increase in plasma CORT. Importantly, however, the graded response to stress occurs only following mild stressors and maximal ACTH and CORT levels are reached rapidly with more severe stressors such as cold, formalin injection, or restraint (Kant et al., 1982). As previously discussed (Chapter IIIa) the maximal CORT response to ACTH is also reached quickly and CORT responses to higher levels of ACTH results in prolonged elevations in CORT.

There also appears to be differential activation of components of the HPA axis depending upon the stressor. CRF mRNA in the PVN and POMC mRNA in the anterior pituitary have been shown to increase with some stressors such as IP injection of hypertonic saline, restraint or swim but not others like cold stress (Harbuz & Lightman, 1989b). Physical stressors such as restraint and swim have been shown to activate CRF mRNA in the PVN alone while other physical stressors such as footshock, IP hypertonic saline, and naloxone-induced morphine withdrawal not only increase CRF mRNA but also proenkephalin A (PEA) mRNA in the PVN (Harbuz & Lightman, 1989a; Harbuz et
Ether stress has been shown to increase PEA mRNA but has no effect on CRF mRNA (Harbuz & Lightman, 1992). Further, it has been demonstrated that increases in cAMP and guanosine 3',5'-monophosphate (cGMP) levels following stress vary with the stressor. Increases in pituitary cAMP following cold, running, formalin injection, restraint and shock appears to be highly correlated with the intensity of the stressor while cerebellar cGMP appears to be associated with an increase in motor activity associated with the stressor (Kant et al., 1982). Increases in cerebellar cGMP were only seen with stressors such as running or shock where activity was also increased whereas cold and restraint did not affect cerebellar cGMP levels.

The exact mechanism(s) for the variation in stress response in hormone secretion, gene induction, and secondary messenger activation are still under investigation. It can be hypothesised that synergistic hormones such as AVP or OT may be selectively released in response to various stressors and potentiate the release of ACTH stimulated by CRF and/or that varying neurotransmitter systems may be activated in response to stressors. In support of this, Gaillet et al. (1991) demonstrated that ascending noradrenergic pathways to the hypothalamus play a major stimulatory role in ether and restraint stress, have a moderate or biphasic involvement in response to insulin-induced hypoglycaemia and have no significant participation in the HPA response to systemic histamine infusion. Therefore, it is possible that the difference in intensity between ether and IP injection stress may result in different levels of CRF and AVP being released as well as a difference in the pathway of activation of the stress response.
As previously mentioned, it has been well-documented that a sex difference exists in basal CORT and stress CORT levels (Critchlow et al., 1963; Kitay, 1961; Weinberg, 1988). The sex difference appears to be related to circulating oestrogen levels as well as perinatal hormone exposure. It has been shown that ovariectomy (OVX) of an adult female results in adrenal atrophy as well as reduction in basal CORT levels while OVX plus oestrogen replacement reinstates the typical sex differences in adrenal weight and basal CORT levels (Kitay, 1963; Le Mevel, 1978; Ramaley, 1976). Further, it has been demonstrated that basal and stress CORT levels vary with the oestrus cycle (Raps et al., 1971; Viau & Meany, 1991). Female rats have the greatest CORT levels during the beginning of proestrus when oestrogen levels are highest and progesterone levels are low as compared to levels during dioestrus and oestrus (Critchlow et al., 1963; Viau & Meany, 1991). Progesterone appears to inhibit the facilitory effects of oestrogen. In women, ACTH and cortisol levels rise towards the end of the follicular phase of the menstrual cycle (Genazzi, 1975) and women have higher post-DEX cortisol levels during the middle 2 weeks of the menstrual cycle as compared to the other weeks of the cycle (Roy-Byrne et al., 1986). Likewise oestrogen in rats appears to affect the negative feedback of CORT. OVX rats given oestrogen replacement have higher and more prolonged responses to footshock, ether and restraint stressors than OVX controls (Burgess & Handa, 1992; Viau & Meany, 1991). Furthermore OVX females are more sensitive to DEX suppression (Ramaley, 1976) than intact females and OVX females are more sensitive to RU 28362 (a specific glucocorticoid receptor agonist) (Burgess & Handa, 1992) than OVX and oestrogen treated females. Consistent with these studies, we found that females had higher stress CORT levels.
The factors which underlie the effects of oestrogen on the HPA axis remain to be determined. As mentioned above, oestrogen affects adrenal size and secretion. OVX decreases pituitary synthesis of and release of ACTH as well as adrenal synthesis of CORT (Kitay, 1963), and this decrease is reversed by oestrogen replacement (Coyne & Kitay, 1969; Kitay, 1963). Oestrogen has also been demonstrated to influence PVN firing and secretion to stress. Extracellular recordings demonstrate an increase in PVN unit firing rates during proestrous and oestrus, whereas in OVX females, the firing rates are only increased after oestrogen replacement and are reduced by progesterone replacement (Negoro et al., 1973b). Further, the percentage of PVN units firing in response to foot pinching are highest during proestrous and are enhanced in OVX females after oestrogen replacement and inhibited after progesterone treatment (Negoro et al., 1973a). In addition, it has been demonstrated that oestrogen alters CRF immunoreactivity and mRNA levels (Bohler et al., 1990; Haas & George, 1988, Swanson & Simmons, 1989) with the highest levels of CRF content being seen during proestrous and dioestrous (Hiroshige & Wada-Okada, 1973). The AVP/OT ratio in the PVN and supraoptic nuclei are also affected by the oestrus cycle; during proestrus the AVP/OT ratio is highest while during oestrus the OT mRNA in the supraoptic nucleus is highest. Furthermore, NE, which is thought to be involved in HPA regulation, is also influenced by oestrogen; during proestrus NE turnover is rapidly increased (Rance et al., 1981). Oestrogen appears to up-regulate \( \alpha_1 \)-adrenergic receptors and down regulate \( \beta \)-adrenergic receptors (Condon et al., 1989, Weiland & Wise, 1989). Low doses of NE enhance CRF release through binding to \( \alpha_1 \)-adrenergic receptors, while high doses inhibit CRF release.
through binding to β-adrenergic receptors (Plotsky, 1987; Plotsky et al., 1989). Thus, it is possible that as oestrogen levels rise, CRF release is enhanced as a result of an increase in α1-adrenergic receptor stimulation and a decrease of β-adrenergic receptor inhibition.

The decrease in HPA axis sensitivity to DEX suppression seen in females is also still under investigation although it appears that it is not a result of altered clearance or adrenal function. Viau & Meany (1991) demonstrated that there was no significant difference in the rate of clearance of ACTH or CORT in females which were OVX alone or OVX and received oestrogen replacement. In addition, Ramaley (1976) demonstrated no difference in the clearance of DEX in OVX or intact females. Although the present study only investigated CORT levels, previous studies have demonstrated elevation in stress ACTH levels in the presence of oestrogen (Burgess & Handa, 1992; Viau & Meany 1991). Thus, the elevated and prolonged levels of ACTH and CORT to stress as well as the desensitisation of the HPA axis to inhibitory feedback of glucocorticoids in the presence of oestrogen appear to indicate that prenatal alcohol exposure may affect the HPA axis by altering feedback inhibition of CORT at the level of the pituitary or higher.

In summary, stress CORT levels appear to be a function of stress intensity as demonstrated by the differential responses to IP injection as compared to ether vapor. In addition, there appears to be a sexual dimorphism in the stress response. Further, although the doses in this experiment were not sufficient to suppress the HPA axis fully, the experiment supports the hypothesis that HPA hyperresponsiveness and/or delays in
recovery from stressors that occur in E animals may result from deficits in feedback inhibition.
Figure 6. Corticosterone Levels in Dexamethasone Treated Males Following IP Poke.

Points represent mean ± SEM.

# Main effect of dose, p < 0.05: 0.5 μg/100 g bw DEX < saline and 0.1 μg/100 g bw DEX at 3 h.

^ Main effect of dose, p < 0.05: 1.0 μg/100 g bw DEX < saline, 0.1 and 0.5 μg/100 g bw DEX at 3 and 6 h.
Figure 7. Corticosterone Levels in Dexamethasone Treated Females Following IP Poke.

Points represent mean ± SEM.
# Main effect of dose, p < 0.05: 0.5 and 1.0 μg/100 g bw DEX < saline and 0.1 μg/100 g bw DEX at 3 and 6 h.
^ Main effect of dose, p < 0.05: 1.0 μg/100 g bw DEX < 0.5 μg/100 g bw DEX at 3 h.
Figure 8. Corticosterone Levels in Dexamethasone Treated Males Following Ether Vapor.

Points represent mean ± SEM.
# Main effect of dose, p’s < 0.05: 0.5 and 1.0 µg/100 g bw DEX < saline and 0.1 µg/100 g bw DEX at 3 h
^ Main effect of dose, p < 0.05: 1.0 µg/100 g bw DEX < saline, 0.1 and 0.5 µg/100 g bw DEX at 3 and 6 h.
Figure 9. Corticosterone Levels in Dexamethasone Treated Females Following Ether Vapor.

Points represent mean ± SEM.

# Main effect of dose, p’s < 0.05: 0.5 and 1.0 μg/100 g bw DEX < saline and 0.1 μg/100 g bw DEX at 3 h (above) and 1.0 μg/100 g bw DEX < saline and 0.1 μg/100 g bw DEX at 6 h (below).

^ Main effect of dose, p < 0.05: 1.0 μg/100 g bw DEX < 0.5 μg/100 g bw DEX at 3 h.

* Group x dose interaction, p < 0.05; following injection of 1.0 μg/100 g bw DEX, E = PF > C at 6 h.
C. FOETAL ETHANOL EXPOSURE ALTERS PITUITARY-ADRENAL SENSITIVITY TO DEXAMETHASONE

C.1 INTRODUCTION

The present study utilised higher doses of DEX and examined a longer time course than the previous study (Chapter IIIB) to explore further the hypothesis that the HPA hyperresponsiveness and/or delays in recovery from stressors that occur in E offspring result from deficits in feedback inhibition of the HPA axis induced by prenatal ethanol exposure. The effects of DEX blockade on basal and stress CORT levels and stress ACTH levels were examined over a 36 h period. In addition, stress CORT and ACTH levels were examined after administration of DEX, during both the trough (AM) and peak (PM) of the CORT circadian rhythm.

C.2 METHODS

Sprague-Dawley male (n = 25) and female (n = 145) rats were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed according to Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were obtained from the tail from nine unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma Diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951). Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d 1 of lactation, dam and pups were weighed
and all litters culled to 10 (five males and five females). Dam and pups were weighed
and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and
housed by sex and by litter until testing at 90-110 days of age. Three replicate breedings,
with 48-49 females in each, were done.

One wk prior to testing, animals were singly housed, divided into four subsets,
and randomly assigned to IP injection dose and test time. Testing order was
counterbalanced across prenatal treatment, sex, and injection dose (n = 6-9 for each of E,
PF, and C, males and females at each dose and time. DEX injections for the first three
subsets of animals occurred at 0730-0900 h on the initial test day (AM groups). Doses of
DEX were based on pilot studies which indicated that females required higher doses of
DEX than males to suppress CORT levels in response to stress. Animals in the first
subset received one of four doses of DEX [males, 0 (saline), 1.0, 5.0, or 15.0 \mu g/100 g
body wt (bw); females, 0, 1.0, 10.0, or 30.0 \mu g/100 g bw] and were tested at 3 or 6 h post-
injection. Animals in the second subset received one of three doses of DEX [males, 0,
5.0, or 15.0 \mu g/100 g bw; females 0, 10.0, or 30.0 \mu g/100 g bw] and were tested at 10 or
26 h post-injection. Animals in the third subset received one of two doses of DEX
[males, 0 or 15.0 \mu g/100 g bw; females, 0 or 30.0 \mu g/100 g bw] and were tested at 36 h
post-injection. DEX injections for the fourth subset of animals occurred at 1500-1700 h
on the initial test day (PM group). Animals received one of three doses of DEX [males, 0,
5.0, or 15.0 \mu g/100 g bw; females 0, 10.0, or 30.0 \mu g/100 g bw], and were tested 3 h post-
injection under red light. All animals were returned to the colony room between injection
and blood sampling.
At the designated sampling time, animals were taken from the colony room to an adjacent laboratory, quickly and lightly anaesthetised with ethyl ether, and blood samples (0.5cc) obtained by cardiac puncture using heparinized syringes (the fourth subset of animals was exposed to ether only; no basal samples were drawn). Twenty min later, animals were rapidly decapitated (within 10-15 sec of touching the cage; Rivier et al., 1982b) and trunk blood collected on ice in 12 x 75 mm plastic test tubes containing 7.5 mg EDTA and 1000 KIU aprotinin (0.2 ml/5cc blood). The blood was centrifuged at 2200 x g for 10 min at 4°C and plasma transferred with plastic pipettes to microcentrifuge tubes for storage at -70°C until assayed for CORT and ACTH. Resting ACTH levels could not be determined because the procedure of etherization and cardiac puncture was too slow to obtain undisturbed levels.

C.3 STATISTICAL ANALYSES

All data were analysed by appropriate analyses of variance (ANOVA) for factors of prenatal treatment, sex, and dose of DEX. Significant main and interaction effects were further analysed by Tukey's paired comparisons.
C.4 RESULTS

Developmental Data

Ethanol intake of the pregnant females was consistently high throughout gestation in all three breedings, averaging 9.7 ± 1.4, 11.15 ± 1.0, 10.7 ± 0.8 g/kg bw/day for wk 1, 2, and 3 of gestation, respectively. Blood alcohol levels were consistent with those reported previously (Weinberg, 1985), averaging 145.4 ± 10.9 mg/dl.

Repeated measures ANOVAs on maternal weight gain during pregnancy and pup weight gain during lactation revealed significant main effects of group (p < 0.001 and p < 0.01, respectively) and days (p's < 0.001), as well as group x days interactions (p's <0.01). Body weights of E and PF dams were significantly less than those of C dams on gestation d 7-21 (p's < 0.001). There were no significant differences among groups for litter size. However, E and PF pups weighed significantly less than C pups on all days measured during lactation (p's < 0.001). There were no significant differences in body weight among E, PF, and C offspring at the time of testing.

CORT and ACTH Levels (AM Groups)

Hormone measures in both male and female offspring tested in adulthood showed dose response relationships for both resting CORT levels and for stress CORT and ACTH
levels following DEX blockade; the higher the dose of DEX, the greater the CORT and ACTH suppression and the longer the time to return to basal levels.

**Undisturbed CORT levels** Significant main effects of dose of DEX were obtained at 3, 6, and 10 h post-injection (p's < 0.01). Both males and females injected with all doses of DEX had significantly lower undisturbed CORT levels than their saline-injected counterparts (Table 3). The only exception was at the 6 h sampling time when females injected with the lowest dose of DEX (1.0 µg/100 g body wt) did not differ from saline injected females. By 26 h, there were no significant differences between DEX- and saline-injected animals. There were no significant differences among E, PF, and C animals in undisturbed CORT levels at any sampling time.

**Stress CORT levels** Significant main effects of dose were obtained both for males (Fig. 10-12) and females (Fig. 13-15) at 3, 6, 10, and 26 h post injection (p's < 0.01). At all of these times, males injected with 5.0 or 15.0 µg/100 g bw DEX had lower CORT levels than males injected with saline (p's < 0.01). There were no significant differences in CORT levels among E, PF, and C males at any time tested. At 3, 6, and 26 h post-injection, females injected with 10.0 or 30.0 µg/100 g bw DEX had lower CORT levels than females injected with saline (p's < 0.01). At 10 h post-injection, only females injected with 30.0 µg/100 g bw DEX had lower CORT levels than saline-injected females (p < 0.01).
Importantly, at 3 h post-injection, analysis of female CORT levels revealed a significant main effect of group (p < 0.05). E females had higher CORT levels than PF and C females (p's < 0.05). At 6 h post-injection, E females injected with 30 µg/100 g bw DEX also had higher CORT levels than PF and C females (p's < 0.05). There were no significant differences in CORT levels among E, PF, and C females at 10, 26, or 36 h post-injection.

**Stress ACTH Levels** Significant main effects of dose were obtained at 3, 6, and 10 h post-injection for both males (Fig. 17 and Fig. 18) and females (Fig. 19 and Fig. 20) (p's < 0.001). At 3 h post-injection, males and females injected with all doses of DEX had lower ACTH levels than their saline-injected counterparts. At 6 and 10 h post-injection, males injected with the two highest doses and females injected with the highest dose of DEX, had lower ACTH levels than those injected with saline (p's < 0.01). At 26 and 36 h post-injection, there were no significant differences for either males (Fig. 18) or females (Fig. 21) in ACTH levels between DEX- and saline-injected animals.

Significant main effects of group were also observed (p's < 0.05). At 26 h post-injection, E and C males had significantly higher ACTH levels than PF males (p's < 0.05), and E and PF females had significantly higher ACTH levels than C females (p's < 0.05).
CORT and ACTH Levels (PM Group)

Stress CORT Levels  Significant main effects of dose were obtained for both males and females (p's < 0.001) (Fig. 22); animals injected with DEX had lower CORT levels than animals injected with saline. For males, there was a significant main effect of group (p < 0.05) and a group x dose interaction (p < 0.01). At 5.0 μg/100 g bw DEX, E males had higher CORT levels than C males; at 15.0 μg/100 g bw DEX, E males had higher CORT levels than PF males (p's < 0.05). Similarly, a significant main effect of group was found for females (p < 0.001). E females injected with 10.0 and 30.0 μg/100 g bw DEX had significantly higher CORT levels than C females (p's < 0.01).

Stress ACTH levels  Significant main effects of dose were obtained for both males and females (p's < 0.001) (Fig. 23); animals injected with DEX had significantly lower ACTH levels than saline-injected animals. For males, there were no significant differences in ACTH levels among E, PF and C animals. In contrast, there was a significant main effect of group for females; at 30 μg/100 g bw DEX, E females had significantly higher ACTH levels than PF and C females (p < 0.05).

C.5 DISCUSSION

The results from these experiments support our hypothesis that HPA hyperresponsiveness and/or delays in recovery from stressors that occur in E offspring may result, at least in part, from deficits in feedback inhibition of the HPA axis induced
by prenatal ethanol exposure. Administration of DEX to block HPA activity significantly suppressed both resting levels of plasma CORT and stress levels of plasma CORT and ACTH in all animals, regardless of prenatal treatment. Importantly, E animals did not differ from PF and C animals in basal CORT levels but exhibited significantly higher stress levels of CORT and/or ACTH than PF and C animals following DEX blockade. Furthermore, males and females exhibited differential responsiveness depending on the time of day when testing occurred. When tested at the trough of the CORT circadian rhythm, reduced sensitivity to DEX suppression of stress hormone levels was observed only in E females; E males were similar to PF and C males in responsiveness. In contrast, when tested at the peak of the CORT circadian rhythm, both E males and E females exhibited reduced sensitivity to DEX suppression of stress hormone levels. Sex differences in HPA responsiveness following DEX were further demonstrated by the finding that E males showed increased stress levels of CORT but not ACTH, whereas E females showed increased stress levels of both CORT and ACTH compared to their respective controls. These data suggest that the insult of prenatal ethanol exposure affects both male and female offspring, but that there may be a sex specific difference in sensitivity of the mechanism(s) underlying HPA hyperresponsiveness. Moreover, consistent with previous studies (Taylor et al., 1983; Weinberg, 1992a; Weinberg, 1992b), it appears that E offspring may not differ from controls under nonstressed conditions, but exhibit significant deficits and/or alterations in responsiveness when challenged with stressors, hormones or pharmacological agents, or when placed in behaviourally aversive or challenging situations.
At this time we cannot rule out the possibility that the pharmacokinetics of DEX were altered in E compared to PF and C animals. However, this appears unlikely as undisturbed levels of CORT following DEX administration did not differ in E, PF, and C animals. Basal levels of ACTH could not be measured in the present study as the method of sampling used was too slow to obtain a reliable measure of undisturbed ACTH. However, previous data from our lab (Weinberg et al., 1996) and others (Lee et al., 1990; Taylor et al., 1986b) have shown that E animals do not differ from PF and C animals in basal ACTH levels.

In contrast to previous work from our lab (Weinberg, 1992b; Weinberg, 1988; Weinberg & Gallo, 1982) and others (Lee et al., 1990; Nelson et al., 1986; Taylor et al., 1982), we did not observe increased CORT or ACTH responses to stress in E compared to PF and C animals in the nontreated (i.e. saline-injected) condition. One possible reason is that the potent physiological stressor used in the present study, i.e. ether stress, probably elicited a maximal response in all animals. Our previous data suggests that the parameters of the test situation, the nature and intensity of the stressor, the time course measured, and the level of stress axis examined all play a role in determining whether E animals differ from controls in stress responsiveness and whether differential effects of foetal ethanol exposure are observed in males and females.

In addition to prenatal ethanol effects, we also noted prenatal nutritional effects as well as an effect of pair-feeding itself. At 26 h post-DEX injection, PF males showed suppressed ACTH levels compared to E and C males whereas E and PF females both
showed increased ACTH levels compared to C females. Previous data demonstrated that although pair-feeding provides an essential nutritional control group, pair-feeding itself is a type of experimental treatment (Weinberg, 1984). For example, pair-feeding can produce a shift in the circadian rhythm of a number of physiologic variables as well as alter body and organ weights and behaviour of both the maternal females and the offspring (Gallo & Weinberg, 1981; Weinberg, 1989; Weinberg & Gallo, 1982). The present data further demonstrate long term effects of pair-feeding and highlight the importance of including an *ad libitum* fed control group in prenatal alcohol studies.

The control of the HPA stress response occurs through multiple feedback loops occurring during three different time domains and at several different levels. CORT feedback inhibition of ACTH and CRF occurs within seconds (fast rate sensitive feedback), over 2-10 h (intermediate feedback), and over hours to days (slow feedback) (Jones & Gillham, 1988; Keller-Wood & Dallman, 1984). Fast rate sensitive feedback is thought to inhibit release of ACTH and CRF but not affect synthesis, whereas intermediate feedback is thought to decrease release of both ACTH and CRF and to decrease CRF synthesis (Keller-Wood & Dallman, 1984). Slow feedback which occurs only in pathologic conditions where CORT is elevated for days has been shown to decrease not only ACTH release but also ACTH synthesis (Schacter *et al.*, 1982). DEX has been shown to bind preferentially to the anterior pituitary (DeKloet *et al.*, 1975). Thus when DEX is given at high doses (300µg/100 g bw), ACTH content is affected to a much greater extent than CRF content (Carnes *et al.*, 1987). Our finding that E animals show less suppression than controls at 3 and 6 h post-DEX injection, suggests that
alterations in HPA responsiveness to stressors in E animals may be mediated through a
defect in feedback inhibition at the level of the anterior pituitary during the intermediate
feedback time domain. In contrast, Taylor et al., (1986b), found that at 10 min post-foot
shock stress (during the fast feedback time domain), ACTH levels in E animals remain
elevated compared to those in controls. Together, these data suggest that deficits in
feedback inhibition may occur during both the fast and intermediate feedback time
domains and that it is release and not synthesis of ACTH that contributes to the elevated
CORT and ACTH levels seen in E animals.

A diurnal variation in both basal and stress-induced HPA hormone release has
been demonstrated (Bradbury et al., 1991; Kant et al., 1986). CORT, ACTH, β-EP and β
-lipotropin release are greater in the AM than in the PM following a variety of stressors
(Bradbury et al., 1991; Kant et al., 1986). The mechanism for this diurnal variation is not
clearly understood at present. Recent data from Bradbury et al., (1991) indicate that
circadian variations in stress-induced CORT and ACTH release are independent of basal
CORT levels. However, a number of other factors may be involved. First, it has been
demonstrated that the rate of CORT elevation required for fast feedback inhibition
(greater than 1.3 µg/dl/min) in the PM is much faster (3-15 min) than in the AM (15-30
min). Thus, although maximal CORT levels after restraint stress in the AM and the PM
may be similar, maximal CORT levels are reached approximately 12 min earlier in the
PM (Bradbury et al., 1991). Second, it has been demonstrated that feedback inhibition in
the PM is less sensitive than in the AM; CORT and ACTH levels are higher in the PM
than the AM after the same amount of DEX or CORT is administered (Gibbs, 1970;
Wilson *et al.*, 1983). Third, data suggest that there is an increased sensitivity of the adrenal to ACTH in the PM compared to the AM (Dallman, *et al.*, 1976; Haus, 1964; Unger, 1964). CORT release in response to exogenous ACTH is 2.5 times greater in the PM than in the AM (Dallman *et al.*, 1976;). Fourth, there appears to be a decrease in tissue absorption, distribution and/or metabolism of CORT in the PM compared to the AM (Gibbs, 1970; Saba *et al.*, 1963; Wilkinson *et al.*, 1979; Wilson *et al.*, 1983). Plasma CORT concentrations 5 min after a CORT injection in adrenalectomized rats are significantly higher in the PM than the AM (Wilson *et al.*, 1983) and the half-life of CORT is 9% greater in the PM than the AM (Gibbs, 1970). Together these data suggest that the "resetting" of feedback inhibition of the HPA axis in the PM was sufficient to unmask the altered sensitivity in E animals to the inhibitory effects of DEX. That is, in the PM, when the HPA axis is less sensitive to glucocorticoid feedback inhibition, stress CORT levels of both E males and E females as well as stress ACTH levels of E females were not effectively suppressed by DEX.

It is possible that the sexual dimorphism of the HPA stress response also underlies the differences in sensitivity to DEX suppression seen in males and females in the AM vs PM. Females have greater diurnal variation in plasma CORT (Ottenweller *et al.*, 1979), higher basal CORT and transcortin levels, and show greater CORT responses to stress than males (Critchlow *et al.*, 1963; Kitay, 1961; Weinberg, 1988) and to ACTH (Osborn *et al.*, 1994), and require higher doses of DEX to produce HPA suppression than males. Furthermore, hippocampal glucocorticoid receptor concentration is higher and binding affinity is lower in females than males (Turner & Weaver, 1985; Weinberg & Petersen,
The sex hormones are thought to influence the HPA axis indirectly through effects on hepatic enzyme systems that inactivate CORT (Glenister & Yates, 1961; Kitay, 1961) and binding proteins (Sandberg & Slaunwhite, 1959; Slaunwhite et al., 1962), as well as through noncompetitive binding to glucocorticoid receptors causing destabilisation of the receptor and an increased rate of CORT dissociation (Chou & Lutzge, 1988; Suthers et al., 1976; Svec et al., 1980). Burgess and Handa (1992) demonstrated that oestrogen elevates and prolongs activation of the HPA axis after ether and footshock stress and interferes with Type II receptor down-regulation in the hippocampus after 4 days of administration of RU 28362, a Type II receptor-specific agonist. This sexual dimorphism may help to explain the differential responses of E males in the AM vs the PM. First, it is possible that in the present study, peak levels of CORT in males were missed in the AM but not in the PM. As noted, peak CORT levels following stress are reached more quickly in the PM than in the AM. Differences between E and control females, on the other hand, may have been seen in the AM because the rate of rise is faster in females, resulting in the detection of differences even if the peak was missed. Second, the adrenal cortex responds linearly to a log dose of ACTH (Keller-Wood & Dallman, 1984). Thus, differences in ACTH which are not statistically significant could result in significant CORT level differences. As the CORT response to a specific dose of ACTH is greater in females than in males (Osborn et al., 1994), small differences in ACTH release could result in larger differences in CORT levels. Furthermore, in the PM, an increased adrenal sensitivity to ACTH could have resulted in higher stress CORT levels in both E males and E females. This latter
possibility is supported by our finding that differences in ACTH levels among E, PF, and C animals were less robust than differences in CORT levels following stress.

It is also possible that altered neurotransmitter release may be involved in mediating the HPA hyperresponsiveness of E animals. For example, following restraint stress, cortical and hypothalamic norepinephrine (NE) content is lower in E animals compared to controls (Rudeen & Weinberg, 1993). NE and epinephrine have been shown to stimulate CRF release in a dose dependent manner (Plotsky, 1987). In addition, it has been shown that depletion of hypothalamic NE and serotonin enhances the inhibitory effects of DEX on the CORT response to ether stress (Feldman & Weidenfeld, 1991). If lower hypothalamic NE levels in E animals is indicative of increased NE turnover post-stress, it is possible that prenatal ethanol effects on NE regulation of CRF secretion may play a role in HPA axis hyperactivity in E offspring. Consistent with this hypothesis, Lee et al (1990) demonstrated increased CRF biosynthesis and expression along with an increased ACTH response to stressors in E animals compared to controls. Thus, altered feedback inhibition of neurotransmitter stimulated CRF secretion may also play a role in the stress hyperresponsiveness of E animals. However, the finding that E animals demonstrate altered responses to physiologic, physical, and neurogenic stressors suggest that more that one neural pathway may be affected by prenatal ethanol exposure (Nelson et al., 1984; Taylor et al., 1982; Taylor et al., 1987; Weinberg & Gallo, 1982; Weinberg, 1988; Weinberg, 1992a).
Finally, these data may be of clinical importance. Children prenatally exposed to alcohol are hyperactive, uninhibited and impulsive in behaviour, and have attention deficits which may reflect an inability to inhibit responses (Streissguth et al., 1983; Streissguth et al., 1985; Streissguth, 1986). These behavioural deficits are particularly noticeable in stressful situations (Streissguth, 1986). Recently, it has been shown that maternal drinking during pregnancy is associated with higher post-stress cortisol levels in infants (Jacobson et al., 1993). CRF, 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 foetal alcohol-exposed children.
TABLE 3. UNDISTURBED CORT-LEVELS (μg/100 ml; Mean ± SEM)
* Main effect of dose, p < 0.01: 0 (saline) > DEX p < 0.01
Table 3.

<table>
<thead>
<tr>
<th>DEX</th>
<th>Males</th>
<th>Time Post-DEX Injection</th>
<th>Females</th>
<th>Time Post-DEX Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µg/100g bw)</td>
<td>3 h</td>
<td>6 h</td>
<td>10 h</td>
<td>26 h</td>
</tr>
<tr>
<td>0</td>
<td>1.7 ± 0.4</td>
<td>2.8 ± 0.5</td>
<td>13.2 ± 1.8</td>
<td>1.0 ± 0.1</td>
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<td>1.0</td>
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<td>0.9 ± 0.2*</td>
<td>______</td>
<td>______</td>
</tr>
<tr>
<td>5.0</td>
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<td>0.7 ± 0.03*</td>
<td>2.7 ± 0.7*</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>15.0</td>
<td>0.7 ± 0.2*</td>
<td>0.7 ± 0.02*</td>
<td>0.7 ± 0.1*</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 10. Stress CORT Levels in Males 3 and 6 h After DEX Injection in the AM.
Points represent mean ± SEM.
* Main Effect of Dose, p < 0.01: 5.0, 15.0 < 0 µg/100 g bw at 3 and 6 h, p’s < 0.01.
Figure 11. Stress CORT Levels in Males 10 and 26 h After DEX Injection in the AM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.01: 5.0, 15.0 < 0 μg/100 g bw at 10 and 26 h, p’s < 0.01.
Figure 12. Stress CORT Levels in Males 36 h After DEX Injection in the AM.
Points represent mean ± SEM.
No significant effects of group or dose.
Figure 13. Stress CORT Levels in Females 3 and 6 h After DEX Injection in the AM.

Points represent mean ± SEM.

* Main Effect of Dose, p < 0.01: 10.0, 30.0 < 0 μg/100g bw at 3, and 6, p’s < 0.01.

# Main Effect of Group, p < 0.05: E > PF = C, p’s < 0.05 at 3 and 6 h.
Figure 14. Stress CORT Levels in Females 10 and 26 h After DEX Injection in the AM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.01: 30.0 < 0 μg/100g bw at 10 h; 10.0, 30.0 < 0 μg/100g bw at 26 h, p's < 0.01.
Figure 15. Stress CORT Levels in Females 36 h After DEX Injection in the AM.
Points represent mean ± SEM.
No significant effects of group or dose.
The graph shows the plasma CORT (ug/100ml) levels at 36 hours for different conditions:

- **CONTROL**
- **PAIR-FED**
- **ETHANOL**

The x-axis represents DEX (ug/100 g bw) with values at 0 and 30.0.
Figure 16. Stress ACTH Levels in Males 3 and 6 h After DEX Injection in the AM.
Points represent mean ± SEM.
* Main Effect of Dose, p < 0.001: 1.0, 5.0, 15.0 < 0 μg/100g bw at 3 h, p’s < 0.01; 5.0, 15.0 < 0 μg/100g bw at 10 h, p’s < 0.01.
Figure 17. Stress ACTH Levels in Males 10 and 26 h After DEX Injection in the AM.

Points represent mean ± SEM.

* Main Effect of Dose, $p < 0.01$: 5.0, 15.0 < 0 μg/100g bw at 10 h, $p$’s < 0.01.

# Main Effect of Group, $p < 0.05$: E = C > PF at 26 h, $p$’s < 0.05.
Figure 18. Stress ACTH Levels in Males 36 h After DEX Injection in the AM.
Points represent mean ± SEM.
No significant effects of group or dose.
Figure 19. Stress ACTH Levels in Females 3 and 6 h After DEX Injection in the AM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.001: 1.0, 10.0, 30.0 < 0 µg/100g bw at 3 h, p's < 0.01; 30.0 < 0 µg/100g bw 10 h, p < 0.01.
Figure 20. Stress ACTH Levels in Females 10 and 26 h After DEX Injection in the AM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.01: 30.0 < 0 μg/100g bw at 10 h, p’s < 0.01.
# Main Effect of Group, p < 0.05: E and PF > C at 26 h, p’s < 0.05.
Figure 21. Stress ACTH Levels in Females 36 h After DEX Injection in the AM.
Points represent mean ± SEM.
No significant effects of group or dose.
Figure 22. Stress CORT Levels in Males and Females 3 h After DEX Injection in the PM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.001: DEX < saline, p’s < 0.001.
# Main Effect Group, p < 0.05: Males E > PF = C, p’s < 0.05; Females E > C, p’s < 0.01.
+ Group x Dose interaction, p < 0.05: Males at 5.0 µg/100g bw, E > C p < 0.05; at 15.0 µg/100g bw, E > PF, p < 0.05.
Figure 23. Stress ACTH Levels in Males and Females 3 h After DEX Injection in the PM.

Points represent mean ± SEM.
* Main Effect of Dose, p < 0.001: DEX < saline, p’s < 0.001.
# Main Effect of Group, p < 0.05: Females at 30.0μg/100g bw, E > PF = C, P’s < 0.05.
D. EFFECTS OF FOETAL ETHANOL EXPOSURE ON CORTICOTROPHIN RELEASEING FACTOR (CRF), ARGinine VASOPRESSIN (AVP), AND GLUCOCORTICOID RECEPTOR (GR) mRNA FOLLOWING DEXAMETHASONE SUPPRESSION.

D.1 INTRODUCTION

Corticotrophin releasing factor was once thought to have only releasing factor functions. However, recent data suggests that CRF may act to co-ordinate the endocrine, autonomic, and behavioural responses to stress (Nemeroff, 1992). CRF is released into the hypophysial portal vasculature by parvocellular neurosecretory neurons in the paraventricular nucleus (PVN) of the hypothalamus and is the principal driving force in regulation of the pituitary-adrenal axis in response to stress (Antoni, 1986; Sawchenko & Swanson, 1990). In addition, CRF mRNA expression has been found in the cerebral cortex, limbic system, cerebellum and spinal cord (DeSouza et al., 1985) suggesting that CRF may play a substantial role in the stress response beyond that of hormone stimulation. Furthermore, intracerebroventricular administration of CRF at low doses results in behavioural responses associated with stress, including increases in feeding, locomotion in a novel environment and shock-induced fighting, as well as decreases in social interaction. In contrast, administration at high doses results in behavioural responses associated with anxiety and maladaptive behaviour, including decreases in feeding, in sexual activity, locomotion in a novel environment and shock-induced
fighting (Dunn & Berridge, 1990). Therefore, CRF may be considered the master hormone in control of the endocrine, autonomic, and behavioural responses to stress.

CRF transcription measured by CRF mRNA levels in the PVN is negatively regulated by glucocorticoids (Jingami et al., 1985, Young et al., 1986). Adrenalectomy increases CRF mRNA levels and glucocorticoid replacement effectively reduces elevated CRF mRNA levels to basal levels. Direct placement of glucocorticoid pellets into the PVN results in a decrease in CRF transcription (Harbuz & Lightman, 1989; Kovacs & Mezey, 1987). Furthermore, Lightman and Harbuz (1993) demonstrated that CRF mRNA expression is dependent on glucocorticoids in adrenalectomized rats; the higher the replacement of dexamethasone or CORT the lower the CRF mRNA level.

Although prenatal ETOH exposure has been shown to affect both fast feedback inhibition (Talyor et al, 1988) and possibly intermediate feedback inhibition (Osborn et al., 1996), the exact mechanism of HPA hyperresponsiveness has yet to be determined. An alternative hypothesis for the HPA hyperresponsiveness seen in E animals may be that of altered CRF biosynthesis and/or secretion by the hypothalamus. In support of this hypothesis, Lee et al., (1990) demonstrated an increase in CORT levels in E compared to C animals following inescapable shock, as well as an increase in paraventricular nucleus (PVN) CRF mRNA in nonstressed E animals compared with C animals.

To our knowledge no studies to date have investigated PVN CRF mRNA in E, PF, and C animals following exposure to a stressor. The present study utilised in situ
hybridization to investigate further the hypothesis that HPA hyperresponsiveness seen in E animals is mediated through alterations in feedback inhibition of glucocorticoids on CRF synthesis in the PVN of the hypothalamus. The effects of DEX blockade on stress CRF, AVP, and GR mRNA levels were examined in E, PF and C males and females.

D.2 METHODS

Sprague-Dawley males (n=25) and females (n=53) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed according to Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were obtained from the tail from 3 unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma Diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951). Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dam and pups were weighed and all litters culled to 10 (five males and five females). Dam and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 90-110 days of age.

One wk prior to testing, animals (n = 6-9 E, PF, and C males and females for each postnatal treatment) were singly housed. Testing order was counterbalanced across prenatal treatment, sex, and injection dose. Animals were assigned to one of 2 doses of DEX (0 (saline) or 15.0 μg/100 g bw for males or 30.0 for females μg/100 g bw), administrated IP in a volume of 3.0 cc. Animals were placed back into their home cage.
following DEX injection and 3 h later were exposed to ether vapour for approximately 45 sec. Animals were then placed in a holding room for 60 min after which time blood samples were collected by decapitation for determination of CORT levels and brains were collected for CRF, AVP, and GR mRNA analysis (See Chapter II: General Methods). A separate group of animals (n = 5 E, PF, and C males and females) was taken directly from the colony room and decapitated between 0800 - 0830 h or 1200 - 1230 h to determine if there were changes in mRNA levels over the period of testing.

**D.3 STATISTICAL ANALYSES**

Data were analysed by appropriate analyses of variance (ANOVAs) for factors of group (E, PF, C), sex, dose of DEX, and site. Because of the complexity of the 4 way ANOVA, ANOVAs for females and males as well as ANOVAs on undisturbed animals were run separately. Significant effects were further analysed with Tukey’s post-hoc tests.

**D.4 RESULTS**

**Developmental Data**

Ethanol intake of pregnant females was consistently high throughout gestation averaging 9.2 ± 0.4, 11.6 ± 0.4, 11.5 ± 0.2 g/kg bw/day for wk 1, 2, and 3 of gestation respectively. Blood alcohol levels were measured in 3 females at 1900 h and were consistent with previous levels (Weinberg, 1985), averaging 159.5 ± 19.6 mg/dl.
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 x days interaction (p < 0.001). Post-hoc tests indicated that E and PF females weighed significantly less than C females on d 7, 14, and 21 (p’s < 0.01). During lactation, a group x days interaction (p < 0.001) was also seen. E females weighed significantly less than C females on lactation d 1 (p < 0.01). There were no significant differences among E, PF and C females on lactation d 8, 15, or 22.

There were no significant differences among groups for litter size or number of stillborn pups. Analysis of pup body weights during lactation showed a significant group x days interaction (p < 0.05). Post hoc tests indicated that E and PF pups weighed significantly less than C pups on d 1 and 8 of age (p’s < 0.01). There were no significant differences in pup weight on lactation d 15 and 22 and no significant difference in weight at the time of testing.

**Experimental Results**

**CORT Levels** For both males and females there was a main effect of dose (p’s < 0.05). Animals injected with DEX had significantly lower CORT levels than animals injected with saline (Fig. 24). There were no significant differences among E, PF, and C males and females.
CRF mRNA. There were no significant differences in undisturbed males or females at 0800-0830 h compared to undisturbed males (Fig. 25) or females (Fig. 26) at 1200-1230 h. There was a significant site x sex interaction (p < 0.01) for undisturbed animals. Undisturbed females demonstrated higher levels of CRF mRNA than undisturbed males in the more anterior section of the PVN (p < 0.05) whereas undisturbed males demonstrated higher mRNA levels at the central section of the PVN (p < 0.01). For stressed males there were significant main effects of dose and site (p's < 0.05) as well as a significant group x site interaction (p < 0.05) (Fig. 27). Males injected with DEX had lower CRF mRNA levels than males injected with saline. Furthermore, the more anterior sections had lower CRF mRNA levels than the more posterior sections. Among groups, there were no significant differences in the more anterior PVN sections; however, in the more posterior sections there was a trend for E males to have higher CRF mRNA levels than PF males (p < 0.1). For stressed females, there were significant main effects of group (p < 0.05) and site (p < 0.001) (Fig. 28). Among groups there were no significant differences in the more anterior PVN sections; however, in the more posterior sections E females had significantly higher CRF mRNA levels than PF females (p < 0.05) and showed a trend toward higher levels than C females (p < 0.1). Again the more anterior sections had lower CRF mRNA levels than the more posterior sections. There were no significant differences between DEX and saline injected animals.

AVP mRNA. There were no significant differences in undisturbed males (Fig. 29) or females (Fig. 30) at 0800-0830 h compared to those at 1200-1230 h. There was however a significant effect of site (p< 0.001). The more anterior PVN sections had
lower AVP mRNA then the more posterior sections. There were no significant effects of DEX or saline injection for either males (Fig. 31) or females (Fig 32).

GR mRNA There were no significant differences in undisturbed (Fig. 33) or stressed (Fig. 34) GR mRNA levels among E, PF and C males or females.

D.5 DISCUSSION

The results of this study demonstrate that overall, males and females injected with DEX had lower CORT levels than males and females injected with saline, and that males injected with DEX had lower CRF mRNA levels than saline injected males. Importantly, there were no significant differences in CRF or AVP mRNA levels measured under undisturbed conditions among E, PF and C animals, and no significant differences in CRF or AVP mRNA between 0800 and 1200 h in undisturbed groups. There were, however, significant differences in CRF and AVP mRNA levels depending upon the site of section. Sections from the anterior aspect of the PVN had lower CRF and AVP mRNA than more central sections. Females had higher CRF mRNA levels in the more anterior sections as compared to males, whereas males had higher CRF mRNA levels in the more central sections as compared to females. Moreover, a significant prenatal treatment effect was seen in the stressed animals. E males showed a trend toward higher stress CRF mRNA levels than PF males. E females had significantly higher stress CRF mRNA levels than PF females and showed a trend toward higher stress CRF mRNA levels than C females. There were no group differences in AVP or GR mRNA levels in the PVN.
These data do not support the hypothesis that HPA hyperresponsiveness and/or delays in recovery from stressors that occur in E animals result from alterations in feedback inhibition at the level of the hypothalamus. Instead the data suggest that the hyperresponsiveness seen in E animals may be due to increased synthesis of CRF.

The results of this study were consistent with the two previous studies in this dissertation indicating that post-ether stress animals given DEX had significantly lower CORT levels than animals injected with saline. In contrast to the previous studies, no significant differences in CORT levels among E, PF and C males and females were seen. A possible reason is that CORT levels were examined 1 h post-stress when the CORT levels were recovering towards basal levels, whereas the previous studies examined CORT levels at 20 min post-stress when the CORT secretion rate is at its peak. This change in sampling time was required in order to examine CRF and AVP mRNA levels. Therefore, the difference in CORT levels may only be present during the peak of the CORT response and not during recovery from stress.

Interestingly, DEX suppression of CRF mRNA appears to further demonstrate the sexual dimorphism of the HPA axis. A decrease in CRF mRNA was seen only in males following DEX injection. In vivo studies have demonstrated that DEX preferentially binds to the pituitary (De Kloet et al., 1975; Spencer et al., 1990). Administration of DEX (200 μg/day) for 7 days to adrenalectomized rats has been shown to reduce ACTH/β-lipotropin precursor mRNA to a much greater extent (19% of intact controls)
than prepro-CRF mRNA (102% of intact controls) (Jingami et al., 1985). In addition, subcutaneous injection of DEX has been shown to produce a selective and time related decrease in available Type II receptors in the pituitary and to have no effect on the Type II receptors in the hippocampus at doses of 1-50 μg/kg bw (Spencer et al., 1995). DEX in the drinking water has been demonstrated to activate pituitary Type II receptors at varying doses (0.3 μg/ml, 0.8 μg/ml, and 10.0 μg/ml), whereas hippocampus and hypothalamus Type II receptors are only activated at an exceedingly high dose of DEX (10 μg/ml) (Miller et al., 1992). Furthermore, low dose DEX (1.5 μg/ml) in the drinking water overnight does not effect Type II binding in the hippocampus but 1.0 μg/ml of DEX in the drinking water for 1,2, and 3 days does significantly reduce Type II activation (Spencer et al., 1990). Thus, it appears that the ability of DEX to cross the blood brain barrier and bind to type II receptors may be a function of dose and time and that in males the increase in sensitivity of the HPA axis to feedback inhibition may be in part due to central effects of glucocorticoids on the hypothalamus.

Moreover, in the present study it was demonstrated that the sex difference in CRF mRNA depended upon the site of analysis. Females had higher CRF mRNA levels in the more anterior sections of the PVN whereas males had higher levels in the more central sections. It has been well demonstrated that there is a significant gender difference in the neuroendocrine response to stress which appears to be associated with the presence of sex-specific gonadal steroids (Critchlow et al., 1963; Kant et al., 1983; Kitay, 1963; Turner & Weaver, 1985). In humans, it has been demonstrated that females have higher
hypothalamic CRF concentrations than males (Frederiksen et al., 1991). In the rat it has been shown that during the afternoon of proestrus there is an increase in CRF mRNA levels (Bohler et al., 1990) and that oestrogen treatment of overectomized rats increases CRF content (Burgess & Handa, 1992). In contrast, Patchev et al., (1995) demonstrated that males have higher CRF mRNA levels in the hypothalamus than females. A possible explanation for this discrepancy may be that previous studies looked at whole content rather than individual sections. Taken together with the our data, it appears that sex differences in CRF mRNA may vary throughout the PVN and the variation may be site specific. Furthermore, there appears to be a variation in the circadian rhythm in CRF mRNA levels of males and females. Female rats demonstrate higher PVN CRF mRNA levels in the AM whereas males demonstrate higher levels in the PM. A possible mechanism for the sex difference is that oestrogen may alter CRF gene expression. Vamvakopoulas and Chrousos (1993) demonstrated five perfect half palindromic oestrogen responsive elements (ERE) on the CRF gene. Half palindromic EREs have been demonstrated to enhance CRF gene activity (Wilson et al., 1992).

In this study it was demonstrated that the anterior section of the PVN contains lower levels of CRF and AVP mRNA than sections taken approximately 40 μm posterior. CRF is synthesised in the parvocellular cells of the PVN, transported to the axon terminals in the external zones of the median eminence, and released into the hypophysial portal vascular system. AVP coexists with some CRF neurons in the parvocellular cells. AVP also coexists in the magnocellular cells of the PVN which
terminate in the posterior pituitary and are involved with fluid balance. Immunohistochemical localisation has demonstrated that the CRF-immunoreactive neurons whose axons project to the median eminence are centred in the dorsal aspect of the medial parvocellular area of the PVN (Kawano et al., 1988; Swanson et al., 1983). It is therefore possible that the lower density of hybridization signal seen in the more anterior section may reflect the fact that this area contains fewer CRF and AVP neurons as compared to the central sections rather than reflecting decreased gene expression. Future research should contain single cell analysis to address this issue.

Sample site may also play a role in the variation in response that one may see from study to study or with different stressors. As demonstrated by this study, there are significant differences in the CRF and AVP mRNA levels of sections as close as 40 μm apart. Therefore, when comparing studies it is important to have an idea of where in the PVN the section was taken and if possible to analyse more than one section. Unfortunately, due to technical problems this was not possible for GR mRNA.

Timing also plays a key role in transcription analysis. In response to ether stress, c-fos mRNA in the medial parvocellular PVN appears to respond within 15 min, peak at 30 min, diminish at 60 min and fall to control levels within 2-3 h (Kovacs & Sawchenko, 1996). Unfortunately, although measurement of c-fos was an original aim of the present study, c-fos could not be measured due to technical problems with the probe. CRF mRNA levels have been seen to increase at 120-180 min (Lightman et al., 1993); however, the time course has not been fully described. Consistent with our data, other
investigators have also failed to detect a reliable up-regulation of CRF mRNA in the PVN following ether stress (Kovacs & Sawchenko, 1996; Watts, 1991). Although statistically significant effects of ether stress on in CRF mRNA levels were seen in E females, it is possible that later sampling or another stressor would better quantify the signal. Further, it appears that ether stress may differential increase CRF and AVP heteronuclear RNA (hnRNA), another measure of neuronal response to stressors, depending on the timing of the sample (Kovacs & Sawchenko, 1996). CRF hnRNA peaks at 5 min whereas AVP hnRNA peaks at 120 min post ether stress (Kovacs & Sawchenko, 1996). Therefore, in future studies measurement of multiple time points following a stressor and measurement of CRF and AVP hnRNA and c-fos as well as CRF and AVP mRNA should be done. Finally, the increase in AVP mRNA following stress is very difficult to quantify because of the intermingling of the magnocellular neurons; thus single cell analysis would have been a better method to investigate AVP changes (Lightman & Young, 1987b).

In contrast with the data of Lee et al., (1990), we did not find differences in basal CRF mRNA levels among E, PF and C animals. However, a significant effect of prenatal treatment was seen in the stressed animals regardless of the injection dose. E males showed a trend toward higher CRF mRNA levels than PF males, and E females had significantly higher CRF mRNA levels than PF females and showed a trend toward higher CRF mRNA levels that C females. A possible mechanism for the altered synthesis may be an alteration in the adrenergic drive to the PVN. Ascending adrenergic fibres from the brainstem have been demonstrated to stimulate CRF neurons (Palkovits, 1987) and microinjection of norepinephrine (NE) has been demonstrated to stimulate both CRF
gene expression in the PVN and CRF secretion into the portal circulation (Itoi et al., 1994). In addition, lesion studies in the PVN have indicated that NE pathways are involved in stress induced CRF mRNA expression but are not involved in maintaining basal CRF mRNA (Harbuz et al., 1991) which may explain why increased CRF mRNA was only seen in E animals under stress. It remains to be determined if the increase in gene expression is reflective of an increase in synthesis and/or release.

Importantly, the finding of altered CRF synthesis in E animals may have implications beyond those relating to strictly endocrine responses to stressors. The autonomic and CRF neurons of the PVN have been demonstrated to be morphologically and functionally linked suggesting that CRF neurons within the PVN not only mediate endocrine responses but also behavioural responses to stressors. Repeated administration of CRF for 5 days has been shown to increase tyrosine hydroxylase levels in the locus coeruleus (LC) (Melia & Duman, 1991) suggesting bi-directional input of CRF from the PVN to NE in the LC. Injection of CRF into the PVN results in a robust autonomic response (Brown & Fisher, 1985) and an increase in gastric secretion (Gunion & Tache, 1987), as well as, induces locomotor activity similar to that seen under stress conditions (Monnikes et al., 1992). Furthermore, administration of CRF monoclonal antibody targeted-toxin into the PVN reverses the decreased exploration of the elevated plus-maze open arms caused by prior exposure to a social stressor (Menzaghi et al., 1994). CRF injection into the PVN also decreases food intake in a manner similar to that of stress induced anorexia (Krahn et al., 1988); α-helical CRF reverses restraint stress-induced anorexia (Krahn et al., 1986). Thus, CRF synthesised in the PVN may act both as a
secretagogue for the anterior pituitary hormones and as an extrapituitary peptide neurotransmitter to co-ordinate the stress response at several body levels.

Finally, these data have clinical significance. As mentioned previously, children prenatally exposed to alcohol are hyperactive, impulsive in behaviour, and have deficits which may reflect an inability to inhibit responses particularly in stressful situations (Streissguth et al., 1983; Streissguth et al., 1990). Interestingly, individuals with affective or anxiety disorders also demonstrate altered HPA axis response to stress including elevated CORT and ACTH levels as well as non-suppressibility of the HPA axis to DEX (Nemeroff, 1992). Elevated CRF levels in the cerebrospinal fluid have been found in depressed patients who do not demonstrate suppression of the HPA axis following DEX administrations (Arato et al., 1986; Nemeroff & Evans., 1984; Risch et al., 1987). Moreover, it has been demonstrated in rats that local administration of CRF into the LC results in an increase in unstimulated neuronal discharge and decreased or unchanged firing with exposure to foot-shock (Valentino & Foote, 1987; Valentino & Foote, 1988) suggesting that elevated CRF disrupts the normal pattern of discharge in the LC. Therefore, it is possible that increased CRF release could result in persistently elevated neuronal discharge rates and decreased responses to phasic sensory stimuli, thus producing alterations in behaviour. Such a mechanism could, at least in part, underlie the behavioural changes such as hyperarousal and decreased attention span seen in children prenatally exposed to alcohol as well as in patients with some affective and anxiety disorders (Valentino & Foote, 1987; Valentino & Foote, 1988).
Figure 24. Stress Corticosterone Levels in Males and Females 3 h After DEX Injection in the AM.

Points represent mean ± SEM.

# Main Effect of Dose, p < 0.001: DEX < saline, p's < 0.001.
Figure 25. Undisturbed CRF mRNA Levels in Males in the AM.
Points represent mean ± SEM.
No significant effects of group or time samples.
Figure 26. Undisturbed CRF mRNA Levels in Females in the AM.

Points represent mean ± SEM.

No significant effects of group or time samples.
Figure 27. Stress CRF mRNA Levels in Males 3 h After DEX Injection in the AM.

Points represent mean ± SEM.

# Main effect of dose, p’s < 0.05: 15.0 μg/100 g bw < vehicle
CONTROL  PAIR-FED  ETHANOL

PVN1

CRF mRNA

SALINE  15.0

DOSE (ug/100 g BW)

PVN5

CRF mRNA

SALINE  15.0

DOSE (ug/100 g BW)
Figure 28. Stress CRF mRNA Levels in Females 3 h After DEX Injection in the AM.

Points represent mean ± SEM.
* Main effect of group, p < 0.05: E < PF, p < 0.05.
PVN1

CRF mRNA

CONTROL  PAIR-FED  ETHANOL

SALINE  30.0

DOSE (ug/100 g BW)

PVN5

CRF mRNA

CONTROL  PAIR-FED  ETHANOL

SALINE  30.0

DOSE (ug/100 g BW)
Figure 29. Undisturbed AVP mRNA Levels in Males in the AM.
Points represent mean ± SEM.
No significant effects of group or time samples.
Figure 30. Undisturbed AVP mRNA Levels in Females in the AM.
Points represent mean ± SEM.
No significant effects of group or time samples.
Figure 31. Stress AVP mRNA Levels in Males 3 h After DEX Injection in the AM.
Points represent mean ± SEM.
No significant effects of group or dose.
Figure 32. **Stress AVP mRNA Levels in Females 3 h After DEX Injection in the AM.**

- Points represent mean ± SEM.
- No significant effects of group or dose.
PVN3

AVP mRNA

SALINE    30.0

DOSE (ug/100 g BW)

PVN7

AVP mRNA

SALINE    30.0

DOSE (ug/100 g BW)
Figure 33. Undisturbed GR mRNA Levels in Males and Females in the AM.
Points represent mean ± SEM.
No significant effects of group or time samples.
CONTROL  PAIR-FED  ETHANOL

Male

GR mRNA

basal 1
basal 2

Female

GR mRNA

basal 1
basal 2

175
Figure 34. Stress GR mRNA Levels in Males and Females 3 h After DEX Injection in the AM.

Points represent mean ± SEM.
No significant effects of group or dose.
CHAPTER IV: BEHAVIOURAL ALTERATIONS IN FOETAL ETHANOL EXPOSED RODENTS.

A. EFFECTS OF FOETAL ETHANOL EXPOSURE ON BEHAVIOUR ON THE ELEVATED PLUS MAZE

A.1 INTRODUCTION

Rodents prenatally exposed to ethanol (E) demonstrate many of the physical findings seen in children exposed to alcohol in utero, including growth deficiencies (Abel & Dintcheff, 1978; Gallo & Weinberg, 1986), changes in brain morphology (Meyer et al., 1990b; West et al., 1989), and soft tissue and skeletal abnormalities (Abel, 1978; Gallo & Weinberg, 1986; Sulik, 1983). Importantly, as in children exposed to alcohol in utero, cognitive (Abel, 1979) and behavioural deficits have also been seen in E offspring. Many of the behavioural changes observed in E offspring appear to reflect hyperactivity and hyperresponsiveness and/or deficits in response inhibition. Increased open field activity (Bond, 1981; Bond, 1986), increased wheel running (Martin et al., 1978), increased startle reactions (Anandam et al., 1980), and increased exploratory behaviour (Bond & DiGiusto, 1977a; Riley & Meyer, 1984), as well as deficits in passive avoidance learning (Bond & DiGiusto, 1977b; Bond & DiGiusto, 1978; Gallo and Weinberg, 1982; Riley et al., 1979a; Riley et al., 1986), taste aversion learning (Riley et al., 1984), reversal
learning (Lochry & Riley, 1980), and nose poking behaviour (Riley et al., 1979b) have all been demonstrated in E offspring.

In addition to altered performance and activity, rodents prenatally exposed to ethanol have been shown to have altered behavioural responses to stressors including increased stress-induced analgesia (Nelson et al., 1985b), increased stress-induced alcohol consumption (Nelson et al., 1983a), and an inability to adapt to a stressful swimming paradigm (Taylor et al., 1983). Interestingly, E animals also demonstrate hyperresponsiveness of the hypothalamic-pituitary adrenal (HPA) axis to stressors including increased or prolonged secretion of adrenocorticotrophin (ACTH), β-endorphin (β-EP) and corticosterone (CORT). Increased HPA responsiveness to cardiac puncture (Taylor et al., 1982), restraint (Talyor et al., 1982; Weinberg, 1988; Weinberg et al., 1992b), noise and shaking, (Taylor et al., 1982), novel environments (Weinberg, 1988), intermittent shock (Nelson et al., 1984; Nelson et al., 1986), ether (Angelogianni & Gianoulakis, 1989; Weinberg & Gallo, 1982) and cold stress (Angelogianni & Gianoulakis, 1989) have been demonstrated in E compared to control offspring. Furthermore, E offspring appear to have deficits in using or responding to environmental cues. Unlike control animals, E animals do not show a differential CORT response to predictable and unpredictable restraint stress (Weinberg et al., 1992a), nor do E animals demonstrate reduced CORT responses to a novel environment when allowed access to water (Weinberg, 1988).
The present study further investigates behavioural and hormonal responses in E animals on the elevated plus maze (+-maze). The +-maze task provides a valid and reliable measure of anxiety/fear as measured by behavioural, physiological, and pharmacological responses (Lister, 1987; Pellow et al., 1985). The task is based on spontaneous behaviour and does not require training of the animal, exposure to noxious stimuli, or manipulation of appetitive behaviours such as food deprivation. In addition, it is sensitive to the anxiolytic effects of benzodiazepine (BDZ)-like agents after acute administration without the interference of sedative side effects on behaviour (Lister, 1987; Pellow et al., 1985). The +-maze comprises an elevated cross-maze with two open and two closed arms. It can be considered an aversive task in that it generates a conflict situation by simultaneously activating two natural tendencies, exploration of a novel environment and avoidance of open spaces (Falter et al., 1992). It has been shown that control or undrugged animals prefer the closed arms of the maze, demonstrating decreased entries onto the open arms and decreased time spent on the open arms as compared to closed arms (Lister, 1987; Pellow et al., 1985). In addition, animals confined to the open arms exhibit higher CORT levels, an index of stress (Selye, 1973) than animals confined to the closed arms (Pellow et al., 1985).

In the present study, we utilised the +-maze as an aversive situation to explore the hypothesis that prenatal ethanol exposure alters behavioural responses to stress. In Experiment 1, animals were exposed to the +-maze on consecutive days to compare behavioural responses of E and control animals and to determine if behaviour changed differentially with repeated exposure. In addition, both behaviour and CORT levels were
measured in animals confined to the open and the closed arms to identify if behavioural changes and HPA activation occur in parallel. In Experiment 2 of this study, animals were exposed to an open field apparatus prior to being placed on the + maze. Previous studies have shown that animals placed in a novel environment before exposure to the + maze tend to increase overall activity in the + maze and increases the likelihood that the open arms are explored (Pellow et al., 1985).

A.2 METHODS

Sprague-Dawley males (n=25) and females (n=82) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed according to Chapter II: General Methods. Two replicate breedings, with 39-43 females in each, were done. Blood samples (0.4-0.6 ml) were obtained from the tail of 3 unanaesthetised females in each breeding at 1900 h on d14 of gestation for determination of ethanol levels (sigma Diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951).

Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dams and pups were weighed and all litters culled to 10 ( five males and five females). Dams and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 60-90 days of age.
This study was completed in 3 experiments all using behavioural tests (+-maze and open field (OF) described in Chapter II: General Methods Part E).

**Experiment 1a** One week prior to testing, animals were singly housed. (n=9-10 for each of E, PF, and C males and females). All testing was done between 0830 and 1200 h with prenatal treatment groups being counterbalanced for order of testing and run times. Animals were tested on two consecutive days, at the same time each day, and all testing was done blind to the animal’s prenatal treatment group. Low level (40 dB) white noise was used to mask any extraneous room noises.

At the time of testing on each of the consecutive test days, animals were taken from the colony room to an adjacent test room and placed on the centre of the +-maze facing an open arm. At the end of the 5 min test, animals were placed in a holding room until all testing was completed, at which time all animals were returned to the colony room. The +-maze was washed with 70% ETOH after each animal.

**Experiment 1b** Animals tested in experiment 1a were retested beginning 2 wk after the conclusion of Experiment 1a, between 0830 and 1200 h. Animals were removed from the colony room and placed on either an open or a closed arm of the maze. The entrances to the other arms were blocked. Behaviours were recorded for the first and last 5 min of a 20 min test. One wk later, testing was repeated. Groups were counterbalanced so that half the animals of each prenatal treatment group were exposed to the open arm first and the other half exposed to the closed arm first. Immediately after testing on each
of the 2 test sessions animals were taken to an adjacent room, quickly and lightly anaesthetised with diethyl ether, and blood samples (0.5 cc) obtained by cardiac puncture using heparinized syringes. The entire sampling procedure was completed within 2 min of removing the animal from the + maze, which is rapid enough to obtain a reliable measure of CORT at the end of the + maze testing, without any effects of disturbance or etherization (Davidson et al., 1968). All blood samples were centrifuged at 2200 x g for 10 min at 4°C and plasma was stored at -20°C until analysed.

Experiment 2. Absolute time on the open arms were relatively low in Experiment 1a, 7.7 ± 1.6 min for males and 7.5 ± 1.6 min for females. Prior exposure to the open field (OF) has been shown to increase + maze activity including time on the open arms (Pellow et al. 1985). Therefore, in Experiment 2 animals were exposed to an OF task for 5 min immediately prior to being tested on the + maze.

One week prior to testing, a separate set of animals were singly housed (n=10 for each of E, PF, and C males and females). All testing was done between 0830 and 1200 h with prenatal treatment groups being counterbalanced for order of testing and run times. All testing was done blind to the animal’s prenatal treatment group. White noise (40 dB) was used to mask extraneous noises.

At the time of testing, animals were taken from the colony room to an adjacent room containing the open field and the + maze. Animals were placed in the centre of the open field facing away from the investigator and behaviour was recorded for 5 min.
Animals were then immediately placed on the centre of the + maze facing an open arm for a 5 min test. Following testing, animals were placed in their home cages in a holding room for 10 min. Animals were then quickly and lightly anaesthetised with diethyl ether and blood samples (0.5 cc) taken by cardiac puncture using heparinized syringes for CORT determination. Blood samples were centrifuged at 2200 x g for 10 min at 4° C. Plasma was stored at -20° C until analysed.

A separate set of animals (n = 5 for each of E, PF, and C males and females) were taken directly from the colony room and blood samples were drawn by cardiac puncture under light ether anaesthesia to determine basal CORT levels.

A.3 STATISTICAL ANALYSES

Principal component factor analysis on standardised scores was used to determine which behavioural measures were related. These factors were then analysed by appropriate analyses of variance (ANOVA) for prenatal treatment, sex, and days. Individual behaviours were further analysed separately by appropriate ANOVAs for prenatal treatment and sex. Significant main and interaction effects were analysed by Newman Keul's paired comparisons.
A.4 RESULTS

Experiment 1

Developmental Data

Ethanol intake of the pregnant females was consistently high throughout gestation, averaging 9.7 ± 1.3, 11.1 ± 1.0, 10.7 ± 0.6 g/kg bw/day for wk 1, 2, and 3 of gestation respectively. Blood alcohol levels were consistent with levels previously reported (Weinberg, 1985), averaging 136.6 ± 15.8 mg/dl.

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 x days interaction (p < 0.01). Post-hoc tests indicated that body weights of E and PF females were significantly less than body weights of C females on gestation d 7-21 (p's < 0.001). There were no significant differences among groups for litter size. Analysis of pup body weights indicated a significant group x days interaction; E and PF pups weighed significantly less than C pups on d 8 and d 22 of lactation (p's < 0.01). There were no significant differences among groups in pup weight on the days of testing.

Experiment 1a: Two consecutive days of testing

Factor analysis revealed 2 factors accounting for 73 % of the variance in males and 68 % of the variance in females. The first factor comprised time on the closed arms negatively related to full closed arm entries, partial open arm entries, and time in the
central area (exploration factor). The second factor comprised time on the open arms, full open arm entries, and number of closed arm rears (fear factor). Time on the open arm and open arm entries have been validated by previous investigators (Lister, 1987; Pellow et al., 1985) as measures of anxiety/fear.

For the exploration factor, there was a significant effect of days for both males and females (p < 0.01); animals had lower levels of exploration on d 2 than on d 1. For males, trends for an effect of group and a group x days interaction were seen (p's < 0.10). There were no significant differences among groups on d 1. On d 2, however, E males had significantly higher levels of exploration than PF (p < 0.01) and C (p < 0.05) males. These data were supported by ANOVAs on the individual behaviours. That is, on d 2, E males made more closed arm entries than PF and C males (p's < 0.05) (Fig. 35), and spent more time on the central area than PF males (p < 0.05) (Fig. 35). There were no significant differences among E, PF and C males in time spent on the closed arms or in number of closed arm entries. For females, a significant group x days interaction was seen for the exploration factor (p < 0.05); E and PF females had higher levels of exploration than C females on d 1 (p's < 0.05) but not on d 2. Individual ANOVAs indicated that E and PF females made more closed arm entries than C females on both d 1 (p's < 0.01) and d 2 (p's <0.05) (Fig. 36) and that E females made more partial open arm entries than C females (p < 0.05) on d 1 (Fig. 36). In addition, on d 1, E and PF females spent more time in the central area (p's < 0.05) and less time on the closed arms than C females (p < 0.10 and p < 0.05 respectively) (Fig. 37).
For the fear factor, there was a significant effect of days for both males and females \((p < 0.01)\); animals had higher levels of fear on d 2 than on d 1. For males, a significant main effect of group was seen \((p < 0.05)\). E males demonstrated lower levels of fear than C males \((p < 0.05)\). ANOVAs on the individual behaviours indicated that E males spent more time on the open arms than C males \((p < 0.05)\) on d 1 and showed a similar trend on d 2 \((p < 0.10)\). In addition, E animals made more closed arm rears than PF and C males on both days \((p's < 0.05)\) (Fig. 38). There were no significant differences among males in number of open arm entries (Fig. 39). For females, an overall group trend was seen for the fear factor \((p < 0.10)\); E females showed a trend toward higher levels of fear than PF females. Individual ANOVAs revealed that on d 2, E females showed a trend toward spending less time on the open arms than C females \((p < 0.10)\) (Fig. 40). In addition, a pair-feeding effect was seen on d 1; E and C females spent less time on the open arm than PF females \((p's < 0.05)\) (Fig. 40).

**Experiment 1b: Confinement to open and closed arms.**

Factor analysis revealed 2 main factors accounting for 63% of variance in males and 61% of variance in females. Factor 1 (open arm activity) consisted of ambulation, turns and rears on the open arm. Factor 2 (closed arm activity) consisted of ambulation, turns, and rears on the closed arm.

Overall, there were no significant differences in open arm activity among E, PF, and C males or females.
Significant main effects of group were seen in both males and females in closed arm activity (p's < 0.01). E males showed significantly more closed arm activity than C males (p < 0.01). Individual ANOVAs indicated that E and PF males showed significantly more ambulation than C males (p < 0.01 and p < 0.05 respectively) (Fig. 41) and E males showed more turns (Fig. 41) and rears than C males (Fig. 42) (p's < 0.01). Similarly, E and PF females demonstrated more closed arm activity than C females (p < 0.01 and p < 0.05 respectively). Individual ANOVAs revealed that E and PF females showed more ambulation (p < 0.01 and p < 0.10, respectively) and turns (p < 0.01 and p < 0.05, respectively) (Fig. 43) and rear more than C females (p < 0.10 and p < 0.05, respectively) (Fig. 44).

Corticosterone Levels

Significant main effects of exposure to open vs closed arm were seen in CORT levels of both males and females (p's < 0.01) (Fig. 45). CORT levels were significantly higher on the open arm than on the closed arm. There were no significant differences among E, PF, and C animals.
Experiment 2: \+-Maze behaviour after exposure to the OF

Developmental Data

Ethanol intake of the pregnant females was consistently high throughout gestation, averaging 9.8 ± 1.4, 11.5 ± 1.0, 10.7 ± 0.8 g/kg bw/day for wk 1, 2, and 3 of gestation respectively. Blood alcohol levels were consistent with levels observed previously (Weinberg, 1985), averaging 120.1 ± 12.6 mg/dl.

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 x days interaction (p < 0.01). Post-hoc tests indicated that body weights of E and PF females were significantly less than body weights of C females on gestation d 7-21 (p's < 0.001). In addition, E females weighed significantly less than PF females on d 21 of gestation (p < 0.05). There were no significant differences among groups for litter size. Body weights for pups showed a significant group x days interaction; E and PF pups weighed significantly less than C pups on d 1, 8, and 22 of lactation (p's < 0.01). There were no significant differences in pup weight on the day of testing.

OF Behaviour

There were no significant differences among E, PF and C animals on OF activity.
+Maze behaviour

Similar to Experiment 1, analysis revealed 2 main factors accounting for 68% of the variance in males and 65% of the variance in females. The first factor consisted of time on the closed arms negatively related to full closed arm entries, partial open arm entries, time in the central area, and closed arm rears (exploration factor). The second factor consisted of time on the open arms, full open arm entries, and rearing on the open arm (fear factor).

For the exploration factor, there was a group trend in males (p < 0.10). E males showed a trend towards lower levels of exploration than C males (p < 0.10). These data were supported by ANOVAs on the individual behaviours. E males spent less time on the closed arms than PF (p < 0.05) and C (p < 0.10) males (Fig. 46). E males showed a trend toward fewer rears than PF and C males (p's < 0.10) (Fig. 46). For females, analysis of the exploration factor revealed a significant main effect of group (p < 0.01). Similar to the data on males, E females had lower levels of exploration than PF (p < 0.05) and C females (p < 0.01). Individual ANOVAs revealed that E females spent less time on the closed arms than PF females (p < 0.05) (Fig. 47) and had fewer closed arm entries than C females (p < 0.01) (Fig. 47). In addition, E and PF females reared less than C females on the closed arm (p's < 0.05) (Fig. 48).
For the fear factor, there were no significant differences among E, PF and C males. In contrast, analysis of females revealed a main effect of group (p < 0.01); E and PF females showed higher levels of fear than C females (p < 0.05 and p < 0.01 respectively). These data were supported by individual ANOVAs which demonstrated that E and PF females reared less on the open arms than C females (Fig. 49), spent less time on the open arms (Fig. 49) and had fewer open arm entries than C females (p’s < 0.05) (Fig. 50).

Corticosterone Levels

There were no significant differences among E, PF and C males or females in basal CORT levels (data not shown). Similarly, following exposure to the +-maze, CORT levels did not differ among E, PF, and C males (Fig. 51). In contrast, there was a significant main effect of group for CORT levels of females following +-maze exposure (p < 0.05) (Fig. 51); E females had significantly higher CORT levels than PF and C females (p’s < 0.05).

A.5 DISCUSSION

The +-maze was designed (Handely & Mithani, 1984) and validated as a test of anxiety for both rats (Pellow et al., 1985; Pellow & File, 1986) and mice (Lister, 1987). Controversy exists, however, regarding interpretation of the anxiety/fear state generated by the +-maze. Although the +-maze has been demonstrated to be sensitive to anxiolytic
compounds which act on the γ aminobutyric acid (GABA) system (Lister, 1987; Pellow et al., 1985; Pellow & File, 1986), novel anxiolytic compounds, which act on the serotonergic or noradrenergic systems, produce variable results (Sanger et al., 1991). For example, buspirone has been demonstrated to have both anxiogenic (Critchley & Handely, 1987; Moser, 1989) and anxiolytic properties (Dunn et al., 1989; Lee & Rodgers, 1990). In addition, prior exposure to stressors can increase (Pellow et al., 1985), decrease (Steenbergen et al., 1991) or not effect the animals’ activity level on the + maze (Falter et al., 1992). Thus, it has been suggested that + maze behaviour is not an indication of generalised anxiety but is a valid measure of situational-dependent anxiety/fear (Falter et al., 1992). It was on this basis that the + maze was chosen for the present study as an aversive task to examine the possible differential effects of prenatal ethanol exposure on stress-related behaviours.

In the present study, factor analysis of + maze behaviour revealed 2 main factors. Importantly, the loading of the variables was relatively consistent between the 2 experiments and with data from previous studies (Lister, 1987; Pellow et al., 1985). The 2 factors were assigned the terms ‘exploration’ and ‘fear’ based on the previous literature (Lister, 1987; Pellow et al., 1985). Data suggest that the open arms of the + maze are more fear- or stress- provoking than the closed arms (Lister, 1987; Pellow et al., 1985). That is, undrugged animals spend less time on the open arms, make fewer open arm entries (Lister, 1987; Pellow et al., 1985) and demonstrate more anxiety/fear behaviours (freezing, immobility, defecation) on the open arms than on the closed arms.
In addition, animals confined to the open arms demonstrate higher CORT levels, an index of stress (Selye, 1973) than those confined to the closed arms (Montgomery, 1958; Pellow et al., 1985). Moreover, comparison of + maze behaviour with exploration and locomotor activity in the holeboard task has demonstrated that open arm entries are not correlated with holeboard exploration or locomotion (Pellow et al., 1985). Furthermore, Pellow et al. (1985) demonstrated that anxiolytic agents such as diazepam increase both time on open arms and number of open arm entries on the + maze while reducing exploration and motor activity on the holeboard. In contrast, sedative agents such as haloperidol have no effect on time spent on the open arms but reduce total arm entries on the + maze as well as both exploratory and locomotor activity on the hole board. Taken together, these studies suggest that behaviour on the open arms (open arm entries and time spent on the open arms) is likely a measure of fear or situational-dependent anxiety which is independent of exploration, whereas behaviour on the closed arms and in the central area (closed arm entries, time on the closed arms, time on the central area and partial open arm entries) is likely a measure of exploration.

The results from the present study indicate that prenatal ethanol exposure differentially alters behavioural and hormonal responses to the elevated + maze in males and females. Both E males and females demonstrated higher levels of exploration (exploratory behaviours) when placed directly on the + maze from their home cages without prior exposure to the OF (open field) compared to C males and females. In addition, when confined to the closed arms of the + maze E males and females
demonstrated higher levels of activity (i.e. more turns, rears and midline crosses) compared to C males and females. Following activation of behaviour by prior OF exposure, however, both E males and females demonstrated lower levels of exploration than C males and females. Interestingly, E females but not E males showed an increase in fear-related behaviours on the + maze compared to controls, regardless of prior OF exposure. Moreover, E females had increased CORT levels following + maze testing after exposure to the OF but not with + maze exposure alone. Thus, these data are consistent with other studies (Barron & Riley, 1990; Osborn et al., 1996; Weinberg, 1988) demonstrating that prenatal ethaonl affects both males and females but that there may be a sex difference in the sensitivity of the mechanism(s) underlying the alterations in behaviour.

Previous studies have also demonstrated alterations in behavioural and hormonal responses to stressors or aversive test situations in E compared to C animals. E animals have been shown to have increased grooming after a 1 min forced swim (Hannigan et al., 1987) and increased reactivity to acoustic startle (Anandam et al., 1980). E animals also demonstrate HPA hyperresponsiveness to a variety of stressors compared to controls (Nelson et al., 1986; Taylor et al., 1981; Weinberg & Gallo, 1982; Weinberg, 1988; Weinberg, 1992b). Importantly, as previously demonstrated (Osborn et al., 1996; Weinberg, 1988, Weinberg et al., 1996), sex differences in the response of E animals to stressors are observed. That is, HPA hyperresponsiveness may be manifested differentially in E males and females depending on the nature of the stressor, the time course measured and the hormonal endpoint (Weinberg, 1988, Weinberg et al, 1996).
Further, it has been shown that altered behavioural response to stress in E animals can be modified by prior exposure to stressors. Hannigan et al. (1987) found that E animals did not demonstrate differences in grooming behaviour in a novel environment but when exposed to a prior stressor such as 1 min forced swim E animals demonstrated increased grooming behaviour in a novel environment. The mechanism(s) for the alterations in behavioural and hormonal responses to stressors is unknown. Under the test conditions of the present study, however, it appears that females may be more sensitive to the effects of in utero alcohol exposure than males as measured by both increased fear and CORT levels following +-maze exposure.

Although it has been demonstrated that environmental conditions such as light intensity, maze height, or prior exposure to a stressor such as immobilisation do not alter +-maze behaviour (Falter et al., 1992), prior exposure to an open field apparatus appears to activate the animals and increase time spent on open arms (Pellow et al., 1985). The mechanism for this behavioural activation is unclear at present. The data from this study support and extend this previous work (Pellow et al., 1985) demonstrating that exposure to the OF immediately prior to +-maze testing markedly increases time spent on the open arms. Both males and females, regardless of their prenatal treatment, increased their time spent on the open arms, from 7.7 ± 1.6 min to 38.0 ± 5.0 min for males, and from 7.5 ± 1.6 min to 32.0 ± 2.7 min for females. Importantly, however, the data indicated that following OF exposure, E animals demonstrate a decrease in exploration compared to
their respective controls suggesting that exposure to an aversive situation prior to testing may differentially affect E animals as compared to controls.

The findings in Experiment 1, that E males and females demonstrated increased exploration as well as increased activity levels when confined to the closed arm of the +maze, are consistent with previous studies which have demonstrated behavioural hyperactivity in a variety of tasks including a novel environment (Shah & West, 1984), open field (Becker & Randall, 1989; Bond & DiGiusto, 1977a; Bronstein et al., 1975; Caul et al., 1979; Fernandez et al., 1983; Means et al., 1984; Melcer et al., 1994; Molina et al., 1984; Riley et al., 1986; Vorhees & Fernandez, 1986), and the holeboard (Riley et al., 1979b). Furthermore, it has been shown that E animals demonstrate facilitated swimming performance in the Biel water maze (Vorhees & Fernandez, 1986). E animals had increased swimming speed but still committed the same number of errors compared to control animals, suggesting that E animals did not better utilise environmental cues to complete the task more quickly, but rather, that an increased swimming speed allowed E animals to finish the task faster. Thus, it is important when examining E animals' behaviour on a task to differentiate hyperactivity from other behaviours. Data from the present study suggest that the behavioural hyperactivity seen in E animals may be situation dependent. When confronted with a more intense stressor (e.g. the open arm) or following activation in the OF, E animals no longer demonstrate hyperactivity and instead show increased fear. These data may provide an explanation for the apparent decrease in fear observed in E males in Experiment 1a. That is, although E males had
increased time on the open arm, they did not show an increase in open arm entries. In addition, they showed increased closed arm entries and rears. Thus it is possible that the increased time on the open arms may in fact reflect a generalised increase in activity level or deficits in response inhibition as suggested by previous authors (Abel, 1982; Barron & Riley, 1990; Becker & Randall, 1989; Caul et al., 1979; Driscoll et al., 1982; Driscoll et al. 1985; Molina et al., 1984; Randall et al., 1986; Riley et al., 1979a; Riley et al., 1979b).

Although the mechanism(s) for the alterations in behavioural and HPA responses to stressors seen in E animals have yet to be determined; it is possible that HPA hyperresponsiveness, may at least in part, mediate the increased fear seen in E females in the present study. CRF (Koob & Britton, 1990), ACTH and CORT (File et al., 1979) have all been shown to affect behaviour in aversive situations. CRF (Dunn & Berridge, 1990) and CORT (File et al., 1979) have been shown to have both suppressive and activating effects on behaviour (Dunn & Berridge, 1990; Majewska, 1992). Following exposure to the +-maze, all animals had higher CORT and lower activity levels on the open arms compared to the closed arms. Further, prior exposure to the OF, which would presumably cause an increase in CORT levels, resulted in behavioural activation so that animals spent more time on the open arms. However, the present data indicated that although E females demonstrated increased fear in both Experiments 1a and 2, they showed an increase in CORT levels only in Experiment 2. Thus while elevated CORT levels may play some role in mediating fear-related behaviours, clearly other factors must
also be involved in the altered behavioural responses to stress seen in foetal ethanol exposed animals.

Unlike previous studies (Becker & Randall, 1989; Bond & DiGusto, 1977a; Caul et al., 1979; Fernandez et al., 1983; Means et al., 1984; Melcer et al., 1994; Molina et al., 1984; Riley et al., 1986; Vorhees & Fernandez, 1986), E animals did not differ from PF and C animals in OF activity. The OF has been used to measure exploration but, because it is an aversive environment, data suggest that it is actually a measure of arousal or emotionality as much as a measure of exploration (Archer, 1975). Thus, it appears in the present study that E animals did not differ from PF and C animals in exploration or arousal as measured by the OF. This, however, does not preclude the possibility that there is an underlying behavioural dysfunction in E animals, as it has been shown that OF activity in E animals varies as a function of age; as E animals mature the increased activity seen at early ages appears to diminish (Bond, 1981). In addition, it appears that increased activity in the OF reappears in E animals if they are pharmacologically challenged (Means et al., 1984) or if they are tested at very old ages (Abel & Dintcheff, 1986). Further, Hannigan et al. (1987), demonstrated that exposure to a prior stressor can increase grooming behaviour in a novel environment. In addition, in the present study it was demonstrated that exploration in E animals was differentially affected by prior exposure to the OF; E animals demonstrated increased exploration compared to controls when placed directly on the + maze but demonstrated decreased exploration compared to controls when placed on the + maze after exposure to the OF. Together these data suggest that E animals demonstrate altered behaviour in response to aversive
environments and that the manifestation of the behaviour may be changed by prior exposure to aversive stimuli.

In addition to prenatal ethanol effects, we also noted prenatal nutritional effects as well as an effect of pair-feeding itself. Prenatal nutritional effects were seen in Experiment 1, where both E and PF females had higher exploration levels than C females when confined to the closed arms of the + maze and in Experiment 2, where both E and PF females demonstrated higher fear levels than C females. These data indicate that alterations in these behaviours were mediated primarily by prenatal nutritional effects rather than specific effects of ethanol. Pair-feeding effects were seen in Experiment 1; PF females spent more time in the open arms than E and C females. Previous data demonstrated that although pair-feeding provides an essential nutritional control group, pair-feeding itself is a type of experimental treatment (Weinberg, 1984). For example, pair-feeding can produce a shift in the circadian rhythm of a number of physiologic variables as well as alter body and organ weights and behaviour of both the maternal females and the offspring (Gallo & Weinberg, 1981; Weinberg, 1989; Weinberg & Gallo, 1982). The present data further demonstrate long term effects of pair-feeding and highlight the importance of including an ad libitum fed control group in prenatal alcohol studies.

Finally, these data may have clinical implications. Children prenatally exposed to alcohol also demonstrate behavioural alterations including hyperactivity, as well as impulsivity and attention deficits which may reflect an inability to inhibit responses
(Streissguth et al., 1983; Streissguth et al., 1985; Streissguth, 1986). These behavioural deficits are particularly noticeable in stressful situations (Streissguth, 1986). Recently, it has been documented that prenatally exposed children demonstrate irrational fears of objects (e.g. a red ball) or places (e.g. the bathtub) (Harris et al., 1993; Harris et al. 1995) suggesting that behavioural deficits extend beyond hyperactivity and altered attention span commonly reported. These types of behaviours in children may be related to the altered behavioural responses to a stressful environment as observed in the present study.
Figure 35. Number of Closed Arm Entries and Time on the Centre for Males.
Points represent mean ± SEM.
* Main effect of group, p's < 0.05: E > PF = C for closed arm entries on d 2; E > PF for time in centre area on d 2.
Figure 36. Number of Closed Arm and Partial Open Arm Entries for Females.
Points represent mean ± SEM.
* Main effects of group, p’s < 0.05: E = PF > C for closed arm entries on d 1 and
d 2; E > C for partial arm entries d 1.
FEMALE

CONTROL  PAIR-FED  ETHANOL

Closed Arms

FULL ENTRIES

DAY 1  DAY 2

*  *

Open Arms

PARTIAL ENTRIES

DAY 1  DAY 2

*
Figure 37. Time on Centre Area and on Closed Arms for Females.
Points represent mean ± SEM.
* Main effects of group, p’s < 0.05: E = PF > C for time on centre area on d 1; PF < C for time on closed arms on d 1.
FEMALE

CONTROL  PAIR-FED  ETHANOL

**Center Area**

TIME (Seconds)

DAY 1  DAY 2

**Closed Arms**

TIME (Seconds)

DAY 1  DAY 2
Figure 38. Time on Open Arms and Number of Closed Arm Rears for Males.

Points represent mean ± SEM.

* Main effects of group, p’s < 0.05: E > C for time on open arms on d 1; E > PF = C for rears on closed arms on d 1 and d 2.
Figure 39. Number of Full Open Arm Entries for Males and Females
Points represent mean ± SEM.
No significant differences among E, PF and C males or females
OPEN ARMS

Male

Female

DAY 1
DAY 2

FULL ENTRIES

CONTROL  PAIR-FED  ETHANOL

FULL ENTRIES

CONTROL  PAIR-FED  ETHANOL

DAY 1
DAY 2
Figure 40. Time on Open Arms for Females.
Points represent mean ± SEM.
* Main effect of group, $p < 0.05$: PF > C for time on open arms on d 1 ($p < 0.05$).
FEMALE

CONTROL  PAIR-FED  ETHANOL

Open Arms

TIME (Seconds)

DAY 1  DAY 2

*
Figure 41. Ambulation and Turns on Closed Arm for Males

Points represent mean ± SEM.

* Main effect of group, p’s < 0.05: E = PF > C for closed arm ambulation (p < 0.01 and p < 0.05 respectively); E > C for closed arm turns (p < 0.01).
MALE

CONTROL  PAIR-FED  ETHANOL

Ambulation on Closed Arm

MIDLINE CROSSES

0-5 MIN  15-20

TIME

0  5  10  15  20  25  30

Turns on Closed Arm

TURNS

0-5 MIN  15-20

TIME

0  5  10  15  20  25  30
Figure 42. Rears on Closed Arm for Males
Points represent mean ± SEM.
* Main effect of group, p < 0.05: E > C for closed arm rears (p < 0.01).
MALE

CONTROL  PAIR-FED  ETHANOL

Closed Arm

REARS

TIME

0-5 MIN  15-20

* *
Figure 43. Ambulation and Turns on Closed Arm for Females

Points represent mean ± SEM.

* Main effect of group, p's < 0.05: E > C for closed arm ambulation (p < 0.01); E = PF > C for closed arm turns (p < 0.01 and p < 0.05 respectively).
FEMALE

CONTROL PAIR-FED ETHANOL

**Ambulation on Closed Arm**

**Turns on Closed Arm**

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.218
Figure 44. Rears on Closed Arm for Females

Points represent mean ± SEM.

* Main effect of group, p < 0.05: E = PF > C for closed arm rears (p < 0.01 and p < 0.05 respectively).
FEMALE

- CONTROL
- PAIR-FED
- ETHANOL

**Closed Arm**

<table>
<thead>
<tr>
<th>TIME</th>
<th>REARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 MIN</td>
<td></td>
</tr>
<tr>
<td>15-20</td>
<td></td>
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</tbody>
</table>

* indicates significant difference.
Figure 45. Corticosterone Levels for Males and Females Following Open or Closed Arm Exposure.

Points represent mean ± SEM.

* Main effect of arm, p's < 0.01: Closed arm > Open arm (p's < 0.01)
Figure 46. Time on Closed Arms and Rears on Closed Arms for Males.
Points represent mean ± SEM.
* Main effect of group, $p < 0.05$: $E < PF = C$ for time on closed arms.
MALE

CONTROL PAIR-FED ETHANOL

Closed Arms

TIME (Seconds)

CONTROL PAIR-FED ETHANOL

Closed Arms

REARS
Figure 47. Time on Closed Arms and Entries on Closed Arms for Females.
Points represent mean ± SEM.
* Main effects of group, p < 0.05: E < PF for time on closed arms (p < 0.05); E <
C for closed arms entries (p < 0.01)
Figure 48. Rears on Closed Arms for Females.

Points represent mean ± SEM.

* Main effect of group, $p < 0.05$: $E = PF < C$ for rears on closed arms ($p$'s < 0.05).
Figure 49. Rears on Open arms and Time on Open Arms for Females.
Points represent mean ± SEM.
* Main effects of group, p’s < 0.05: E = PF < C for rears on open arms (p’s < 0.05); E = PF < C for time on open arms (p < 0.05 and p < 0.01).
Figure 50. Number of Open Arm Entries for Females.
Points represent mean ± SEM.
* Main effect of group, p's < 0.05: E = PF < C for number of open arm entries (p < 0.05 and p < 0.01).
Figure 51. Corticosterone Levels for Male and Females Following OF and +-maze Exposure.

Points represent mean ± SEM.

* Main effect of group, $p < 0.05$: E < PF = C ($p's < 0.05$) for females.
B. FOETAL ETHANOL EFFECTS ON CRF SENSITIVITY MEASURED BY THE ELEVATED PLUS MAZE.

B.1 INTRODUCTION

Selye (1936) defined the stress response as a non-specific response to any demand on the body which results in various physiological changes including activation of the hypothalamic-pituitary-adrenal axis. Corticotrophin releasing factor (CRF) is the predominant releasing hormone which regulates the pituitary-adrenal axis resulting in adrenocorticotropic hormone (ACTH) secretion from the pituitary which in turn stimulates secretion of corticosterone (CORT) from the adrenal gland, which has many metabolic and immunological effects (Munck & Naray-Fejes-Toth, 1994). Importantly, CRF appears to have behavioural and physiological effects outside those of HPA axis regulation, suggesting that it may have a direct neurotransmitter role in the co-ordination of behavioural, autonomic, and metabolic responses to stressors.

During the preweaning period, rodents prenatally exposed to ethanol (E) exhibit suppressed or blunted HPA responses to a variety of stressors (Angelogianni & Gianoulakis, 1989; Taylor et al., 1986a; Weinberg et al, 1986; Weinberg et al, 1989) but, as adults, E animals demonstrate increased HPA responses to stressors (Taylor et al., 1982; Weinberg, 1988; Weinberg, 1992b). In addition, hypothalamic CRF content appears depressed in E neonates (Redei et al., 1989) but in adulthood both basal CRF mRNA (Lee & Rivier, 1993a) and stress CRF mRNA (Osborn et al., 1995) levels are
elevated in E animals. Further, adult E offspring demonstrate hyperactivity (Bond, 1981; Meyer & Riley, 1986), deficits in response inhibition (Driscoll et al., 1985; Riley et al., 1979a; Riley et al., 1979b), and increased anxiety-like behaviours in stressful environments (Anandam et al., 1980; Hannigan et al., 1987). As discussed previously (Chapter I: C.2) intracerebroventricular (ICV) infusion of CRF results in behaviours resembling stress-induced behaviour (Dunn & Berridge, 1990; Koob & Britton, 1990) and appears to increase the sensitivity of the rat to stressful aspects of the environment. Therefore, central alterations in CRF synthesis and/or sensitivity may play a role in the mechanism(s) underlying HPA and behavioural hyperactivity seen in animals prenatally exposed to ethanol (ETOH).

This study utilised the elevated plus maze (+-maze) to examine behavioural responses of E, pair-fed, and control males and females to intracerebroventricularly (ICV) administered CRF, α-helical CRF, or saline, or subcutaneously (SC) administrated CRF or saline.

**B.2 METHODS**

Sprague-Dawley males (n=25) and females (n=53) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed as described in Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were obtained
from the tails from 3 unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951).

Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dams and pups were weighed and all litters culled to 10 (five males and five females). Dams and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 90-110 days of age.

One wk prior to testing, animals were singly housed and randomly assigned to experimental treatment groups. Testing order was counterbalanced across prenatal treatment, sex, and experimental treatment groups (n=9-10 for each of E, PF, and C, males and females for each experimental treatment). Animals were implanted with ICV cannulae 5-7 d prior to testing as described in the Chapter II: General Methods. All testing of animals occurred at 0730-1100 h on the test day. White noise (40 dB) was used to mask any extraneous background noises.

Animals were taken from the colony room and infused or injected according to 1 of 5 experimental treatment conditions: 1) 10 µg/rat ICV CRF; 2) 5.0 µg/rat ICV α-helical CRF; 3) ICV saline in an injection volume of 5 µl; 4) 10 µg/rat SC CRF; 5) SC saline in an injection volume of 2.0 cc. ICV infusion rate was 2.5 µl/min. Animals remained in their home cages in the infusion room for 20 min at which time they were taken to an adjacent room containing the open field (OF) and the +-maze. As described
in Chapter IVA: Methods, animals were placed in the OF for a 5 min test and then in the + maze for a 5 min test. As in Chapter IV.A behaviour in the OF was scored immediately and + maze behaviour was video taped and scored by 2 investigators independently. Behaviour measured on the OF included ambulation in the central 4 squares and the 12 outer squares and number of rears. Behaviour measured on the + maze included time spent in open arms, in the closed arms and in the central area, full open and closed arm entries, partial open arm entries and number of rears in the open and closed arms.

B.3 STATISTICAL ANALYSES

Principal component factor analysis on standardised scores was used to determine which behavioural measures were related. These factors were then analysed by appropriate analyses of variance (ANOVA) for prenatal treatment and sex. Individual behaviours were further analysed separately by appropriate ANOVAs for prenatal treatment and sex. Significant main and interaction effects were analysed by Newman Keul's paired comparisons.
B.4 RESULTS

Developmental Data

Ethanol intake of pregnant females was consistently high throughout gestation with 9.2 ± 0.4, 11.6 ± 0.4, 11.5 ± 0.2 g/kg bw/day for wk 1, 2, and 3 of gestation respectively. Blood alcohol levels were consistent with previous levels (Weinberg, 1985), averaging 159.5 ± 19.6 mg/dl.

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 x days interaction (p < 0.001). Post-hoc tests indicated that E and PF females weighed significantly less than C females on d 7, 14, and 21 of gestation (p’s < 0.01). During lactation, a group x days interaction (p < 0.001) was also seen. E females weighed significantly less than C females on d 1 of lactation (p < 0.01). There were no significant differences among E, PF and C females on d 8, 15, or 22 of lactation.

There were no significant differences among groups for litter size or number of still born pups. Body weights for pups showed a significant group x days interaction (p < 0.05). Post hoc tests indicated that E and PF pups weighed significantly less than C pups on d 1 and 8 of lactation (p’s < 0.01). There were no significant differences in pup weight on d 15 and 22 of lactation and no significant differences in weight at the time of testing at 60-90 days of age.
Experimental Results

Open Field

Analysis of ambulation revealed a main effect of sex (p < 0.001): males had lower ambulation scores in both the inner and outer areas and made significantly fewer rears than females (p's < 0.001) (Fig. 52). For both males and females, there were no significant effects of either prenatal or experimental treatment on OF ambulation or rearing.

Plus Maze

Factor analysis revealed 2 main factors accounting for 61 % of the variance in males and 68 % of the variance in females. Factor 1 (fear factor) consisted of time on open arms, full open arm entries, negatively related to time on closed arms. Factor 2 (exploration factor) consisted of partial open arm entries, full closed arm entries, time on central area, and number of closed arm rears.

Overall, a group x sex x treatment ANOVA revealed main effects of sex for both the fear and exploration factors (p's < 0.01). Males had significantly higher fear scores (p < 0.01) and showed a trend towards lower exploration levels than females (p< 0.10). This was supported by ANOVAs on individual behaviours; males spent significantly less time on open arms and more time on closed arms than females (p's< 0.05) (Fig. 53). Males
also made less full closed arm entries (p < 0.05), less closed arm rears than females (p < 0.001) (Fig. 54) and spent less time in the central area (p < 0.05) (Fig. 55).

For females, there was a significant main effect of experimental treatment for both the fear factor (p < 0.05) and the exploration factor (p < 0.01). Post-hoc analysis indicated that overall, females given ICV CRF had higher fear levels than females given ICV saline (p's < 0.05) and lower exploration levels than females in any other postnatal experimental group (p's < 0.01). This was supported by ANOVAs on individual behaviours. Females given ICV CRF had fewer full open arm entries and spent more time on the closed arms compared to females given ICV saline (p's < 0.05) (Fig. 56). Consistent with these data, ICV CRF also had fewer closed arm entries than females given ICV saline (p < 0.01) and marginally fewer closed arm entries compared to females given SC saline (p < 0.10) (Fig. 57). In addition, females given ICV CRF spent less time on the central area than females given ICV or SC saline or SC CRF (p’s < 0.05) (Fig. 58).

For females, there was also a significant main effect of prenatal group alone for the exploration factor and a group trend for the fear factor. Post-hoc analysis indicated that overall, exploration levels were lower in E than in C females (p < 0.05) and marginally lower in PF than in C females (p < 0.10). This was supported by ANOVAs on individual behaviours. E and PF females made fewer closed arm entries than C females (p < 0.05 and p < 0.10 respectively) (Fig. 59) and E females made significantly fewer closed arm rears than PF and C females (p’s < 0.05) (Fig. 59). Post-hoc analysis of the fear factor indicated that E females showed a trend towards elevated fear scores compared
to C females (p < 0.10), supported by the finding that E females spent more time on closed arms than C females (p < 0.05) (Fig. 60).

For males, there were no significant effects of either postnatal experimental or prenatal treatment. Further, for both females and males there were no significant prenatal x experimental interactions.

B.5 DISCUSSION

These data do not support the hypothesis that behavioural hyperactivity seen in E animals is a result of a differential sensitivity to CRF; however, it appears that there may have been a difficulty with the dose of CRF and α-helical CRF chosen. The dose of ICV CRF (10 μg/rat) appeared to only increase fear responses in the females and did not affect the males. In addition, the dose of ICV α-helical CRF (5.0 μg/rat) had no effect on females or males. Previous literature has indicated that the above doses of CRF and α-helical CRF do result in significant alterations in +maze behaviour (Koob et al., 1993). There are a number of possible reasons for the lack of behavioural responses to ICV CRF and α-helical CRF in this study. First, it has been recently demonstrated that the behavioural response to exogenously administered CRF and α-helical CRF depends on the baseline state of arousal and stress level of the animal (Heinrichs et al., 1994). At low levels CRF appears to behaviourally activate the animal; e.g. increase ambulation, rearing and grooming in a familiar environment but the same dose in a novel environment appears to suppress activity (Dunn & Berridge, 1990; Koob et al., 1993). Furthermore, it
has been shown that although α-helical CRF increases exploration of the open arms on the + maze (Koob et al., 1993) and is effective in reversing the decreased time on the open arms following swim stress and restraint (Heinrichs et al., 1994), its effectiveness is variable. In a recent study, Heinrichs et al. (1994) demonstrated that ICV administered α-helical CRF at a low dose (1 μg) was effective in increasing open arm time but at higher doses (5 μg and 25 μg) was ineffective in altering open arm time following social stress, swim stress, or restraint stress. It is therefore possible that the lack of effect of CRF and α-helical CRF in the present study was a result of incorrect dose selection for the stressor chosen. Second, the lack of response to the exogenous substances may reflect methodological differences. File et al. (1992) demonstrated that handling can modify rat’s behavioural responses on the + maze as well as neurochemical responses to anxiolytic agents. Further, it appears housing conditions may also influence behaviour; group housing appears to increase time spent on open arm to about 50% (Heinrichs et al., 1994) which is significantly higher than what we saw in our animals (28%). Our animals were only handled once a week during cage changing, once during the surgery and were singly housed 1 week prior to the study. It is possible that these differences may have altered + maze activity and sensitivity to CRF and α-helical CRF. Third, there was difficulty in getting the CRF and α-helical CRF into solution and it was necessary to vortex gently. It was later suggested that the vortexing can inactivate CRF and α-helical CRF (communication with Dr. C. Rivier). Thus it is possible that the CRF and α-helical CRF used in this study were partially inactivated.
Despite the relative ineffectiveness of the peptides in this study, the data indicated significant effects of both prenatal treatment and postnatal experimental treatment on behaviour on the + maze. The results of this study support those of previous work (Alonso et al., 1991; Gonzalez & Leret, 1994; Imhof et al., 1993; Leret et al., 1994; Meng & Drugan, 1993; Rodgers & Cole, 1993; Slob et al., 1981; Steenbergen et al., 1991; Zimmerberg & Farley, 1993;) which demonstrated a sexually dimorphic response to the OF and + maze. In this study, females demonstrated higher levels of activity on the OF and + maze as well as reduced fear on the + maze compared to males. In addition, it was demonstrated that ICV CRF altered female but not male behaviour on the + maze. Overall, females infused with ICV CRF demonstrated increased fear levels compared to ICV saline infused females and decreased exploration levels compared to all other treatment groups. Importantly, as in previous studies in this dissertation, E females also showed increased fear levels and decreased exploration levels compared to control females reinforcing that the behavioural changes observed in prenatally ethanol exposed animals on the + maze is a robust phenomenon. Further, as with the HPA hyperresponsiveness in E animals, the mechanism(s) underlying differences in + maze behaviour appears to be differentially sensitive in E males and E females.

It has been well documented that in rodents, a sex difference occurs in a variety of nonreproductive behaviours (Beatty, 1979). The results from the present study support previous studies which demonstrated that females have greater ambulatory and rearing activity in the OF (Alonso et al., 1991; Archer, 1975; Beatty & Fessler, 1976; Beatty & Holzer, 1978; Blizard et al., 1975; Masur et al., 1980; Meng & Drugan, 1993; Slob et al.,
1981) and have decreased fear levels and increased exploration levels on the + maze compared to males (Gonzalez & Leret, 1994; Imhof et al., 1993; Johnston & File, 1991; Leret et al., 1994; Rodgers & Cole, 1993; Steenbergen et al., 1991; Zimmerberg & Farley, 1993). Further, sex differences in behavioural responses to aversive stimuli have been demonstrated on a number of behavioural tasks. Males demonstrated a larger decrease in locomotion on the OF when tested 1 h after inescapable shock (IS) (Heinsbroek et al., 1988) and 24 h after a single restraint stress as compared to females (Kennett et al., 1986). Males and females demonstrated decreased direct exploration (head-dipping in the hole board) 24 h after IS but only males demonstrated a decrease 72 h after IS and only males demonstrated a decrease in ambulation and rearing on the holeboard 24 h after IS (Steenbergen et al., 1991). Furthermore, it has been shown that on the + maze, IS reduces rearing in males but not females 24 h later and that males tended to have less open arm entries and spend less time on the open arms than females following IS (Steenbergen et al., 1991). These studies suggest that prior exposure to aversive stimuli may differentially alter behaviour in males and females. Thus it is possible that our experimental manipulations, which include handling during infusion and exposure to a novel environment during the 20 min holding period as well as OF exposure, may have altered + maze behaviour in males and females differentially.

Interestingly, in the first two studies of this dissertation on the + maze, no sex differences were found. Sex differences in + maze behaviour appear to be a function of age (Imhof et al., 1993). Imhof et al. (1993) demonstrated that males and females do not differ in + maze behaviour at 60 days of age but that decreased activity levels and open
arm time are seen in males at 90 days of age and in females at 120 days of age. In the first 2 studies, animals were tested at 60 to 90 days of age with a mean age of 71.4 days. In contrast, in the present study animals were tested at 60 to 104 days with a mean age of 89.4 days. This increase in age was due to the number of animals required in the present study; testing was extended over a greater number of days. It is possible that the differences in age of testing between these studies may have influenced the finding of sex differences in + maze behaviour, suggesting that there is a critical period during which gender related behaviours can be detected on the + maze. In addition, the + maze has been behaviourally, physiologically, and pharmacologically validated using only males. Thus, when interpreting results it is possible that the + maze may not measure the same variables in males and females and that in testing anxiolytic or anxiogenic agents ‘false’ positives may occur if testing males and females beyond the critical ages of 90 and 120 days of age, respectively.

A possible mechanism for this sexual dimorphism on + maze behaviour is prenatal and postnatal exposure to gonadal steroids. Oestrogen and progesterone have been shown to have both early neural organisational effects in neonates and activational effects in adults on the + maze (Leret et al., 1994; Zimmerberg & Farley, 1993), whereas, testosterone appears to be involved with neural organisational effects prenatally (Gonzalez & Leret, 1994) and neonatally (Gray et al., 1965; Pfaff & Keiner, 1973; Pfaff & Zigmond, 1971; Swanson, 1967) but does not appear to have activational effects on adult + maze behaviour (Zimmerberg & Farely, 1993). Females deprived of oestrogen neonatally (Leret et al., 1994; Zimmerberg & Farley, 1993) and prepubertally
(Zimmerberg & Farley, 1993) demonstrated increased anxiety responses on the + maze and thus appeared more similar to males. Further, females which had been ovariectomised neonatally and received oestrogen replacement as adults demonstrated anxiety responses similar to males but had activity levels similar to females on the + maze (Leret et al., 1994). Finally, neonatal treatment with the antiandrogen, flutamide, or the aromatase inhibitor, LY43578, or pubertal orchiectomy did not alter male performance on the + maze (Gonzalez & Leret, 1994; Zimmerberg & Farley, 1993)

These data suggest that the sex differences seen in + maze behaviour may be a result of normal gonadal steroid exposure during development as well as during adulthood.

In summary, the results of this study demonstrate a sex difference in OF and + maze behaviour and suggest that there may be a sex difference in sensitivity to ICV administered CRF. In addition, this study further supports the previous study which demonstrated prenatal ethanol exposure altered adult behaviour on the + maze. Further, similar to the HPA hyperresponsiveness seen in E animals, behavioural alterations to stress seen in E animals appear to have a sexual dimorphism in the sensitivity of the underlying dysfunction. In the present study, only E females demonstrated increased fear and decreased exploration on the + maze as compared to control females, whereas in the previous study (Chapter IVA) both E males and females demonstrated alterations in fear and exploration levels compared to control males and females. Finally, the mechanism(s) for the altered behaviours on the + maze have yet to be determined. The results of this study are inconclusive regarding the hypothesis that alteration in + maze behaviour seen in E animals is a result of increased sensitivity to CRF. This is possibly due to the fact...
that only one dose of CRF and α-helical CRF was used in this study with only marginal significance being seen in female behaviour after CRF administration, and that the peptides may have been partially inactivated by vortexing. Presently, a replication of this study is being conducted in Dr. Weinberg's laboratory. Multiple doses of CRF as well as a more potent CRF antagonist, [D-Phe$^{12}$, Nle$^{31-38}$, C$^{9}$ MeLeu$^{37}$] h/rCRF$_{12-41}$, are being tested to better address the possibility that there is a differential sensitivity in E animals to centrally administered CRF compared to controls.
Figure 52. Ambulation and Rearing on the Open Field for Males and Females.
Points represent mean ± SEM.
*** Main effect of sex, p’s < 0.001: Males < Females for ambulation on outer and inner area and for rearing (p’s < 0.001).
OPEN FIELD

Ambulation

Squares Crossed

Outer Area  Inner Area

Rearing

Rears

Male  Female
Figure 53. Time on Open Arms and Closed Arms for Males and Females.  
Points represent mean ± SEM.  
* Main effects of sex, p’s < 0.05: Males < Females for time on open arms (p < 0.05); Males > Females for time on closed arms (p < 0.05).
PLUS MAZE

[Graph showing time (seconds) for Open Arms and Closed Arms for Male and Female]

Open Arms

Closed Arms
Figure 54. Entries onto Closed arms and Rears on Closed Arms for Males and Females.

Points represent mean ± SEM.
* Main effect of sex, \( p < 0.05 \): Males < Females for number of closed arm entries (\( p < 0.05 \)).
*** Main effect of sex, \( p < 0.001 \): Males < Females for number of closed arm rears (\( p < 0.001 \)).
PLUS MAZE

Closed Arm Entries

Entries

Male

Female

Closed Arm Rear

Rears

Male

Female
Figure 55. Time in Central Area for Males and Females.
Points represent mean ± SEM.
* Main effect of sex, p < 0.05: Males < Females for time on central area (p < 0.05).
Figure 56. Number of Open Arm Entries and Time on Closed Arms for Females Given ICV CRF, ICV hCRF, ICV Saline, SC CRF, or SC Saline.

Points represent mean ± SEM.

* Mains effect of treatment, p's < 0.05: ICV CRF-treated females < ICV saline-treated females for number of open arm entries (p < 0.05); ICV CRF-treated females > ICV saline-treated females for time on closed arms (p < 0.05).
Figure 57. Number of Closed Arm Entries for Females Given ICV CRF, ICV hCRF, ICV Saline, SC CRF, or SC Saline.

Points represent mean ± SEM.

** Main effect of treatment, p < 0.01: ICV CRF-treated females < ICV saline-treated females for number of closed arm entries (p < 0.01).
FEMALE

Closed Arm Entries

Experimental Treatment
Figure 58. Time in Central Area for Females Given ICV CRF, ICV hCRF, ICV Saline, SC CRF, or SC Saline.

Points represent mean ± SEM.

* Main effect of treatment, p < 0.05: ICV CRF-treated females < ICV saline-treated = SC CRF-treated = SC saline-treated females for time on central area (p’s < 0.05).
FEMALE

Central Area

Experimental Treatment

Time (seconds)
Figure 59. Number of Closed Arm Entries and Rears for Females.
Points represent mean ± SEM.
* Main effects of group, p's < 0.05: E < C for number of closed arm entries (p < 0.05); E < PF = C for number of closed arm rears (p's < 0.05).
FEMALE

Closed Arm Entries

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Pair-fed</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Entries</td>
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<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Rearing

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Pair-fed</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rears</td>
<td>15</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 60. Time on Closed Arms for Females.
Points represent mean ± SEM.
* Main effect of group, p’s < 0.05: E > C for time on closed arms (p < 0.05).
FEMALE

- **Control**
- **Pair-fed**
- **Ethanol**

**Closed Arms**

Time (seconds)

Groups

*
C. FOETAL ETHANOL EFFECTS ON BENZODIAZEPINE (BZD) SENSITIVITY MEASURED BY THE ELEVATED PLUS MAZE.

C.1 INTRODUCTION

The previous elevated plus maze (+-maze) studies in this dissertation have demonstrated that prenatal ethanol exposure (E) differentially alters behavioural responses to aversive environments. Interestingly, the alterations in behavioural responses seen in E animals may be changed by prior stressor exposure. Both E males and females demonstrate an increase in exploratory-related behaviours compared to control (C) males and females when placed directly on the +-maze but following behavioural activation by open field (OF) exposure, E animals demonstrate a decreased in exploratory-related behaviours compared to C animals. In addition, E females but not E males demonstrated increased fear-related behaviours compared to C females regardless of prior OF exposure and an increase in corticosterone (CORT) levels, an index of stress levels (Selye, 1973) compared to pairfed (PF) and C females. Although the mechanism(s) for the alterations in behaviour have yet to be determined, these data suggest that prenatal ethanol exposure affects both males and females but there may be a sex difference in the sensitivity of the mechanism(s) underlying the alterations in behaviour.
As discussed previously, the +-maze is used to test anxiolytic and anxiogenic agents and is particularly sensitive to agents that act via the γ amino-butyric acid (GABA) system such as the benzodiazepines (BZD) (Pellow et al., 1985). To date no studies have examined BZD sensitivity in E animals; however, a number of studies (Ledig et al., 1988; Moloney & Leonard 1984; and Rawat, 1977) have demonstrated alterations in the GABA-ergic system in E animals. Therefore, the present study was designed to examine further effects of prenatal ethanol exposure on anxiety/fear-related behaviour and to compare behavioural responses of E, PF and C animals following administration of a BZD. Animals were injected with either saline or BZD and then tested on the +-maze after prior exposure to the OF. As discussed previously, exposure to the OF prior to +-maze testing increases the time spent on the open arms of the +-maze. This study was run in parallel with the study in Chapter IVB: Foetal Ethanol Effects on CRF Sensitivity Measured by the Elevated Plus Maze using animals from the same breeding.

C.2 METHODS

Sprague-Dawley males (n=25) and females (n=53) were obtained from Canadian Breeding Farms, St. Constant, PQ. Animals were bred and fed as described in Chapter II: General Methods. On d 12-14 of gestation, blood samples (0.4-0.6 ml) were obtained from the tails from 3 unanaesthetised females at 1900 h for determination of blood ethanol levels (Sigma diagnostic Kit 332-UV, based on Bonnischsen & Theorell, 1951).
Females were undisturbed except for weighing and cage cleaning on d 1, 7, 14, and 21 of gestation. At birth, designated d1 of lactation, dams and pups were weighed and all litters culled to 10 (five males and five females). Dams and pups were weighed and cages cleaned on d 1, 8, 15, and 22 of lactation. On d 22, pups were weaned and housed by sex and by litter until testing at 60-90 days of age.

One wk prior to testing, animals were singly housed and randomly assigned to experimental treatment groups. Testing order was counterbalanced across prenatal treatment, sex, and experimental treatment (n=5-7 for each of E, PF, and C males and females for each experimental treatment). All testing of animals occurred at 0730-1100 h on the test day. White noise (40 dB) was used to mask any extraneous background noises.

Animals were taken from the colony room to an adjacent holding room and given either a subcutaneous injection of saline or 0.15 mg/kg body wt Diazemuls (diazepam injectable emulsion, Kabi Pharmacia Inc., PQ, Canada) (BZD) in an injection volume of 2.0 cc. Twenty min later, animals were taken from the holding room to an adjacent room containing the open field (OF) and the +-maze. Animals were placed in the OF for 5 min and then in the +-maze for 5 min and behaviours scored as in Chapter IVA.

Following exposure to the OF and +-maze, animals were taken from the testing room and housed in their home cages without water for 10 min in a holding room. Animals were quickly and lightly anaesthetised with Metofane (Janssen Pharmaceutica,
Mississauga, ON, Canada) and blood samples (0.5 cc) taken by cardiac puncture using heparinized syringes. The sampling procedure was completed within 2 min of touching the cage, which is rapid enough to obtain a reliable measure of corticosterone (CORT) at the end of the behavioural testing without any effect of disturbance resulting from the blood sampling procedure itself (Davidson et al., 1968). Blood samples were centrifuged at 2200 g for 10 min at 4° C, plasma collected, and stored at -70° C.

C.3 STATISTICAL ANALYSES

Principal component factor analysis on standardised scores was used to determine which behavioural measures were related. These factors were then analysed by appropriate analyses of variance (ANOVA) for sex, prenatal treatment (i.e. group) and experimental treatment (i.e. treatment). Individual behaviours were further analysed separately by appropriate ANOVAs for sex, prenatal treatment (i.e. group) and experimental treatment. Significant main and interaction effects were analysed by Newman Keul's paired comparisons.

C.4 RESULTS

Developmental Data

See Chapter IVB; Developmental Data
Experimental Results

Open Field

Analysis of OF behaviour revealed a significant main effect of sex (p < 0.001). Overall, females had higher ambulation scores in both the central and outer areas and made significantly more rears than males (p's < 0.001). There were no significant effects of experimental treatment (i.e. saline or BZD injection) on ambulation for either males or females (Fig. 61 and Fig. 62). There was, however, a main effect of experimental treatment on rearing (p < 0.0001); males and females injected with BZD reared significantly less than males and females injected with saline (Fig. 63). For males, there was also a main effect of group for ambulation in the central area (p < 0.05); overall, E males showed significantly less central ambulation than PF males (Fig. 61). There were no significant differences among E, PF, and C males in rearing and no significant differences among E, PF and C females in any OF measure.

Plus Maze

As animals in this study spent more time in the central area than they did in previous studies, time in the open and closed arms are expressed as a percentage of time on the open and closed arms [Percentage of time on the open arms (%Topen) = (time in open arms / (time in open arms + time in closed arms)) x 100; Percentage of time on the
closed arms (%Tclosed) = (time in closed arms / (time in open arms + time in closed arms)) x 100].

Factor analysis revealed 2 main factors accounting for 72 % of the variance in males and 84 % of the variance in females. Factor 1 consisted of %Topen and full open arm entries, negatively related to %Tclosed (fear factor). Factor 2 consisted of partial open arm entries, full closed arm entries, time on central area, and number of closed arm rears (exploration factor).

A main effect of sex was seen for both the fear and exploration factors (p's < 0.01): females had significantly lower fear levels and higher exploration levels than males. This was supported by ANOVAs on individual behaviours. Females had higher %Topen and made more open arm entries than males (p's < 0.01). Overall, females also made more closed arm entries and more closed arm rears and spent less time in the central area (p's < 0.01) than males.

For males, group x treatment ANOVAs revealed significant main effects of experimental treatment for both the fear and exploration factors (p's < 0.001); males given BZD had lower fear and exploration scores than males given saline (p's < 0.001). This was supported by ANOVAs on individual behaviours. Overall, BZD-treated males had a higher %Topen (p < 0.01) (Fig. 64), more open arm entries (p < 0.05) (Fig. 65) and a lower %Tclosed (p < 0.01) (Fig. 66) than saline treated males. Males given BZD also
had fewer closed arm entries (p < 0.05) (Fig. 67) and fewer closed arm rears than males given saline (p < 0.01) (Fig. 69).

There were no significant differences among E, PF, and C male for either the fear or exploration factors; however, ANOVAs on individual behaviours indicated that BZD treatment differentially affected E compared to PF and C males (p's < 0.05). Following BZD, E males had a higher %Topen (p's < 0.05) (Fig. 64) and fewer open arm entries (p's < 0.05) (Fig. 65) as well as lower %Tclosed (p's < 0.05) (Fig. 66) and fewer closed arm entries than BZD-treated PF and C males (p's < 0.05) (Fig. 67). In addition, E males given BZD also spent more time on the central area than PF and C males given BZD (p's < 0.01) (Fig. 68). There were no significant differences among saline-treated E, PF and C males on any + maze measure (Fig. 69 and Fig. 70).

For females, group x experimental treatment ANOVAs similarly revealed significant main effects of experimental treatment for both the fear and exploration factors (p's < 0.001). Consistent with data on males, females given BZD had decreased fear and decreased exploration scores compared to females given saline (p's < 0.01). This was supported by ANOVAs on individual behaviours. BZD-treated females had a higher %Topen (Fig 64), lower %Tclosed (Fig. 66) and made more open arm entries (Fig 65) than saline-treated females (p's < 0.01). In addition, BZD-treated females made fewer closed arm entries (Fig 67), fewer partial arm entries (Fig 70), fewer closed arm rears (Fig 69) and spent less time on the central area compared to saline-treated females (Fig 68) (p's < 0.01).
There were no significant differences among E, PF, and C female for either the fear or exploration factors; however, ANOVAs on individual behaviours indicated that BZD treatment differentially affected E compared to PF and C females (p's < 0.05). Following BZD, E females had a higher %Topen (Fig 64) and lower %Tclosed (Fig 66) than C females (p's < 0.05) and showed a similar trend compared to PF females (p's < 0.10). There were no significant differences among saline treated E, PF and C females on any of the + maze behaviours.

Corticosterone

As expected there was a main effect of sex (p < 0.0001); females had higher CORT levels than males. There were no significant differences among E, PF, and C males. However, there was a main effect of experimental treatment (p < 0.05) (Fig. 71); BZD treated males had lower CORT levels than saline-treated males. For females, there were no significant differences in CORT levels among E, PF, and C females or between BZD and saline treated females (Fig. 71).

C.5 DISCUSSION

Consistent with the data from the previous studies (Alonso et al., 1991; Gonzalez & Leret, 1994; Imhof et al., 1993; Leret et al., 1994; Meng & Drugan, 1993; Rodgers & Cole, 1993; Slob et al., 1981; Steenbergen et al., 1991), a sexually dimorphic response to both the OF and + maze was demonstrated. Females demonstrated increased exploration
on both the OF and + maze as well as decreased fear on the + maze compared to males. BZD decreased fear and exploration behaviours on the + maze in both males and females regardless of group importantly. BZD treatment differentially affected E males and females compared to their PF and C counterparts. Both E males and females treated with BZD had a higher %Topen and lower %Tclosed, reflecting decreased fear, than their PF and C counterparts. Further, BZD-treated E males demonstrated decreased open and closed arm entries, and also spent significantly more time in the central area than BZD-treated PF and C males. No significant differences in + maze behaviours were found among E, PF and C males and females injected with saline. These data support previous work demonstrating that the + maze provided a reliable measure of anxiety/fear. Although E, PF and C males and females did not differ in + maze behaviour following saline treatment, in this paradigm prenatal ethanol exposure appears to alter BZD sensitivity as measured by altered behavioural responses on the + maze.

The results of the present study also support previous work demonstrating that BZD treatment significantly decrease anxiety/fear on the + maze (Lister, 1987; Pellow et al, 1985; Pellow & File, 1986). Overall, BZD-treated males and females had an increased %Topen, increased open arm entries and decreased %Tclosed compared to saline-treated males and females regardless of their group. Importantly, these differences were not due to altered activity levels, as BZD treatment had no affect on total ambulation in the OF. In addition, BZD-treated males demonstrated lower CORT levels, an index of stress, than saline-treated males. BZD has been shown to bind to a BZD site
on the γ aminobutyric acid, (GABA) receptor and to potentiate GABA inhibitor function (Olsen & Tobin; 1990). GABA is the major inhibitory neurotransmitter in the central nervous system (CNS), binding to 2 types of receptors; the GABA receptor which is coupled to a chloride channel and the GABA receptor which is G-protein coupled. Binding of the GABA receptor results in opening of the associated chloride channel leading to increased chloride transport and hyperpolarization of the neuronal membrane (Schofield et al, 1987). The GABA receptor is found on almost every neuron in the CNS and appears to play an important role in controlling neuronal excitation (Schofield et al, 1987). Thus, in the present experiment, BZD may have acted by potentiating the inhibitory effects of the GABA-ergic system to reduce fear on the + maze.

As in the previous + maze study, females demonstrated increased exploration on the OF and + maze and decreased fear on the + maze compared to males, supporting and extending the results of studies demonstrating a sexual dimorphism in nonreproductive behaviours (Beatty, 1979). Recently, it has been demonstrated that females are less sensitive to the activity-suppressant effects of the BZD inverse agonist FG 7142 (FG) suggesting that there may be a sex difference in the response of the GABA-ergic system. The mechanism for this sexual dimorphism is unknown but may be related to gonadal steroids and corticosterone levels. Metabolites of progesterone, deoxycorticosterone and testosterone have all been shown to bind to GABA receptors and alter the binding affinity for its' ligand (Majewska, 1992). Bitran et al (1991) demonstrated that 3a-hydroxy-5a(b)-pregnan-20-one, a metabolite of progesterone, has anxiolytic properties on
behaviour on the + maze. In addition, it has been shown that oestrogen and progesterone increase BZD binding sites and enhance GABA-activated chloride ion flux in vivo (Wilson, 1992). Further, pregnenolone sulphate (PS) and dehydroepiandrosterone sulphate (DHEAS) both act as non-competitive antagonists of the \( \text{GABA}_A \) receptor and inhibit GABA-induced currents (Majewska, 1990; Majewska, 1988). Interestingly, the desulphated forms of PS and DHEAS act very differently. Pregnenolone does not affect the \( \text{GABA}_A \) receptor (Harrison et al, 1987) but dehydroepiandrosterone inhibits GABA-induced currents (Demirgoren et al., 1991). Glucocorticoids also interact with the \( \text{GABA}_A \) receptor but their interaction appears to be more complex. Glucocorticoids at nanomolar concentrations potentiate GABA binding to the \( \text{GABA}_A \) receptor but at micromolar concentrations reduce GABA binding to the \( \text{GABA}_A \) receptor (Majewska et al., 1985). In addition, a metabolite of the corticosterone precursor, \( \alpha-5 \alpha \)-tetrahydrodeoxycorticosterone, modulates GABA activity in a manner similar to that of barbiturates (Majewska, 1992) and has been shown to have anxiolytic effects similar to BZD in rodents (Crawley et al., 1986). Thus, it is possible that sex differences in behaviour on the OF and + maze are modulated by differential levels of neurosteroids which result from activity of gonadal steroids and/or glucocorticoids.

Unlike the 2 prior + maze studies in this thesis, prenatal ethanol exposure does not alter + maze behaviour in undrugged animals. As previously discussed, E animals' behaviour on the + maze was differentially affected by prior exposure to an aversive environment (i.e. OF); E animals demonstrated increased exploration compared to
controls when placed directly on the +-maze but demonstrated decreased exploration compared to controls when placed on the +-maze after exposure to the OF. Further, it has been demonstrated that novelty-induced grooming responses in E animals differ from controls following a swim stress but do not differ with novelty stress alone (Hannigan et al., 1987). In addition, Hannigan et al. (1987) demonstrated that prior exposure to the novelty stress abolished the increased novelty-induced grooming seen in E animals following swim stress. Taken together, these data indicate that alterations in behavioural responses to stress seen in E animals may be very specific, depending upon the nature, intensity, and timing of the stressor. Thus, it is possible that our experimental manipulations including handling during the injection, the injection and the exposure to the novel environment during the 20 min holding period as well as the OF may have abolished the alterations in +-maze behaviour previously seen in E animals.

Importantly, E males and females appear to be more sensitive to BZD than PF and C males and females. Following BZD but not saline treatment, E males and females had significantly increased time on the open arms and decreased time on the closed arms compared to PF and C males and females. In addition, BZD-treated E males also had decreased open and closed arm entries and spent more time in the central area than BZD-treated PF and C males. These latter behaviours did not reflect a decrease in activity levels, but rather reflected the increased time spent exploring the open arms and central area. This interpretation is supported by the finding that there were no significant differences in OF ambulation among E, PF and C animals.
No other studies to date have examined BZD sensitivity in E animals. However, a number of studies have demonstrated alterations in the GABA-ergic system and neurosteroid sensitivity following prenatal ethanol exposure. Ledig et al. (1988) found that in 3 wk old pups, prenatal ethanol exposure decreased GABA levels in the thalamus, pons, cerebellum and hippocampus, increased GABA levels in the frontal cortex, olfactory bulbs, anterior colliculus and amygdala and caused no changes in GABA levels in the posterior colliculus, occipital cortex, temporal cortex, hypothalamus, septum or striatum. Rawat (1977) demonstrated increased cerebral GABA content in neonates suckling on ethanol-consuming mothers. In contrast, Moloney & Leonard (1984) found alterations in GABA levels of prenatally and postnatally ethanol exposed only pups if ethanol was still in the pup’s system. Furthermore, Ledig et al. (1993) found increased GABA turnover in the frontal cortex and olfactory bulb and decreased GABA turnover in the hypothalamus and olfactory tubercles in 2 month old rats born to mothers who consumed 20% (v/v) ethanol during the month before pregnancy only. Together the results from these studies suggest that prenatal alcohol exposure may alter the GABA content in specific brain sites and appears to have variable effects on the GABA-ergic system depending upon the alcohol exposure regimen, age at testing and the site studied.

The increase in BZD sensitivity in adult E males and females in this study suggest that the alterations in GABA levels in neonates may have functional implications in adults. Janiri et al. (1994), using microiontophoresis, demonstrated increased somatosensory and frontal cortex responses to GABA and decreased responses to
acetylcholine in adult E animals compared to control animals. In contrast, Zimmerberg et al. (1995) found that E neonates were less sensitive to the neuroactive steroid allopregnanolone, a GABA$_A$ agonist, as measured by ultrasonic vocalisation. Neuroactive steroids bind to a site on the GABA$_A$ receptor and affect ligand binding affinity by either increasing or decreasing receptor affinity (Majewska, 1992). Furthermore, Savage et al. (1995) demonstrated that prenatal ethanol exposure enhanced the positive modulatory effects of flunitrazepam and the negative effects of the inhibitory neurosteroid, 5 α-pregnon-3 β-ol-20-one sulphate while attenuating the effect of the benzodiazepine inverse agonist FG on GABA$_A$ receptor-stimulated chloride flux into vesicles prepared from the hippocampus. These changes in GABA-ergic activity may actually reflect alterations in the number of GABA-ergic neurons present in the animals as suggested by data of Lee et al. (1992) who demonstrated that after a single injection of ethanol, chick embryos had decreased cortical cholinergic neurons and increased cortical GABA-ergic neurons. Thus, it is possible that the increased sensitivity to BZD in E animals may reflect an alteration in the GABA-ergic system induced by prenatal alcohol exposure.

Finally, these data may have clinical implications. Prenatal alcohol exposure results in hyperactive, uninhibited and impulsive behaviour, attention deficits, as well as deficits in learning and memory (Streissguth et al., 1983; Streissguth et al., 1985; Streissguth, 1986). BZD receptor antagonists and inverse agonists have been shown to modulate learning and memory (Izquierdo & Medina, 1991). Anxiolytic agents which
enhance GABA binding impair memory whereas anxiogenic agents which inhibit GABA binding enhance memory (Izquierdo & Medina, 1991). Recently, it has been shown that BZD impairs learning and memory in FAS animals to a greater extent than in control animals (Petkov et al., 1991). Thus, it is possible that alterations in the GABA-ergic system either directly or through altered neurosteroid activity may play a role in the behavioural and/or cognitive alterations seen in children prenatally exposed to alcohol.
Figure 61. Ambulation on Central Area on the Open Field for Males and Females.
Points represent mean ± SEM.
* Main effect of group, p < 0.05: E < PF for males.
No significant differences among E, PF and C females.
OPEN FIELD

Male

Female
Figure 62. Ambulation on Outer Area on the Open Field for Males and Females. Points represent mean ± SEM. No significant differences among E, PF and C males or females.
OPEN FIELD

<table>
<thead>
<tr>
<th>Control</th>
<th>Pair-fed</th>
<th>Ethanol</th>
</tr>
</thead>
</table>

**Male**

- Ambulation Outer
- BZD
- Saline

**Female**

- Ambulation Outer
- BZD
- Saline

TREATMENT
Figure 63. Rears on the Open Field for Males and Females.
Points represent mean ± SEM.
* Main effect of experimental treatment, p’s < 0.0001: BZD-treated males and females < saline-treated males and females (p’s < 0.0001).
OPEN FIELD

Male

Control  Pair-fed  Ethanol

Rears

0  10  20  30  40

BZD  Saline

TREATMENT

Female

Control  Pair-fed  Ethanol

Rears

0  10  20  30  40

BZD  Saline

TREATMENT
Figure 64. Percent Time on Open Arms for Males and Females.
Points represent mean ± SEM.
# Main effect of experimental treatment, p’s < 0.01: BZD-treated males and females > saline-treated males and females (p’s < 0.01).
* Group x experimental treatment interaction, (p’s < 0.05); BZD-treated E males > BZD-treated PF and C males (p’s < 0.05); BZD-treated E females > BZD-treated C (p < 0.05) and PF (p < 10) females.
OPEN ARMS

Male

Female

Control  Pair-fed  Ethanol

% Time

BZD  Saline

TREATMENT

BZD  Saline

TREATMENT

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Figure 65. Number of Open Arms Entries for Males and Females.
Points represent mean ± SEM.
# Main effects of experimental treatment, p’s < 0.05: BZD-treated males and females < saline-treated males and females (p’s < 0.05).
* Group x experimental treatment interaction, (p’s < 0.05); BZD-treated E males < BZD-treated PF and C males (p’s < 0.05).
OPEN ARMS

Male

Female

Control
Pair-fed
Ethanol

BZD
Saline

TREATMENT

Full Entries

# #

# #

# #

0 3 6 9 12

0 3 6 9 12

291
Figure 66. Percent Time on Closed Arms for Males and Females.
Points represent mean ± SEM.
# Main effects of experimental treatment, p's < 0.01: BZD-treated males and females < saline-treated males and females (p's < 0.01).
* Group x experimental treatment interaction, (p's < 0.05); BZD-treated E males < BZD-treated PF and C males (p's < 0.05); BZD-treated E females < BZD-treated C females (p < 0.05).
CLOSED ARMS

![Graph showing data for male and female subjects with BZD Saline treatment comparisons.](image)

- Male and Female bars are color-coded: Control (black), Pair-fed (crosshatched), Ethanol (double-crosshatched).
- % Time axis ranging from 0 to 120.

**Note:** The graph illustrates the percentage of time spent in different conditions across treatments for male and female subjects.
Figure 67. Number of Closed Arm Entries for Males and Females.

Points represent mean ± SEM.

# Main effects of experimental treatment, p’s < 0.01: BZD-treated males and females < saline-treated males and females (p’s < 0.01).

* Group x experimental treatment interaction, (p’s < 0.05); BZD-treated E males < BZD-treated PF and C males (p’s < 0.05).
CLOSED ARMS

![Graph showing differences in Full Entries between BZD and Saline treatments for Male and Female subjects.]

- **Male**: Control, Pair-fed, Ethanol
- **Female**: Control, Pair-fed, Ethanol

Legend:
- Control
- Pair-fed
- Ethanol

Graphs illustrate the number of Full Entries for BZD and Saline treatments in both Male and Female subjects, with symbols indicating significant differences.
Figure 68. Time on Central Area for Males and Females.
Points represent mean ± SEM.
# Main effect of experimental treatment, p < 0.01: BZD-treated females < saline-treated females (p < 0.01).
* Group x experimental treatment interaction, (p’s < 0.01); BZD-treated E males > BZD-treated PF and C males (p’s < 0.01).
Figure 69. Number of Closed Arm Rears for Males and Females.
Points represent mean ± SEM.
# Main effects of experimental treatment, p’s < 0.01: BZD-treated males and females < saline-treated males and females (p’s < 0.01).
CLOSED ARMS

Male

Female

Rears

BZD

Saline

TREATMENT

Control

Pair-fed

Ethanol

Rears

BZD

Saline

TREATMENT

Control

Pair-fed

Ethanol

299
Figure 70. Number of Partial Open Arm Entries for Males and Females.
Points represent mean ± SEM.
# Main effects of experimental treatment, p’s < 0.01: BZD-treated males and females < saline-treated females (p’s < 0.01).
PARTIAL OPEN ARM

Control  Pair-fed  Ethanol

Male

TREATMENT

Female

TREATMENT
Figure 71. Corticosterone Levels for Males and Females Following +-Maze exposure.

Points represent mean ± SEM.

# Main effects of experimental treatment, p < 0.05: BZD-treated males < saline-treated males (p < 0.05).
CORTICOSTERONE

Control

Pair-fed

Ethanol

Male

BZD Saline

TREATMENT

Plasma CORT (ug/100ml)

0 20 40 60 80 100

#

#

TREATMENT

BZD Saline

Female

Control

Pair-fed

Ethanol

BZD Saline

Plasma CORT (ug/100ml)

0 20 40 60 80 100

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CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS

The studies in this thesis focused on two major areas: 1) possible mechanisms which may mediate the hypothalamic-pituitary-adrenal (HPA) hyperresponsiveness seen in prenatal ethanol exposed (E) animals; and 2) an examination of possible mechanisms which may mediate the alterations in behavioural responses to stressful environments seen in E animals. Specifically, the HPA hyperresponsiveness experiments examined the effects of prenatal ethanol exposure on: a) adrenal responsiveness to adrenocorticotrophin (ACTH); b) the effects of dexamethasone (DEX) blockade on basal and stress corticosterone (CORT) levels and stress ACTH levels over a 36 h period; c) corticotrophin releasing factor (CRF), arginine vasopression (AVP) and glucocorticoid receptor (GR) mRNA expression in the hypothalamus following stress. The behavioural experiments examined the effects of prenatal ethanol exposure on: a) behaviour on the elevated plus maze (+-maze) and b) investigated the possible role of CRF and γ aminobutyric acid (GABA) in mediating the altered behavioural responses seen in E animals.

The first set of studies focused on investigation of mechanisms underlying HPA hyperresponsiveness seen in E animals. One possible mechanism for the enhanced HPA response to stress seen in E animals may be a greater sensitivity of the adrenal gland to ACTH. Previous studies (Lee et al., 1990; Lee & Rivier., 1994; Taylor et al., 1982) have suggested that in E animals neither the adrenal gland or the pituitary are hyperresponsive
to secretagogues but these studies did not evaluate a full dose response curve in both males and females. The present study utilised 5 doses of ACTH and examined the CORT response over a 4 h period. We found that there were no significant differences in CORT levels following ACTH infusion among E, PF and C animals, when sensitivity is tested at the trough of the CORT circadian cycle. The data did, however, demonstrate the expected sex difference (Kitay, 1961; Skelton & Bernardis, 1966) in CORT responses; females have greater CORT responses to the same dose of exogenous ACTH than males regardless of their group. However, the pattern of CORT secretion over time was similar in males and females. Thus, our data suggest that during the trough of the circadian cycle, prenatal ethanol exposure does not exert long-term effect on adrenal sensitivity to ACTH.

The second possible mechanism underlying the HPA hyperresponsiveness in E offspring examined in this thesis was a deficit in feedback inhibition of the HPA axis. Nelson et al., (1985b) demonstrated that E animals appear to have an accelerated rebound of basal CORT levels following a high dose of the synthetic glucocorticoid, dexamethasone-21-phosphate (DEX). Clinically, the DEX suppression test has been used to evaluate HPA axis function in a number of psychiatric conditions and it appears that feedback inhibition of CORT is altered in a number of affective disorders (Nemeroff et al., 1988). Our studies of feedback inhibition of CORT supported the hypothesis that HPA hyperresponsiveness and/or delays in HPA recovery from stressors that occur in E animals may result, in part, from delays in feedback inhibition of the HPA axis induced by prenatal ethanol exposure. However, differential responsiveness was seen in males.
and females depending on the time of day when testing occurred. During the trough of the CORT circadian cycle, E males had greater CORT responses to ether vapour than C males at 3 h following injection of low doses of dexamethasone (DEX) (0.1 and 0.5 μg/100 g body weight (bw). There were no significant differences among E, PF and C males at 3 or 6 h following injection of high dose DEX and no significant differences in ACTH levels among E, PF, and C males at any time or dose tested. In contrast, at 6 h post-injection, E females given a low dose of DEX (1.0 μg/100 g bw) had higher CORT responses to ether vapour than C females given the same dose of DEX. At high doses of DEX, E females had higher CORT responses to ether vapour than PF and C animals at 3 h following 10.0 or 30.0 μg/100 g bw DEX and at 6 h following 30.0 μg/100 g bw DEX. Importantly, during the peak of the circadian cycle, 3 h post injection of high doses of DEX (5.0 or 15.0 μg/100 g bw DEX for males and 10.0 or 30.0 μg/100 g bw DEX for females) both E males and females demonstrated increased CORT responses to ether vapour compared to C males and females. In addition, E females given 30.0 μg/100 g bw DEX had higher ACTH responses to ether vapour than PF and C females. Together these data suggest that in the PM, when the HPA axis is less sensitive to glucocorticoid feedback inhibition, stress CORT levels of both E males and E females as well as stress ACTH levels of E females were not effectively suppressed by DEX. Thus our data suggest that the insult of prenatal ethanol exposure affects both male and female offspring, but that there may be a sex-specific difference in the sensitivity of the mechanism(s) underlying HPA hyperresponsiveness (Osborn et al., 1996).
A third possible mechanism examined in this thesis for the HPA hyperresponsiveness seen in E animals may be an alteration in CRF biosynthesis and/or secretion of CRF by the hypothalamus. CRF transcription measured by CRF mRNA expression in the PVN has been shown to be negatively regulated by glucocorticoids (Jingami et al., 1985; Young et al., 1986). Lee et al. (1990) demonstrated increased CORT levels in E animals following inescapable shock, as well as an increase in paraventricular nucleus (PVN) CRF mRNA in nonstressed E animals compared to controls. We examined the effects of prenatal ethanol exposure on CRF, AVP and GR mRNA expression in the hypothalamus following ether stress in DEX-treated animals. There were no significant differences in basal CRF, AVP or GR mRNA levels among E, PF, and C males or females. In addition, there were no significant differences in stressed AVP or GR levels among E, PF and C males or females. DEX had little effect on CRF mRNA expression suggesting DEX does not exert significant feedback inhibition at the levels of the hypothalamus. Importantly, E males showed a trend toward higher stress CRF mRNA levels than PF males and E females had significantly higher stress CRF mRNA levels than PF females and showed a trend toward higher stress CRF mRNA levels than C females. Thus, our data and that of Lee et al. (1990) suggest that prenatal ethanol exposure may have a long-term effect on the HPA axis including alterations in synthesis of CRF mRNA. It remains to be determined if the increase in gene expression is reflective of an increase in synthesis and/or release.

The second set of studies focused on mechanisms underlying the behavioural alterations seen in E animals. Adult E animals demonstrate hyperactivity (Bond, 1981;
Meyer & Riley, 1986), deficits in response inhibition (Driscoll et al., 1985; Riley et al., 1979a; Riley et al., 1979b), and increased anxiety-like behaviours in stressful environments (Anandam et al., 1980; Hannigan et al., 1987). The initial set of studies utilised the elevated plus maze (+-maze) to investigate behavioural alterations seen in E animals. The +-maze was utilised as it has been demonstrated to be a valid and reliable measure of anxiety/fear as measured by behavioural, physiological, and pharmacological responses (Lister, 1987; Pellow et al., 1985). E males and females both demonstrated increased exploration on the +-maze when placed directly on the maze compared to C males and females. In addition, E males demonstrated decreased fear levels compared to C males while E females demonstrated increased fear levels compared to PF females. In contrast, when exposed to the open field (OF) prior to the +-maze, a procedure which has been shown to behaviourally activate the rodents (Pellow et al., 1985), both E males and females demonstrated decreased exploration. Further, E females continued to demonstrate increased fear compared to C females whereas the decreased fear found in E males compared to C males was no longer apparent. Furthermore, there were no significant differences in CORT levels among E, PF, and C males or females when confined to the open or closed arm but when exposed to both the OF and the +-maze E females had higher CORT levels than PF and C females. Thus these data indicate that E males and females demonstrate behavioural differences on the +-maze compared to PF and C males and females and that these alterations in behaviour can be modified by prior experience. There does, however, appear to be a sexual dimorphism in the mechanisms mediating the behaviour; E males demonstrated no alterations or decreased fear whereas E females demonstrated increased fear. In addition, it appears the altered behaviour seen
in E animals can occur in parallel with HPA hyperresponsiveness or in isolation suggesting that the elevated CORT levels in E animals may not be mediating the alterations in +-maze behaviour.

The second set of behavioural studies investigated possible mechanisms for altered behaviour seen on the +-maze. Both the GABA-ergic system (Ledig et al., 1988; Ledig et al., 1993; Moloney & Leonard, 1984; Rawat, 1977) and hypothalamic CRF mRNA (Lee et al., 1990; Osborn et al., 1995) has been shown to be altered by prenatal ethanol exposure. In addition, the GABA agonist benzodiazepine (BZD) (File et al., 1992; Pellow et al., 1985) and CRF agonists/CRF antagonists (Heinrichs et al., 1994; Koob et al., 1993) have been widely tested on the +-maze. The studies in this dissertation investigated the effects of prenatal ethanol exposure on BZD sensitivity and on central and peripheral CRF sensitivity as measured by the +-maze.

The data indicated that E males and females appear to be differentially sensitive to benzodiazapine (BZD) compared to their PF and C counterparts. Both E males and females spent more time on the open arms and less time on the closed arms following BZD treatment compared to PF and C males and females. These data suggest that the altered behaviour on the +-maze may in part be mediated by alterations in the GABA-ergic system. In contrast, the study investigating central CRF sensitivity suggested no significant differences in sensitivity among E, PF and C animals. However it appeared that methodological issues may have influenced the results. First, the doses of CRF and
α-helical CRF used may have been subthreshold and therefore had no or minimal effect on + maze behaviour. Second, the method of getting the CRF and α-helical CRF into solution may have partially inactivated it. Furthermore, recent studies have indicated that α-helical CRF may possess anxiogenic-like as well as anxiolytic-like effects depending upon the endogenous level of CRF (Menzaghi et al., 1994). Therefore, it is premature at this time to reject the hypothesis that altered behavioural responses to stress may be mediated by altered sensitivity to central CRF in E animals. This study is currently being replicated in Dr. Weinberg’s laboratory.

Together, the data presented in this thesis suggest that several physiological mechanisms may interact to produce the HPA hyperresponsiveness and altered behaviour seen in E animals. Alterations both in feedback inhibition of CORT and in CRF synthesis may mediate the HPA hyperresponsiveness. In addition, the altered behaviour in E compared to PF and C animals on the + maze may in part be mediated by alteration in the GABA system. Although these studies have revealed several possible mechanisms by which prenatal ethanol exposure may affect HPA function and behaviour later in life, many questions still remain.

1.) Research is required to investigate the possibility that E animals may be differentially sensitive to ACTH. As mentioned previously, the adrenal gland markedly changes its sensitivity to ACTH during the circadian cycle, being maximally sensitive during lights-off and minimally sensitive during lights on (Kaneko et al., 1981). Further,
our data indicate that there was a differential responsiveness of the HPA following DEX suppression depending upon the time of day tested. That is, during the trough of the CORT circadian rhythm only, E females demonstrated increased stress CORT levels, whereas in the peak of the circadian cycle, both E males and E females demonstrated elevated CORT levels compared to their respective controls. Therefore, it is possible that differential adrenal sensitivity to ACTH in E compared to PF and C animals might be observed at the peak of the circadian rhythm. An additional study should be conducted in which CORT responses to ACTH are examined during the peak of the CORT circadian cycle.

2.) Although these data support the hypothesis that HPA hyperresponsiveness seen in E animals may result from deficits in feedback inhibition of CORT, it still remains to be determined at what level(s) and during what time frame(s) these deficits occur. The present study suggests that the deficits occur at the level of the pituitary during the intermediate feedback domain, as DEX binds preferentially to the pituitary (DeKloet et al., 1975) and exerts the majority of its effects at the pituitary level (Spencer et al., 1995). In support of this suggestion, a recent study in our lab has demonstrated that during the trough of the CORT circadian cycle, DEX-suppressed E females have increased ACTH responses to exogenously administered CRF compared to DEX-suppressed PF and C females (Yu et al., 1996). As previously discussed, Taylor et al. (1986b) found there are deficits in fast feedback inhibition in E animals. Thus, future studies should further investigate the deficits in feedback inhibition using other suppressing agents such as exogenous CORT which has been shown to provide feedback.
input to both the pituitary and higher brain centres (Dallman et al., 1987). As well, feedback inhibition of CORT should be investigated during all 3 time domains (Keller-Wood & Dallman, 1984).

3.) It is possible that the HPA hyperresponsiveness seen in E animals is a result of increased synthesis of CRF. As previously mentioned, Lee et al. (1990) demonstrated elevated basal levels of CRF mRNA in E animals and data from the present thesis indicated elevated stress levels of CRF mRNA in E animals compared to controls, even though the sampling time was not optimal. Future studies should evaluate CRF mRNA in both undisturbed and stressed E, PF and C animals. The time of sampling post-stress should be 120-180 min which has been shown to be the time of peak CRF mRNA expression (Lightman et al., 1993). Studies should also evaluate CRF mRNA in both exogenous CORT suppressed and non-suppressed animals to differentiate between CRF mRNA expression in basal conditions and during feedback inhibition of glucocorticoids.

4.) It still remains to be determined if increased sensitivity to central CRF plays a role in the altered behavioural responses to stressful environments seen in E animals. Presently, a study is being run in our lab utilising multiple doses of CRF as well as a more specific CRF antagonist, [D-Phe12, Nle31,38, Cα MeLeu37] h/ rCRF12-41 (D-Phe CRF) (Menzaghi et al., 1994) to address this question more completely. D-Phe CRF, unlike α-helical CRF, does not have any CRF agonistic activity and has an extended duration of
action making it a more effective agent with which to examine the role of CRF in behavioural responses to stress.

5.) It is possible that the alteration in behaviour on the +-maze seen in E animals is mediated by the GABA-ergic system. Although the study in this thesis demonstrated a differential sensitivity to BZD in E animals compared to PF and C animals, the study only evaluated 1 dose in a small number of animals. A more complete investigation utilising a variety of doses should be conducted on a larger number of animals, in order to evaluate better the possibility of altered sensitivity to BZD in E compared to PF and C animals. Further, other GABA-ergic agents such as neurosteroids should be utilised to test the hypothesis that the alteration in behaviour seen in E animals is a result of deficits in the GABA-ergic system.

In addition to these future lines of research, the present data may have clinical implications; however, caution must be used when extrapolating data from animal studies to the human condition. As discussed previously, developmental and biological differences may exist between humans and rodents which may affect the way in which prenatal alcohol exposure affects the developing brain. Nevertheless, a number of similarities have been demonstrated between children prenatally exposed to alcohol and rodents prenatally exposed to ethanol. Children prenatally exposed to alcohol are hyperactive, have uninhibited and impulsive behaviour and have attention deficits which may reflect an inability to inhibit responses (Streissguth et al., 1983; Streissguth et al.,
Similarly, rodents prenatally exposed to ethanol demonstrate behavioural hyperactivity and deficits in response inhibition in a variety of tasks (Becker & Randall, 1989; Bond & DiGiusto, 1977b; Caul et al., 1979; Fernandez et al., 1983; Means et al., 1984; Melcer et al., 1994; Molina et al., 1984; Riley et al., 1986; Shah & West, 1984; Vorhees & Fernandez, 1986). Furthermore, both children (Jackson et al., 1993) and rodents (Lee & Rivier., 1993b; Nelson et al., 1986; Taylor et al., 1982; Weinberg 1992b; Weinberg, 1988; Weinberg & Gallo, 1982) prenatally exposed to alcohol demonstrate elevated CORT levels following stress. Glucocorticoids (McEwen et al., 1986) and CRF (Nemeroff, 1992) are known to modulate behaviour during stress; thus, it is possible that sustained increases in hormones of the HPA axis could play a role in mediating the hyperactivity and behavioural arousal observed in foetal alcohol-exposed children. The studies in this dissertation attempting to investigate this possibility, however, were inconclusive. Further studies must address this issue.

Prenatal ethanol exposure not only results in HPA and behavioural hyperactivity, but also produces cognitive deficits in humans (Streissguth et al., 1983; Streissguth et al., 1985; Streissguth, 1986) and rodents (Abel, 1979; Barron et al., 1988; Blanchard et al., 1990). It has been documented that high levels of CORT both acutely (Diamond & Rose, 1994; Kant; 1993) and chronically (Luine et al., 1994) can decrease cognitive function in rodents. Similarly, cold stress has been associated with a decrease in cognitive function in humans (Sharma & Panwar, 1987). Further, major depression, dementia of the Alzheimer type, and Cushing’s syndrome all result in increased cortisol levels along with
decreases in cognitive function (Martignoni et al., 1992). Thus it is possible that sustained increases in hormones of the HPA axis could play a role in mediating the decreased cognitive function observed in foetal alcohol-exposed children.

Finally, studies have demonstrated alterations in the cholinergic (Janiri et al., 1994; Rudeen & Weinberg, 1993) as well as GABA-ergic systems (Janiri et al., 1994; Ledig, 1988; Savage et al., 1995; Zimmerberg et al., 1995) in prenatally alcohol-exposed animals. Cholinergic and BZD receptor agonists and antagonists have been shown to modulate learning and memory (Izquierdo & Medina, 1991). Anxiolytic agents which enhance GABA binding impair memory whereas anxiogenic agents which inhibit GABA binding enhance memory (Izquierdo & Medina, 1995). It has been shown that cholinergic agonists improve learning and memory while BZD impairs learning and memory in E animals to a greater extent than in control animals (Petkov et al., 1991). Thus, it is possible that alterations in the cholinergic and GABA-ergic systems may play a role in the behavioural and/or cognitive deficits seen in children prenatally exposed to alcohol.

In summary, these experiments demonstrate that prenatal ethanol exposure has long term effects on both HPA and behavioural responses to stress. Further, they indicate that both alterations in feedback inhibition of CORT and increased synthesis of CRF may modulate the HPA hyperresponsiveness to stress seen in E animals. In addition, they suggest that the alterations in behavioural responses to stress seen in E animals may be modulated by alterations in the GABA-ergic system.
REFERENCES


Armario, A., Montero, J.L. and Balasch, J. (1986b) Sensitivity of corticosterone and some metabolic variables to graded levels of low intensity stresses in adult male rats. *Physiol Behav* **37**, 559-561.


Negoro, H., Visessuwan, S. and Holland, R.C. (1973b) Unit activity in the paraventricular nucleus of female rats at different stages of the reproductive cycle and after ovariectomy, with or without oestrogen or progesterone treatment. *J Endocrinol* 59, 545-558.


