EFFECTS OF PRENATAL ETHANOL EXPOSURE IN RATS ON MULTIPLE ENDPOINTS OF CENTRAL SEROTONERGIC FUNCTION

by

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ABSTRACT

Maternal consumption of alcohol during pregnancy produces a wide range of abnormalities in the offspring. The main objective of this thesis was to examine long-term functional changes in central 5-HT receptor function of female and male rats prenatally exposed to ethanol. Serotonin receptor function was assessed through pharmacological challenge with the 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptor agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), respectively. Adult offspring of Sprague-Dawley rats from prenatal ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) dams were examined.

The first study (Chapter III) investigated 8-OH-DPAT-induced hypothermia and DOI-induced Wet Dog Shakes (WDS). Both E females and males showed a greater hypothermic response to 8-OH-DPAT than PF and C animals. In addition, E females and males also showed less of a differential response to a low and high dose of 8-OH-DPAT than PF and C animals. In response to DOI, E females but not males, showed a significantly greater rate of WDS than PF and C females.

The second study (Chapter IV) examined expression of anxiety-like behaviour and the anxiolytic response to 8-OH-DPAT in the novelty-induced suppression of feeding task. There was a marked drop in the number of E animals feeding in the novel environment compared to PF and C animals. The observed increase in anxiety-like behaviour in E females was ameliorated by 8-OH-DPAT, suggesting that expression of anxiety-like behaviour in this task is partially mediated by the 5-HT\textsubscript{1A} receptor.

The third study (Chapter V) examined 8-OH-DPAT- and DOI-induced increases in plasma adrenocorticotropic hormone (ACTH) and corticosterone. Corticotropin releasing hormone (CRH) mRNA and 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptor mRNA expression was also measured. E females had an attenuated ACTH response to 8-OH-DPAT, but a potentiated ACTH response to DOI, in comparison to PF and C females. Furthermore, 8-OH-DPAT increased
5-HT\textsubscript{1A} mRNA expression in the hippocampus in E females compared to PF and C females. E males also showed increased CRH mRNA levels in response to DOI.

These data indicate that prenatal ethanol exposure results in long-term effects on 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptor-mediated behavioural and physiological function in adult animals and that some of these effects may be sex specific.
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LIST OF ABBREVIATIONS

ACTH – adrenocorticotropic
ANOVA – analysis of variance
ARBD – alcohol-related birth defects
ARND – alcohol-related neurodevelopmental disorder
°C – degrees Celsius
C – prenatal control diet condition (ad libitum-fed)
cc – cubic centimetre
CORT - corticosterone
cm – centimeter
CNS – central nervous system
CRH – corticotropin releasing hormone
DOI - (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride
dl – decilitre
DG – dentate gyrus
E – prenatal ethanol diet condition
FAE- Fetal Alcohol Effects
FAS – Fetal Alcohol Syndrome
Fig. – figure
g – gram(s)
GR – glucocorticoid receptor
hr – hour
HPA – hypothalamic-pituitary-adrenal
kg – kilogram
mCPP – meta-chlorophenylpiperazine
MDMA – 3,4-methylenedioxymethamphetamine
min – minute(s)
mRNA – messenger ribonucleic acid
NaCl – sodium chloride
ng – nanograms
PCPA – para-chlorophenylalanine
PF – prenatal pair-fed diet condition
pg – picograms
PN – postnatal
PVN – paraventricular nucleus
sc – subcutaneous
SEM – standard error of the mean
WDS – wet dog shakes
5-HIAA – 5-hydroxyindoleacetic acid
5-HT – 5-hydroxytryptamine (Serotonin)
5-HTP – 5-hydroxytryptophan
5,7-DHT – 5,7,-dihydroxytryptamine
8-OH-DPAT – 8-hydroxy-2-(di-n-propylamino)tetratin
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"If we knew what we were doing we wouldn’t call it research"

Einstein
CHAPTER I: GENERAL INTRODUCTION

Maternal consumption of alcohol during pregnancy has serious consequences for the developing fetus. From 1968-1973, Fetal Alcohol Syndrome (FAS) became clinically recognized (Jones et al. 1973; Lemoine et al. 1968). Despite knowledge of this preventable disorder for the past three decades, the syndrome remains a major cause of developmental disability. The USA has the highest incidence of FAS in the world (Abel 1998a). Furthermore, research suggests that as many as 49% of women consume alcohol prior to, or during pregnancy (Abel 1998b), with effects on offspring ranging from subtle to severe.

Since prenatal ethanol exposure affects a developing organism, many systems can be severely affected. Serotonin is a neurotransmitter and neurotrophic factor that appears early in development and is thus susceptible to prenatal insult. The experiments in this thesis utilized a rodent model of prenatal ethanol exposure to investigate the long-term impact of prenatal alcohol on the function of the serotonergic neurotransmitter system.

1.A. Effects of Prenatal Alcohol Exposure

Children of mothers who consumed large amounts of alcohol during pregnancy may show numerous physical, physiological and functional deficits. A diagnosis of Fetal Alcohol Syndrome is assigned to a subset of individuals meeting the following four criteria: 1) confirmed maternal alcohol exposure; 2) facial anomalies, (i.e., flat midface, deficient philtrum, thin upper lip, low set ears, short palpebral fissures and depressed nasal bridge); 3) pre and/or postnatal growth retardation (e.g., low birth weight); and 4) central nervous system neurodevelopmental abnormalities (e.g., decreased cranial size at birth, structural brain abnormalities, impairment in fine motor skills). A child may also receive a diagnosis of “FAS without confirmed maternal alcohol exposure” if the first criterion is not met.
Although behavioural abnormalities are not required to be present for diagnosis of FAS, parents and guardians often describe children with FAS in behavioural terms. The recently developed Fetal Alcohol Behavior Scale captures these behavioural descriptors in a 36-item scale (Streissguth et al. 1998). Examples of the items included in this scale are “poor attention”, “overstimulated”, “noise sensitive”, “mood swings”, “overreacts”, “overly friendly” and “fidgety”. Children with FAS also tend to be hyperactive and impulsive. These children also typically have a lower IQ and show poorer learning and memory skills compared with developmentally normal children (Streissguth 1986; Streissguth et al. 1985). A diagnosis of Partial FAS may be assigned if there is confirmed maternal alcohol consumption and some, but not all, of the criteria of FAS are present. There are two categories of Alcohol-Related Effects [formerly Fetal Alcohol Effects (FAE)]. Alcohol-Related Birth Defects (ARBD) and Alcohol-Related Neurodevelopmental Disorder (ARND) may be assigned together or separately in those with a history of maternal alcohol consumption. ARBD is assigned to individuals with cardiac, skeletal, renal, ocular, auditory and other anomalies, malformations or dysplasias. ARND is assigned to individuals with central nervous system developmental abnormalities including decreased cranial size, structural brain morphology, and neurological hard or soft signs (e.g., impaired motor skills) as well as the same pattern of behavioural or cognitive abnormalities as observed in children with FAS.

Several animal models of prenatal alcohol exposure have been developed that effectively model many of the characteristics observed in children with FAS, including behavioural and cognitive abnormalities. Rodents prenatally exposed to ethanol (E) show body and brain growth deficiencies (Gallo and Weinberg 1986; Abel and Dintcheff 1978), decreased brain myelination (Özer et al. 2000), spatial learning and memory deficits (Berman and Hannigan 2000), and numerous physiological changes including hormonal
hyperresponsiveness to stressors and immune challenge (Kim et al. 1999a; Weinberg et al. 1996), as well as immune and neurotransmitter deficits (Tran and Kelly 1999; Giberson and Weinberg 1993; Rudeen and Weinberg 1993). Mice and rats both show prenatal ethanol-induced craniofacial anomalies (Ismail and Janjua 2001). Therefore, rodents exposed to ethanol prenatally can provide a valuable model for investigation into the causes of the behavioural and cognitive deficits reported in children with FAS.

Because rodents do not readily consume ethanol, it is most effectively administered with minimal stress to the animal, in conjunction with a nutritionally complete liquid diet. Ethanol administered in this form, with all the nutrients required by pregnant dams, results in high blood alcohol levels and adequate nutritional status (Weinberg 1985). However, a nutritional control group is often included in experiments using this model. Pair-fed dams receive a liquid diet with maltose dextrin substituted for ethanol, in an amount matched to an ethanol-consuming female. This control group is necessary because the high caloric content of ethanol can displace nutrients, resulting in malnutrition. The inclusion of the pair-fed control group allows experimenters to isolate the teratogenic effects of prenatal ethanol from the effects of malnutrition. In addition, a third control group is included consisting of dams fed standard laboratory rat chow ad libitum throughout pregnancy. Thus, offspring from ethanol-fed (E), pair-fed control (PF) and ad libitum-fed control (C) dams were used in the experiments contained in this thesis.

1.B. Effects of Prenatal Ethanol Exposure on the Neurotransmitter Serotonin

One of the first neurotransmitter systems to develop in the brain, and therefore a neurotransmitter susceptible to early prenatal insult, is the serotonergic system. Serotonin or 5-hydroxytryptamine (5-HT) is synthesized in two steps. First, tryptophan is converted to 5-
hydroxytryptophan (5-HTP) through the action of the rate-limiting enzyme, tryptophan hydroxylase. Second, 5-HTP is converted to 5-HT by 5-HTP decarboxylase. The major metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), is produced by the action of monoamine oxidase. Serotonin is produced by serotonergic cell bodies, almost all of which are located in the raphe nuclei. The raphe nuclei have diffuse efferent projections and there are 14 subtypes of serotonin receptors widely distributed throughout the brain, which contribute to the diversity of function of the system. Serotonergic agents are used clinically to treat a variety of conditions including anxiety, depression, alcoholism, migraines, eating disorders and gastrointestinal upset.

There are several compelling reasons to examine the effects of prenatal ethanol on the 5-HT system. First, the 5-HT system is greatly affected by prenatal ethanol exposure. In comparison to control animals there are decreased levels of brain 5-HT and 5-HIAA in fetal (Maier et al. 1996), preweanling (Druse et al. 1991), and adult (Kršiak et al. 1977) E animals. A decrease in whole brain 5-HT under basal conditions was also found in 1, 2 and 6 month old mice prenatally exposed to ethanol (Elis et al. 1976; Elis et al. 1978) indicating that the effects of prenatal ethanol exposure on 5-HT levels are long lasting. Whole brain 5-HT content is also decreased in rats following prenatal ethanol exposure (Maier et al. 1996), and more specifically, decreased 5-HT has also been found in the brainstem and cortical regions (Druse et al. 1991). Prenatal ethanol reduces the number of 5-HT containing neurons in the brain. The number of 5-HT immunoreactive neurons in the dorsal and median raphe nuclei (Zhou et al. 2001; Tajuddin and Druse 1999) and the medial forebrain bundle, a major serotonergic projection pathway (Sari et al. 2001), are greatly reduced by prenatal ethanol exposure. Serotonin transporters are also affected by prenatal ethanol exposure. In comparison to PF and C animals, 5-HT transporter levels are decreased in cortex, hippocampus and the dorsal raphe nucleus, but increased in the medial amygdala
nucleus and dorsomedial and ventromedial hypothalamus (Zafar et al. 2000). A decrease in 5-HT transporter sites in the frontal and parietal cortices, lateral hypothalamus, substantia nigra, septum, and striatum has also been found in E rats (Kim and Druse 1996).

Of all of the 5-HT receptor subtypes, only the \( 5\text{-HT}_{1A} \) and \( 5\text{-HT}_{2A} \) have been examined in E animals and these studies report regional changes in \( 5\text{-HT}_{1A} \) receptors. Receptor binding studies have found an increase in \( 5\text{-HT}_{1A} \) receptors in E compared to PF and C animals in the dentate gyrus of the hippocampus and in brain stem, but a decrease in the motor and somatosensory areas of the cortex (Druse et al. 1991). In addition, normal developmental changes in \( 5\text{-HT}_{1A} \) receptor number are altered by prenatal ethanol exposure. Normally, during the preweaning period, the first three weeks of life, there is a decrease in \( 5\text{-HT}_{1A} \) receptors in the raphe nuclei and an increase in the cortex; however these changes are delayed by prenatal ethanol exposure. Less is known regarding prenatal ethanol effects on \( 5\text{-HT}_{2A} \) receptors. However, these receptors do not seem to be affected in cortical areas in young animals (Druse et al. 1991).

Second, although currently speculative, the prenatal ethanol-induced decrease in 5-HT, its receptors and transporter may be implicated in the resulting craniofacial abnormalities, the hallmark feature of FAS. In rodents, normal craniofacial development appears to depend on a certain range of 5-HT levels and the presence of the \( 5\text{-HT}_{1A} \) receptor (Moiseiwitsch and Lauder 1995). Neural crest cells contain \( 5\text{-HT}_{1A} \) receptors and the appropriate dose of 5-HT stimulates normal migration of these cells (Moiseiwitsch and Lauder 1995). In addition, inhibiting 5-HT uptake into craniofacial epithelia results in craniofacial defects in whole embryonic mice cultures (Shuey et al. 1992). Therefore, prenatal ethanol-induced alterations in 5-HT levels or the \( 5\text{-HT}_{1A} \) receptor may affect craniofacial development in E offspring.
Third, behavioural and physiological abnormalities that occur following prenatal ethanol exposure in humans and rodents are consistent with and suggest altered 5-HT function. Individuals with FAS or other related diagnoses show an increased incidence of psychiatric disorders, including depression and anxiety (Famy et al. 1998). The role of 5-HT in the regulation of mood is complex, and it is conceivable that prenatal ethanol-induced alterations in 5-HT or any of its receptors may contribute to depression, anxiety or substance abuse (Tollefson 1989; Kahn et al. 1988). In addition, in response to stressors infants prenatally exposed to ethanol (Jacobson et al. 1999) and E rodents (Weinberg et al. 1996), show increased activation of the hypothalamic-pituitary-adrenal (HPA) axis. Stressors stimulate release of corticotropin releasing hormone (CRH) from the hypothalamus that in turn stimulates adrenocorticotropin (ACTH) release from the pituitary. The final step in the HPA axis cascade is release of corticosteroids, such as corticosterone (CORT), from the adrenal gland in response to ACTH. Serotonin is an important neurotransmitter involved in regulation of the HPA axis (Chaouloff 1993). Therefore, altered HPA function following prenatal ethanol exposure may in part be due to the aforementioned alterations in the 5-HT system. In comparison to PF and C animals E animals show deficits in response inhibition (Riley et al. 1979) that could indicate perseverative behaviour and/or impulsivity. Both clinical and experimental studies correlate decreased 5-HT levels with impulsive behaviour (Ho et al. 1998). Similarly, E mice exhibit increased aggression (Kršiak et al. 1977), also associated with decreases in 5-HT levels (Dee Higley et al. 1996). Impaired sexual behaviour in E males (Ward et al. 1994) may also implicate altered 5-HT function, as this neurotransmitter and its receptors are known to influence sexual behaviour (Gorzalka et al. 1990). E animals also show increased locomotor activity (Kaneko et al. 1993), suggestive of altered 5-HT$_{1A}$ receptor function (Mignon and Wolf 2002).
I.C. Thesis Objective

Although prenatal ethanol-induced changes in 5-HT neurotransmitter levels, the 5-HT$_{1A}$ receptor and neuronal number are well documented, little is known about what functional consequences arise for the organism from these alterations. There are five published reports of 5-HT mediated function in rats prenatally exposed to ethanol, three reporting no alterations, and two reporting an increase in 5-HT-mediated function in E animals. Haenlein et al. (1983) reported that E animals were similar to control animals in discriminating 5-HTP, the precursor to 5-HT, from saline. The authors concluded that central 5-HT receptor function was not altered. Similarly, Dicerbo and Hannigan (1991) and Bond (1986) examined locomotor activity in response to administration of various serotonergic agents in E, PF and C animals and found that activity levels were not differentially affected. Fulginiti et al. (1992) found that acute prenatal ethanol exposure resulted in increased behavioural responses (e.g., wet dog shakes, forepaw treading) to 5-HT agonists in rats 45 days old, but effects were diminished in 90-day-old rats. Finally, Grace et al. (1986) found that prenatal ethanol exposure during the first week of gestation but not the second, third, or all three weeks resulted in an increased suppression of ethanol consumption in response to administration of the 5-HT uptake inhibitor, zimelidine. This latter study indicates increased receptor sensitivity in response to zimelidine-induced 5-HT release. These studies were limited in that both sexes were not always examined and the length of prenatal ethanol exposure varied between 1 and 14 days.

Increased 5-HT receptor function, due to increased number, affinity or other mechanisms, might be expected as a compensatory response for 5-HT deficits. E animals show many similarities to animals that receive lesions of the serotonergic system prenatally, neonatally or in adulthood. These studies are conducted to examine the role of 5-HT in a
particular behaviour or physiological process or to model the effects of drugs of abuse. Similar to the prenatal ethanol-induced decrease in 5-HT levels, administration of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) or para-chlorophenylalanine (PCPA), an inhibitor of 5-HT synthesis, results in long term depletion of 5-HT levels. Breese and Cooper (1975) found that administration of 5,7-DHT resulted in decreases in 5-HT in several brain regions of greater than 50%, levels comparable to the 20-50% decreases in 5-HT and 5-HIAA found in E animals (Rathbun and Druse 1985). In addition, both ethanol- and PCPA-treated dams have offspring with lower birth weights (Shemer et al. 1998; Weinberg 1995). Animals with serotonergic lesions also show increased 5-HT receptor-mediated behavioural and physiological function. For example, lesioned adult male rats show an increase in aggressive behaviour and male mice show an increase in muricidal behaviour compared to nonlesioned animals (Breese and Cooper, 1975). Administration of 3,4-methylenedioxymethamphetamine (MDMA) to adult animals, another neurotoxin, was reported to increase anxiety-like behaviour in the elevated plus-maze and social interaction test (Gurtman et al. 2002) and tryptophan depletion resulted in an increase in anxiety-like behaviour in the open field (Blokland et al. 2002). Following 5-HT depletion an enhancement of 5-HTP-induced suppression of operant behaviour (Breese and Cooper 1975), increased hormonal responses to RU 24969, a 5-HT1A/B agonist (Van de Kar et al. 1989), increased 8-OH-DPAT-induced hypothermia (Aguirre et al. 1998) and locomotor activity (Mignon and Wolf 2002) have also been observed. Increased 5-HT receptor function in 5-HT depleted animals is termed "supersensitivity" and is thought to reflect a compensatory response to decreased 5-HT stimulatory input. In summary, prenatal, neonatal or adulthood depletion of 5-HT results in increased 5-HT receptor-mediated function. This may suggest that E offspring, with prenatal-ethanol-induced depletion of 5-
HT levels should also display increased 5-HT receptor-mediated function or supersensitivity.

The objective of this thesis is to examine the effect of prenatal ethanol exposure on long-term central 5-HT function. We hypothesized that the effects of prenatal ethanol exposure on the 5-HT system would parallel the effects of prenatal, neonatal and adult depletion of 5-HT with neurotoxins. This thesis proposes that supersensitive 5-HT receptors may also exist in E animals as a result of deficits in 5-HT neurotransmitter levels early in development, resulting in increased 5-HT function in adult animals. If present, supersensitive 5-HT receptors may be an explanation for the aforementioned behavioural and physiological abnormalities in E animals. For example, in comparison to PF and C animals, E animals exhibit increased levels of HPA hormones in response to a variety of stressors (Weinberg et al. 1996). 5-HT receptors can mediate increases in stress hormones and importantly, increased 5-HT levels are observed in several brain regions in response to stress. Therefore, increased hormonal responses to stressors in E animals may reflect increased 5-HT receptor sensitivity to stress-induced increases in 5-HT.

Two of the 14 receptor subtypes were chosen for examination for this thesis, 5-HT$_{1A}$ and 5-HT$_{2A}$ for several reasons. These receptors are more effectively studied than other 5-HT receptors as there are selective 5-HT$_{1A}$ and 5-HT$_{2A}$ agonists, allowing for pharmacological experimentation. The 5-HT$_{1A}$ receptor is involved in mediating the neurotrophic effects of 5-HT and the 5-HT$_{2A}$ receptor is involved in cell proliferation, synaptogenesis apoptosis (Azmitia 2001). The 5-HT$_{1A}$ receptor develops early in gestation and achieves peak levels on gestation day 15 in rats (Hillion et al. 1994), making this receptor susceptible to prenatal ethanol exposure as discussed above. The 5-HT$_{2A}$ receptor appears later in development than the 5-HT$_{1A}$ receptor (Morilak and Ciarnello 1993), but is still affected by prenatal events, such as prenatal stress (Peters 1988). Interestingly, 5-
HT₁A and 5-HT₂A receptor activation often exert opposite effects. 5-HT₁A activation results in hypothermia, a decrease in anxiety-like behaviour and hyperphagia while 5-HT₂A activation results in hyperthermia, an increase in anxiety-like behaviour and hypophagia. Although the two receptors differ in many functions, they do not have completely opposing roles. For example, both 5-HT₁A and 5-HT₂A agonists are capable of inducing an increase in stress hormones.

Central 5-HT₁A and 5-HT₂A receptor function was assessed through pharmacological challenge with serotonergic agonists. Because the serotonergic system is involved in numerous physiological and psychological processes, administration of various 5-HT agonists results in several measurable behavioural and physiological responses, including hyperthermia and hypothermia, wet dog shakes, locomotor activity, and several other behaviours collectively referred to as the 5-HT behavioural syndrome. These responses can provide an indication of central 5-HT function. The experiments in this thesis utilized the the 5-HT₁A and 5-HT₂A/C agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and (+)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) respectively.

Experiments in this thesis were designed to address three specific aims. Specific aim 1 (Chapter III) examined 5-HT₁A and 5-HT₂A mediated physiological and behavioural function in adult E, PF and C animals. We tested the hypothesis that 5-HT₁A mediated hypothermia and 5-HT₂A mediated wet dog shakes are increased by prenatal ethanol exposure. Specific aim 2 (Chapter IV) examined 5-HT mediated anxiety and anxiolytic response to the 5-HT₁A agonist 8-OH-DPAT. We tested the hypothesis that E animals would show a greater suppression of feeding (anxiety-like behaviour) in a novel environment compared to PF and C animals. We also tested the hypothesis that E animals would show a greater anxiolytic effect to the 5-HT₁A agonist 8-OH-DPAT, in the novelty-
induced suppression of feeding task compared to PF and C animals. Specific aim 3 (Chapter V) examined neuroendocrine responses to 5-HT receptor stimulation. We tested the hypothesis that 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor-mediated neuroendocrine responses are increased in E compared to PF and C animals. We also tested the hypothesis that 5-HT$_{1A}$ and 5-HT$_{2A}$ mRNA expression is increased in E compared to PF and C animals.

Although DOI is a 5-HT$_{2A/C}$ agonist, the dependent measures in this thesis reflect 5-HT$_{2A}$ receptor function. DOI-induced wet dog shakes are mediated through the drug's actions at the 5-HT$_{2A}$ receptor (Willins & Meltzer 1997). Similarly, DOI-induced increases in ACTH and CORT are also mediated through the 5-HT$_{2A}$ receptor (2001).
CHAPTER II: GENERAL METHODS

2.A. Breeding and Feeding of Animals

Sprague-Dawley breeding female (200 - 250g) and male rats (250 - 275g) were obtained from the Animal Care Centre, University of British Columbia. A 12:12 hr light-dark cycle was maintained with lights on from 06:00 – 18:00 hr. Animal room temperature was maintained at 21°C. Females were bred in hanging stainless steel cages (25 x 18 x 18 cm), with mesh front and floor. During mating animals were allowed ad libitum access to standard laboratory chow (Jamieson’s Pet Food Distributors Ltd., Delta, B.C.) and water. On the first day of gestation, determined by the presence of a vaginal plug, female rats were housed in polycarbonate cages (24 x 16 x 46 cm) lined with wood shavings and randomly assigned to one of three prenatal treatment groups: 1) Ethanol-fed (E) females received a liquid diet containing 36% ethanol-derived calories; 2) Pair-fed (PF) females received a liquid diet identical in nutrients and calories to that of E females, with maltose-dextrin substituted for ethanol, in an amount matched to that consumed by an E female, per kg body weight per day of gestation; 3) Ad libitum-fed control (C) females received unrestricted access to standard laboratory chow and water. Diets were formulated to meet the nutritional requirements of pregnant dams (Weinberg and Bezio 1987) and were prepared by Bio-Serv, Inc (Frenchtown, NJ). Animals were maintained on experimental diets until gestation day 21. Diet bottles were weighed and replaced daily at approximately 16:00 hr. As the corticoid rhythm in animals receiving a reduced ration, such as PF animals, entrains to the time of feeding, animals were fed within 2 hours of lights off, permitting maintenance of a normal corticoid rhythm (Gallo and Weinberg 1981). Animals were tested in adulthood (60-150 days of age). No more than 1 female and 1 male were tested from one
litter. All experiments utilized 8-12 animals for from each prenatal treatment condition for each experimental testing condition except for the in situ hybridization study in Chapter IV. For analysis of mRNA, 5-8 animals from each prenatal treatment condition for each experimental testing condition were utilized. In all chapters, the number of animals in each testing condition is listed in the table and figure legends. All animal use procedures were in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

2.B. Drugs

8-OH-DPAT and DOI were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). All doses of drugs for all experiments were determined through pilot studies of published doses for each response examined. Drugs were dissolved in physiological saline (0.9% NaCl) on the day of usage. All drugs and saline were injected subcutaneously (s.c, dorsal flank) at a volume of 1 ml/kg while rats were loosely restrained. Animals were weighed 1 day prior to testing to determine body weight. As E animals may be more responsive to the stressful effects of drug injection, saline (vehicle) injection served as an appropriate control in all experiments.
CHAPTER III: EFFECTS OF PRENATAL ETHANOL EXPOSURE ON
SEROTONERGIC MEDIATED PHYSIOLOGICAL AND BEHAVIOURAL
FUNCTION

3.A. Introduction

E animals show behavioural and physiological changes consistent with altered 5-HT function. For example, E animals show response perseveration (Riley et al. 1979), increased aggression (Kršiak et al. 1977), decreased thermoregulatory ability (Jänicke and Coper 1993), altered sexual behavior and increased locomotor activity (Kaneko et al. 1993). However, these experiments have focused primarily on young animals, with less consideration of the long-term effects in adult animals. As well, previous studies have examined prenatal ethanol effects mainly in males. The purpose of the present study was to examine the long-term effects prenatal ethanol exposure on 5-HT receptor mediated behavioral and physiological responses in adult female and male rats. 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced hypothermia and (+)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) -induced wet dogs shakes (WDS) were studied in adult female and male offspring from ethanol exposed (E), pair-fed control (PF) and in ad libitum-fed control (C) dams as indices of central 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor function, respectively (Schreiber et al. 1995; Watson and Gorzalka 1990).

3.B. Methods

3.B.1. Experiment 1. 5-HT$_{1A}$ Receptor Function: 8-OH-DPAT-Induced Hypothermia

Animals were housed under a 12:12 hr light-dark cycle with lights off from 06:00 –
18:00 hrs for one month prior to testing at 70 - 90 days of age. This experiment was conducted 6 hrs after lights off, when 8-OH-DPAT-induced hypothermic responses are maximal (Lu and Nagayma 1997). All testing occurred in naive animals under red-light conditions. Core body temperatures were measured using a rectal thermistor thermometer (Cole-Parmer Instrument Co, Vernon Hills, IL) and flexible probe (YSI Series 400 Probe, Yellow Springs Instrument Co., Yellow Springs, OH) under constant environmental conditions. Procedures, including time points, were based on the protocol described in Weinberg et al. (1995). Animals were randomly assigned to receive an injection of saline, or 8-OH-DPAT (0.125 mg/kg or 0.500 mg/kg) on the day of testing. No more than one female or one male from any one litter was assigned to any test condition in order to control for litter effects. Basal (pre-injection) temperatures were recorded and then 8-OH-DPAT was injected and rectal temperature recorded at 30, 60, 90, 120 and 180 min post-injection. Rats were loosely restrained by the tail and the probe was inserted 5 cm into the rectum and allowed to equilibrate for 45 sec before temperature recording. Animals were placed individually into a clean new cage following injection and remained in a room adjacent to the colony room, with the same ambient temperature, for the duration of the experiment. Saline injection provided a control for the effects of injection over the 180 min testing period.

3.B.2. Experiment 2. 5-HT₂A Receptor Function: DOI-Induced Wet Dog Shakes

Animals were housed under a 12:12 hr light cycle with lights on from 06:00 – 18:00 hrs and animals were tested at 45 - 60 days of age since DOI-induced WDS decline in intensity after 60 days of age (Darmani and Ahmad 1999). All testing with DOI occurred approximately two hrs prior to lights off, since the WDS response to DOI is maximal at this time.
time (Nagayama and Lu 1996). WDS are a paroxysmal shudder of the head, neck and trunk, occurring either spontaneously or in response to certain stimuli, such as administration of 5-HT precursors, daily adrenocorticotropic (ACTH) treatment, or repeated stress (Kuroda et al 1992; Watson and Gorzalka 1990; Kennett et al. 1985; Yap and Taylor 1983; Bedard and Pycock 1977). WDS are believed to be part of the grooming repertoire and occur spontaneously, although at a low rate (Wei 1981). WDS were measured according to the methods of Hanson and Gorzalka (1999). Naive rats (one female or one male from each litter) received an injection of DOI (1 mg/kg s.c.) or isotonic saline (0.9% NaCl). Immediately after injection rats were placed in a holding cage. Recording of WDS began 30 min post-injection and continued for a period of 30 min. A score of one was assigned to each occurrence of a WDS.

3.B.3. Drugs

8-OH-DPAT and DOI were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). 8-OH-DPAT was administered at one of two doses, 0.125 mg/kg or 0.500 mg/kg, and DOI was administered at a dose of 1 mg/kg. Pilot studies in our laboratory of doses of 8-OH-DPAT ranging from 0.125 mg/kg to 1 mg/kg indicated that 0.125 mg/kg and 0.500 mg/kg represent low and high doses for induction of hypothermia (unpublished data). The dose of DOI was chosen based on the effective dose for induction of WDS (Hanson and Gorzalka 1999; Takao et al. 1997; Nankai et al. 1995) and because higher doses of the drug produce behavioural effects non-conducive to observation of WDS (unpublished observations). On each day of testing, drugs were dissolved in physiological saline (0.9% NaCl). All drugs and saline were injected subcutaneously (s.c, dorsal flank) at a volume of 1 ml/kg while rats were loosely restrained. As E animals may be more responsive to the
stressful effects of drug injection, vehicle (0/9% NaCl) injection served as an appropriate control in both experiments.

3.B.4. Statistical Analysis

Analyses of variance (ANOVA) were performed for the factors of sex, prenatal treatment, dose and time, with time as a repeated measure where appropriate. Significant main and interaction effects were further analyzed by Newman-Keuls post hoc analysis. Initial analysis revealed an effect of prenatal treatment on basal core body temperatures $[F(2, 83) = 6.72, p < 0.005]$. Basal temperatures in PF (38.38 ± 0.09 °C) females were significantly lower than in E (38.74 ± 0.06 °C) or C (38.62 ± 0.05 °C) females (p's < 0.005). In addition, repeated measures ANOVA on thermal responses to saline injection indicated an interaction between prenatal treatment and time for females $[F(8, 84) = 2.81, p < 0.01]$, and males $[F(8, 92) = 2.11, p < 0.05]$, reflecting a greater hyperthermic response in E and/or PF compared to C females (p's ≤ 0.01) (Fig. 1A) and in E and PF compared to C males (E = PF > C; p's ≤ 0.05) (Fig. 1B). Therefore, temperature data from experiment 1 were normalized and analyzed in two ways. First, each animal's basal pre-injection temperature was subtracted from the temperature at each post-injection time point and change from basal temperatures was analyzed. Second, to account for the hyperthermic effects of injection stress, temperatures in 8-OH-DPAT injected animals were also normalized to their saline injected counterparts. This was accomplished by subtracting the mean of each saline injected E, PF and C group from the temperature values of each animal injected with 8-OH-DPAT and then dividing by the standard deviation of the saline injected group.
3C. Results

3.C.1. Developmental Effects of Prenatal Ethanol Exposure

There was no effect of prenatal ethanol on the number of liveborn or stillborn pups. However, there were effects of prenatal ethanol on pup pre-weaning weight gain. A significant interaction between prenatal treatment and age for both females [F (6, 162) = 3.27, p < 0.005] and males [F (6, 162) = 2.27, p < 0.05] indicated that prenatal ethanol and pair-feeding resulted in lower average pup weights on postnatal day 21 (Table 1). This result is consistent with previous findings from our laboratory of decreased birth and pre-weaning body weights (Hofmann et al. 1999). There were no significant body weight differences on the day of testing for either experiment. However, for experiment 1 there was a trend (p = 0.06) toward decreased body weight in E compared to C females (Table 1).

3.C.2. Experiment 1: 5-HT\textsubscript{1A} Receptor Function

Response to 8-OH-DPAT: Females. Analysis of change from basal temperatures indicated that all animals showed a similar hypothermic response following administration of 0.500 mg/kg 8-OH-DPAT; however, effects of prenatal treatment emerged following 0.125 mg/kg 8-OH-DPAT [F (4, 77) = 2.73, p < 0.05]. At 30 min post-injection, where the peak hypothermic effect of 8-OH-DPAT is observed (Lu and Nagayama 1996; Cryan et al. 1999), there was a significantly greater drop in temperature in E (-1.58 ± 0.28 °C) compared to PF (-0.93 ± 0.17 °C) and C (-1.04 ± 0.19 °C) females (p's ≤ 0.05).

Analysis of data normalized to account for the hyperthermic effects of saline injection revealed a significant interaction among prenatal treatment, drug and time [F (8,
Table 1. The effects of prenatal ethanol exposure on mean pup weights at postnatal (PN) days 1, 21 and at the time of testing (g, mean ± SEM). * On PN 21 ethanol-fed (E) and pair-fed exposed (PF) offspring weighed significantly less than ad libitum-fed (C) offspring (p < 0.05). A trend (# p = 0.06) was observed at the time of testing (PN70-90) toward a decreased body weight in E compared to C females.

<table>
<thead>
<tr>
<th></th>
<th>PN1 Female</th>
<th>PN1 Male</th>
<th>PN21 Female</th>
<th>PN21 Male</th>
<th>PN70-90 Female</th>
<th>PN70-90 Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>5.5 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>*43.2 ± 1.7</td>
<td>*46.6 ± 1.5</td>
<td>#279.6 ± 4.8</td>
<td>474.8 ± 5.3</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>5.8 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>*46.5 ± 1.3</td>
<td>*48.4 ± 1.5</td>
<td>286.7 ± 4.5</td>
<td>478.8 ± 6.4</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>6.5 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>52.2 ± 5.6</td>
<td>55.5 ± 1.6</td>
<td>295.6 ± 4.8</td>
<td>491.8 ± 6.1</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Following administration of 0.125 mg/kg 8-OH-DPAT, E females showed greater hypothermia at 30 and 60 min post-injection compared to PF and C females (E > PF = C; p’s < 0.001). Similarly, 30 and 60 min following 0.500 mg/kg 8-OH-DPAT, E females showed a greater hypothermic response than PF and C females (E > PF = C; p’s < 0.0005), while at 60 min PF females also showed a greater response than C females (E > PF > C; p’s < 0.05).

Dose Response. Normalized data were further analyzed by the factors of drug and time for each prenatal treatment group. We found a differential hypothermic effect of the low and high doses of 8-OH-DPAT on E, PF and C females. At 30 and 60 min post-injection E [F (4, 80) = 4.58, p < 0.005], PF [F (4, 76) = 18.24, p < 0.0005] and C [F (4, 68) = 4.05, p = 0.005] females all showed a greater hypothermic response to the higher dose of 8-OH-DPAT (0.500 mg/kg) compared to the lower dose (0.125 mg/kg; all p’s < 0.05). However, at 90 and 120 min post-injection E females no longer show a differential response, whereas, PF and C females continue to show a greater hypothermic response to the higher compared to the lower dose of 8-OH-DPAT (Fig. 2).

Response to 8-OH-DPAT: Males. Unlike females, initial analysis on basal temperature data from males showed no differences (not shown). However, due to prenatal treatment effects on the hyperthermic response to saline, and for consistency of analysis and comparison with the data from females, data from males were also normalized in the two ways described in the statistical analysis section.

An ANOVA on change from basal temperatures revealed no significant effects of prenatal treatment. Analyses of normalized data to account for the hyperthermic effects of injection revealed significant interactions between prenatal treatment and time [F (8, 216) =
Figure 1. Hyperthermic response to saline (1 ml/kg) injection in females and males expressed as change from basal (pre-injection) temperatures (n's = 8-10). There was a significant interaction between prenatal treatment and time for females (p < 0.01) and males (p < 0.05). Pair-fed (PF) females had a significantly greater hyperthermic response than ethanol-fed (E) and ad libitum-fed (C) females (^ p's < 0.05). E and/or PF animals are significantly different from C animals (* p's < 0.05).
20.76, p < 0.0001] and dose of 8-OH-DPAT and time [F (4, 216) = 8.33, p < 0.0001] (Fig. 3). Following administration of both doses of 8-OH-DPAT, E males showed greater hypothermia at 30 (E > PF > C, p’s < 0.001) and 90 (E > PF = C, p’s ≤ 0.005) min post-injection compared to PF and C males. However, at 60, 120 and 180 min post-injection, E and PF males both showed significantly increased hypothermia compared to C males (p’s < 0.05), suggesting that nutritional effects played a role in this response. At 120 min post-injection E and PF showed a similar hypothermic response (E = PF > C); however, at 60 and 180 min, PF showed the greatest hypothermic response (PF > E > C, p’s < 0.05), an effect of pair-feeding in itself.

**Dose Response.** Similar to females, analysis of normalized data revealed a differential hypothermic effect of the low and high doses of 8-OH-DPAT on E, PF and C males. At 30 min post-injection E [F (4, 76) = 2.81, p < 0.05], PF [F (4, 68) = 5.63, p < 0.0001] and C [F (4, 72) = 2.41, p = 0.05] males all showed a greater hypothermic response to the higher dose of 8-OH-DPAT (0.500 mg/kg) compared to the lower dose (0.125 mg/kg; all p’s < 0.05). However, at 60 and 90 min post-injection E males no longer show a differential response whereas PF and C males continue to show a greater hypothermic response to the higher than to the lower dose of 8-OH-DPAT (Fig. 3).

3.C.3. **Experiment 2: 5-HT₁A Receptor Function**

An ANOVA revealed an interaction between prenatal treatment and drug [F(2, 49) = 5.79, p < 0.01] for females. Post hoc analysis revealed that E females showed a
**Figure 2.** Hypothermic response to 8-OH-DPAT (0.125 mg/kg or 0.500 mg/kg) in females expressed as normalized scores (n’s = 8-10 per group). There was a significant interaction between prenatal treatment, drug and time (p < 0.05). At 30 min for both doses of 8-OH-DPAT and at 60 min for 0.125 mg/kg, ethanol-fed (E) females had a greater hypothermic response than pair-fed (PF) and ad libitum-fed (C) females (E > PF = C). For 0.500 mg/kg, at 60 min, E > PF > C (p’s < 0.05). For each prenatal treatment group, there was a significant interaction between drug and time (p’s ≤ 0.05). The hypothermic response to the two doses of 8-OH-DPAT was significantly different for PF and C females at 30, 60, 90 and 120 min post injection, but only 30 and 60 min post injection for E females (* p’s < 0.05).
Figure 3. Hypothermic response to low and high doses of 8-OH-DPAT (0.125 mg/kg or 0.500 mg/kg) in males expressed as normalized scores (n’s = 8-10 per group). There was a significant interaction between drug and time (p’s < 0.001), as well as prenatal treatment (ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C)) and time (p’s < 0.001). For both doses of 8-OH-DPAT, at 30 min E > PF > C and at 60 and 180 min PF > E > C. At 90 min E > PF = C and at 120 min E = PF > C (p’s < 0.05). For each prenatal treatment group, there was a significant interaction between drug and time (p’s ≤ 0.05). The hypothermic responses to the two doses of 8-OH-DPAT were significantly different at 30, 60 and 90 min post injection for PF and C males, but only 30 min for E males (* p’s < 0.05).
Figure 4. Number of wet dog shakes (WDS) over a 30 min period in response to DOI (1 mg/kg) in females and males (n's = 9-10). There was a significant interaction between prenatal treatment and drug (p < 0.01) effect of prenatal treatment for females, but not males. Ethanol-fed (E) females showed a significantly greater number of DOI-induced wet dog shakes compared to pair-fed (PF) and ad libitum-fed (C) females (* p's < 0.005).
significantly greater number of DOI- but not saline-induced WDS compared to PF and C females (p’s = 0.005) (Fig. 4). There was no effect of prenatal treatment following saline or DOI injection for males (Fig. 4). However, a sex difference was observed in animals injected with saline [F(1, 42) = 4.11, p = 0.05]; there was a significantly greater number of saline-induced WDS in males (0.67 ± 0.22) compared to females (0.21 ± 0.08) (Figure 5). As expected, there was also a significantly greater number of DOI-induced WDS compared to saline-induced (spontaneous) WDS in both females [F (1, 49) = 156.542, p < 0.001] and males [F(1, 49) = 136.98, p < 0.001] (data not shown).

3.D. Discussion

We found that 1) both E females and males have an increased sensitivity to the hypothermic effects of 8-OH-DPAT as evidenced by a greater drop in temperature compared to that in PF and C animals. 2) Over time, E females and males show less of a differential response to the low and high doses of 8-OH-DPAT compared to that in PF and C animals. 3) E females show increased sensitivity to DOI-induced WDS compared to PF and C females, whereas E males do not show this effect. These data indicate that prenatal ethanol exposure results in long-term effects on 5-HT mediated behavioural and physiological function in adult animals and that some of these effects may be sex specific.

The mechanisms underlying the effects of prenatal ethanol exposure on 5-HT_{1A} and 5-HT_{2A} receptor function are presently unclear. One possible explanation for the above findings is that prenatal ethanol results in an increased number of 5-HT_{1A} and 5-HT_{2A} receptors and/or function. The finding that E females and males show less of a differential response to the two doses of 8-OH-DPAT, although they also show an increased
Figure 5. Sex differences in saline-induced (1 ml/kg) wet dog shakes (WDS). Across all prenatal treatment conditions, males had a significantly higher number of saline-induced wet dog shakes compared to females (* p = 0.05).
hypothermic response, supports this suggestion. This finding may indicate a shift in 5-HT$_{1A}$ receptor function that is apparent with the low dose of 8-OH-DPAT but reaches a maximum effect with the higher dose.

It is unlikely that an increase in drug transport in E animals contributes to the increased response to 8-OH-DPAT and DOI. Research from our laboratory found a blunted hormonal response to 8-OH-DPAT in E females (Hofmann et al. 2001). This finding, taken together with other findings of altered 5-HT function in E animals, does not point to alterations in drug transport as an explanation for the present findings.

Deficiencies in 5-HT early in development may have important consequences for brain maturation since 5-HT has been shown to be a neurotrophic factor (Whitaker-Azmitia et al. 1996). Decreased brain levels of 5-HT have been found in rat E fetuses as early as gestation day 15 (Druse et al. 1991). 5-HT promotes the development of 5-HT neurons by stimulating release of the growth factor S100β from glial cells, mediated through 5-HT$_{1A}$ receptors (Azmitia et al. 1990). Eriksen et al. (2000) found that prenatal ethanol resulted in a decrease in S100β immunopositive cells in fetal and postnatal brains of E animals. The authors hypothesized that decreases in 5-HT associated with prenatal ethanol result in subsequent reductions in production and release of S100β and therefore may result in developmental deficiencies of the 5-HT system (Eriksen et al. 2000).

In experiment 1 we found effects of prenatal ethanol exposure on 5-HT mediated hypothermia in both females and males. However, in experiment 2 the effects of prenatal ethanol were limited to females. Female specific effects in other 5-HT measures following prenatal/postnatal ethanol have also been found. Zafar et al. (2000) found that 21 day old E females had a transient decrease in 5-HT transporter binding sites in the hypothalamus compared to control females, though 21 day old male offspring were not affected. Clausing et al. (1996) found a decrease in striatal 5-HT and 5-HIAA in 20 day old E females, but not
E males. A third study found that females exposed to ethanol in the early postnatal period (third trimester equivalent) showed increased basal levels of septal 5-HT and 5-HIAA in adulthood (Kelly 1996). These results illustrate the complex interaction between prenatal/postnatal ethanol exposure, sex and effects of brain 5-HT levels. Furthermore, these results suggest that on some indices of 5-HT function, females may be more vulnerable than males to the disruptive effects of prenatal ethanol.

Another sex difference was found in experiment 2, though irrespective of prenatal treatment. Males showed a greater number of saline-induced (spontaneous) WDS than females. This finding is consistent with previous experiments showing an increased rate of WDS in males, either spontaneous or DOI-induced (Gorzalka et al. 1998). It has also been found that gonadectomy decreases 5-HT2A mRNA in the hypothalamus, possibly indicating an interaction between the 5-HT2A receptor and testosterone (Zhang et al. 1999).

In addition to the long term effects of prenatal ethanol, we also found that E and PF females and males showed greater stress-induced hyperthermia than C females and males, suggesting that this effect may be partially mediated by caloric restriction. The finding that saline injection results in hyperthermia, and does so preferentially in E and PF animals, is not unexpected. Injection stress activates the HPA axis and induces expression of stress-responsive genes, as shown by our laboratory and others, and differential responsiveness to stress is known to occur in E animals (Dent et al. 2000; Wong et al. 2000; Hofmann et al. 1999; Kim et al. 1999b). Effects of pair-feeding independent of prenatal ethanol effects were also observed. Although a previous experiment in our laboratory (Weinberg et al. 1995) and previous work by Nelson et al. (1986) did not find differences in basal temperatures among E, PF and C animals, the present experiment found that PF females had slightly but significantly lower basal temperatures than E and C females. However, the physiological relevance of the minor difference in basal temperatures is questionable. PF
dams receive an amount of diet matched to E dams in g/kg body weight for the same day of gestation, with maltose-dextrin isocalorically substituted for ethanol. Therefore they receive a ration that is less than they would consume ad libitum. Although both groups are receiving the same number of calories, PF dams experience mild food deprivation, while E dams do not. In this respect, pair-feeding may be a type of prenatal stressor. Together, these findings are important in understanding altered responses to stress in E and PF animals. It is possible that these alterations occur through different mechanisms (i.e., prenatal ethanol, nutritional, or prenatal stress effects) that are difficult to distinguish from each other.

The increased 5-HT receptor mediated function found in the present study is consistent with other changes observed in E animals such as increased hormonal responses to stress and deficits in response inhibition (Nelson et al. 1984b; Riley et al. 1979), functions that are in part, mediated by the 5-HT system. Clinically, the 5-HT system has been studied for its involvement in psychiatric illness. Abnormal 5-HT neurotransmission is associated with major affective disorders, particularly depression (Lopez et al. 1998). 5-HT abnormalities have also been associated with anxiety and substance abuse (Tollefson 1989; Kahn et al. 1988), an important fact in light of recent studies reporting a significant incidence of mental illness in adults with FAS and Partial FAS. In these individuals depression was the second most common mental illness, preceded by alcohol and/or drug dependence (Famy et al. 1998). Others have also reported an association between prenatal alcohol exposure and self- and parental-reported depressive symptoms in children and adults (O’Connor and Kasari 2000; Roebuck et al. 1999; Shah et al. 1999) as well as increased drug dependence in adult adoptees with fetal alcohol exposure (Yates et al. 1998). Children with FAS also tend to be hyperactive, impulsive, emotionally labile and easily distracted and show poorer learning and memory skills compared with developmentally normal children (Streissguth 1986; Streissguth et al. 1985). Importantly, impulsivity,
learning and memory are correlated with 5-HT function (McAllister-Williams et al. 1998). Thus prenatal ethanol-induced alterations of the 5-HT system may have several important consequences for the children impacted by its effects.
CHAPTER IV: EFFECTS OF PRENATAL ETHANOL EXPOSURE ON
NOVELTY-INDUCED SUPPRESSION OF FEEDING AND ANXIOLYTIC
RESPONSE TO 8-OH-DPAT

4.A. Introduction

Behavioural and physiological responses in E animals have been examined in a variety of tasks that may indicate increased reactivity to stimuli or hyperactivity in comparison to PF and C animals. These tests have employed conditioned (taste aversion, avoidance) and unconditioned (exploratory, stress-induced, startle reflexes) stimuli. Behaviourally, E animals show an increased startle response to acoustic stimuli (Potter and Berntson 1987), increased grooming following brief swim stress (Hannigan et al. 1987), increased footshock-induced alcohol consumption (Nelson et al. 1983), increased shock-elicted aggression (Davis et al. 1984), greater avoidance of a shocked arm in a Y maze (Osborne et al. 1980), and increased consummatory responses in conditioned taste aversion tasks (Clausing et al. 1995; Driscoll et al. 1990; Riley et al. 1984). In addition, tasks including aversive or novel stimuli can also evoke a stress response. Physiologically, E animals show increased stress-induced hyperthermia (Hofmann et al. 2002a), as well as increased levels of adrenocorticotropin and corticosterone to a variety of stressors such as novelty (Kakihana et al. 1980), cold stress (Angelogianni and Gianoulakis 1989), and ether vapours (Hofmann et al. 1999). However, there have been contradictory results in some of the behavioural tests. Means et al. (1986) failed to find an effect of prenatal ethanol exposure on avoidance behaviour in response to a novel alley or approach of novel objects in an open field. Similarly, our laboratory has found no effect of prenatal ethanol exposure in a conditioned taste aversion task (Gabriel et al. 2002). Others have found that E animals
exhibit an increased number of entries into a shocked arm of the Y-maze (Fernandez et al. 1983), suggestive of a decrease in reactivity to stimuli.

Responses in two more popular tests of anxiety, the open field and the elevated plus-maze, have also been examined. Data have shown that prenatal ethanol exposure results in increased activity in the open field, as measured by increased ambulation and/or rears (Fernandez et al. 1983; Osborne et al. 1980; Bond and Di Gusto 1976; Branchey and Friedhoff 1976). Furthermore, Becker and Randall (1989) found this effect in E animals to be independent of light levels. The authors have typically concluded that their results reflect increased locomotor activity in E animals, parallel to the hyperactivity observed in children with FAS or ARMD. Indeed, increased activity is one of the most common behavioural effects observed in E animals (Riley et al. 1990). A confound for both the open field and elevated plus-maze, is that indices of activity level and emotionality often cannot be separated from exploratory behaviour (Weinberg 1977). This confound may contribute to the contradictory results that have been found in the open field as others have failed to find an effect of prenatal ethanol exposure on activity in the open field (Nelson et al. 1988; Mothes et al. 1996; Osborn et al. 1998a; Osborn et al. 1998b), or an effect was observed in females only (Grant et al. 1983).

Our laboratory has conducted the only two experiments employing the elevated plus-maze to study the effects of prenatal ethanol exposure on anxiety-like behaviour (Osborn et al. 1998a, Osborn et al. 1998b). Results from this widely used and validated test of anxiety are also inconclusive. Anxiety-like behaviour in the elevated plus-maze following prenatal ethanol exposure appears to depend on sex, the variable examined, and whether the animal is naive to the test or has had prior experience with the open field (Osborn et al. 1998a, Osborn et al. 1998b). An additional confound in the elevated plus-maze is that behaviour of female rats in this test appears to reflect activity, rather than
anxiety, while the reverse is true for males (Fernandes et al. 1999). Osborn et al. (1998a) found that E males spent more time on the open arms of the plus-maze on an initial exposure to the test, however, on second exposure, they showed an increase in closed arm entries, although still a trend toward increased time spent in the open arms. Exposure to the plus-maze after testing in the open field revealed a different pattern of prenatal ethanol effects. E males and females spent less time in the closed arms, although E females had higher levels of CORT (Osborn et al. 1998a). Interestingly though, E females and males showed a greater anxiolytic response to diazepam in the plus-maze compared to PF and C animals following prior open field exposure (Osborn et al. 1998b).

In light of recent studies that reveal the increased incidence psychiatric disorders, including anxiety, in individuals affected with FAS or related diagnoses, it is of significant clinical relevance to experimentally model the affective disturbances observed in these individuals. Recent studies make it apparent that earlier findings in stress and anxiety tests should be revisited.

The main purpose of the present study was to examine the effect of prenatal ethanol exposure on anxiety-like behaviour in a novel anxiety task. We measured anxiety-like behaviour in the novelty-induced suppression of feeding task (Muller-Gass et al. 2000), a test of anxiety that does not utilize exploration as an index of anxiety, to address the conflicting results found following prenatal ethanol exposure in the elevated plus-maze, open field and other behavioural tests. The test is based on an animal’s natural reluctance to approach and consume a familiar, palatable food in a novel environment. This effect is termed hyponeophagia and is thought to reflect anxiety as the animal is placed in a conflict situation (i.e., fear of novel setting vs. drive to consume palatable food) (Poschel 1971). Animals were habituated in their home cage to No Name™ graham crumbs for a period of
21 days, and habituation was assessed. Subsequently, animals were exposed to the bedding-free cage and feeding behaviour was observed following 8-OH-DPAT or saline treatment.

Our laboratory has previously observed increased 5-HT$_{1A}$ receptor-mediated physiological function in E animals. Following prenatal ethanol exposure, adult females and males show increased hypothermia in response to the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Chapter III; Hofmann et al. 2002a). The 5-HT$_{1A}$ receptor has recently been found to play an especially important role in expression of anxiety-like behaviour (Gross et al. 2002). For example, 5-HT$_{1A}$ knock out mice exhibit increased anxiety in several tasks, including the novelty-induced suppression of feeding task (Gross et al. 2000). In addition, clinically and in the laboratory, 5-HT$_{1A}$ agonists have anxiolytic effects. Therefore, a second purpose of the present study was to examine the anxiolytic effects 8-OH-DPAT in E, PF and C animals. Importantly, although anxiety disorders are more prevalent in females than in males (Palanza, 2001; Blanchard et al. 1995), the majority of research on anxiety and anxiolytics has focused on males as test subjects. Therefore, this study included females in addition to males as test subjects.

Adult female and male offspring from ethanol exposed (E), pair-fed control (PF) and ad libitum-fed control (C) dams were tested in a modified novelty-induced suppression of feeding task. Most novelty-induced suppression of feeding tasks assess neophobia by placing food deprived animals in a novel environment, usually the open field. The present study utilized non-deprived animals and a home cage without bedding as the novel environment, thus assessing hyponeophagia. Since at some doses, 8-OH-DPAT appears to enhance feeding behaviour through an increase in appetite (i.e., orexigenic effects) additional conditions and measures were added to control for this possible confound.
4.B. Methods

4.B.1. Prenatal Ethanol Exposure

Prenatal ethanol exposure was conducted as described in Chapter II.

4.B.2. Animals

Animals were housed under a normal light cycle with lights on from 06:00 – 18:00 hrs. On postnatal day 22 pups were weaned and re-housed with same sex littermates until 2 days prior to the start of the habituation period, when animals were singly housed for the duration of the 21 day habituation period. Habituation to the graham crumbs began when the animals were approximately 90-120 days of age.

4.B.3. Drugs

8-OH-DPAT was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). 8-OH-DPAT (0.06 mg/kg) or vehicle (0.9% NaCl) was administered. The dosage for 8-OH-DPAT was determined through pilot studies in our laboratory. 8-OH-DPAT was dissolved in vehicle on the day of usage and injected subcutaneously (dorsal flank) at a volume of 1 ml/kg while rats were loosely restrained.

4.B.4. Novelty-Induced Suppression of Feeding

Habituation. Animals were first habituated to a novel, palatable food in their home cage (No Name™ graham crumbs) daily, for 15 min/day, for 21 days. Each day,
approximately 4 hrs after lights on, the rat was gently removed from the cage by the tail and a clear dish (Anchor Hocking glassware, 10.5 cm top, 6.5 cm base, 4 cm height) containing 10 g of the food was placed into the centre of the cage before the rat was returned to the cage. The food was removed from the cage after 15 min. Animals were food deprived for 24 hr prior to the first habituation day to facilitate consumption of the graham crumbs. Chow was replaced after this initial deprivation and exposure to the food, and rats were maintained on ad libitum access to chow for the remainder of the habituation period. Animals were weighed on day 7, 14 and 21 of habituation and the amount of the graham crumbs consumed was measured at the end of each habituation period on these days.

**Baseline-Feeding Behaviour.** On the last day of habituation (day 21) animals were observed in their home cage following the placement of the food in their cage to determine baseline-feeding behaviour. Variables included latency to begin feeding (min), duration of feeding (min), number of feeding bouts, and amount consumed (g/100 g body weight). From these measures, two additional measures were assessed; amount consumed per min (g/min) (i.e., feeding rate) and amount consumed per feeding bout. A feeding bout was defined as a continuous period of feeding temporally separated from other activities by 10 seconds or more.

**Behavioural Testing in Novel Environment.** On the day following the habituation period (day 22), animals were randomly assigned to receive either 8-OH-DPAT or saline and 30 min later presented with the food in either their home cage or a novel cage (new home cage without bedding). Thus animals were in one of four conditions: 1) saline treatment in their home cage, 2) 8-OH-DPAT treatment in the home cage, 3) saline treatment in the novel cage, or 4) 8-OH-DPAT treatment in the novel cage. Observers blind
**Figure 6.** The experimental design for novelty-induced suppression of feeding. Animals were habituated to a palatable food for 15 min/day for 21 days. Baseline feeding behaviour was measured on day 21 in the home cage. On day 22, animals were treated with saline (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg) and feeding behaviour was measured in the home cage or the novel environment.
to prenatal treatment and drug condition again recorded the same measures taken for baseline feeding. Analysis of home cage feeding behaviour served as a control for the possible appetite enhancing effects of 8-OH-DPAT. As the home cage is not expected to be anxiogenic, any drug effects observed in this condition are not due to the anxiolytic effects of the drug, but rather due to effects of the drug on appetite. In addition, each animal’s chow was weighed prior to and after observation in the novel and home cages to provide an additional measure of drug effects on appetite.

4.B.5. Statistical Analysis

Analyses of variance (ANOVA) for the factors of sex, prenatal treatment and injection type (saline or 8-OH-DPAT) where appropriate were performed. Repeated measures ANOVA were also performed to compare baseline- and novel environment-feeding behaviour. Significant main and interaction effects were further analyzed by Newman-Keuls post hoc analysis.

4.C. Results

4.C.1. Developmental Effects of Prenatal Ethanol Exposure

Ethanol intake of pregnant females was consistently high throughout gestation, averaging $11.36 \pm 1.55$, $12.66 \pm 1.31$ and $12.28 \pm 1.17$ g/kg body weight for weeks 1, 2 and 3 of gestation respectively. Repeated measures ANOVA revealed significant differences in dam weight gain throughout the gestation period $[F(6, 123) = 4.20, p < 0.001]$. There were no differences between prenatal treatment groups on day 1 or 7 of gestation; however, on
day 14 both E and PF dams weighed significantly less than C dams (p's < 0.05). On day 21 of gestation, E dams weighed significantly less than C dams, while PF dams did not differ from either group (p = 0.01). While there were no differences in the number of pups born to the dams, there was a significant effect of prenatal treatment on total litter weight [F(2, 33) = 4.08, p < 0.05]. The total mass of the litters was less for E and PF compared to C dams (p's < 0.05). There were significant interactions between prenatal treatment and age for both females [F(6,114) = 7.22, p < 0.0001] and males [F(6,117) = 7.09, p < 0.0001] on mean pup weight (Table 2). There were no significant differences between prenatal treatment conditions on weight at birth; however, at day 13 E and PF males weighed significantly less than C males (p's < 0.05). At day 21, both E and PF females and males weighed significantly less than C females and males (p's ≤ 0.01). All pups also showed a significant increase in weight across the preweaning period (p ≤ 0.0001). No weight differences existed between prenatal treatment groups at the time of behavioural testing (postnatal days 120 - 150).

4.C.2. Anxiogenic Effects of Novel Environment

The novel environment did indeed produce anxiety in all animals as indicated by the comparison of baseline (day 21 of habituation) to novel environment feeding behaviours (Figs. 7 and 9). A repeated measures ANOVA revealed that for both saline- and 8-OH-DPAT-treated animals there was a significant increase in the latency to feed (min) in the novel environment compared to baseline [F(1,63) = 7.68, p = 0.007] (p’s < 0.0002).
Table 2. The effects of prenatal ethanol exposure on mean pup weight on postnatal (PN) days 1, 21 and time of testing (PN 120 - 150) (g, mean ± SEM). * On PN 21, E and PF females and males weighed significantly less than C females and males (E + PF < C; p’s ≤ 0.01). There were 12 litters per prenatal treatment condition.

<table>
<thead>
<tr>
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<th>PN1</th>
<th>PN21</th>
<th>PN90-120</th>
<th>PN120-150</th>
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<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
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<tr>
<td>E</td>
<td>5.3 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>*42.7 ± 1.6</td>
<td>*44.9 ± 1.8</td>
</tr>
<tr>
<td>PF</td>
<td>5.8 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>*46.2 ± 1.7</td>
<td>*48.4 ± 1.8</td>
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<tr>
<td>C</td>
<td>6.5 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>52.0 ± 1.3</td>
<td>55.5 ± 1.3</td>
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Figure 7. Baseline-feeding behaviour on day 21 of habituation in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) (A.) females and (B.) males. E and PF females had a significantly slower rate of feeding than C females (* p = 0.002). E and PF males had a significantly longer feeding latency (min) than C males (E = PF > C) (* p’s < 0.05). In addition, E males had a significantly shorter duration of feeding (min) and consumed less food (g/100 g body weight) than PF and C males (E < PF = C) (* p’s < 0.05). E males also had a slower feeding rate than PF males, who in turn had a slower rate than C males (E < PF < C, *, ^ p’s < 0.001). Each bar represents the mean +/- SEM for 48 animals.

**Females.** During the habituation period, the amount of food consumed (g/100 g body weight) was recorded on day 7, 14 and 21. A repeated measures ANOVA indicated that there was no increase in food consumption from day 7 to 21. On day 21, there were no effects of prenatal treatment condition on latency to feed, duration of feeding, the amount consumed or number of feeding bouts ((Fig. 7). However, there was an effect of prenatal treatment on feeding rate \[F(2, 141) = 6.92, p = 0.001\] (Fig. 7). Post hoc analysis revealed that E and PF females had a slower feeding rate than C females (p’s = 0.002).

**Males.** Unlike females, a repeated measures ANOVA revealed a main effect of prenatal treatment condition on the amount of food consumed over habituation days 7, 14 and 21 \[F(2, 276) = 12.38, p = 0.00001\]. E males consumed less food than PF and C males across all days (p’s < 0.05). On day 21, there were also main effects of prenatal treatment condition on latency to feed \[F(2, 138) = 3.73, p < 0.05\], duration of feeding \[F(2, 138) = 5.55, p = 0.005\], feeding rate \[F(2, 138) = 10.89, p < 0.0005\], and a trend for differences in the number of feeding bouts (p = 0.06). Post hoc analysis revealed that both E and PF males had a longer latency to begin feeding compared to C males (p’s < 0.05); however, only E males had a shorter duration of feeding compared to PF and C males (p’s < 0.01). In addition, E males had a slower rate of feeding than PF males who in turn had a slower feeding rate than C males (i.e., E < PF < C; p’s < 0.001).

Females and males were similar in baseline feeding behaviour, with the exception of amount consumed \[F(1, 280) = 36.51, p < 0.0001\] and feeding rate \[F(1, 279) = 14.98, p = 0.0001\]. Although, E females consumed more food than males (per 100g/ body weight), males consumed the food at a faster rate.
4.C.4. Control for Orexigenic Effects of 8-OH-DPAT

Each animal’s chow was weighed on day 20, 21, 22 and 23, determine whether the chosen dose of 8-OH-DPAT had orexigenic effects. Thus a comparison was made of baseline chow consumption prior to drug treatment and the 24 hr period following drug treatment. For both females and males there was no increase in amount of chow consumed before and after saline or 8-OH-DPAT treatment in either the home or novel environment. In 8-OH-DPAT-treated females, there was actually a decrease in consumption of chow compared to basal consumption [F(1, 118) = 7.06, p = 0.009].

As a second control for possible orexigenic effects of 8-OH-DPAT, a group of saline- or 8-OH-DPAT-treated E, PF and C females and males were observed for feeding behaviour in their home cage. 8-OH-DPAT treatment did not result in an increase in the amount of food consumed in the home cage for females or males (Fig. 8). For females, there were no effects of 8-OH-DPAT treatment on any of the measures, except for duration of feeding [F(1,67) = 4.43, p = 0.04], which was significantly longer in 8-OH-DPAT-compared to saline-treated females. For males, there were no significant effects of 8-OH-DPAT treatment on any of the measures.

4.C.5. Novelty-Induced Suppression of Feeding

Females.

Latency to feed (min): There was a significant main effect of prenatal treatment [F(2,63) = 4.55, p = 0.014]. E had a significantly longer latency to begin feeding compared to PF and C females (p’s < 0.05), primarily due to the large increase in latency to feed in
Figure 8. The amount of food consumed (g/100 g body weight) in the home cage (day 22) in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males following saline (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg) treatment. There was no significant effect of the drug on the amount of food consumed indicating that the drug did not have orexigenic effects. Each bar represents the mean +/- SEM for 12 animals.
saline-treated E females. Importantly, 8-OH-DPAT treatment in E females, resulted in a normalization of latency values comparable to those of PF and C females (Fig. 9A).

Feeding duration (min): There were main effects of prenatal treatment [F(2,63) = 3.30, p = 0.04] and drug [F(1,63) = 15.28, p = 0.0002]. Post hoc analysis revealed that E spent significantly less time feeding than PF, while C females did not differ from either group (p’s < 0.05). In addition, 8-OH-DPAT treatment resulted in an equal increase in feeding duration across all prenatal treatment conditions (Fig. 9A). Amount consumed (g/100 g body weight): There was a significant effect of drug [F(1,59) = 10.68, p = 0.001] and a trend toward a prenatal treatment effect (p = 0.06). 8-OH-DPAT treatment resulted in a significant increase in the amount of the food consumed by all females (Fig. 9A). In addition, E females showed a tendency to consume less food than PF and C females.

Feeding bouts: There was a significant effect of drug [F(1,63) = 14.23, p = 0.0004] on the number of feeding bouts. Regardless of prenatal treatment condition, 8-OH-DPAT treatment resulted in a significant increase in feeding bouts compared to saline treatment (Fig. 9A).

Percentage of rats feeding: There was a main effect of prenatal treatment [F(2,63) = 3.58, p < 0.05] and of drug treatment [F(1,63) = 6.27, p = 0.01] (Table 3). Regardless of drug treatment, there was a lower percentage of E females feeding in the novel environment in comparison to PF and C females (p’s < 0.05). In addition, regardless of prenatal treatment, there was a higher percentage of 8-OH-DPAT-treated females feeding in comparison to saline-treated.

Feeding rate (g/min): There was a significant interaction between prenatal treatment and drug [F(2,59) = 3.12, p = 0.05]. 8-OH-DPAT treatment significantly increased feeding rate compared to saline treatment in E females only, resulting in a local feeding rate comparable to that of PF and C females (p = 0.05) (Fig. 9A).
Amount consumed (g/100 g body weight) per feeding bout: There were significant effects of prenatal treatment \[F(2,60) = 3.65, p = 0.03\] and drug \[F(2,60) = 6.50, p = 0.01\]. Post hoc analysis revealed that E females consumed less food per feeding bout compared to PF and C females (p's < 0.05). In addition, 8-OH-DPAT-treated females consumed significantly more per feeding bout than saline-treated females (data not shown).

Males.

There were no significant differences due to prenatal treatment or drug treatment (saline or 8-OH-DPAT) in latency to begin feeding, duration of feeding, amount consumed, number feeding bouts, feeding rate or amount consumed per feeding bout.

Percentage of rats feeding: There was a drop in the percentage of animals feeding in the novel environment (saline-treated) compared to baseline levels (Table 3). E males showed a 43% decrease in the number of rats feeding in the novel environment compared to baseline levels compared to a 28% and 30% decrease in PF and C males, respectively.

4.D. Discussion

The present study utilized novelty-induced suppression of feeding to assess anxiety-like behaviour and the anxiolytic effect of 8-OH-DPAT in animals prenatally exposed to ethanol. After 21 days exposure to the palatable food, E males, but not E females, exhibited a deficit in habituating to repeated presentation of the palatable food. However, when challenged with a novel environment, E females showed greater suppression of feeding as reflected by an increased latency to consume and a decreased duration of feeding. Both E females and E males showed a large decrease in the percent of animals feeding in the novel environment (vs. baseline-feeding) compared to PF and C animals, though this effect was
**Figure 9.** Feeding behaviour in the novel environment in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males following saline (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg) treatment. E females had a longer latency to begin feeding (min) than PF or C females, due to the increased latency in saline-treated E females (E > PF = C; * p’s < 0.05). E females also had a shorter duration of feeding (min) compared to PF females (E < PF; * p’s < 0.05). 8-OH-DPAT treated females consumed more food (g/100 g body weight) and had more feeding bouts than saline-treated females (# p < 0.05). Only 8-OH-DPAT-treated E females had an increased feeding rate (# p = 0.005). Each bar represents the mean +/- SEM for 12 animals.
Table 3. Percent (%) of ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) animals feeding in the home cage on day 21 (baseline) and in the novel environment on day 22 following saline (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg). There was a decrease in the percentage of animals feeding in the novel environment in comparison to baseline feeding levels. The greatest decrease in percent of animals feeding was observed for E females and E males. 8-OH-DPAT treatment resulted in an increase in the percent of animals feeding in the novel environment, in comparison to saline treatment for females only (n = 12 per group).

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<th>Baseline Feeding Day 21 (Home cage)</th>
<th>Novel Environment Day 22</th>
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<tr>
<td></td>
<td>Females (%)</td>
<td>Males (%)</td>
<td>Females (%)</td>
<td>Males (%)</td>
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<td></td>
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<td>Saline</td>
<td>8-OH-DPAT</td>
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<tr>
<td>E</td>
<td>96</td>
<td>93</td>
<td>33</td>
<td>89</td>
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<tr>
<td>PF</td>
<td>98</td>
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especially obvious in E females. Treatment with 8-OH-DPAT resulted in an increase in some feeding behaviours in E females that was comparable to levels of PF and C animals. These findings indicate that prenatal ethanol exposure in rodents results in increased anxiety-like behaviour in adulthood, that the 5-HT₁₅ receptor may in part mediate this effect and that prenatal ethanol exposure results in greater anxiety-like behaviour in females than in males. In this task, we found that E males showed decreased baseline-feeding behaviour compared to PF and C males and during habituation to the palatable food, E males consumed less than PF and C males. Furthermore, on day 21 of habituation, E males had a shorter duration of feeding and exhibited a decreased feeding rate compared to PF and C males. Taken together, these findings suggest a decreased or delayed ability to habituate to repeated stimuli in E males.

Unlike males, there were no differences in baseline-feeding behaviour among E, PF and C females. Exposure to the novel environment however, resulted in greater suppression of feeding in E compared to PF and C females. E males also exhibited a greater suppression of feeding in the novel environment than PF and C males, although to a lesser extent than E females. Importantly, in almost all of the measures, except duration of feeding, treatment with the anxiolytic 8-OH-DPAT normalized feeding behaviour in E females to levels comparable to those of PF and C females. This normalization effect by 8-OH-DPAT suggests that prenatal-ethanol induced hyponeophagia is at least in part, mediated by 5-HT₁₅ receptors.

The novelty-induced suppression of feeding task is a simple task with many advantages over traditional tests of anxiety. The small size of the testing environment (i.e., standard housing cage without bedding) allows assessment of anxiety-like behaviour independent of the effects of exploration or activity, and behavioural testing in the home cage can serve as a control for extraneous drug effects. In addition the test does not require
food deprivation of animals or extensive and chronic handling. We found that all animals exhibited a suppression of feeding in the novel environment in comparison to their home cage, as evidenced by increases in latency to begin feeding, decreased feeding duration and decreased food consumption. Although 8-OH-DPAT-induced increases in feeding is the index of anxiety levels in this task, the effect observed was not due to orexigenic effects of the drug. Interestingly, some measures were more sensitive to 8-OH-DPAT treatment than others. For example, 8-OH-DPAT did not appear to be effective in reducing the latency to begin feeding in PF and C females and males. This may suggest that latency to feed is not the best measure of anxiety. Briton and Briton (1981) suggest that the most sensitive measure of anxiolytic drug effects on novelty-induced suppression of feeding is the amount of food consumed per feeding bout. Consistent with this, the present study found that 8-OH-DPAT-treated females consumed more food per feeding bout than saline-treated females.

In the present study novelty-induced suppression of feeding was most strongly indicated by the percent of animals feeding. Others have also used this variable as a measure of hyponeophagia (Caldji et al. 2000). We found a large decrease in the percentage of E animals feeding in the novel environment in comparison to PF and C animals. In E females but not E males, 8-OH-DPAT treatment increased the percentage of animals feeding to levels comparable to PF and C females. Our laboratory has previously reported that E females and males show decreased levels of anxiety compared to control animals when administered the benzodiazepine, diazepam (Osborn et al. 1998b). Benzodiazepines increase whole brain levels of 5-HT and 5-HIAA levels, therefore this result may indirectly implicate an altered serotonergic system in E animals (Chase et al. 1970). We have also found that E females have increased levels of CORT following plus-maze exposure. Recent research in normal rodents has found that adrenalectomy results in decreased
responsiveness to anxiolytics that exert their actions through 5-HT\textsubscript{1A} receptors: ipsapirone and buspirone (Lopez-Rubalcava 1999). This suggests that glucocorticoids contribute to the anxiolytic effects of serotonergic agonists. It is possible that since E animals respond to stressful situations, such as the elevated plus-maze with increased levels of corticosterone (Osborn et al. 1998a) that responses to certain drugs in E animals may be enhanced.

There was also a large sex difference in percent feeding in the novel environment. Suppression of feeding was similar for saline-treated females and males, a result in agreement with other hyponeophagia tasks (Shephard and Broadhurst 1982). However, 8-OH-DPAT-treated females and males showed clear differences. An anxiolytic effect of 8-OH-DPAT was observed only in females. This data clearly indicates the importance of including females in experiments utilizing animal tests of anxiolytic drug effects. Analysis of other feeding behaviours in this study also supported the conclusion that females exhibit a greater sensitivity to 8-OH-DPAT compared to males, a finding also reported by Blanchard et al. (1992).

Some of the effects found in the present study may be mediated by nutritional deficits associated with maternal consumption of ethanol. E and PF males had a longer baseline latency to begin feeding compared to C males. Baseline feeding rate was also decreased in E and PF males and females compared to C animals. In addition, E and PF females and males showed a developmental delay in weight gain during the preweaning period. However, no weight differences were observed at the time of behavioural testing, indicating that E and PF animals are capable of overcoming this early life deficit.

Prenatal ethanol-induced alterations in the serotonergic system have important consequences for the offspring, as serotonin is involved in many behavioural and physiological functions. The neurotrophic actions of 5-HT are exerted through 5-HT\textsubscript{1A} receptors. Activation of the 5-HT\textsubscript{1A} receptor results in growth cone elongation, neurite
outgrowth and release of the growth factor S100β from glial cells (Azmitia 2001). Prenatal ethanol exposure results in a decrease in fetal 5-HT (Druse et al. 1991) and subsequently results in a decrease in 5-HT₁A stimulation and therefore abnormal development of the 5-HT system. In addition, in normal rats, there is a developmental increase in central 5-HT₁A receptors from postnatal days 19 to 35. In E rats, this developmental increase in 5-HT₁A receptors does not occur in the septum and cortex (Kim et al. 1997). This developmental alteration in 5-HT₁A receptors may be an important mechanism underlying the increased anxiety observed in E animals. Gross et al. (2002) have recently reported on the contribution of postnatal 5-HT₁A receptors in the septum and cortex in expression of normal anxiety-like behaviour in mice. E animals do exhibit a reduction in 5-HT₁A receptor binding (Tajuddin and Druse 1989b) and 5-HT innervation of the frontal cortex (Zhou et al. 2002). In addition, it has recently been suggested that moderate 5-HT depletion with neurotoxins can be anxiogenic (Green and McGregor, 2002). Decreased 5-HT levels in E offspring have been well documented (Clausing et al. 1996; Druse et al. 1991; Elis et al. 1976). Therefore, early prenatal ethanol-induced alterations in 5-HT and the 5-HT₁A receptor in E animals may contribute to expression of anxiety-like behaviour later in life.

The modified novelty-induced suppression of feeding task used to assess anxiety-like behaviour in the present experiment has advantages over previous versions of this task and other tests of anxiety. Typically, novelty-induced suppression of feeding is examined in a large, open field arena; an unfamiliar environment for the rat. In addition, the animals are typically food deprived. The results of this experiment further support the conclusions of Muller-Gass et al. (2000) that this test is an effective, simple task to measure anxiety-like behaviour and anxiolytic drug effects.

Finally, it is clinically relevant to establish an experimental model of the affective disturbances observed in individuals affected with FAS or ARND. In addition, serotonergic
anxiolytics are highly prescribed in the treatment of anxiety disorders. Importantly, therapeutic efficacy of these drugs may depend on their ability to alter 5-HT receptors, release, metabolism or reuptake; alterations that may be affected by prenatal alcohol exposure. Therefore, understanding of 5-HT function in animal model of prenatal ethanol exposure may facilitate treatment of this affected population.
5.A. Introduction

Our laboratory has found that prenatal ethanol exposure results in long-term effects on the organism's ability to respond and adapt to stressors, as measured by alterations in hypothalamic-pituitary-adrenal (HPA) function. Exposure to stressors results in release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, which in turn stimulates release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH subsequently stimulates release of the glucocorticoid, corticosterone (CORT) from the adrenal glands. In rodents prenatally exposed to ethanol (E animals), altered HPA activity can be observed through several stages of maturation. Shortly after birth E animals show elevated basal levels of brain, plasma (Taylor et al. 1982; Kakihana et al. 1980) and adrenal CORT and elevated plasma CORT levels during stress (Weinberg 1989) in comparison to control animals. During the preweaning period, normal animals exhibit a period of reduced adrenocortical activity referred to as the stress hyporesponsive period (Sapolsky and Meaney 1986). E animals show an even greater HPA hyporesponsiveness to stressors compared to control animals during this period, with decreased ACTH and CORT levels following stressors such as ether, novelty, saline injection, cold stress, and ethanol and morphine challenge (Angelogianni and Gianoulakis 1989; Taylor et al. 1986; Kakihana et al. 1980). In contrast, in adulthood E animals exhibit HPA hyperresponsiveness, with increased HPA responses following cardiac puncture, noise/shake, (Taylor et al. 1982b), restraint (Gabriel et al. 2001), footshock stress (Ogilvie and Rivier, 1997) and immune challenge (Kim et al. 1999a). In addition, following chronic
or repeated stress, E animals have been shown to exhibit delayed or deficient recovery to basal levels, as reflected by prolonged plasma elevations of CORT and ACTH (Kim et al. 1999; Weinberg et al., 1996; Taylor et al. 1986).

Serotonin is one of the neurotransmitters involved in regulation of the HPA axis. 5-HT microinjected into the PVN results in an increase in ACTH levels and CRH mRNA in the PVN, effects blocked by pretreatment with a 5-HT1A antagonist (Kageyama et al. 1998). The 5-HT1A and 5-HT2A receptors are the primary subtypes mediating the effects of 5-HT on the HPA axis (Contesse et al. 2000), although other receptor subtypes may play a role. 5-HT also regulates hippocampal corticosteroid receptors. Conversely, CORT influences levels of 5-HT receptors. Adrenalectomy results in an increase in 5-HT1A receptors in the hippocampus. Importantly, increasing evidence implicates the complex interactions between the 5-HT and HPA systems in the psychopathology of affective disorders, which show an increased prevalence in individuals prenatally exposed to alcohol.

The purpose of the present study was to investigate whether prenatal ethanol exposure alters 5-HT receptor-mediated endocrine responses. This was accomplished by measuring plasma ACTH and CORT, and CRH mRNA levels in response to systemic administration of the 5-HT1A agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and the 5-HT2A agonist (+)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride. Previously, we have observed increased 8-OH-DPAT-induced hypothermia and DOI-induced wet dog shakes in E animals (Chapter III, Hofmann et al. 2002a) suggesting increased 5-HT1A and 5-HT2A receptor function. One possibility for increased 5-HT1A and 5-HT2A receptor function in E animals may be an increase in receptor number. In situ hybridization to detect 5-HT1A and 5-HT2A receptor mRNA levels can provide an indication of receptor protein levels and therefore receptor number (Lanfumey and Hamon 2000; Cornea-Hébert et al. 1999; Pompeiano et al. 1992). Therefore, in addition to
examining HPA responses to DOI and 8-OH-DPAT, we investigated whether prenatal ethanol exposure alters 5-HT$_{1A}$ and 5-HT$_{2A}$ mRNA in the hippocampus and the medial prefrontal cortex, brain regions important in HPA and mood regulation. As well, the medial prefrontal cortex mediates DOI-induced wet dog shakes (Willins and Meltzer 1997). Adult female and male offspring from ethanol exposed (E), pair-fed control (PF) and in ad libitum-fed control (C) dams were utilized in this experiment.

5.B. Methods

5.B.1. Prenatal Ethanol Exposure

Prenatal ethanol exposure was conducted as described in the Chapter II.

5.B.2. Drugs

8-OH-DPAT and DOI were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). 8-OH-DPAT (0.2 mg/kg), DOI (0.3 mg/kg), or vehicle (0.9% NaCl) was administered to E, PF and C females and males. On the day of testing, drugs were dissolved in physiological saline (0.9% NaCl). All drugs were injected subcutaneously (dorsal flank) at a volume of 1 ml/kg while rats were loosely restrained.

5.B.3. Surgeries and Blood Sampling

At approximately 90 – 120 days of age, animals were implanted with indwelling jugular cannulae under halothane anesthesia and singly housed until blood sampling, two
days later. Cannulae were inserted into the left internal jugular vein, with the free ends of the cannulae exteriorized dorsally between the scapulae and capped. On the day of blood sampling, a syringe was attached to the free end of the cannula, lines were checked to ensure patency and animals were placed in holding containers, undisturbed, for 2 hrs. A basal blood sample (0.4 cc) was then taken, followed by injection of 8-OH-DPAT, DOI or saline. Blood samples (0.4 cc) were taken at 15, 30, 60 and 120 min after injection. All blood samples were collected on ice in polystyrene 5 ml tubes containing a .2 ml solution of ethylenediaminetetraacetic acid (0.5 mg/ml) and aprotinin (3.75 mg/ml) and immediately centrifuged at 3500 rpm for 10 min at 4 °C. Plasma was collected in polypropylene microcentrifuge tubes and stored at -70 °C.

5.B.4. Radioimmunoassays

Plasma ACTH levels were measured using a commercial kit (DiaSorin, Inc., Stillwater, MN). All reagent volumes were halved and 50 μl of plasma per sample were used. Inter and intra-assay coefficients of variance were 14.35% and 7.62% respectively. Sensitivity was 15 pg/ml with a high degree of cross reactivity for rat ACTH.

Total plasma CORT levels (bound plus free) were measured using our adaptation of the methods of Kaneko et al. (1981). Dextran-coated charcoal was used to adsorb and precipitate free steroids after incubation. Rat plasma CORT antibody was used in this assay. Inter and intra-assay coefficients of variance were 9.38% and 2.41% respectively. Sensitivity of the assay was 0.05 ng/dl.
5.5. In Situ Hybridization

Non-cannulated animals, littermates of the cannulated animals, were terminated at 150 days of age, 3 hrs following administration of 8-OH-DPAT (0.2 mg/kg) or DOI (0.3 mg/kg). Brains were removed for analysis of 5-HT1A and 5-HT2A receptor and CRH mRNA expression. Brains were quickly removed following decapitation and rapidly frozen on dry ice and stored at -70 °C until sectioning. Coronal sections (16 μm) through the medial prefrontal cortex, PVN and dorsal hippocampus were taken using a cryostat and thaw mounted onto poly-l-lysine (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) coated slides and stored at -70 °C until processing. In situ hybridization of 5-HT1A and 5-HT2A receptor and CRH mRNA was performed using [35S]-deoxyadenosine 5'-triphosphate (Amersham Biosciences Inc., Piscataway, NJ) labeled oligonucleotide probes. 5-HT1A and 5-HT2A oligonucleotide probes were synthesized according to the sequence published by Chen et al. (1995). CRH oligonucleotide probes were synthesized according to the sequence published by Jingami et al. (1985). Sections were post-fixed with formalin. Hybridization buffer containing dithiothreitol and probe was applied to slides which were then cover slipped and incubated overnight at 37 °C. Following hybridization, slides were washed several times in saline sodium citrate (SSC), dipped in distilled water and dehydrated in 70% ethanol. Air-dried slides were exposed to autoradiographic film (Biomax, Kodak) Exposure times were 21 days for 5-HT1A, 33 days for 5-HT2A, and 5 days for CRH. The films were developed and analyzed by image densitometry with Scion Image analysis (National Institutes of Health, Bethesda, MD). The measurement of 5-HT2A mRNA in the medial prefrontal cortex included the infralimbic, prelimbic and anterior cingulate regions.
The CA1, CA2, CA3 and dentate gyrus of the dorsal hippocampus were measured for 5-HT\textsubscript{1A} mRNA and the PVN for CRH mRNA.

**5.B.6. Statistical Analysis**

Analyses of variance (ANOVA) for the factors of sex, prenatal treatment, drug treatment and time, with time as a repeated measure where appropriate, were performed. Significant main and interaction effects were further analyzed by Newman-Keuls post hoc analysis.

**5.C. Results**

**5.C.1. Developmental Effects of Prenatal Ethanol Exposure**

Ethanol intake of pregnant females was fairly consistent throughout gestation, averaging 9.53 ± 1.17, 11.91 ± 1.16 and 9.06 ± 0.85 g/kg body weight for weeks 1, 2 and 3 of gestation respectively. There was no effect of prenatal ethanol on the number of liveborn or stillborn pups. However, a significant interaction between prenatal treatment and age on for both females [F (6, 162) = 3.27, p < 0.005] and males [F (6, 162) = 2.27, p < 0.05] indicated that prenatal ethanol and pair-feeding resulted in lower average pup weights on postnatal day 21 (Table 4). On postnatal days 90-120 (cannulated animals) there were significant differences in body weight among E, PF and C females [F(2, 89) = 4.52, p < 0.005], but not males. Post hoc analysis revealed that E females weighed significantly less than PF and C females (p's < 0.05). On postnatal days 120-150 (non cannulated animals) there was a significant effect of prenatal ethanol exposure for both females [F(2, 75) = 4.08, p < 0.05] and males [F(2, 59) = 4.31, p < 0.05]. PF females weighed significantly less than
Table 4. The effects of prenatal ethanol exposure on mean pup weights at postnatal (PN) days 1, 21, 90–120 (age of cannulated animals) and 120-150 (age of non-cannulated animals) (g, mean ± SEM). On PN21 ethanol-fed (E) and pair-fed (PF) animals weighed significantly less than ad libitum-fed (C) animals (* p’s < 0.05). On PN days 90–120 E females weighed significantly less than PF and C females (* p’s ≤ 0.05). On PN days 120–150 PF females weighed significantly less than C females (* p < 0.02), while E males weighed significantly less than C (* p < 0.05) and showed a trend toward lower body weights compared to PF males (p = 0.07). There were 12 litters in each prenatal treatment condition.

<table>
<thead>
<tr>
<th></th>
<th>PN 1</th>
<th></th>
<th>PN 21</th>
<th></th>
<th>PN 90-120</th>
<th></th>
<th>PN 120-150</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>E</td>
<td>5.5 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>*43.2 ± 1.7</td>
<td>*46.6 ± 1.5</td>
<td>*296.4 ± 6.3</td>
<td>532.1 ± 10.2</td>
<td>359.3 ± 8.2</td>
</tr>
<tr>
<td>PF</td>
<td>5.8 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>*46.5 ± 1.3</td>
<td>*48.4 ± 1.5</td>
<td>318.3 ± 6.8</td>
<td>538.4 ± 10.2</td>
<td>*345.8 ± 10.2</td>
</tr>
<tr>
<td>C</td>
<td>6.5 ± 0.1</td>
<td>6.9 ± 0.1</td>
<td>52.2 ± 5.6</td>
<td>55.5 ± 1.6</td>
<td>317.2 ± 6.5</td>
<td>560.5 ± 10.2</td>
<td>381.5 ± 8.1</td>
</tr>
</tbody>
</table>
C females (p < 0.05), while E females did not differ from either. In contrast, E males weighed significantly less than C (p < 0.05) and showed a trend toward a lower weight than PF (p = 0.07).

5.C.2. Plasma ACTH Levels

Females. There was no effect of prenatal treatment condition on basal ACTH levels. In comparison to saline, 8-OH-DPAT and DOI treatment resulted in an increase in ACTH levels. There was an interaction between time and drug [F(6, 225) = 41.57, p < 0.0001]. In comparison to saline, 8-OH-DPAT treatment resulted in increased ACTH levels. DOI did not significantly increase ACTH levels in comparison to saline. Post hoc analysis revealed that at 15 and 30 min post injection, 8-OH-DPAT-induced increases in ACTH were greater than the response to saline and DOI (p’s < 0.05).

Further analysis within each drug treatment condition, revealed differential effects of prenatal treatment. For 8-OH-DPAT there was an interaction between prenatal treatment and time [F(8, 100) = 3.00, p < 0.005]. E females had a significantly blunted ACTH response to 8-OH-DPAT at 15 (E < PF < C) and 30 (E < C) min post injection (p’s < 0.05). PF females did not differ from either E or C females at 30 min (Fig. 10). In contrast to the blunted ACTH response to 8-OH-DPAT, E females showed an augmented ACTH response to DOI, indicated by an effect of prenatal treatment [F(2, 23) = 3.82, p < 0.05]. Overall, DOI-treated E females had significantly higher ACTH levels compared to PF and C females, who did not differ from each other (p’s < 0.05). Finally, there was a main effect of saline-treatment [F(8, 100) = 3.00, p < 0.005] on ACTH levels. Across all prenatal treatment conditions, saline treatment resulted in a small but significant increase in ACTH levels at 15 and 30 min post injection (p’s < 0.01), in comparison to basal levels.
Males. There was an effect of prenatal treatment on basal ACTH levels, \( F(2, 77) = 4.26, p = 0.005 \). E males had significantly higher ACTH levels than C males \( (p = 0.05) \) (Table 5). In comparison to saline, 8-OH-DPAT and DOI treatment resulted in an increase in ACTH levels. There was an interaction between time and drug \( F(6, 219) = 26.97, p < 0.0001 \). 8-OH-DPAT resulted in increased ACTH at 15 min post injection in comparison with saline \( (p < 0.05) \). DOI resulted in increased ACTH levels at 60 and 120 min post injection in comparison to saline \( (p's < 0.05) \). In addition, drug induced increases in ACTH occurred earlier for 8-OH-DPAT than for DOI. At 15 min post injection, 8-OH-DPAT resulted in increased ACTH in comparison to DOI, but at 120 min, DOI-induced ACTH increases were greater than 8-OH-DPAT \( (p's < 0.05) \).

In contrast to females, further analysis within a drug treatment condition did not reveal significant effects of prenatal treatment at any time point following 8-OH-DPAT or DOI treatment, although patterns of responsiveness similar to that of females were observed for both 8-OH-DPAT and DOI (Fig. 11). Analyses did reveal an interaction between prenatal treatment and time \( F(8, 84) = 2.61, p < 0.02 \) on ACTH levels following saline treatment. PF males showed a significantly higher ACTH response 15 min following saline treatment compared to E males \( (p = 0.0002) \) while C males did not differ from either (Fig. 11).

Sex Differences. There was a main effect of sex on basal ACTH levels \( F(1, 159) = 6.67, p < 0.02 \). As expected, females had significantly higher basal ACTH levels than males, regardless of prenatal treatment. Following 8-OH-DPAT treatment, a sex and time interaction emerged \( F(3, 141) = 7.77, p < 0.0001 \). Females had higher ACTH levels than males at 15 and 30 min post injection but not at the other time points \( (p's < 0.0002) \). No sex differences emerged in response to DOI treatment.
Table 5. Basal plasma adrenocorticotropic hormone (ACTH) (± SEM) levels for ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males. E males had significantly higher basal levels of ACTH than C males, while ACTH levels in PF males were intermediate to those in E and C males (* p < 0.05).

<table>
<thead>
<tr>
<th>Prenatal Treatment Condition</th>
<th>Basal Plasma ACTH(pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>E</td>
<td>51.0±5.7</td>
</tr>
<tr>
<td>PF</td>
<td>43.1±3.7</td>
</tr>
<tr>
<td>C</td>
<td>47.6±2.9</td>
</tr>
</tbody>
</table>
Figure 10. Plasma adrenocorticotropic hormone (ACTH) levels in response to saline (1 ml/kg), 8-OH-DPAT (0.2 mg/kg) or DOI (0.3 mg/kg) treatment in females. There were no differences among prenatal treatment conditions (ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C)) in response to saline treatment. Following 8-OH-DPAT treatment there was a significant blunting of the ACTH response in E females at 15 (* E < PF < C) and 30 (^ E < C) min (p's ≤ 0.05). In response to DOI treatment there was an overall augmented ACTH response to DOI in E females (* E > PF = C; p's ≤ 0.05). 8-OH-DPAT treatment resulted in increased ACTH levels in comparison to saline treatment at 15 and 30 (PF and C only) min (p's < 0.05). DOI treatment resulted in increased ACTH levels compared to saline treatment at 60 and 120 min (p's < 0.001). (n = 8-10 per group).
**Figure 11.** Plasma adrenocorticotropic hormone (ACTH) levels in response to saline (1 ml/kg), 8-OH-DPAT (0.2 mg/kg) or DOI (0.3 mg/kg) treatment in males. In response to saline treatment there was an effect of prenatal treatment condition (ethanol-fed (E), pair-fed (PF), ad libitum-fed (C)) at 15 min post injection (PF > E; * p = 0.0002). No significant effects of prenatal treatment condition were found following 8-OH-DPAT or DOI treatment. 8-OH-DPAT treatment resulted in increased ACTH levels in comparison to saline treatment at 15 and 30 min (p’s < 0.001). DOI treatment resulted in increased ACTH levels compared to saline treatment at 60 and 120 min (p’s < 0.05). (n = 8-10 per group).
5.C.3. Plasma CORT Levels

**Females.** There was no effect of prenatal treatment condition on basal CORT levels. Similar to drug-induced increases in ACTH, 8-OH-DPAT and DOI treatment resulted in significant increases in CORT. There was an interaction between drug and time \([F(6, 222) = 34.51, p < 0.0005]\). 8-OH-DPAT treatment resulted in an increase in CORT at 30 and 60 min post injection in comparison to saline (p’s < 0.001). DOI treatment resulted in an increase in CORT at 60 and 120 min post injection in comparison to saline (p’s = 0.0001). At 60 and 120 min post injection, DOI-induced ACTH increases were greater than 8-OH-DPAT-induced increases (p’s < 0.05).

Further analysis within each drug treatment condition revealed no significant effects of prenatal treatment on CORT levels at any time point following 8-OH-DPAT or DOI treatment (Fig. 12). However, there was a main effect of saline treatment \([F(4, 84) = 61.89, p < 0.0001]\) on CORT levels. In comparison to basal levels, saline treatment increased CORT levels at 15 and 30 min post injection (p’s < 0.0001). However, there were no differential effects of prenatal treatment on CORT.

**Males.** There was no effect of prenatal treatment condition on basal CORT levels. In comparison to saline, 8-OH-DPAT and DOI treatment resulted in significant increases in CORT. There was an interaction between drug and time \([F(6, 189) = 42.26, p < 0.0001]\). 8-OH-DPAT treatment resulted in increased CORT levels at 60 min post injection in comparison to saline (p < 0.05). DOI treatment resulted in increased CORT levels at 60 and 120 min post injection in comparison to saline (p’s < 0.001). DOI treatment resulted in increased CORT levels at 120 min post injection in comparison to saline (p < 0.005).
Further analysis within a drug treatment condition revealed no significant effects of prenatal treatment on CORT levels at any time point following 8-OH-DPAT or DOI treatment (Fig. 13). However, there was a main effect of saline treatment \[F(4, 64) = 52.06, p < 0.0001\] on CORT levels. Across all prenatal treatment conditions, saline treatment increased CORT levels at 15, 30 and 60 min post injection (\(p's < 0.0005\)), in comparison to basal levels.

**Sex Differences.** As expected, there was a main effect of sex on basal CORT levels \[F(1, 156) = 40.77, p < 0.00001\]. Females had significantly higher basal CORT levels than males, regardless of prenatal treatment. Sex and time interactions were observed following saline \[F(3, 114) = 22.35, p < 0.00001\], 8-OH-DPAT \[F(3, 126) = 11.71, p = 0.0001\] and DOI \[F(3, 135) = 3.05, p < 0.05\] treatment. Post hoc analysis revealed that females had higher CORT levels than males at 15 and 30 min post saline injection (\(p's < 0.001\)), and at all time points following treatment with 8-OH-DPAT or DOI (\(p's < 0.001\)).

**5.C.4. CRH mRNA Levels in the PVN**

**Females.** There was a trend toward increased CRH mRNA levels in C compared to E and PF females following DOI treatment \[F(1, 17) = 2.86, p = 0.08\]. However, there were no effects of prenatal treatment following 8-OH-DPAT or saline treatment (Fig. 14).

**Males.** There was a main effect drug treatment \[F(2, 47) = 3.17, p = 0.05\]. DOI-treated males had significantly higher levels of mRNA than saline-treated males, an effect due primarily to the elevated levels of mRNA among E males (Fig. 14). There were no differences following 8-OH-DPAT or saline treatment.
Figure 12. Plasma corticosterone (CORT) levels in response to saline (1 ml/kg), 8-OH-DPAT (0.2 mg/kg) or DOI (0.3 mg/kg) treatment in females. No significant effects of prenatal treatment condition were found at any time point examined. 8-OH-DPAT treatment resulted in increased CORT levels in comparison to saline treatment at 15, 30 and 60 min (p’s < 0.05). DOI treatment resulted in increased CORT levels compared to saline treatment at 60 and 120 min (p’s < 0.001). (n = 8-10 per group).
**Figure 13.** Plasma corticosterone (CORT) levels in response to saline (1 ml/kg), 8-OH-DPAT (0.2 mg/kg) or DOI (0.3 mg/kg) treatment in males. No significant effects of prenatal treatment condition were found at any time point examined. 8-OH-DPAT treatment resulted in increased CORT levels in comparison to saline treatment at 30 and 60 min (p’s < 0.05). DOI treatment resulted in increased CORT levels compared to saline treatment at 60 and 120 min (p’s < 0.0005). (n = 8-10 per group)
Sex differences. There were no sex differences among saline-treated females and males. However, following 8-OH-DPAT treatment males showed higher CRH mRNA levels than females [F(1, 34) = 5.84, p < 0.05]. Following DOI treatment, there was a significant interaction between prenatal treatment and sex [F(2, 3) = 4.20, p < 0.05]. E males had higher levels of CRH mRNA than E females (p < 0.05).

5.C.5. 5-HT₁A Receptor mRNA Levels in the Dorsal Hippocampus

Females. There were no effects of prenatal treatment on 5-HT₁A mRNA levels in saline- or DOI-treated females. Among 8-OH-DPAT-treated females, mRNA levels were higher in E compared to PF females in the CA1 subfield [F(2, 14) = 3.61, p = 0.05] (p = 0.05) and higher levels in E compared to C females in the CA2 subfield [F(2, 14) = 3.50, p = 0.05] (p = 0.05) (Fig. 15). A repeated measures ANOVA also revealed a significant difference in 5-HT₁A mRNA levels among hippocampal subfields [F(3, 129) = 187.18, p < 0.0001]. Levels of 5-HT₁A mRNA in descending order was DG > CA1 > CA3 > CA2 (p's < 0.0005).

Males. There were no main effects of drug treatment on 5-HT₁A receptor mRNA levels. Within saline-, 8-OH-DPAT- and DOI-treated males there were no differences in 5-HT₁A mRNA due to prenatal treatment condition. As in females, there was a significant difference in 5-HT₁A mRNA levels between hippocampal subfields [F(3, 123) = 185.8, p < 0.0001], except for regions CA1 and CA3, which showed equal levels of expression. Levels of 5-HT₁A mRNA in descending order was DG > CA1 = CA3 > CA2 (Fig. 16).
Figure 14. Corticotropin releasing hormone (CRH) mRNA levels in the paraventricular nucleus of ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males. There was a trend toward higher levels of mRNA among DOI-treated C compared to E and PF females (\(^ p = 0.08 \)). DOI-treated males had higher levels of mRNA compared to saline-treated males (* \( p = 0.05 \)), an effect primarily due to elevated levels among E males. In addition, males had higher levels of mRNA than females. (\( n = 5 - 8 \) per group).
Figure 15. 5-HT₁A receptor mRNA levels in various regions of the dorsal hippocampus in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females following saline, 8-OH-DPAT (0.3 mg/kg) or DOI (0.2 mg/kg) treatment. Following 8-OH-DPAT treatment, E females had higher levels of mRNA in the CA1 subfield (* E > PF) and in the CA2 subfield (^ E > C) (p's = 0.05). There was no effect of drug treatment. There was a main effect of hippocampal subfield on mRNA levels (DG > CA1 > CA3 > CA2) (p's < 0.0005). (n = 5 - 8 per group).
Figure 16. 5-HT_{1A} receptor mRNA levels in various regions of the dorsal hippocampus in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) males following saline (1 ml/kg), 8-OH-DPAT (0.3 mg/kg) or DOI (0.2 mg/kg) treatment. There were no significant effects of prenatal or drug treatment. There was a main effect of hippocampal subfield on mRNA levels (DG > CA1 = CA3 > CA2) (p's < 0.0005). (n = 5 - 8 per group).
Sex Differences. Females had higher levels of mRNA in the CA1 subfield than males \([F(3, 252) = 5.83, p < 0.01]\).

5.C.6. 5-HT2A Receptor mRNA Levels in the Medial Prefrontal Cortex

There were no significant differences among prenatal treatment conditions, drug treatment or sex on 5-HT2A mRNA levels in the medial prefrontal cortex (Fig. 17).

5.D. Discussion

The purpose of the present study was to investigate whether 5-HT receptor-mediated endocrine responses are altered by prenatal ethanol exposure. We found that prenatal ethanol exposure in females results in a decreased 5-HT1A receptor-mediated but increased 5-HT2A receptor-mediated ACTH response in comparison to PF and C females. We also found that following 8-OH-DPAT treatment there was an increase in 5-HT1A mRNA in the subregions of the hippocampus in E females. In contrast, E males did not differ from PF and C males in plasma ACTH and CORT responses to 8-OH-DPAT or DOI, but showed increased CRH mRNA in response to DOI-treatment, contributing to an overall increase in CRH mRNA in DOI- compared to saline-treated males. These results indicate that prenatal ethanol exposure alters neuroendocrine responses to 5-HT1A and 5-HT2A agonists in a sex specific manner. Furthermore, these findings may indicate an altered interaction between the HPA axis and 5-HT in E animals and altered responsiveness of the 5-HT1A receptor to stimulation.
**Figure 17.** 5-HT$_2$A receptor mRNA levels in the medial prefrontal cortex of ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males. There were no significant differences among prenatal treatment conditions. (n = 5 - 8 per group).
Basal levels of ACTH were not altered in females. However, among males significantly higher basal ACTH levels were found in E compared to C males, while PF did not differ from either. This finding was unexpected as previous research in our laboratory and others have not consistently observed any effects of prenatal ethanol exposure on basal hormone levels. This may reflect residual effects of surgery in E males. However, it is also possible that this represents a novel finding. Interestingly, a recent experiment in our laboratory found elevated basal ACTH levels in naive and untreated, E compared to PF and C females (unpublished observations). Our data suggest differential central regulation of basal HPA activity in E females and males compared to their respective control counterparts and may be related to deficits in HPA feedback regulation (Glavas et al. 2000). These findings support further investigation into basal HPA axis function following prenatal ethanol exposure.

Previous studies in our laboratory found an increased hypothermic and anxiolytic response to 8-OH-DPAT in E compared to PF and C animals, as well as an increase in DOI-induced wet dog shakes, suggesting that prenatal ethanol exposure increases central 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} function (Chapter III; Chapter IV; Hofmann et al. 2002a; Hofmann et al. 2002b). Therefore, we hypothesized that E animals would also show increased hormonal responses to 8-OH-DPAT and DOI. We found that administration of the 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} agonists 8-OH-DPAT and DOI generally resulted in significant increases in ACTH and CORT in comparison to basal levels and saline treatment, consistent with several previous studies examining the endocrine effects of these agonists (Rittenhouse et al. 1994; Pan and Gilbert 1992; Lorens and Van de Kar 1987). Interestingly, we found that E females showed an increased ACTH response to DOI, but a blunted ACTH response to 8-OH-DPAT compared to PF and C females. E males showed a similar pattern of increased responses to DOI, but the differences were not significant. Our laboratory (Hofmann et al.
2002a, Hofmann et al. 2002b) has previously found that the effects of prenatal ethanol exposure on functioning of the 5-HT system are especially obvious in females compared to males. It appears that females are more vulnerable to the effects of in utero exposure to ethanol on 5-HT receptor mediated responses, through unknown mechanisms.

Furthermore, examination of hormone levels over time indicates that the effects of prenatal ethanol exposure are not due to a shift, advanced or delayed, in the ACTH response to the agonists, but rather to an effect on peak levels. No effects of prenatal treatment on CORT levels were observed. As small ACTH increases can result in maximal CORT increases, CORT levels may be a less sensitive indicator of neuroendocrine alterations (Rittenhouse et al. 1994). It is presently unclear why prenatal ethanol exposure would result in selective increases in 5-HT1A receptor-mediated function (i.e., increased hypothermic but decreased ACTH response). However, an answer likely requires consideration of the presence of pre- and post-synaptic 5-HT1A receptors and the complexity of the entire 5-HT system. Alternatively, it is possible that the blunted hormonal response to 8-OH-DPAT actually reflects increased 5-HT1A receptor function. 5-HT can have inhibitory effects, or hyperpolarize the neuron on which it’s located (via 5-HT1A receptors). In the hippocampus, 5-HT1A receptors are located on pyramidal neurons mediating feedback inhibition of the HPA axis. Therefore a blunted HPA response to 5-HT1A stimulation might be expected with increased function of this receptor in E animals.

Saline treatment also stimulated increases in ACTH and CORT release in comparison to basal levels, indicating that the injection procedure itself was a stressor. However, the magnitude of the hormone response to saline injection was small in comparison to that induced by 8-OH-DPAT and DOI treatment. Furthermore, the hormone response to saline injection was similar among prenatal treatment conditions, with the one exception that PF males showed a significantly greater ACTH, response than E males at 15
min post saline injection. Saline injection served as a control for the possible stressful effects of the injection procedure, which is an especially important control in experiments with E animals that show increased hormonal hyperresponsiveness to a variety of stressors. Effects due to pair-feeding apart from prenatal ethanol effects are not unique to this experiment and may represent methodological issues associated with the pair-feeding procedure. PF dams receive an amount of diet matched to E dams in g/kg body weight for the same day of gestation, with maltose-dextrin isocalorically substituted for ethanol. Therefore, they receive a ration that is less than they would consume ad libitum. Although both groups are receiving the same number of calories, PF dams experience mild food deprivation, while E dams do not. In this respect, pair-feeding may be a type of prenatal stressor and prenatal exposure to stressors is known to impact adult functioning of the HPA axis (Hofmann et al. 1999; Vallee et al. 1997). Despite the effect of pair-feeding on saline-induced ACTH levels, PF females and males showed a similar pattern of ACTH and CORT release to 8-OH-DPAT and DOI treatment to C females and males, indicating that these HPA responses to 5-HT stimulation are normal.

In addition to examining the ACTH and CORT responses to 8-OH-DPAT and DOI, we also examined CRH mRNA levels in the PVN. There was no effect of prenatal ethanol treatment following saline injection. This is consistent with another study in our laboratory (unpublished findings) and others (Aird et al. 1997) that prenatal ethanol exposure does not alter basal levels of CRH mRNA. Increased basal levels in E females and E males (Lee et al 1990) or only in E males (Glavas et al. 2000; Redei et al. 1993) and not E females (unpublished observations) have also been reported. The discrepancies in basal CRH mRNA may reflect experimental differences, such as time of testing, age of animal or method of ethanol administration. Following stressors, however, CRH mRNA appears to be increased in E animals (Lee et al. 2000). We expected to see an increased CRH mRNA
in response to DOI treatment in E females in parallel to the increased ACTH response. Interestingly, this effect appeared in E males instead. It is important to note that overall, DOI-treated males had higher levels of CRH mRNA than saline-treated males, though the effect was largest in E males. Van de Kar et al. (2001) reported an increase in Fos immunoreactivity in the PVN following DOI treatment. Our work further supports their conclusion that DOI-induced increases in ACTH are mediated by 5-HT<sub>2A</sub> activation in the PVN and that endocrine responses to DOI can be used to assess central 5-HT<sub>2A</sub> receptor function (Van de Kar et al. 2001). We cannot conclusively say that DOI-induced increases in ACTH in E females are not due to increased CRH levels as we did not measure peptide levels, though there appears to be a good correlation between CRH mRNA levels and peptide (Herman and Morrison 1996). There are other mechanisms through which 5-HT agonists could differentially increase ACTH in E females. For example, arginine vasopressin acts synergistically with CRH to stimulate release of ACTH from the pituitary. Since the agonists were administered systemically, we cannot rule out direct actions of these drugs at the pituitary or increased sensitivity of the pituitary to CRH. However, if the latter possibility were true, we would expect to see an increased ACTH response to both DOI and 8-OH-DPAT in E females compared to PF and C females.

Regardless of prenatal treatment condition, we found the amount of 5-HT<sub>1A</sub> mRNA in the various subregions of the hippocampus to be consistent with previous research (Chen et al. 1995, Mendelson and McEwen, 1991). In general, 5-HT<sub>1A</sub> levels were highest in the dentate gyrus and lowest in CA2. In addition, females had higher levels of 5-HT<sub>1A</sub> mRNA in CA1 compared to males, a result also reported by Mendelson and McEwen (1991). We examined 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor mRNA to provide an indication of basal levels of receptor expression and receptor function. As we have observed increased 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor function in E animals, we hypothesized that 5-HT receptor activation might
result in increased mRNA synthesis. We found that 5-HT$_{1A}$ mRNA in CA1 and CA2 was increased in E but not PF and C females following 8-OH-DPAT treatment. This may suggest that E animals show an increased sensitivity of the 5-HT$_{1A}$ receptor to stimulation, at least in the hippocampus. The physiological significance of this finding remains to be determined, though likely has implications for HPA feedback and affective states (Lopez et al. 1998; McAllister-Williams and Young 1998; Jacobson and Sapolsky 1991).

There were no differences in 5-HT$_{1A}$ or 5-HT$_{2A}$ mRNA among prenatal treatment conditions following saline treatment. This may reflect a lack of basal differences in 5-HT receptors, at least in the hippocampus and prefrontal cortex. As we have previously observed an increase in DOI-induced WDS in E females, and the medial prefrontal cortex is thought to mediate DOI-induced wet dog shakes (Willins and Meltzer 1997), this brain region was a logical choice for examination. However, it is possible that an increase in 5-HT receptors do not mediate the observed increases in 5-HT receptor mediated function. Van de Kar et al. (1998) has reported that increased receptor function can occur in the absence of changes in the number of receptors. In this case, increased coupling to second messenger systems may be a possible explanation for increased function (Li et al. 1997). It is possible that increased G-protein coupling at the 5-HT$_{2A}$ receptor is increased in E animals. However a preliminary study in E animals suggests that only minor changes in G-proteins results from prenatal ethanol exposure (Druse 1994).

Infants, and non-human primates prenatally exposed to ethanol exhibit hormonal hyperresponsiveness to stressors (Jacobson et al. 1999, Schneider et al. 2002). As prenatal ethanol exposure potentiated the ACTH response to a 5-HT$_{2A}$ agonist, there may be a role for 5-HT$_{2A}$ receptors in hormonal hyperresponsiveness to stressors observed in E animals. Exposure to stressors results in increases in 5-HT in several brain regions (Funada and Hara, 2001, Kirby et al. 1997). It could be predicted that stress-induced increases in 5-HT
result in hormonal hyperresponsiveness to stressors in E animals via increased 5-HT$_{2A}$ mediated hormone function. Interestingly, our laboratory has found other sex differences in HPA axis functioning among E females and males. HPA hyperresponsiveness to stressors has been observed in both E females and males, though the effects depend on the time course, type and duration of stressor, and the hormonal endpoint measured (Weinberg et al. 1996). Clearly, further research is needed to clarify the potential role of 5-HT$_{2A}$ receptors in mediating hormonal hyperresponsiveness to stressors in E offspring.

The HPA axis is an important system in the stress response and has been implicated in the pathophysiology of affective disorders (Holsboer 2000; McAllister-Williams and Young 1998). Depressed individuals have elevated basal levels of ACTH and cortisol (Maes et al. 1991), blunted neuroendocrine responses to pharmacological challenge (Weizman et al. 1988), decreased dexamethasone suppression (Rybakowski and Twardowska 1999; Maes et al. 1995) and hyperactivity of CRH neurons, as evidenced by the effectiveness of CRH antagonists in treating depression (Keck and Holsboer 2001; Holsboer 1999). Treatment with 5-HT agents results in a normalization of many of these neuroendocrine abnormalities in individuals with depression (Holsboer and Barden 1996) and is often a requirement for therapeutic improvement. In the present experiment, we have begun to explore the interaction between 5-HT and the HPA axis in E animals, as it is highly relevant to individuals with FAS, who have an increased incidence of affective disorders. In individuals with a history of prenatal alcohol exposure, depression was the second most common mental illness, preceded by alcohol and/or drug dependence (Famy et al. 1998). Children with prenatal alcohol exposure score higher on the anxious/depressed subscale of the Achenbach Child Behavior Checklist (Sood et al. 2001). Others have also reported an association between prenatal alcohol exposure and self- and parental-reported depressive symptoms (Roebuck et al. 1999; Shah et al. 1999; O'Connor and Kasari 2000)
as well as increased drug dependence in adult adoptees with fetal alcohol exposure (Yates et al. 1998; Mattson and Riley 2000).

In summary, 5-HT neuroendocrine challenge has been used clinically as a marker of clinically depressed individuals (Riedel et al. 2002) and as a marker of clinical improvement following drug treatment (Bhagwagar et al. 2002; Raap and Van de Kar 1999). The results of the present study suggest that hormonal responses to 5-HT agonists may be used as a marker of central 5-HT alterations induced by prenatal ethanol exposure or that individuals affected by prenatal ethanol exposure may respond differently than other clinical populations to serotonergic drug treatment.
CHAPTER VI: GENERAL DISCUSSION

6.A. Summary and Discussion

The major objectives of this thesis were to investigate the long-term consequences of prenatal ethanol exposure on serotonergic receptor-mediated behavioural (i.e., wet dog shakes, anxiety-like behaviour) and physiological (i.e., hypothermia, HPA response) function. The hypothesis was that increased 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor function would be observed in E animals, as a result of a prenatal ethanol-induced decrease in 5-HT levels. This hypothesis was based on reports of supersensitivity of 5-HT receptors in the offspring of dams treated with 5-HT depleting drugs during pregnancy, or effects of neonatal or adult lesions of the serotonergic system.

The first study (Chapter III) investigated 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor-mediated behavioural and physiological function in adult E animals. We tested the hypothesis that E animals would show increased 8-OH-DPAT-induced hypothermia and DOI-induced WDS compared to PF and C animals. We found that both E females and males showed a greater hypothermic response to 8-OH-DPAT than PF and C animals. In addition, E females and males also showed less of a differential response to low and high doses of 8-OH-DPAT than PF and C animals. In response to DOI, E females but not males, showed a significantly greater rate of WDS than PF and C females. That is, E females and males showed increased 5-HT$_{1A}$ mediated function, while only E females showed increased 5-HT$_{2A}$ mediated function. These data indicate that prenatal ethanol exposure results in long-term effects on 5-HT-receptor mediated behavioural and physiological function in adult animals and that some of these effects may be sex specific.
Previous research supports the role of the hypothalamus in mediating 8-OH-DPAT induced hypothermia (Stockmeier et al. 1992) and the medial prefrontal cortex in mediating DOI-induced wet dog shakes (Willins and Meltzer 1997). Therefore, the 5-HT receptor supersensitivity in E animals may be localized to the hypothalamus and the medial prefrontal cortex although involvement of other brain regions can not be excluded as the drugs were administered systemically. Therefore, it is more likely that these physiological and behavioural assays provide preliminary data on central 5-HT_{1A} and 5-HT_{2A} receptor function rather than on region specific receptor function in general, following prenatal ethanol exposure.

The second study (Chapter IV) investigated the impact of prenatal ethanol exposure on anxiety-like behaviour, using suppression of feeding as a marker of anxiety in a novel environment. We also investigated the possibility that prenatal ethanol exposure alters sensitivity to the anxiolytic effects of 8-OH-DPAT. We hypothesized that E animals would show increased anxiety-like behaviour and an increased anxiolytic response to 8-OH-DPAT in comparison to PF and C animals. While there were few differences under home cage conditions following saline and 8-OH-DPAT treatment, there was a marked drop in the number of E animals consuming the familiar, palatable food in the novel environment compared to PF and C animals. E animals showed increased anxiety-like behaviour in the novelty-induced suppression of feeding task, an effect ameliorated by 8-OH-DPAT, suggesting that prenatal ethanol exposure may impact 5-HT mediated anxiety-like behaviour in adulthood.

The findings from this study share similarities with findings from studies with animals with genetic deletions of the 5-HT_{1A} receptor. 5-HT_{1A} knock-out mice have a significantly longer latency to begin feeding in a novel environment (Gross et al. 2002). Gross et al. (2002) also showed that 5-HT_{1A} receptor expression, especially in the cortex
and hippocampus, during the preweaning period was required for normal adult expression of anxiety-like behaviour. Druse et al. (1991) showed that the normal developmental increase in 5-HT$_{1A}$ receptors is delayed in E animals during the late preweaning period. Therefore, it is plausible that prenatal ethanol induced alterations in 5-HT$_{1A}$ receptor expression in E animals impact anxiety-like behaviour in adulthood.

It is also possible that altered mother and E pup interactions in the early postnatal period may impact expression of adult anxiety-like behaviour in E animals, due to ethanol effects on the pup, rather than possible residual effects ethanol consumption on the dam. For example, E exposed pups have a longer nipple attachment latency (Rockwood and Riley 1990) and are not as effective in eliciting retrieval behaviour from dams compared to control pups (Ness and Franchina 1990). Decreased maternal behaviour, such as decreased licking and grooming can result in increased anxiety-like behaviour in adult animals (Caldji et al. 1998). Therefore, it is possible that the reduction in E pup-elicited maternal care affects expression of anxiety-like behaviour in adulthood.

The third study (Chapter V) investigated the effects of prenatal ethanol exposure on central 5-HT receptor-mediated hormonal function and on 5-HT receptor mRNA and CRH mRNA levels. We found that E females had an attenuated ACTH response to 8-OH-DPAT but an augmented ACTH response to DOI, in comparison to PF and C females. Furthermore, there were no prenatal ethanol-induced alterations in 5-HT receptor mRNA in saline-treated animals. 8-OH-DPAT treatment resulted in increased 5-HT$_{1A}$ mRNA levels in the CA1 and CA2 subfields of the hippocampus in E females compared to PF and C females. E males showed increased CRH mRNA levels in response to DOI, contributing to an overall increase in CRH mRNA in DOI-treated versus saline-treated males. These data demonstrate that prenatal ethanol exposure can augment central 5-HT$_{2A}$ but attenuate 5-HT$_{1A}$ receptor-mediated neuroendocrine responses in a sex specific manner. Furthermore,
the data indicate increased responsiveness of the 5-HT\textsubscript{1A} receptor in E females to the stimulatory effects of 8-OH-DPAT.

It is possible that the blunted hormonal response to 8-OH-DPAT actually reflects increased 5-HT\textsubscript{1A} receptor function. 5-HT has inhibitory effects and can hyperpolarize the neuron on which it is located (via 5-HT\textsubscript{1A} receptors). In the hippocampus, 5-HT\textsubscript{1A} receptors are located on pyramidal neurons that mediate feedback inhibition of the HPA axis. Therefore a blunted HPA response to 5-HT\textsubscript{1A} stimulation might be expected with increased function of this receptor in E animals.

No differences in 5-HT receptor mRNA were found among prenatal treatment conditions following saline treatment. Although this experiment was limited in the brain regions examined and did not examine 5-HT receptor protein levels, this preliminary examination of 5-HT receptor mRNA may suggest that 5-HT receptor number is not altered by prenatal ethanol exposure. Further experiments should use immunohistochemistry to examine 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptor number to rule out alteration in receptor number. Van de Kar et al. (1998) has reported that increased receptor function can occur in the absence of changes in the number of receptors. In this case, increased coupling to second messenger systems may be a possible explanation for increased function. Chapter III reported an increase in DOI-induced WDS in E females, compared to PF and C females, although others have reported no increase 5-HT\textsubscript{2A} receptor number in cortical areas in 19 and 35 day old rats as determined by binding studies are not altered by prenatal ethanol exposure (Tajuddin and Druse 1989a). Patel et al (1996) found that neurotoxin-induced denervation of the hippocampus in neonates resulted in increased 5-HT\textsubscript{1A} receptors in some regions of the hippocampus, similar to increases observed in E offspring (Druse et al. 1991), providing a molecular basis for supersensitivity. It is possible that increased G-
protein coupling at the 5-HT\textsubscript{2A} receptor is increased in E animals. However, Druse et al. (1994) found only minor changes in G-proteins results from prenatal ethanol exposure.

In a review of experiments comparing both hypothermic and neuroendocrine responses to meta-chlorophenylpiperazine, a 5-HT\textsubscript{2C} agonist, in neuropsychiatric patients and normal controls, Murphy et al. (1996) found nonconcordant results. Although psychiatric patients exhibited increased hyperthermic responses to m-CPP compared to controls, they did not necessarily show increased hormonal responses. While Chapter III reported an increased 5-HT\textsubscript{1A} mediated hypothermic response in E compared to PF and C animals, Chapter V did not find an increased 5-HT\textsubscript{1A} mediated hormonal response in E compared to PF and C animals, but instead found the opposite, a blunted hormonal response in E females. These results suggest caution in drawing conclusions on the basis of single endpoint studies utilizing pharmacological challenge to assess 5-HT receptor function.

The results of this thesis support the hypothesis that 5-HT receptor supersensitivity exists in E animals and may be a result of prenatal ethanol-induced decreases in 5-HT. This conclusion is supported by findings of studies in which central 5-HT levels were depleted with neurotoxins. The experiments in this thesis found that E offspring exhibited increased 8-OH-DPAT-induced hypothermia as well as increased DOI-induced WDS in comparison to control animals. Similarly, following 5-HT depletion, increased 8-OH-DPAT-induced hypothermia and increased behavioural responses to 5-HT agonists have been found in comparison to non-depleted animals (Aguirre et al. 1998; Pranzatelli et al. 1988; Goodwin et al. 1987). Furthermore, E animals also showed increased stress-induced (saline injection) hyperthermia and increased basal ACTH in comparison to control animals. Chung et al. (1999) found increases stress-induced hyperthermia and increased basal CORT levels in 5-HT depleted animals. E offspring also exhibited both blunted and augmented ACTH
responses to 8-OH-DPAT and DOI, respectively. In 5-HT depleted animals, blunted and augmented neuroendocrine responses to various challenges, including 5-HT agonists, stressors and immune challenge have also been observed (Van de Kar et al. 1989; Laflamme et al. 1999; Quattrone et al. 1981). For example, increased plasma renin, ACTH, CORT and prolactin levels were observed in 5-HT depleted animals in comparison to control animals following administration of RU 24969, primarily a 5-HT1A/B agonist (Van de Kar et al. 1989). However, a blunted CRH mRNA and CORT response to lipopolysaccaride (LPS) (i.e., immune challenge) was observed following 5-HT depletion (Laflamme et al. 1999). Finally, 5-HT depletion also resulted in a blunted ACTH response to restraint stress, but not other stressors or LPS (Jørgensen et al. 1998).

The mechanism underlying supersensitivity is not known, though some suggest that it reflects an increase in receptor number. While some have found increased, decreased or no change in 5-HT receptor mRNA in response to 5-HT depletion (Garcia-Osta et al. 2000; Van de Kar 1998; Patel et. al. 1996). Garcia-Osta et al. (2000) reported an increase in 5-HT1A mRNA in the cortex but a decrease in the hippocampus. Thus, 5-HT depletion-induced changes in receptor mRNA are likely dependent on brain region and other experimental factors (Frankfurt et al. 1993). E animals did not exhibit an increase in basal (i.e., following saline injection) 5-HT1A or 5-HT2A mRNA. These findings may also support the conclusion that an increase in 5-HT receptor number does not underly supersensitivity. However, following 8-OH-DPAT treatment, E females exhibited an increase in hippocampal 5-HT1A mRNA. It is unclear if this is a property of supersensitive receptors, and may suggest other altered cellular events in hippocampal neurons of E animals. Furthermore, hippocampal neuron responses to 8-OH-DPAT may be somewhat different from responses to the natural ligand (i.e, 5-HT), especially as 8-OH-DPAT may act as a partial agonist in the hippocampus (Sanders-Bush and Conn 1987).
In summary, these results are among the first to examine the long term functional consequences of altered neurotransmitter levels in E animals, and are the first to specifically examine 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptor function in adult female and male animals. The importance of examination of prenatal ethanol exposure in both sexes is highlighted by the findings of this thesis; 5-HT receptor mediated function in females is especially affected by prenatal ethanol exposure. In addition, the experiments of this thesis represented a preliminary attempt to correlate prenatal ethanol-induced changes in 5-HT with clinically relevant behavioural and physiological function, such as increased hormonal responsiveness to stressors and anxiety-like behaviour. Finally, in all experiments adult E females and males (i.e., 60-150 days of age) were utilized. These results are the first to report long term functional alterations in 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A} receptors in E offspring. This finding is important, as some effects of prenatal ethanol exposure are attenuated with increasing age.

\textbf{6.B. Future Directions}

The results from the experiments in this thesis reveal that E animals exhibit long-term 5-HT receptor-mediated functional alterations. Furthermore, responses to agonists tend to be increased in E animals, possibly suggesting the presence of supersensitive 5-HT receptors. Further experiments including dose responses of drugs are needed to provide definitive proof of supersensitive 5-HT receptors in E animals. A leftward shift in the dose response curve to 8-OH-DPAT or DOI would further support the hypothesis of supersensitive 5-HT receptors in E animals.

There is a reciprocal relationship between the 5-HT system and the HPA axis. 5-HT can stimulate the HPA axis to result in an increase in stress hormones, and glucocorticoids released from the adrenal gland can suppress 5-HT receptor expression. Our laboratory is
particularly interested in the possible role of 5-HT receptors in mediating hormonal hyperresponsiveness to stressors in E animals. The results from Chapter V revealed augmented 5-HT$_{2A}$ but blunted 5-HT$_{1A}$ receptor-mediated increases in ACTH in E animals. However, it is not clear whether activation of 5-HT receptors during exposure to a stressor contributes to hormonal hyperresponsiveness in E animals. Future experiments might also include administration of 5-HT$_{2A}$ or 5-HT$_{1A}$ antagonists prior to stressor exposure in E animals to determine the role of these receptors in stress-induced hormonal increases. The absence of hyperresponsiveness in E animals following receptor blockade would indicate the involvement of this receptor in this response.

It is also possible that increased HPA activity in E animals has consequences for functioning of the 5-HT system. Chronic stress or chronic administration of CORT has suppressive effects on 5-HT$_{1A}$ receptor mRNA levels (Nishi and Azmitia 1996; Chalmers et al. 1993). Elevated CORT levels resulting from ethanol-induced activation of the maternal and/or the fetal HPA axis (Weinberg and Bezio 1987; Weinberg and Gallo 1982) could potentially suppress fetal levels of 5-HT$_{1A}$ mRNA. This effect could be detrimental to the developing fetus, as 5-HT$_{1A}$ receptors mediate the neurotrophic actions of 5-HT (Azmitia 2001). Our laboratory has also shown that glucocorticoid receptors are altered by prenatal ethanol exposure. Basal levels of glucocorticoid receptor (GR) mRNA are increased E compared to PF and C females. Meijer et al. (1998) reported that following GR activation with high levels of CORT, there was increased 5-HT$_{1A}$ receptor-mediated behaviour (i.e., locomotion). Furthermore, 10 day administration of ACTH, which may result in prolonged GR activation, resulted in increased DOI-induced WDS. These data may suggest that increased GR activation in E females mediates increased 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor-mediated function. As elevated stress hormones can be detrimental to an organism and increasing evidence indicates the importance of the interaction between the 5-HT and HPA
systems in affective disorders, answers to these research questions are highly relevant to individuals with FAS or alcohol-related diagnoses.

In all three experiments in this thesis, the effects of prenatal ethanol exposure on 5-HT receptor mediated function was greater in females than in males. There are many reports of sex differences in animals prenatally exposed to ethanol, including numerous reports from our laboratory on sex differences in HPA function (Hofmann et al. 1999; Weinberg et al. 1996). There are several possible explanations for sex differences in 5-HT₁A and 5-HT₂A receptor function following prenatal ethanol exposure, that involve direct and indirect actions of ethanol. In general, there are sex differences in several aspects of the 5-HT system, including 5-HT levels and receptor number. For example, estradiol and progesterone can increase 5-HT2A mRNA expression (Birzniece et al. 2002) and increased levels of 5-HT have been observed in several brain regions in females (Borisova et al. 1996; Carlsson & Carlsson, 1988). However, these explanations are speculative and sex differences following prenatal ethanol exposure remain an important topic for future investigation. Although little is known about prenatal ethanol-induced sex differences in 5-HT and 5-HIAA levels, it could be speculated that prenatal ethanol-induced decreases in 5-HT are greater in E females contributing to greater adulthood expression of 5-HT receptor supersensitivity in comparison to E males. Consistent with hypothesis, Clausing et al. (1996) found decreased levels of striatal 5-HT and 5-HIAA in 20 day old E females but not E males in comparison to control animals. Prenatal ethanol exposure may indirectly impact the 5-HT system through its actions on other systems. For example, prenatal ethanol exposure affects the HPA axis as well as the hypothalamic-pituitary-gonadal (HPG) axis. Both glucocorticoids and gonadal steroids can influence the level of 5-HT and its receptors, thereby providing an indirect pathway for prenatal ethanol exposure to affect central 5-HT
function. Further research in our laboratory will investigate the impact of prenatal ethanol exposure on the HPA and HPG axes.

FAS is entirely preventable and yet this syndrome remains one of the leading causes of developmental disability. Research evaluating various types of postnatal manipulations for their effectiveness in attenuating the effects of prenatal ethanol exposure has yielded equivocal results. Intensive motor training in rodents exposed to ethanol during the human third trimester equivalent (i.e., early postnatal period) results in improvements in motor training and synaptogenesis in the cerebellum (Klintsova et al. 2002). Environmental enrichment in rodents models of prenatal ethanol exposure has had limited effects. It appears that prenatal ethanol exposure impairs the neuroplastic response to environmental enrichment (Hannigan & Berman 2000). In addition, it is unclear how environmental enrichment therapies may be applied to children with FAS. There are anecdotal reports that suggest that children with FAS may actually respond negatively to increased stimulation (Hannigan & Berman 2000; Streissguth 1997). Data from this thesis would suggest that future research should focus on identifying conditions under which pre and/or postnatal treatments can produce significant functional or neuroanatomical improvements in the serotonergic system E animals. Tajuddin and Druse (2001;1999) and have successfully ameliorated deficits in several aspects of the 5-HT system with administration of the 5-HT$_{1A}$ agonist buspirone in conjunction with prenatal ethanol exposure without affecting 5-HT receptors or neurotransmitter levels in control animals. Additional studies could examine the observed benefits of prenatal 5-HT agonist administration to functioning of adult E offspring. For example, increased anxiety-like behaviour in E offspring may be ameliorated by maternal 5-HT$_{1A}$ agonist co-administration with ethanol. These experiments could offer important insight into ameliorating the effects of a highly addictive drug that remains a major cause of developmental disability in children of alcoholic women.
6.C. Clinical Relevance

In Chapter IV it was observed that E males showed a deficit in habituation to repeated food presentation. Similarly, deficits in habituation to repeated stimuli presentation have been observed in human neonates exposed prenatally to alcohol (Pytkowicz Streissguth et al. 1983). Importantly, early habituation responses are related to cognitive and intellectual functioning in later childhood (Pytkowicz Streissguth et al. 1983). Deficits in cognitive function and intellectual ability have been well documented in children with FAS and these deficits are also modelled by prenatal ethanol exposure in rodents.

The results of Chapter IV also indicated that E animals exhibit an increase in anxiety-like behaviour, an effect that was especially obvious in E females. An increased prevalence of psychiatric disorders, including anxiety disorders, in individuals with prenatal alcohol exposure has only recently been investigated. Roebuck et al. (1999) found a significant increase in scores on the anxiety subscale of the Personality Inventory for Children in children prenatally exposed to alcohol. Similarly, Mattson and Riley (2000) found a near significant increase in scores on the Anxious/Depressed scale of the Child Behavior Checklist in children with FAS and prenatal exposure to alcohol. A study in adults with FAS or Fetal Alcohol Effects found a high prevalence of anxiety disorders, including posttraumatic stress disorder, panic disorder, generalized anxiety disorder and phobia (Famy et al. 1998), with some individuals presenting with more than one anxiety disorder (Famy et al. 1998). The results of this thesis provide experimental evidence from a rodent model that are consistent with observations in children and may suggest that anxiety disorders are a long term effect of prenatal ethanol exposure.
The results of Chapter V may also be relevant to the psychiatric disorders observed in individuals with prenatal alcohol exposure. Altered hormonal responses indicative of blunted 5-HT_{1A}, but augmented 5-HT_{2A} receptor function were observed in E females. Similar hormonal alterations have been reported in individuals with depression (Weizman et al. 1988). Hormonal alterations in depressed individuals may reflect a decrease in 5-HT_{1A} but an increase in 5-HT_{2A} receptor number. However, decreased 5-HT_{1A} and increased 5-HT_{2A} receptor binding is more clearly established in suicidal individuals or those who have committed suicide (D’haenen 2001). Finally, 5-HT_{1A} and 5-HT_{2A} receptor supersensitivity in E animals may be consistent with the occurrence of clinical depression. Aprison and Hingtgen (1981) suggest that a subgroup of depressed individuals have supersensitive 5-HT receptors. They further suggest that development of depression would depend on a precipitating factor such as stress (Aprison & Hingtgen 1981). This later suggestion is especially relevant to individuals with FAS, as prenatal alcohol exposure increases hormonal responding to stressors.

A major finding from this thesis is the presence of sex differences in 5-HT_{1A} and 5-HT_{2A} receptor function in E offspring. In general, E females were more affected than E males. This finding may imply that sex differences may also exist in humans prenatally exposed to alcohol. Early research on FAS suggested that there was an altered sex ratio in individuals with FAS, though later findings did not support this conclusion (Qazi & Masakawa 1976). There has been no systematic, thorough examination of sex differences following prenatal alcohol exposure in humans. Furthermore, the studies that have included sex as a variable in statistical analysis were limited in sample size, or examined young children, in which sex differences might not be yet expected to exist. For example, there have been reports of no sex difference in growth parameters (Pytkowicz Streissguth et al. 1991), a correlation between maternal depression and childhood depression in girls exposed
prenatally to alcohol, but not boys (O'Connor & Kasari 2000) and the observation of a correlation between prenatal alcohol exposure and child aggressive behaviour in boys, but not girls (Yumoto et al. 2001). Therefore, there is evidence from experimental research and some clinical evidence that warrant further examination of sex differences in adults with prenatal alcohol exposure.

6.D. Conclusions

The teratogenic effects of prenatal ethanol may be exerted through mechanisms other than decreased levels of 5-HT, and the prenatally ethanol exposed offspring exhibit alterations in a variety of processes at the molecular, cellular and neurochemical level (for review see Goodlett & Horn 2001). Other neurotransmitters, hormones, second messenger systems, nutritional deficiencies, prostaglandins, oxidative damage and fetal hypoxia may also be involved in mediating the effects of prenatal ethanol exposure. It is likely that multiple factors interact in the effects of prenatal ethanol exposure on the offspring. The results of this thesis do not preclude the involvement of other factors in the abnormalities observed following prenatal ethanol exposure, or suggest that the other 12 5-HT receptor subtypes are similarly affected.

The experiments in this thesis generally revealed increased long term functioning of central 5-HT₁A and 5-HT₂A receptors in E compared to PF and C animals, possibly suggesting that supersensitive 5-HT receptors may exist in adult E animals. In addition, E animals showed increased anxiety-like behaviour in the novelty-induced suppression of feeding task, an effect ameliorated by 8-OH-DPAT, suggesting that prenatal ethanol exposure may impact 5-HT mediated anxiety-like behaviour in adulthood. The observed increases in 5-HT₁A receptor function in E animals may not be the result of increased levels
of these receptors, although the 5-HT$_{1A}$ receptor may have an increased sensitivity to modulatory effects.

Increased prenatal ethanol-induced receptor-mediated function may be unique to the serotonergic system. Prenatal ethanol exposure does alter other neurotransmitter systems, however, functioning of the adult receptor for these neurotransmitters may not be increased. Maier et al. (1996) have reported decreased whole brain dopamine, increased γ-aminobutyric acid, and no change in norepinephrine following prenatal ethanol exposure. Clonidine, an alpha-adrenergic agonist, induces increases in CORT in normal animals. However, in preweanling E females and males, this agonist failed to induced a CORT increase (McGivern 1986). Likewise, adult E males did not show quinpirole-induced yawning, a D$_3$ receptor-mediated behaviour. Therefore, increased responses to 5-HT agonists in E animals in comparison to PF and C animals, does not necessarily reflect an overall increase in neurotransmitter receptor function.

The findings of this thesis may be generalizable to the effects of prenatal exposure to other drugs that influence 5-HT levels. Although there are distinctly different effects arising from prenatal ethanol and other drugs of abuse, early neonatal cocaine also results in increased DOI-induced head twitch response in mice (Darmani and Ahmad 2000). The authors suggest that withdrawal from cocaine results in a decrease in 5-HT levels that may later lead to supersensitivity of the 5-HT$_{2A}$ receptor (Darmani and Ahmad 2000). As well, prenatal cocaine results in increased DOI-induced ACTH levels, although decreased p-choroamphetamine-induced ACTH and CORT levels in females and males (Battaglia et al. 2000; Cabrera et al. 1994; Cabrera et al. 1993). These neuroendocrine abnormalities in offspring prenatally exposed to cocaine were not accompanied by altered radioligand binding to 5-HT$_{1A}$ or 5-HT$_{2A/C}$ receptors (Battaglia et al. 2000; Cabrera et al. 1993). Together, results from studies on prenatal ethanol and cocaine exposure may suggest that
prenatal exposure to drugs that affect the 5-HT system in particular, may have numerous detrimental effects for the offspring.

Even early in prenatal development, 5-HT receptors appear to be functional (Lauder et al. 2000) and both rat and human fetuses express high levels of the 5-HT$_{1A}$ receptor (Bar-Peled et al. 1991; del Olmo et al. 1998). Furthermore, the role of 5-HT in human behavioural and physiological function is similar to the role it plays in rodents. 5-HT agonists and antagonists are used to treat a variety of conditions and disorders in humans, based on screening in rodent models. Extending the comparison further, chronic consumption of alcohol in adult humans appears to result in decreases in 5-HT levels (Tollefson 1989) as it does in the offspring of ethanol consuming dams (Maier et al. 1996). Therefore, the effects of prenatal ethanol exposure on 5-HT receptor function in rodents is relevant to individuals with FAS. Prenatal alcohol-induced perturbations in 5-HT levels may play a role in some of the many abnormalities in children and adults of women who consumed alcohol during pregnancy. Prenatal alcohol effects on the serotonergic system in the fetus may in part mediate observed abnormalities.
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