EFFECTS OF PRENATAL ETHANOL EXPOSURE ON COGNITION/BEHAVIOR AND HYPOTHALAMIC-PITUITARY-ADRENAL STRESS RESPONSE IN ADULT RATS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience Program)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

January 1998

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Vancouver, Canada

Date Jan 12, 1998

DE-6 (2/88)

ABSTRACT

The maternal consumption of alcohol during pregnancy produces a wide range of abnormalities in the offspring. The main purpose of this thesis was to investigate the (1) cognitive deficits and behavioral abnormalities and (2) hypothalamic-pituitary-adrenal (HPA) stress hyperresponsiveness produced by prenatal ethanol exposure. Sprague-Dawley rats from prenatal ethanol (E), pair-fed (PF) and *ad libitum* fed control (C) treatment groups were tested as adults.

The purpose of the first study was to assess the effects of prenatal ethanol exposure on cognitive and behavioral function. Two memory tasks that placed different cognitive demands on the subject were used: the object-recognition nonrecurring-items delayed-nonmatching-to-sample (DNMS) task and the spatial-navigation Morris water maze task. There were no significant effects of prenatal ethanol exposure in performance of the DNMS task at retention delays ranging from 4 to 300 s, or in reacquisition of the task following a 10 week rest period. Furthermore, prenatal ethanol exposure did not produce significant increases in distractibility or response perseveration in the DNMS apparatus. However, when the same rats were tested on the water maze task, prenatal ethanol exposure produced performance deficits. E rats took significantly longer than PF and C rats to find the submerged platform over the course of the training sessions. There are two likely explanations for the differences in performance on these two tasks, that are not mutually exclusive. First, the brain areas underlying spatial cognitive abilities may be more vulnerable to the teratogenic effects of prenatal ethanol exposure than those underlying object-recognition abilities. Second, E animals, which display HPA hyperresponsiveness to stressors, may be differentially responsive to the stress involved in performance of these two tasks.

The main purpose of the second study was to determine the mechanism of HPA hyperresponsiveness produced by prenatal ethanol exposure. It tested the hypothesis that decreased corticosteroid receptor densities at HPA feedback sites may be responsible, at least partially, for the deficient feedback inhibition and resultant HPA hyperresponsiveness produced by prenatal ethanol exposure. Densities of both types of corticosteroid receptors, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), were measured at the hippocampus, prefrontal cortex, hypothalamus, and anterior pituitary. Furthermore, differential downregulation of corticosteroid receptors following one of two intermittent chronic stress regimens (two of six stressors daily for 18 days), and differential upregulation of receptors 7 days following adrenalectomy (ADX) compared to that at the usual 24 h were examined. Prenatal ethanol exposure did not produce changes in basal or nonstressed corticosteroid receptor densities, nor differentially downregulate hippocampal GRs under conditions of the more stressful Stress Regimen I, or differentially upregulate hippocampal GRs 7 days following ADX. However, there were small changes in hippocampal GRs among E, PF and C rats under the conditions of the milder Stress Regimen II. Given the nature of these changes in receptor densities, however, it would appear that these changes do not play a critical role in mediating the feedback deficits. Thus, the hypothesis that decreased corticosteroid receptor densities, as measured by the whole site cytosolic binding assay, underlie the deficits in feedback inhibition and resultant HPA hyperresponsiveness following prenatal ethanol exposure was not supported.

The main purpose of the third study was to test the hypothesis that prenatal ethanol exposure would produce HPA hyperresponsiveness to chronic cold stress (4 °C for 0, 1 and 3 days), and increased sensitization to a novel acute stressor (IP isotonic saline injection) superimposed during the chronic cold stress. Exposure to cold stress resulted in elevated

corticosterone (CORT) levels in E males, and elevated adrenocorticotropin (ACTH) levels in E males and females. However, prenatal ethanol exposure did not increase sensitization to the acute stressor, nor affect corticotropin-releasing factor (CRF) and vasopressin (VP) mRNA levels in the parvocellular nucleus (PVN) of the hypothalamus under basal conditions or following chronic cold stress. Thus, although prenatal ethanol exposure produced HPA hyperresponsiveness, it was not observed at all levels of the HPA axis examined or under all experimental conditions. This study extends the finding of HPA hyperresponsiveness following prenatal ethanol exposure to chronic cold stress. This study was also a preliminary exploration of the effects of prenatal ethanol exposure on the hypothalamic-pituitary-thyroid (HPT) response to chronic cold stress. Cold stress increased TRH mRNA levels in both males and females, but there were no signifcant differences among the E, PF and C groups.

In addition, a fourth study of this thesis made an important methodological point regarding the use of the gel filtration technique using Sephadex gel beads. Gel filtration is a commonly used method to separate particles based on molecular size, and was used in the corticosteroid receptor assay (second study of this thesis). The measured receptor densities were significantly higher with previously used gel columns compared to new gel columns, and the measured densities became stable after just one use of the columns. The change in measured densities between new versus used gel columns is sizable enough to produce serious confounding of experimental results if attention is not given to whether new or used gel columns are used. It is possible that this feature of Sephadex gel filtration beads may generalize to other types of gel filtration systems.

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LIST OF ABBREVIATIONS

ACh - acetycholine

ACTH - adrenocorticotropin

ADH - alcohol dehydrogenase

ADX - adrenalectomy

ALD - aldosterone

ANG - angiotensin II

ANOVA - analysis of variance

ARBD - alcohol-related birth defects

ARND - alcohol-related neurodevelopmental disorders

BAL - blood alcohol level

C - prenatal control diet condition

CBG - corticosterone-binding globulin or transcortin

CCK - cholecystokinin

CNS - central nervous system

CORT - corticosterone

CRF - corticotrophin-releasing factor

DA - dopamine

DEX - dexamethasone

DNA - deoxyribonucleic acid

DNMS - delayed-nonmatching-to-sample

DTT - DL-dithiothreitol

E - prenatal ethanol diet condition

EP - epinephrine

FAE - fetal alcohol effects

FAS - fetal alcohol syndrome

G - gestation day

GABA - γ-aminobutyric acid

GR - glucocorticoid receptor

GREs - glucocorticoid responsive elements

hnRNA - heteronuclear RNA

h - hour

HPA - hypothalamic-pituitary-adrenal

hsp90 - heat shock protein

IOD - integrated optical density

IP - intraperitoneal

IQ - intelligence quotient

L - lactation or preweaning day

LC - locus coeruleus

LTP - long-term potentiation

min - minutes

MR - mineralocorticoid receptor

mRNA - messenger ribonucleic acid

NE - norepinephrine

NLS - nuclear localization signal

NMDA - N-methyl-D-aspartate

OD - optical density

OT - oxytocin

oz - ounce

PBS - phosphate-buffered saline

PF - prenatal pair-fed diet condition

POMC - proopiomelanocortin

PVN - paraventricular nucleus

RIA - radioimmunoassay

s - second

SC - subcutaneously

SEM - standard error of the mean

SS - sympathetic nervous system

SSC - 300 mM NaCl/30 mM sodium citrate

TBG - thyroxine-binding globulin

TRH - thyrotropin-releasing hormone

TSH - thyrotropin

T3 - triiodothyronine

T4 - thyroxine

VP - vasopressin

β-EP - β-endorphin

5-HT - serotonin

[^{1}H]-ALD - unlabelled aldosterone

[^{3}H]-ALD - [1,2,6,7-^{3}H(N)]-aldosterone

[^{1}H]-CORT - unlabelled corticosterone

[^{3}H]-CORT - [1,2,6,7-^{3}H]-corticosterone

[^{1}H]-DEX - unlabelled dexamethasone

[^{3}H]-DEX - [1,2,4-^{3}H]-dexamethasone

ACKNOWLEDGMENTS

I would like to thank my advisor Joanne Weinberg for her guidance, support and patience. She has been not only a mentor, but a good friend. I thank the members of my supervisory committee (Ken Baimbridge, John Church and Boris Gorzalka) for their support and helpful insights. I thank the whole Weinberg crew: Wayne Yu, Glenn Edin, Linda Ellis, Pamela Giberson, Katherine Keiver, Catherine Yu, Kara Gabriel, Jill Osborn, Candace Hoffman, Maria Glavas, Wendy Simms, Lawrence Chan, Brigitte Lenger, and Kim Arkanstall. You have all been family to me, and we have shared some grand adventures together!!! I thank the many students who have assisted me in my crazy studies. I thank Wayne Vogl for the intellectual banter over the many coffees. And I thank the many rats who have made the ultimate sacrifice in the name of science; I hope that my karmic debt will not be too high.

I am forever indebted to my family for their love and support: my father, my sisters Junie and Nadia, and Dave. I give warm thanks to Julie, a very special person, who made it so much easier; I am so glad our paths finally met.

I dedicate this dissertation to my mother who has always been with me in spirit, and to my grandmother whose wish I have now fulfilled.

FORWARD

Portions of this thesis have been previously published as follows:

Kim, C. K., Kalynchuk, L. E., Kornecook, T. J., Mumby, D. G., Afshar Dadgar, N., Pinel, J. P. J., & Weinberg, J. (1997). Object-recognition and spatial learning and memory in rats prenatally exposed to ethanol. *Behavioral Neuroscience, 111,* 985-995.

Kim, C. K., Yu, W., Edin, G., Ellis, L., Osborn, J. A., & Weinberg, J. (accepted). Effects of prenatal ethanol exposure on corticosteroid receptor densities in rats. *Psychoneuroendocrinology.*

Kim, C. K., Yu, W., Giberson, P. K., Zoeller, T., & Weinberg, J. (submitted). Effects of chronic cold stress on HPA stress response in rats prenatally exposed to ethanol. *Alcoholism: Clinical and Experimental Research.*

CHAPTER I: GENERAL INTRODUCTION

The maternal consumption of alcohol during pregnancy has serious adverse effects on the developing fetus. It affects virtually every system of the organism, producing a wide range of abnormalities that impacts the individual's health and ability to function in society. The term fetal alcohol syndrome (FAS) has been used to describe this condition (Jones & Smith, 1973a).

The main purpose of this thesis was to examine (1) cognitive deficits and behavioral abnormalities and (2) hypothalamic-pituitary-adrenal (HPA) hyperresponsiveness to stressors produced by prenatal ethanol exposure. Accordingly, this General Introduction is divided into five sections: Section A provides the general background to the clinical effects of prenatal alcohol exposure; Section B describes rodent models of prenatal ethanol exposure; Section C discusses the effects of prenatal ethanol exposure on cognition and behavior; and Section D discusses the effects of prenatal ethanol exposure on the HPA stress response. The General Introduction concludes with the general rationale and objectives of this thesis.

A. Fetal Alcohol Syndrome

The fact that maternal consumption of alcohol during pregnancy produces abnormalities in the offspring has been appreciated for thousands of years. There are references to this in the religious texts of the Bible and Talmud, and in the writings of ancient Carthage, Greece and Rome (Colangelo & Jones, 1982; Warner & Rosett, 1975). However, it was not until Lemoine et al. (1968) and Jones et al. (1973a; 1973b) independently documented a set of abnormalities in children of mothers who had chronically consumed high doses of alcohol during pregnancy that there was widespread acceptance of alcohol's teratogenic potential. The term FAS was used to

describe this set of abnormalities resulting from prenatal alcohol exposure (Jones & Smith, 1973a). Since these initial landmark studies, thousands of scientific reports have confirmed the teratogenic potential of *in utero* alcohol exposure (Streissguth et al., 1991).

1. Diagnosis

The diagnostic criteria for FAS includes the following: (1) a characteristic pattern of facial anomalies such as short palpebral fissures, flattened philtrum, thin upper lip and flat maxillary area; (2) pre and/or postnatal growth retardation; and (3) central nervous system (CNS) neurodevelopmental anomalies such as decreased cranial size, structural brain abnormalities and neurological problems (Stratton et al., 1996). The adverse effects of prenatal alcohol exposure fall on a continuum. FAS generally describes the most severe condition resulting from prenatal alcohol exposure. The terms fetal alcohol effects (FAE), partial FAS, alcohol-related birth defects (ARBD) and alcohol-related neurodevelopmental disorders (ARND) have been used to describe cases where all three diagnostic criteria of FAS are not met, yet there are still symptoms of prenatal alcohol exposure (Sokol & Clarren, 1989; Stratton et al., 1996). In the present thesis, the term FAE will be used to denote collectively the individuals in which there are symptoms of prenatal alcohol exposure but not all criteria of FAS are met.

2. Clinical Features

Alcohol is an insidious teratogen to the developing fetus that produces structural and functional abnormalities of both brain and body. Prenatal exposure to alcohol has been shown to produce abnormalities in virtually every system of the organism (Abel, 1984; 1996; Rosett & Weiner, 1984). Table 1 outlines many of the abnormalities resulting from *in utero* alcohol exposure that are observed in clinical populations, and serves to emphasize the range of the abnormalities produced by this potent teratogen. Specific abnormalities produced by prenatal

alcohol exposure that are relevant to the present research will be discussed in detail in the appropriate sections of this thesis.

The debilitating condition of individuals with FAS is well recognized (Streissguth, 1986; Streissguth et al., 1980). What is less appreciated is that even individuals with more subtle symptoms associated with prenatal alcohol exposure, those diagnosed with FAE, are often equally debilitated. Some individuals diagnosed with FAE have normal scores on standard intelligence tests, yet are often unable to function in society, primarily because of their maladaptive behaviors such as hyperactivity, distractibility, response perseveration, impaired habituation and poor attention span (Coles, 1992; Streissguth, 1986; Streissguth et al., 1991).

FAS/FAE are not just childhood disorders; there is a predictable long-term progression of the disorders into adulthood. In a comprehensive follow-up examination (Streissguth et al., 1991) of 61 individuals diagnosed with FAS/FAE, now 12-40 years of age, it was found that they continued to suffer many adverse effects, making them unable to function in society. In fact, none were independent in terms of both housing and income. Although the characteristic facial anomalies became more subtle, short stature and microcephaly were still evident. More importantly, cognitive deficits and behavioral abnormalities were among the most detrimental long-term effects. Intellectual performance measured by IQ scores were shown to be decreased: 42% of individuals scored below 70, the cut-off for the mental retardation classification. These individuals also did very poorly on other measures of cognitive performance such as academic functioning, which was at early grade school level (grades 2-4) despite a mean chronological age of 17 years. Mean adaptive functioning measured by tests of daily living and socialization skills was equivalent to that of 7 year olds. Furthermore, these individuals displayed many

maladaptive behaviors, the most commonly reported being increased distractibility, poor judgment, difficulty perceiving social cues, and problems with comprehension and abstraction.

3. Incidence and Epidemiology

FAS/FAE have been identified in children from all ethnic groups and socio-economic classes. However, it appears that certain ethnic groups (Native and African Americans) and individuals from lower socio-economic classes have higher rates of alcohol abuse, and thus an increased number of diagnosed cases. The reported prevalence varies significantly with the social drinking habits of a population under study. For example, reported incidence of FAS/FAE in some Native Canadian villages have been as high as 25-189 per 1000 (Asante & Nelms-Matzke, 1985; Robinson et al., 1987). However, overall estimates of FAS incidence range between 0.33-4.7 per 1000 live births (Abel & Sokol, 1991). Additionally, 3-4 in 1000 may be affected with FAE, and an unknown number of individuals may have more subtle effects on the CNS whose consequences for behavior and development are still under investigation (Coles, 1992).

Prenatal alcohol exposure is now recognized as the leading known cause of mental retardation in the Western world, surpassing Down's syndrome and spina bifida (Abel & Sokol, 1986). It may account for 2.3% of all institutionalized mentally retarded patients in the US (Abel & Sokol, 1991). The resulting effects of prenatal alcohol exposure produce enormous social and financial costs, with conservative estimates of $75-321 million annually in the US for FAS alone (Abel & Sokol, 1991; Streissguth et al., 1991).

4. Pharmacokinetics of Alcohol

Alcohol is lipid soluble and thus rapidly absorbed from the stomach and gastrointestinal tract following ingestion, and evenly distributed throughout the fluids and tissues of the body

and brain (Julien, 1981). It also readily crosses the placental barrier producing approximately equal maternal and fetal blood alcohol levels (BALs)(Waltman & Iniquez, 1972). The fetus is limited in its ability to metabolize alcohol due to a lack of hepatic alcohol dehydrogenase (ADH), the major metabolizing enzyme for alcohol. Therefore, the elimination of alcohol from the fetus is through passive diffusion of alcohol across the placenta and then maternal elimination (Waltman & Iniquez, 1972). In addition, the rate of alcohol elimination from amniotic fluid is approximately half that from maternal blood, resulting in relatively high alcohol concentrations in amniotic fluid when alcohol levels are low or eliminated from maternal blood. Thus, amniotic fluid may act as a reservoir for alcohol, such that the fetus can be exposed for a longer period than would be predicted on the basis of maternal alcohol concentration (Brien et al., 1983).

5. Teratogenic Effects of Alcohol

FAS does not occur in all infants exposed prenatally to alcohol. It occurs in approximately one-third of infants born to women with chronic alcoholism; the remaining two-thirds may show partial (FAE) or no apparent symptoms (Olegård et al., 1979; Streissguth & LaDue, 1987). Thus, many factors seem to be involved in the teratogenesis of *in utero* alcohol exposure.

The dose/duration of alcohol exposure has been identified as an important factor in alcohol teratogenesis (Coles, 1992; Schenker et al., 1990). Human epidemiological studies have reported a dose dependency (Ernhart et al., 1989), and animal studies have definitively confirmed a dose-response curve in the teratogenic effects of prenatal alcohol exposure (Chernoff, 1977; Randall et al., 1977). The minimum dose required to produce deficits or the amount of alcohol that can be "safely" consumed during pregnancy have not been established. Clinical studies have shown that as little as 1 oz of absolute alcohol per day was associated with intellectual deficits, and two

drinks per day produced a seven point decrement in IQ scores (Streissguth et al., 1989; 1990). Importantly, what is more critical than the absolute amount and duration of alcohol exposure is the BALs achieved. Indeed, binge drinking (condensed exposure producing high BALs) is more harmful to the fetus than the same amount of alcohol consumed at a steady rate over an extended period of time (Pierce & West, 1986).

Another important variable in the teratogenesis of prenatal alcohol exposure is the fetal developmental stage at which the alcohol exposure occurs. The different profiles of alcohol related birth defects are related to the critical periods for specific aspects of fetal development. First trimester alcohol exposure (during organogenesis) results mainly in organ and musculoskeletal anomalies; whereas, second and third trimester exposure results primarily in growth, intellectual and behavioral abnormalities (Schenker et al., 1990).

In addition, a host of other factors may contribute to and interact with the impact of alcohol on the fetus: physiological variables affecting uptake and metabolism of alcohol; alcohol's effects on uptake, utilization and metabolism of nutrients; maternal nutritional status, health and age; parity; genetic vulnerability; and the use of other substances such as cocaine, narcotics, marijuana and nicotine (Coles, 1992; Schenker et al., 1990).

B. Rodent Models of Prenatal Ethanol Exposure

Human studies have intrinsic limitations in that they cannot control for the many confounding variables (as discussed above) that can affect fetal development independent of ethanol per se, and they cannot address issues of the underlying mechanisms of the teratogenesis. Thus, animal models of prenatal ethanol exposure have been developed, using a variety of animal species and a variety of methods of ethanol delivery.

Many animal species have been used to study the effects of prenatal ethanol exposure including the monkey, sheep, dog, chick, guinea pig, rabbit, mouse and rat (Colangelo & Jones, 1982; Driscoll et al., 1990; Streissguth et al., 1990). However, rodent models (particularly the rat) are by far the most commonly used because of the ease of handling, short gestation periods, and relatively low cost to purchase, house and feed (Keane & Leonard, 1989). The metabolism of ethanol is similar in humans and rats, although rats metabolize ethanol more quickly. The fetal development of humans and rats follow similar stages, but differ in the timing of birth. The human third trimester gestation equivalent in rats is the postnatal period shortly after birth (West, 1987; West et al., 1989). Accordingly, both prenatal ethanol exposure and postnatal ethanol exposure models in rats have been developed to address different types of research questions. In this thesis, the rat prenatal ethanol exposure model was used; this models human first and second trimester ethanol exposure. This rat model is appropriate for the study of cognition and behavior since the neuronal network for these functions begin to develop *in utero*, and is also appropriate for the study of the HPA axis since it develops and begins to function during prenatal life of the rat (Eguchi, 1969).

Many different ways of administering ethanol to the developing fetus have been utilized, including injection, inhalation, gastric intubation, and via drinking fluid or liquid diet. No method is ideal and each has its advantages and drawbacks. Injection, inhalation and intubation methods allow for controlled doses of ethanol to be administered and high BALs to be achieved, but they require handling and/or confinement, both of which are stressors. Prenatal stress itself is capable of producing hyperresponsiveness of the HPA axis and behavioral abnormalities in the offspring (Suchecki & Neto, 1991). Ethanol in the drinking fluid is nonstressful, but because the

taste of ethanol is aversive, animals reduce their liquid intake; this leads not only to low BALs but reduced water and food intake (Weiner, 1980).

The liquid diet method of delivering ethanol provides all the required nutrients and fluids as well as the ethanol. It is a non-stressful way to achieve high BALs with adequate nutritional intake (Lieber & De Carli, 1989; Weinberg, 1985). Typically in these models, in addition to the prenatal ethanol (E) and *ad libitum* fed control (C) conditions, there is a nutritional control condition in which the nutritional intake is matched with that of the E condition [pair-fed (PF)]. The PF condition is necessary because food intake and therefore nutrient intake is typically reduced in ethanol consuming animals. This results because ethanol derived "empty" calories displace nutrition rich regular foods, and because ethanol consumption leads to less overall food intake compared to consumption of ethanol free diet (Weinberg, 1985). Thus, primary malnutrition, the simple reduction of nutrient intake resulting from ethanol intake, is controlled by the PF condition. However, it does not control for secondary malnutrition, resulting from ethanol altering the uptake, utilization, metabolism and placental transport of nutrients (Weinberg, 1985). In this thesis, the liquid diet method was used to deliver ethanol to pregnant rat dams.

1. Effects of Prenatal Ethanol Exposure in Rodent Models

Rodent models of prenatal ethanol exposure have replicated many of the effects found in humans with FAS/FAE, and have thus proven useful in the study of prenatal ethanol effects (Driscoll et al., 1990; Schwetz et al., 1978; Streissguth et al., 1980). In fact, a comparison of prenatal ethanol effects (mainly behavioral and cognitive abnormalities) in humans and animal models (mostly rodents) have demonstrated good congruence with respect to qualitative endpoints (Driscoll et al., 1990). Quantitatively, the magnitude of the observed effects were

dose-related for both humans and animals. The BALs required to produce an effect was also similar between humans and animals, although the dose of ethanol differed across species (Driscoll et al., 1990). Rodent models have demonstrated increased fetal deaths and/or resorptions, retarded pre and postnatal growth and development, various physical malformations, changes in brain morphology, impaired cognitive abilities, and various behavioral abnormalities such as hyperactivity and response inhibition (Abel, 1980; Colangelo & Jones, 1982; Meyer & Riley, 1986; Randall, 1987; Riley et al., 1986; Streissguth et al., 1980; West & Pierce, 1986). Specific abnormalities produced by prenatal ethanol exposure that are relevant to the present research will be discussed in detail in the appropriate sections of this thesis.

C. Cognitive and Behavioral Abnormalities

This section provides the background for the experiments studying the effects of prenatal ethanol exposure on cognition and behavior (Chapter III). Accordingly, Section 1 describes the two types of memory studied in this thesis, Section 2 describes the two memory tasks that were used in the present experiments, and Sections 3 and 4 outline the cognitive deficits and behavioral abnormalities resulting from prenatal ethanol exposure.

1. Types of Memory

Cognition is a general term for mental functions, and includes not only learning and memory but other mental processes such as language and attention (Howard, 1983). Learning is defined as the induction of behavior-related neural changes by sensory experience, and memory as the brain's ability to store and retrieve the learned effects of its experiences (Pinel, 1997). Learning and memory are conceptual distinctions that are often difficult to separate

operationally; thus, the term memory will be used in this thesis to represent both learning and memory processes.

Research in the past several decades has demonstrated that memory is not a unitary phenomenon. There is now a general consensus that multiple forms of memory exist, and several noteworthy variants of the multiple memory systems notion have been proposed (Cohen & Squire, 1980; Hirsh, 1974; Honig, 1978; Mishkin et al., 1984; Schacter & Tulving, 1982). One division of memory that has proven useful and has been supported by experimental data is between spatial and nonspatial memory. Spatial memory refers to memory for spatial locations, presumably represented in the form of a cognitive or spatial map (O'Keefe & Nadel, 1978). A cognitive map is the integrated information about a number of spatial locations, similar to an aerial view or plan of the space that is being represented (Pearce, 1997). The hippocampus is an important brain structure for spatial memory, as is the entorhinal cortex and subiculum (O'Keefe, 1993; Olton et al., 1978). Strong support for the concept of a cognitive map comes from the discovery of hippocampal neurons called "place cells" (O'Keefe & Dostrovsky, 1971). After an animal is familiarized to an environment, these place cells come to respond (measured by single cell electrical recording) only when the animal passes through a specific location of the familiar environment (i.e., in the place field of that neuron). Nonspatial memory refers to memory that does not involve spatial location; there are many types of nonspatial memory, but the one of interest in this thesis is that for object-recognition.

2. DNMS and Morris Water Maze Tasks

In this thesis, two memory tasks that place very different cognitive demands on the subject were used: (1) the object-recognition nonrecurring-items delayed-nonmatching-to-sample (DNMS) task, and (2) the spatial-navigation Morris water maze task.

The nonspatial object-recognition DNMS task was first developed for use in humans by Gaffen (1974), and then adapted for monkeys by Mishkin (1978). On a typical trial of the monkey DNMS task, the monkey is presented with an unfamiliar object (sample object). During this first phase of the trial, the monkey pushes the sample object aside in order to obtain food reward, which is concealed beneath it. Next, an opaque screen is lowered in front of the monkey while the now-familiar sample object and an unfamiliar object are positioned over respective food wells. After a retention delay, the screen is raised, and the monkey's task is to remember the sample object so that it can select the new object (nonmatching rule) to obtain food reward. Different objects are used on each trial (nonrecurring-items procedure). The DNMS task has become perhaps the most widely used task to study memory in humans and monkeys, and has provided invaluable information about the processes governing memory.

Recently, this task has been adapted for use in rats (Mumby et al., 1990). In this task, rats make the object-discrimination on the basis of visual cues as do humans and monkeys, since they do not seem to use olfactory or tactile cues (Mumby et al., 1995a). This rat DNMS model has proven to be comparable to the monkey model in terms of rate of acquisition of the nonmatching rule and performance over various retention delays (Mumby et al., 1990). Importantly, the findings of the rat model have replicated the pattern of deficits following various brain lesions in humans and monkeys. Performance on the DNMS task was disrupted by rhinal cortical (Mumby & Pinel, 1994), mediodorsal thalamic (Mumby et al., 1993) or ischemic (Wood et al., 1993) lesions, and relatively unaffected by hippocampal or amygdala (Duva et al., in press; Mumby et al., 1992; 1995b) lesions.

The Morris water maze is a widely used test of spatial-navigation memory in rats (Morris, 1981). Rats are placed into a featureless circular pool filled with opaque water from which they

can escape by locating and climbing onto a submerged platform. Distal visual cues available in the testing room are used to form a cognitive map, and this map is used to navigate and locate the hidden platform regardless of the initial starting point. Performance of this task is disrupted by bilateral lesions to the hippocampus (Morris et al., 1982), a structure that plays a major role in spatial memory (O'Keefe, 1993).

3. Effects of Prenatal Ethanol Exposure on Cognition

Among the most consistent features of FAS/FAE are cognitive deficits (Conry, 1990; Coles, 1992; Streissguth, 1986; Streissguth & LaDue, 1987; Streissguth et al., 1980; 1991). Intellectual functioning measured by IQ scores are typically below normal; a recent comprehensive study showed individuals with FAS and FAE to have mean IQ scores of 66 and 73, respectively, with the range from 20 (severely retarded) to 105 (Streissguth et al., 1991). These individuals also do very poorly on other measures of cognitive performance such as academic functioning (Conry, 1990; Streissguth et al., 1991; 1994a; 1994b).

Experiments in laboratory animals have demonstrated cognitive deficits on various memory tasks following prenatal ethanol exposure. Nonspatial memory tasks include the following: active avoidance in which animals must perform a task to avoid punishment such as electric shock (Abel, 1979; Abel et al., 1983; Blanchard & Riley, 1988; Bond & DiGiusto, 1978; Randall et al., 1986; Shaywitz et al., 1979); passive avoidance in which animals are required to withhold a normally preferred response to avoid punishment such as electric shock (Becker & Randall, 1989; Driscoll et al., 1982; Molina et al., 1984; Riley et al., 1979a); odor aversion and taste aversion in which animals learn to avoid a certain odour or taste associated with prior illness (Barron et al., 1988; Driscoll et al., 1985; Riley et al., 1984; Riley et al., 1979a); odor association in which animals learn to move to one side of a two-sided chamber that has had an

odor associated with food reward (Barron et al., 1988); and operant lever response in which animals learn to lever press for food reward (Gentry & Middaugh, 1988; Viglieccа et al., 1989).

Impairments have also been demonstrated on various spatial memory tasks in animals prenatally exposed to ethanol: Morris water maze task (Blanchard et al., 1987; 1990; Gianoulakis, 1990); radial arm maze task in which animals must learn which arms of a maze have been baited with food (Omoto et al., 1993; Reyes et al., 1989); T-maze task in which animals must choose the appropriate goal area to obtain a reward (Lochry et al., 1985; Riley et al., 1979b; Zimmerberg et al., 1991); and spatial lever response task in which animals must depress a particular lever to obtain food reward (Zimmerberg et al., 1989).

4. Effects of Prenatal Ethanol Exposure on Behavior

Among the most detrimental effects of FAS/FAE are maladaptive behaviors (Coles, 1992; Streissguth, 1986; Streissguth & LaDue, 1987; Streissguth et al., 1980; 1991). In fact, maladaptive behaviors along with cognitive deficits, are the primary reason that individuals diagnosed with FAS/FAE are unable to function in society (Streissguth, 1996; Streissguth et al., 1991). In a recent study of 61 adults and adolescents diagnosed with FAS/FAE (Streissguth et al., 1991), none were independent in terms of both housing and income. The detrimental behaviors include hyperactivity, distractibility, response perseveration, impaired habituation and poor attention span (Coles, 1992; Streissguth, 1986; Streissguth et al., 1991).

Experiments in laboratory animals have confirmed many of the behavioral effects of prenatal ethanol exposure that have been observed in clinical populations. Behaviors reflecting hyperactivity and hyperresponsiveness have been observed in animals prenatally exposed to ethanol. These include increased open field activity measured by the amount of ambulations and rearings (Bond & DiGiusto, 1976; 1977); increased exploratory behavior measured by the

number of head-dips (Plonsky & Riley, 1983; Riley et al., 1979c) and nose-pokes (Riley et al., 1979c); increased startle reaction to a loud tone (Anandam et al., 1980); and increased wheel running activity (Martin et al., 1978).

Behaviors reflecting response perseveration and lack of inhibition have been demonstrated in animals prenatally exposed ethanol. These include deficits in passive avoidance (Becker & Randall, 1989; Driscoll et al., 1982; Molina et al., 1984; Riley et al., 1979a); decreased taste aversion (Driscoll et al., 1985; Riley et al., 1984; Riley et al., 1979a); decreased reversal performance in which animals must perform a previously incorrect response in order to obtain a reward (Riley et al., 1979b); and decreased spontaneous alternations, a natural tendency of rats to alternate responses such as visiting alternate arms of an apparatus (Abel, 1982; Riley et al., 1979b).

Increased distractibility has been difficult to measure in animal studies. In one study (Means et al., 1986), rats were tested on a 2-choice visual discrimination maze. The two goal-box choices each had discrete markings on the walls and floor, and the rats were trained to enter one of the goal-boxes. The measure of distraction was the degree of disruption in observed performance by reversing the markings on the floor of the goal-boxes. No significant effects of prenatal ethanol exposure were found on this distraction task.

D. HPA Stress Hyperresponsiveness

This section provides the background for the experiments studying the effects of prenatal ethanol exposure on HPA hyperresponsiveness to stressors (Chapters IV and V). Accordingly, Section 1 describes the HPA stress response, Section 2 describes the effects of prenatal ethanol

exposure on the HPA stress response, and Section 3 describes the possible mechanisms underlying this HPA hyperresponsiveness.

1. HPA Stress Response

Stress, Stressor and Stress Response

The term stress has been defined in a variety of ways in scientific and popular literature. It will be defined here as the state of threatened homeostasis; stressors will be defined as the threatening forces, and the adaptive or stress response as the way the organism deals with the threat to homeostasis (Johnson et al., 1992).

The constancy of the internal milieu or homeostasis is critical for the survival and independent existence of organisms, and the preservation of this internal environment requires continuous adaptation to stressors (Aguilera, 1994). The organism's stress response varies with the type of stressor, which can be broadly classified as physical (e.g., electric shock) or psychological (e.g., thesis defense). It also depends on the duration (acute versus chronic) and intensity of the stressor, if there has been prior exposure to that specific or to different stressors, health of the organism, genetic makeup and early life experiences (Aguilera, 1994; Johnson et al., 1992; Sapolsky, 1992). The stress response involves the release of many hormones in the body, and thus produces widespread effects throughout the whole organism (Asterita, 1985; Sapolsky, 1992). The acute stress response is advantageous for survival of the organism, and helps the organism withstand the stressor with a variety of adaptive physiological (energy mobilization, increase in cardiovascular and respiratory tone, suppression of anabolic processes, suppression of inflammatory/immune function, stress-induced analgesia) and behavioral (sharpened cognition, increased arousal and alertness, selective memory enhancement, focused attention, altered sensory threshold) responses (Chrousos & Gold, 1992; Johnson et al., 1992).

However, chronic stress is maladaptive; it can lead to a variety of pathological conditions such as gastrointestinal ulceration, immunosuppression, weight loss, fatigue, myopathy, steroid diabetes, hypertension, psychogenic dwarfism, reproductive dysfunction and neuronal death (Sapolsky, 1992; Stratakis & Chrousos, 1995). Furthermore, a dysregulation of the stress response has been implicated in a variety of psychiatric disorders such as depression, panic disorder, obsessive-compulsive disorder and anorexia nervosa (Chrousos & Gold, 1992; Johnson et al., 1992).

HPA Axis

There are two principle stress systems. (1) The HPA system which is composed of the hypothalamus which releases corticotropin-releasing factor (CRF), the anterior pituitary which releases adrenocorticotropin (ACTH) and β-endorphin (β-EP), and the adrenal cortex that releases corticosterone (CORT, the main glucocorticoid in rats; cortisol is the main glucocorticoid in humans). (2) The sympathetic nervous system/locus coeruleus (SS/LC) which releases norepinephrine (NE) from sympathetic nerves and NE and epinephrine (EP) from the adrenal medulla (Chrousos & Gold, 1992; Johnson et al., 1992). These two systems are functionally interrelated: CRF stimulates while glucocorticoids inhibit the SS/LC system; conversely, NE and EP stimulate CRF and ACTH release from the HPA system (Johnson et al., 1992).

The HPA system is the focus of the present research. During the stress response, CRF, the main secretagouge of ACTH, is synthesized and released from the parvocellular division of the paraventricular nucleus (PVN) of the hypothalamus (Dunn & Berridge, 1990; Owens & Nemeroff, 1991; Reisine et al., 1986). CRF release/synthesis is stimulated by EP, NE, acetycholine (ACh), serotonin (5-HT), dopamine (DA), angiotensin II (ANG), and enkephalins;

and inhibited by CRF itself, ACTH, CORT, γ-aminobutyric acid (GABA), β-EP, dynorphin and α-melanocyte stimulating hormone (Dunn & Berridge, 1990; Owens & Nemeroff, 1991; Reisine et al., 1986).

The CRF neurons of the PVN project to the median eminence where they terminate on the capillaries of the hypothalamo-hypophyseal portal vessels that reach the anterior pituitary (Dunn & Berridge, 1990; Owens & Nemeroff, 1991; Reisine et al., 1986). CRF promotes the synthesis of proopiomelanocortin (POMC) and stimulates the release of the POMC-derived peptides (ACTH and β-EP) from the anterior pituitary (Dunn & Berridge, 1990; Owens & Nemeroff, 1991; Reisine et al., 1986).

Vasopressin (VP), better know for its role in maintaining body water balance, is another important peptide involved in the release of ACTH and β-EP. VP is released from the parvocellular (colocalized with neurons that release CRF) and magnocellular divisions of the PVN (Antoni, 1993; Reisine et al., 1986) and released into the hypothalamo-hypophyseal portal vessels. It is a weak secretagogue by itself, but acts synergistically with CRF. During some types of repeated or chronic stress, the rate of CRF release is maintained, whereas VP secretion rate and colocalization in CRF nerve terminals are markedly increased. In this way, an increase in VP synthesis and release, resulting in an increased VP:CRF ratio may be critical for maintaining pituitary responsiveness during chronic stress (Aguilera, 1994).

Oxytocin (OT), EP, NE, ACh, 5-HT, cholecystokinin (CCK), ANG, and vasoactive intestinal polypeptide can also stimulate ACTH release by themselves or synergistically with CRF; ACTH is inhibited by ACTH itself, CORT and β-EP (Johnson et al., 1992; Owens & Nemeroff, 1991; Reisine et al., 1986).

ACTH is transported via the systemic circulation to the adrenal gland and acts on the adrenal cortex to stimulate the synthesize and release of CORT. CRF, ANG and interleukin-1-β can also have stimulatory effects on CORT synthesis/release, while β-EP has inhibitory effects (Johnson et al., 1992; Owens & Nemeroff, 1991 Reisine et al., 1986).

CORT is the final product in the HPA axis pathway. It is released into the systemic circulation and is normally 95% bound, primarily to corticosterone-binding globulin (CBG) and to a lesser extent to albumin, in the blood (Berne & Levy, 1993; Sapolsky, 1992). Because of its lipophilic nature, the free fraction readily passes through cell membranes and binds to receptors located primarily in the cytoplasm of target cells. These receptors are normally bound to proteins, most notably to a heat shock protein (hsp90); however, once CORT binds to the receptor, the hsp90 dissociates. The receptor then undergoes a conformational change, exposing nuclear localization signal (NLS) binding proteins, which are involved in the translocation of the receptor/ligand complex to the nucleus. The molecular mechanism of this translocation is unknown, but may be ATP-dependent and involve the cell cytoskeleton (Beato et al., 1996). At the nucleus, the CORT/receptor complex may bind to DNA and stimulate gene transcription, protein synthesis and concomitant modification of cell function, or it may repress transcription. The way that the CORT/receptor complex produces enhanced or decreased transcription is not well understood (Beato, 1989; Beato et al., 1996; McEwen et al., 1997; Starr et al., 1996). However, the receptors have been shown to interact with specific genomic sites, termed glucocorticoid responsive elements (GREs), in at least three ways--simple, tethering and composite. Simple interactions involve the receptors binding to the GREs as dimers to activate transcription. In this type of interaction, the receptors are the sole DNA-binding factors that are necessary for transcription. In contrast, certain nonreceptor transcriptional regulators are

required for tethering and composite interactions. Tethering interactions involve receptors binding not to the DNA, but to nonreceptor transcriptional activators, causing repression of transcription. Composite interactions involve receptors interacting with both specific DNA sequences and with DNA-bound nonreceptor factors, causing increased or decreased transcription depending on the composition of the nonreceptor factors. Thus, the enhanced or decreased transcription ultimately leads to many of the observed actions of CORT on the physiology and behavior of the organism (as discussed above).

Negative Feedback Regulation of HPA Axis

There are at least three distinct time domains in which negative feedback by CORT operates during and following stress (Keller-Wood & Dallman, 1984; Dallman et al., 1987): fast feedback (seconds to minutes), early delayed or intermediate feedback (2-10 h), and late delayed or slow feedback (hours to days). Fast feedback (Abe & Critchlow, 1977) is sensitive to the rate of increase in CORT and occurs while plasma levels of CORT are increasing. It occurs within seconds or minutes of CORT increase and is no longer effective by 20-30 min. The feedback results from the inhibition of CRF and ACTH release. Delayed feedback (intermediate and slow) is not rate sensitive but depends on the level of CORT achieved, the dose and duration of CORT exposure, and the interval since the stressor. It requires between 45-120 min to develop, with the maximum inhibition at 2-4 h, and attenuation of inhibition after 6-12 h. Intermediate feedback occurs during exposure to CORT for 2-10 h and involves the inhibition of CRF and ACTH release and CRF synthesis. Slow feedback results during constant CORT exposure for 12 h or more and involves the inhibition of synthesis and release of both CRF and ACTH.

Thus, fast rate sensitive feedback probably controls the rate and magnitude of CORT responses to acute stimuli, whereas intermediate feedback may limit the response of the system

to repeated stimulation within a relatively short period of time (hours), and slow feedback may limit the response of the system during prolonged stress (Keller-Wood & Dallman, 1984).

Corticosteroid Receptors in HPA Feedback Regulation

HPA negative feedback regulation is accomplished by CORT binding to its receptors at feedback sites. There are two major classes of intracellular corticosteroid receptors (Beaumont & Fanestil, 1983; Reul & De Kloet, 1985): mineralocorticoid receptor (MR; Type I) and glucocorticoid receptor (GR; Type II). They vary in distribution throughout the brain. GRs are widely distributed throughout the brain, with high concentrations in the limbic system (hippocampus, septum), PVN and supraoptic nuclei of the hypothalamus, cerebral cortex and anterior pituitary. MRs have a more restricted topography with high densities in the hippocampus, septum and amygdala (De Kloet, 1991).

GRs and MRs vary in their binding affinities for various endogenous and synthetic steroids. CORT and aldosterone (ALD) bind with high affinity to MRs, while dexamethasone (DEX) binds with high affinity to GRs. Interestingly, the binding affinity of CORT to MRs is 5-10 times greater than that for GRs (Reul & de Kloet, 1985). The differential binding affinities to CORT result in differential GR and MR occupancy during the phases of the circadian rhythm and during basal versus stress conditions. There is high MR and low GR occupancy at the circadian trough, and high GR occupancy occurring only with high CORT levels such as during a stress response or at the circadian peak (Reul & de Kloet, 1985). The observed differential occupancies led to the suggestion that the MR system was involved in tonic (permissive) influence of certain brain mechanisms, and the GR system was involved in feedback action on stress-activated brain mechanisms (Reul & de Kloet, 1985). However, recent evidence suggests that MRs may also play a role in feedback regulation of CORT (Bradbury et al., 1994; Ratka et

al., 1989; Sapolsky et al., 1990). HPA feedback by CORT occurs at the hippocampus (Herman et al., 1989; Sapolsky et al., 1990), prefrontal cortex (Diorio et al., 1993), PVN of the hypothalamus (Kovacs et al., 1986; Sawchenko, 1987), and anterior pituitary (Abou-Samra et al., 1986; Dallman et al., 1985).

Importantly, the brain corticosteroid receptor system shows dynamic down and upregulation in density in response to varying levels of circulating glucocorticoids. Prolonged elevation of CORT by chronic stress (Eldridge et al., 1989; Sapolsky et al., 1984) or exogenous CORT administration (Chao et al., 1989; Lowy, 1991; Reul et al., 1987a; Sapolsky & McEwen, 1985; Sapolsky et al., 1985; Spencer et al., 1991) produces corticosteroid receptor downregulation. Conversely, removal of endogenous glucocorticoids via adrenalectomy (ADX) produces receptor upregulation (Chao et al., 1989; Eldridge et al., 1989; Lowy, 1991; Luttge & Rupp, 1989; Luttge et al., 1989; McEwen et al., 1974; Olpe & McEwen, 1976; Reul et al., 1987a; 1987b; Spencer et al., 1991; Turner, 1986). Furthermore, mRNAs for corticosteroid receptors have been shown to be downregulated with glucocorticoid administration (Herman et al., 1989; Kalinyak et al., 1987) and upregulated following ADX (Herman et al., 1989; Kalinyak et al., 1987; Pfeiffer et al., 1991; Reul et al., 1989; Sheppard et al., 1990). Moreover, studies using cell lines have also demonstrated downregulation of corticosteroid receptor proteins and/or their mRNAs with glucocorticoid administration (Burnstein et al., 1990; Rosewicz et al., 1988; Vedeckis et al., 1989).

GRs and MRs mediate feedback, but an understanding of the way they mediate the feedback effects at the various CNS feedback sites, in the various feedback time domains is far from complete. The anterior pituitary may mediate early and intermediate feedback (Abou-Samra et al., 1986; Dayanithi & Antoni, 1989) probably via GRs (Dayanithi & Antoni, 1989);

MRs may also play a role in this feedback (Ratka et al., 1989). The hippocampus may mediate feedback via GRs, in conjunction with hippocampal MRs and hypothalamic GRs (Sapolsky et al., 1990); but hippocampal GRs may not mediate fast feedback (De Kloet et al., 1988). It has also been suggested that GRs in the parvocellular PVN of the hypothalamus may mediate fast and intermediate feedback (Dallman et al., 1987; De Kloet, 1991). A possible mechanism by which CORT inhibits HPA activity has been proposed at the level of the anterior pituitary. POMC transcription is activated by cAMP, and glucocorticoids inhibit this induction by cAMP (Akerblom et al., 1988). The inhibition may be accomplished by the CORT/receptor complex competing with the positive factors for overlapping targets sites on the POMC promotor (Drouin et al., 1993).

In addition to the classic slow-acting genomic effects of CORT mediated by the intracellular receptors, there are fast-acting nongenomic effects mediated by membrane receptors (Orchinik et al., 1995). Little is known of these receptors in the mammalian CNS (Orchinik et al., 1995), and their role is thought to be relatively minor compared to the intracellular receptors (Sapolsky, 1992). However, it has been proposed that they may play a role in fast feedback of the HPA axis (De Kloet, 1991).

The efficacy of HPA negative feedback regulation has been shown to be positively related to the density of corticosteroid receptors at the feedback sites. That is, alterations in corticosteroid receptor densities produce alterations in CORT negative feedback regulation with resultant changes in HPA responsiveness. The following are three examples where this relationship has been demonstrated.

First, "early handling" or infantile stimulation involves the separation of pups from the dams for up to 15 min daily during the preweaning period (Meaney et al., 1993; 1996). This

treatment produces permanent changes in HPA functioning. As adults, the handled animals display lower ACTH and CORT levels in response to stressors and quicker recovery to basal levels following stressors compared to nonhandled animals. They also demonstrate increased HPA negative feedback efficacy as demonstrated by the DEX suppression test. This synthetic glucocorticoid acts to block the release of ACTH from the pituitary and subsequently of CORT from the adrenal cortex. Handled rats broke through the DEX blockade more slowly, thus displaying lower CORT levels compared to nonhandled rats. The decreased stress response of handled animals appear to result from the more efficient negative feedback regulation of the HPA axis mediated by an increase in GR densities at the hippocampus and prefrontal cortex (Meaney et al., 1993; 1996).

Second, old animals display greater ACTH and CORT responses to stressors and delayed recovery to basal levels following stressors, and less efficient HPA negative feedback as measured by the DEX suppression test compared to younger animals. The increased stress response of older animals results from the less efficient negative feedback of the HPA axis mediated by the progressive loss of hippocampal GRs and MRs, and hypothalamic and pituitary GRs with aging (De Kloet, 1991; Sapolsky, 1992).

Third, prolonged CORT implantation has been shown to produce downregulation of corticosteroid receptors (this study was prior to the GR/MR distinction) in the hippocampus, with resultant increase in CORT response to stressors, and less efficient HPA negative feedback as measured by the DEX suppression test (Sapolsky et al., 1985).

2. Effects of Prenatal Ethanol Exposure on HPA Stress Response

Among the many effects of fetal ethanol exposure is HPA hyperresponsiveness to stressors, characterized by increased release of HPA hormones and delayed recovery back to

basal levels following stress. Although clinical studies have established that alcohol consumption markedly alters HPA function in chronic alcoholics (Merry & Marks, 1973), few clinical studies have investigated the effects of drinking during pregnancy on the HPA axis of the developing child. An early case study found that plasma cortisol levels were within the normal range in 9-14 year old children with FAS (Root et al., 1975). However, a recent study found that maternal drinking at conception and during pregnancy was associated with higher poststress cortisol levels in infants (Jacobson et al., 1993). Moreover, there is evidence that alcohol can have stimulatory effects on the HPA axis of the newborn. A case study reported pseudo-Cushing's syndrome, defined by an excess of circulating free cortisol, in an infant exposed to alcohol via breast milk (Binkiewicz et al., 1978).

HPA hyperresponsiveness has been found to be a robust phenomenon in rodent models of prenatal ethanol exposure. In general, E, PF and C rats do not differ in basal or nonstressed levels of CRF, ACTH, β-EP and CORT (Angelogianni & Gianoulakis, 1989, Lee et al., 1990; Nelson et al., 1986; Taylor et al., 1981; 1982; 1983; 1986; Weinberg, 1988; 1992a). However, under conditions of stress, increased secretion of these hormones become apparent. From weaning age through to adulthood, E rats display increased plasma CORT response to various physiological and psychological stressors such as footshock, cardiac puncture, noise and shake, restraint, swim, ether and cold (Angelogianni & Gianoulakis, 1989; Nelson et al., 1986; Taylor et al., 1982; 1983; Weinberg, 1988; 1992a, Weinberg et al., 1996), and to challenges with drugs such as ethanol and morphine (Nelson et al., 1986; Taylor et al., 1981; 1983). E rats also display increased plasma ACTH response following footshock or restraint (Lee et al., 1990; Nelson et al., 1985; 1986; Ogilvie & Rivier, 1997; Taylor et al., 1986; Weinberg et al., 1996), and

increased β-EP response following ether or cold stress (Angelogianni & Gianoulakis, 1989; Weinberg et al., 1996).

Furthermore, E rats displayed less attenuation in CORT response compared to controls when given the opportunity to engage in a consummatory behavior during novel cage stress (Weinberg, 1988), and were less discriminating in CORT response to predictable versus unpredictable restraint stress (Weinberg 1992b). These studies suggest deficits in the ability of E animals to use or respond to environmental cues. Increased CORT response to a forced swim test has also been observed following early postnatal ethanol exposure (Kelly et al., 1991).

Although both male and female rodents have been shown to exhibit increased HPA responsiveness following prenatal ethanol exposure, it appears that ethanol may differentially affect male and female offspring depending upon the parameters of the test situation, the nature and intensity of the stressor used, the time course measured, and the level of the stress axis measured (Halasz et al., 1993; Redei et al., 1993; Weinberg, 1994; Weinberg et al., 1996).

3. Mechanisms of Fetal Ethanol Effects on HPA Hyperresponsiveness

Ethanol may produce the effects on the fetal HPA axis by acting directly on the fetal endocrine glands and/or brain/pituitary sites (Weinberg, 1993). Ethanol consumed by the pregnant female can readily cross the placenta (Waltman & Iniquez, 1972) and have direct effects on the fetal HPA axis, which is functional before birth (Eguchi, 1969). Alternately, ethanol may affect the fetal HPA axis indirectly through disruption of the maternal HPA function (Anderson, 1981; Redei et al., 1993). The pregnant female and the fetus constitute an interrelated functional unit and CORT can cross the placenta in both directions (Eguchi, 1969). Thus, an ethanol-induced increase in circulating levels of maternal CORT could affect activity

of the fetal HPA axis. At present, there is evidence to support both the direct and indirect effects of ethanol in altering the fetal HPA axis (Weinberg, 1993).

The sites and mechanisms underlying HPA hyperresponsiveness of E offspring are presently unknown. The hyperresponsiveness may be a result of many factors including: (1) hypersecretion of CRF and/or VP, (2) increase in anterior pituitary or adrenal sensitivity to their respective secretogogues, (3) alterations in the neurotransmitters that affect the stress response, and (4) deficits in negative feedback control of HPA activity. One or more of these mechanisms may be at work and evidence for one does not preclude the possibility of the other factors.

First, there may be hypersecretion of CRF and/or VP from the PVN of E animals. Increased basal levels of CRF mRNA in the hypothalamus (Redei et al., 1993) and specifically in the PVN (Lee et al., 1990) of E rats have been demonstrated. However, Osborn et al. (1995) did not find changes in CRF or VP mRNA levels in the PVN following prenatal ethanol exposure. Furthermore, prenatal ethanol exposure produced no changes in basal hypothalamic CRF levels (Angelogianni & Gianoulakis, 1989), rather both E and PF rats displayed lower basal median eminence CRF levels compared to C rats (Lee & Rivier, 1994).

Second, there may be increased sensitivity of the anterior pituitary and adrenal cortex to their respective secretagogues in E animals. Increased basal levels of POMC mRNA have been reported in the anterior pituitary of E rats (Redei et al., 1993). CRF or VP administrations did not produce differential anterior pituitary ACTH release under basal conditions (Lee et al., 1990; Lee & Rivier, 1993; Taylor et al., 1988), but an increased ACTH response to CRF in DEX suppressed E animals was recently reported (Yu et al., 1996). ACTH administration did not produce differential adrenal cortical CORT release (Lee & Rivier, 1994; Osborn et al., 1994).

Third, it is also possible that altered neurotransmitter systems may be involved in mediating the HPA hyperresponsiveness of E animals. NE content in the cortex and hypothalamus was found to be lower in E animals compared to controls following restraint stress (Rudeen & Weinberg, 1993). NE has been shown to stimulate CRF release in a dose-dependent manner (Plotsky, 1987). If lower hypothalamic NE levels in E animals is indicative of increased NE turnover, it is possible that prenatal ethanol effects on NE regulation of CRF secretion may play a role in HPA hyperactivity. The GABA system also appears to be altered in E animals, as demonstrated by altered responsiveness to the anxiolytic effects of benzodiazepines (Yu et al., 1995). CORT can modulate $GABA_A$ receptor activity and in turn GABA has a role in regulating HPA activity (Jones et al., 1984; Majewska et al., 1985). Thus, prenatal ethanol exposure may affect the HPA axis via alterations in the GABA system.

Fourth, there is evidence for deficits in feedback control of the HPA axis following prenatal ethanol exposure from studies examining ACTH and CORT responses following DEX suppression. E rats showed increased basal CORT levels following DEX injection (Nelson et al., 1985), as well as increased ACTH and CORT responses to acute ether stress following DEX suppression (Osborn et al., 1996) compared to controls. These studies suggest deficits in the intermediate feedback domain. There has also been a suggestion of impairment in the fast feedback domain in E rats; an increased ACTH peak at approximately 10 min following footshock has been observed (Taylor et al., 1986; 1988).

E. General Rationale and Purpose

It is well established that maternal consumption of alcohol during pregnancy has serious adverse consequences for the developing fetus. It affects virtually every system of the organism,

producing a wide range of abnormalities. The general rationale of this thesis was to obtain a better understanding of (1) the cognitive deficits and behavioral abnormalities and (2) the HPA hyperresponsiveness to stressors that are produced by prenatal ethanol exposure. Accordingly, the first study examined cognitive deficits and behavioral abnormalities, the second and third studies examined HPA hyperresponsiveness, and the fourth study made an important methodological point regarding a technique that was used in this thesis. The purpose of each of the four studies of this thesis is outlined below:

(1) Cognitive deficits and behavioral abnormalities are among the most detrimental consequences of prenatal alcohol exposure in clinical populations. This study assessed the effects of prenatal ethanol exposure on cognitive function using two memory tasks that placed very different cognitive demands on the subject. The newly developed object-recognition DNMS task for rats and the well established spatial-navigation Morris water maze task were used. In addition, behavioral effects of prenatal ethanol exposure (distractibility and response perseveration) were assessed in the DNMS apparatus. Thus, the purpose of this study (Chapter III) was to test the hypothesis that prenatal ethanol exposure would produce performance deficits on both the DNMS and water maze tasks, and produce increased distractibility and response perseveration.

(2) Prenatal ethanol exposure produces HPA hyperresponsiveness to stressors. HPA hyperresponsiveness has been shown to produce severe adverse physiological and behavioral consequences which could compromise health and possibly even survival of the organism. Importantly, it has been proposed that HPA hyperresponsiveness may be responsible for some of the behavioral abnormalities and immune deficits that occur following prenatal ethanol exposure. The mechanisms underlying HPA hyperresponsiveness following prenatal ethanol

exposure are not well understood; however, it appears to be mediated in part by deficits in negative feedback regulation of the HPA axis. Furthermore, it has been demonstrated that the efficacy of HPA feedback is related to the density of corticosteroid receptors at the feedback sites; there is increased feedback efficacy with greater receptor density. Thus, the purpose of this study (Chapter IV) was to test the hypothesis that the impaired negative feedback regulation of the HPA axis that occurs following prenatal ethanol exposure was due to decreased levels of corticosteroid receptors at HPA feedback sites.

(3) Previous studies that have examined HPA hyperresponsiveness in E animals have used acute stressors or repeated episodes of the same acute stressor over several days. The present study investigated the effects of prenatal ethanol exposure on HPA response to chronic cold stress, a continuous stressor. Thus, the purpose of this study (Chapter V) was to test the hypothesis that prenatal ethanol exposure would produce HPA hyperresponsiveness to chronic cold stress. Furthermore, this was also a preliminary exploration of the effects of prenatal ethanol exposure on the hypothalamic-adrenal-thyroid (HPT) response to chronic cold stress.

(4) Gel filtration, a commonly used method to separate particles based on molecular size, was used in the corticosteroid receptor assay in the experiments of the second study (Chapter IV). The purpose of this study (Chapter VI) was to make an important methodological point regarding the use of the gel filtration technique.

Table 1

Clinical Features of Prenatal Alcohol Exposure

Endocrine abnormalites

Cognitive deficits

Behavioral abnormalities

Facial dysmorphology

Reduced pre and postnatal body weight, height and head circumference

Developmental delays

Microcephaly

Brain morphological changes

Cardiac malformations

Genital and renal malformations

Respiratory problems

Skeletal defects

Motor problems

Perceptual disturbances

Immune deficiency

Electroencephalogram abnormalities

Seizure susceptibility

Feeding difficulties

This list was compiled from Abel (1980), Colangelo and Jones (1982), Coles (1992), Driscoll et al. (1990), Meyer and Riley (1986), Randall (1987), Streissguth (1986), Streissguth and LaDue (1987), Streissguth et al. (1980) and Weinberg (1993).

CHAPTER II: GENERAL METHODS

This section describes the methods common to most of the present experiments. Specific additions or changes to this general methodology are described in the Methods section of each study.

A. Breeding and Feeding

Sprague-Dawley female rats (250-275 g; Canadian Breeding Farms, St. Constant, PQ) were bred with Sprague-Dawley males in suspended stainless-steel mesh cages (36 x 24 x 18 cm). During this time, rats were maintained on *ad libitum* access to standard rat chow (Ralston Purina, Woodstock, ON) and water. The colony room had controlled temperature (21 °C) and lighting (lights on 0700-1900 h). Waxed paper was placed under each cage containing a male and female, and was checked daily. The presence of a vaginal plug indicated day 1 of gestation (G). Pregnant females were then singly housed into polycarbonate cages (46 x 24 x 20 cm) lined with bedding and assigned to one of three groups: (1) Ethanol (E) - *ad libitum* access to liquid ethanol diet (36% ethanol-derived calories); (2) Pair-Fed (PF) - liquid control diet (maltose-dextrin isocalorically substituted for ethanol), with each dam fed the amount consumed by an E dam (g/kg body weight/day of gestation); and (3) Control (C) - *ad libitum* access to standard rat chow and water.

This liquid diet method of ethanol administration is a well established standard procedure used extensively by this and other laboratories, and designed to deliver doses of ethanol effective at producing abnormalities in the offspring while providing adequate nutrition. The E and PF diets were previously developed in this laboratory to provide adequate nutrition to pregnant dams regardless of ethanol intake (Weinberg, 1985) and were prepared by Bio-Serv

Inc. (Frenchtown, NJ). Although E and PF dams on these diets have somewhat reduced caloric and protein intake compared to C dams, their intake still exceed the minimum nutritional requirements (Weinberg, 1985). See Table 2 for nutritional details of the E and PF diets.

Fresh diet was placed on the cages each day in the late afternoon, just prior to lights off. At the same time, the bottles from the previous day were removed and weighed to determine the amount that had been consumed. The diets were given at this time because rats, being nocturnal, have a major eating bout immediately after lights off. Experimental diets were replaced with *ad libitum* access to rat chow and water on G22. Birth occurred at approximately G23.

Dams were weighed on G1, G7, G14 and G21. At birth, designated day 1 of lactation (D), dams and pups were weighed and the litters were culled to ten (five males and five females, when possible). The dams and pups were weighed again on D8, D15 and D22. Weaning occurred on D22, after which the offspring were group-housed by litter and sex in polycarbonate cages (46 x 24 x 20 cm) until testing.

B. Experimental Subjects

The subjects were offspring from the E, PF and C groups. They were tested as young adults, starting at a minimum age of 60 days. To control for litter effects, one male and one female per litter were randomly selected for testing for each experimental condition. Rats from the various treatment conditions were tested in pseudorandom order. In all studies, the number of subjects per condition are reported in the figure captions or in the tables.

C. Experimental Manipulations

Throughout the studies, all rats were housed in rooms with controlled temperature (21 °C) and lighting (lights on 0700-1900 h). All experimental manipulations occurred during the light-phase.

D. Blood Sampling

All blood sampling occurred in the morning, within 1.5-3.5 h of lights on, which corresponds to the diurnal CORT trough for the rat. Animals were left undisturbed overnight to prevent artificial elevations in hormone levels due to disturbance. At the time of sampling, each animal was quietly removed from the colony room to an adjacent sampling room to prevent disturbance of other animals. Two methods were used to collect blood.

Cardiac puncture

This method was used to collect blood for measurement of plasma CORT or CBG levels. Sampling occurred within 2 min of moving the animal's cage, which is rapid enough to obtain reliable nonstressed measures of CORT (Weinberg, 1992a; Weinberg et al., 1996). However, it is not adequate for measurement of nonstressed levels of ACTH, since detectable increases can be noted within seconds. Because the animal is not sacrificed by this technique, it allows for repeated samplings from the same animal. In the present experiments, when repeated samplings were taken from the same animal, they were separated by 19 days.

Animals were lightly anesthetized with ethyl ether (Fisher Scientific Ltd., Vancouver, BC) and 0.5 cc blood was withdrawn from the heart using a 1 cc heparinized syringe with 25G 5/8 needle (Becton Dickinson, Franklin Lakes, NJ). The blood was centrifuged at 2200 x g for 10 min at 4 °C, and the plasma was stored in microcentrifuge tubes at -20 °C until assayed.

Decapitation

This method was used to collect blood for measurement of plasma ACTH as well as CORT and CBG levels. Sampling occurred within 15 s of touching the animal's cage, which is rapid enough to obtain reliable nonstressed measures of ACTH (Weinberg et al., 1996).

Animals were decapitated with a guillotine and trunk blood was collected on ice in plastic tubes (12 x 75 mm) containing 7.5 mg ethylenediaminetetraacetic acid (Sigma Chemical Co., St Louis, MO) and 1000 KIU aprotinin (ICN Biochemical Inc., Aurora, OH). The blood was centrifuged at 2200 x g for 10 min at 4 °C, and the plasma was stored in microcentrifuge tubes at -70 °C until assayed.

E. Radioimmunoassays (RIAs)

CORT and CBG

Steroids. Labelled [1,2,6,7-^{3}H]-corticosterone ([^{3}H]-CORT), 71.7 Ci/mmol, was from Dupont Inc. (Mississauga, ON); unlabelled corticosterone ([^{1}H]-CORT) from Sigma Chemical; and CORT antiserum from Immunocorp Sciences Inc. (Montreal, PQ).

CORT. Total plasma CORT (bound plus free) was measured using an adaptation (Weinberg & Bezio, 1987) of Kaneko et al. (1981). CORT was extracted from plasma in absolute ethanol, and dextran-coated charcoal (Fisher Scientific) was used to absorb and precipitate free steroids after incubation. Samples were counted with liquid scintillation counting, and expressed in μg/100 ml. The intra and interassay coefficients of variation were 3.0% and 3.9%, respectively.

CBG. Total plasma CBG binding capacity was measured using an adaptation (D'Agostino & Henning, 1981) of Martin et al. (1977). Plasma was stripped of endogenous CORT using dextran-coated charcoal. A premix containing plasma and [^{3}H]-CORT was added to tubes containing either no [^{1}H]-CORT (to estimate total binding) or 100-fold molar excess of [^{1}H]-CORT (to estimate nonspecific binding). Dextran-coated charcoal was used to separate free and bound steroid after incubation. Bound [^{3}H]-CORT was determined by liquid scintillation counting. The binding capacity of CBG, expressed as μg CORT bound/100 ml plasma, was

calculated as the difference between total binding and nonspecific binding. The intra and interassay coefficients of variation were 5.4 % and 7.4 %, respectively.

ACTH

Plasma ACTH was assayed by a RIA kit from Incstar Inc. (Stillwater, MA). All blood and plasma for ACTH measurement were handled with plastic, since ACTH can bind to glass. The midrange intra and interassay coefficients of variation were 3.9 % and 6.5 %, respectively.

F. Statistical Analyses

Separate analysis of variance (ANOVA) were run for males and females because the relevant comparisons were within each sex. A separate overall ANOVA was run to compare between the sexes. Post hoc comparisons were made with the Tukey test. In all cases, the criterion of statistical significance was $p<0.05$, two-tailed. Only data from those rats that completed an experiment were included in the analyses. The F and P values are provided only when statistical significance was achieved.

Table 2

Nutritional Content of E and PF Liquid Diets From Bio-Serv

	E Diet (kcal/L)	PF Diet (kcal/L)
Protein	258	258
Fat	255	255
Carbohydrate	118	486
Ethanol	368	0
Total	999	999

The actual values have a variability of less than or equal to 10% of the listed theoretical values due to analytical and sampling variability, and moisture levels.

CHAPTER III: EFFECTS OF PRENATAL ETHANOL EXPOSURE ON COGNITION AND BEHAVIOR

Introduction

Among the most detrimental consequences of prenatal alcohol exposure in clinical populations are cognitive deficits and behavioral abnormalities (Conry, 1990; Coles, 1992; Streissguth, 1986; Streissguth & LaDue, 1987; Streissguth et al., 1980; 1991). Indeed, it is these effects that most often compromise the ability of individuals diagnosed with FAS/FAE to function in society. Individuals diagnosed with FAS/FAE have IQ scores that are typically below normal, often in the range for the mental retardation classification (Coles, 1992; Streissguth, 1986). They also do poorly on various other measures of cognitive performance such as academic functioning (Conry, 1990; Streissguth et al., 1994a; 1994b). Furthermore, a vast array of behavioral abnormalities such as increased distractibility, response perseveration, hyperactivity, poor attention span, and impaired habituation have been observed following prenatal alcohol exposure (Streissguth, 1986; Streissguth et al., 1991).

Experiments in laboratory animals have demonstrated cognitive deficits following prenatal ethanol exposure on a number of nonspatial memory tasks such as active and passive avoidance, odor and taste aversion, odor association, and operant lever response (Abel, 1979; Becker & Randall, 1989; Driscoll et al., 1985; Gentry & Middaugh, 1988). Deficits have also been found on spatial tasks such as the Morris water maze, radial arm maze, T-maze and spatial lever response (Gianoulakis, 1990; Reyes et al., 1989; Zimmerberg et al., 1991). In addition, behavioral abnormalities such as hyperactivity (Anandam et al., 1980; Bond & DiGiusto, 1976;

1977; Martin et al., 1978; Riley et al., 1979c) and response perseveration (Abel, 1982; Becker & Randall, 1989; Riley et al., 1979a; 1979b) have been found in E animals.

The main purpose of the present study was to assess the effects of prenatal ethanol exposure on cognitive function. For this reason, the same rats were tested on two memory tasks that placed very different cognitive demands on the subject: the object-recognition nonrecurring-items DNMS task (Mumby et al., 1990), and the spatial-navigation Morris water maze task (Morris, 1981). The DNMS task, which is perhaps the most widely used task to study memory function in humans and monkeys, has recently been adapted for use in rats (Mumby et al., 1990). Rats are presented with an object, which is then removed for an imposed retention delay, and subsequently presented again together with a novel object; choosing the novel object results in food reward. Rats do not seem to use olfactory or tactile cues to perform the object-discrimination, thus they make the discrimination on the basis of visual cues, as do humans and monkeys (Mumby et al., 1990). This rat DNMS model has proven to be comparable to the monkey model in terms of (1) rate of acquisition of the nonmatching rule (Mumby et al., 1990), (2) performance over various retention delays (Mumby et al., 1990), and (3) pattern of deficits following rhinal cortical (Mumby & Pinel, 1994), mediodorsal thalamic (Mumby et al., 1993), hippocampal (Mumby et al., 1995b; Mumby et al., 1992), amygdala (Mumby et al., 1992; 1995b) and ischemic (Wood et al., 1993) lesions. Thus, unlike the tasks used in previous examinations of memory in E rats, this task has direct human and monkey equivalents. In addition, distractibility and response perseveration were assessed in the DNMS apparatus. This is the first time the DNMS task has been used in the study of E rats.

In contrast, the Morris water maze is a commonly used test of spatial memory (Morris, 1981). Rats are placed into a featureless circular pool filled with opaque water from which they

can escape by locating and climbing onto a submerged platform. Distal visual cues available in the testing room are used to form a cognitive map, which is then used to navigate and locate the hidden platform regardless of the initial starting point. Performance of this task is disrupted by bilateral lesions to the hippocampus (Morris et al., 1982), a structure that plays a major role in spatial memory (O'Keefe, 1993). Furthermore, the hippocampus is known to be very vulnerable to damage by prenatal ethanol exposure (Hammer, 1986; Ward & West, 1992; West & Pierce, 1986). Moreover, this task was previously shown to uncover spatial memory deficits in rats that have been prenatally exposed to ethanol (Blanchard et al., 1987; 1990; Gianoulakis, 1990).

In the present study, it was hypothesized that prenatal ethanol exposure would produce performance deficits on both the DNMS and water maze tasks, and produce increased distractibility and response perseveration on the DNMS task.

Developmental Data

The following data are from E, PF and C dams during gestation and lactation (preweaning), and of the offspring of these dams during the period from birth to weaning. These offspring were tested as adults in the present study.

Ethanol Intake

Ethanol intake by pregnant female E rats (n = 21) was consistently high throughout gestation, averaging 9.2 $\pm$ 0.3, 11.6 $\pm$ 0.2 and 11.7 $\pm$ 0.2 g/kg body weight/day, respectively, for the 3 weeks of gestation. The overall mean for the 3 weeks was 10.8 $\pm$ 0.3 g/kg body weight/day. This laboratory has shown that intake at these levels result in maximal or near maximal BALs of 145-155 mg/dl (Weinberg, 1985; Osborn et al., 1996).

Maternal Weights

Analysis of maternal body weights during gestation (Table 3) indicated significant effects of group [$F(2,60) = 35.2$, $p<0.001$] and day [$F(3,180) = 1959.3$, $p<0.001$], and a group x day interaction [$F(6,180) = 33.9$, $p<0.001$]. Post hoc tests revealed that all dams displayed the expected increase in body weights as gestation progressed (p's<0.05). On G7 and G14, weights of PF dams were lower than E dams, and both were lower than C dams (p's<0.05). On G21, weights of E and PF dams did not differ from each other, and both were lower than those of C dams (p's<0.05).

Analysis of maternal body weights during lactation (Table 3) indicated a significant effect of day [$F(3,180) = 346.9$, $p<0.001$] and a group x day interaction [$F(6,180) = 5.4$, $p<0.001$]. Post hoc tests revealed that weights of all dams increased and then stabilized as lactation progressed (p's<0.05). Weights of E and C dams did not differ, but weights of PF dams were lower than those of C dams on D1, and lower than those of E dams on D8 (p's<0.05).

Pup Data

There were no significant differences among E, PF and C groups in the number of live or stillborn offspring. Analysis of male pup weights from birth to weaning (Table 4) revealed a significant effect of day [$F(3,177) = 1959.3$, $p<0.001$]. Post hoc tests revealed that all pup weights progressively increased with age (p's<0.05). On D1, weights of E and PF pups were lower than those of C pups (p's<0.05). On subsequent days, E and PF pups displayed catch-up growth and did not differ from C pups.

Experiment 1: DNMS Task

Methods

Subjects

The subjects were 21 adult male offspring from the E, PF and C groups (n = 7 per group). They were individually housed in suspended stainless-steel mesh cages (24 x 18 x 18 cm) throughout the experiment. At the start of the study, there were no significant differences among the groups in body weights. Each rat's body weight was reduced to 85-90% of its *ad libitum* fed value by reducing its daily ration of rat chow; body weights were maintained at these reduced levels throughout the experiment. Training commenced after the rats had been on the restricted feeding regimen for 14 days. The rats were 4 months of age at the start and 12 months by the end of Experiment 1. The rats of the three groups were tested in pseudorandom order, and the experimenters were unaware of their prenatal dietary treatments.

DNMS Apparatus

Figure 1 is a photograph of the DNMS apparatus for rats (Mumby et al., 1990). The aluminum apparatus (60 cm long x 20 cm wide x 40 cm high, elevated 70 cm from the floor), consists of a runway, which is separated from identical goal areas at each end by opaque plexiglass guillotine doors; the central area is enclosed by the doors. Each goal area contains two food wells into which food pellets (45 mg, Bio-Serv Inc., Frenchtown, NJ) can be delivered by hand through plastic tubes that are mounted on the outside of the apparatus. The two food wells are separated by a divider wall (9 x 9 cm) that protrudes from the center of the end wall. The sides of the goal areas are open to allow the experimenter to place stimulus objects over the food wells and to remove them quickly.

Test objects were selected from a collection of over 350 objects of various shapes, textures, and colors, similar to the "junk" objects in the monkey DNMS task (Mishkin & Appenzeller, 1987). Each object was large enough to cover a food well but small and light enough to be displaced easily by the rats. No objects with obvious scents were included.

Procedures

All testing occurred during the light-phase, between 14 and 21 h after the rat's most recent meal. Each rat was tested no more than once per day and no less than three times per week, with no more than two consecutive days without testing. The rats were not handled during a session once they had been placed in the apparatus. Each rat progressed through two phases of training: (1) habituation and (2) acquisition of a simple object-discrimination task.

Habituation. The habituation phase consisted of six 20 min sessions. In Sessions 1 and 2, each rat was placed in the apparatus and allowed to explore and find food pellets inside the food wells. In Sessions 3 and 4, the rats were trained to run back and forth between the goal areas by alternately baiting a single well at each end. The guillotine doors remained open during Sessions 1 through 4. In Sessions 5 and 6, the guillotine doors were closed, and the experimenter shaped the rats to approach the doors by baiting a food well on the other side and raising the door when the rat approached it.

Acquisition of object-discrimination. Following the habituation phase, each rat received five 25 trial object-discrimination sessions. The same two objects served as stimuli on all trials of each session. One of the objects was designated S+ (reward) and the other object was designated S- (no reward). To begin each session, the rat was placed in the center of the apparatus; one door was open and the other closed. The S+ and S- objects were each placed over the food wells behind the closed door. The position of S+ (left or right) varied from trial to trial according to a pseudorandom pattern. The experimenter then opened the door to expose the two objects. When the rat approached and displaced an object, the far door was lowered. A food pellet was delivered to the food well only if S+ was displaced. The experimenter then positioned S+ and S- at the other end of the apparatus in preparation for the next trial. The duration of the

intertrial interval typically varied between 15 and 20 s. Two special procedures were employed on the first discrimination session but not thereafter: (1) The well under the S+ was prebaited so that the rat could smell the food, to encourage them to displace the objects, and (2) if the rats displaced S-, they were allowed to then displace S+ to obtain a reward before the experimenter removed the objects.

Experiment 1A

There were three parts to Experiment 1A: (1) acquisition of the DNMS task at a 4 s retention delay; (2) testing at retention delays of 15, 60 and 300 s; and (3) reacquisition of the DNMS task at a 4 s retention delay after a 10 week rest period.

Methods

Acquisition at 4 s retention delay. The acquisition of the DNMS task followed the training phase. The 350 test objects were divided into seven sets of 50. Different sets were used on successive sessions, and different pairs of objects were used for each of the trials within a session. Each rat received 20 trials per DNMS session.

To begin each session, the rat was placed in the apparatus, and the doors were lowered to enclose it in the central starting area. Before each trial, a food pellet was placed in one of the food wells, the sample object was placed over it, and the novel object was placed over one of the food wells at the other end of the apparatus. The location of the sample and novel objects varied from trial to trial according to a pseudorandom pattern.

To begin a trial, the experimenter raised the first door to allow access to the sample object, which the rat approached and displaced from the food well. While the rat ate the pellet, the experimenter removed the sample object and positioned it over the vacant food well at the other end of the apparatus. After the prescribed retention delay, the second door was raised and the rat

was allowed to displace either the sample object or the novel object. If the novel object was displaced, it was considered a correct choice and a food pellet was delivered to the exposed food well. A rat was considered to have displaced an object if it moved the object enough to expose the food well. The experimenter then removed the objects, and the rat was allowed to return to the central starting area. The doors were closed to confine the rat there until the next trial, which began after new sample and novel objects were positioned. See Figure 2 for a diagrammatic representation of a typical DNMS trial. The intertrial interval typically varied between 20 and 30 s. The rats were permitted to make corrections during the first two DNMS sessions but not thereafter.

The retention delay, defined as the time between the displacement of the sample object by the rat and the raising of the door to reveal the sample and novel objects, was 4 s. Each rat was trained at this 4 s retention delay until it reached a performance criterion of at least 17 of 20 correct trials on two consecutive sessions. The dependent measures were the number of trials required to reach the performance criterion, and the percent of correct choices out of the total number of trials required to reach this criterion.

Retention delays of 15, 60 and 300 s. Once the performance criterion was reached at the 4 s retention delay, the delay was progressively increased to 15, 60 and finally 300 s. Each rat was tested at each delay for eight sessions; each session consisted of 20 trials. The dependent measure was the percent of correct choices at each retention delay.

Reacquisition at 4 s retention delay. Following the last session at the 300 s retention delay, each rat was given a 10 week rest period, and then was retrained at the 4 s retention delay until a performance criterion of at least 17 of 20 correct trials on two consecutive sessions was

reached. The dependent measures were the number of trials required to reach the performance criterion and the percent of correct choices on the trials required to reach this criterion.

Results

Acquisition at 4 s retention delay. There were no significant differences among E, PF and C rats in the number of trials required to reach the performance criterion, or in the percent of correct choices on the trials required to reach this criterion (Figure 3).

Retention delays of 15, 60 and 300 s. The percent of correct choices of E, PF and C rats significantly declined with progressively longer retention delays [$F(2,36) = 88.9$, $p<0.001$; 15 s > 60 s > 300 s, p's<0.05](Figure 4). However, there were no significant differences among groups in the percent of correct choices at each delay.

Reacquisition at 4 s retention delay. Following the 10 week rest period, there were no significant differences among E, PF and C rats in the number of trials required to reattain the performance criterion, or in the percent of correct choices on the trials required to reach the criterion (Figure 5).

Furthermore, a comparison of the performance to learn the task initially at the 4 s delay and then to relearn it following the 10 week rest period showed that all groups took fewer trials to reach the performance criterion [$F(1,18) = 62.4$, $p<0.001$] with a higher percent of correct choices [$F(1,18) = 86.4$, $p<0.001$] in relearning than in initial learning of the task. However, there were no significant differences among E, PF and C rats in performance between initial learning and relearning.

Experiment 1B

The effects of distraction on performance of the DNMS task was examined by presenting distractor objects during the retention delay. Response perseveration was examined by first

training the rats to perform an object-discrimination between two objects, and then reversing the discrimination of the same two objects.

Methods

Distraction task. Immediately following Experiment 1A, each rat was retested on the DNMS task at a 60 s retention delay, during which the rat was exposed to two novel distractor objects. A trial began as usual with the rat in the central start area, then the first door was opened, and the rat displaced the sample object, after which the object was removed. After 20 s, the second door was opened and the rat was allowed to displace a distractor object to receive a food pellet. The first door was then closed. After another 20 s, the first door was reopened and the rat was allowed to displace another distractor object to receive a food pellet. The second door was then closed. After another 20 s, the second door was reopened and the rat was presented with the original sample object and a novel object. Food reward was delivered only if the novel object was displaced. Each rat received five sessions of 20 trials without distractor items and five sessions with distractor items; these were alternated across consecutive sessions. The dependent measures were the percent of correct choices on the sessions with and without distractor items.

Response perseveration. The object-discrimination phase was similar to that used in the acquisition of object-discrimination training. The same two objects served as stimuli on all trials of each session. One of the objects was designated S+ (reward) and the other object was designated S- (no reward). Training continued until the performance criterion of at least 22 correct trials out of 25 on two consecutive sessions was reached. The object-discrimination reversal phase immediately followed, in which the contingency was reversed so that the object previously designated S+ was now designated S-, and vice versa. The performance criterion was

again at least 22 correct trials out of 25 on two consecutive sessions. The dependent measures for both object-discrimination and object-discrimination reversal were the number of trials required to reach the performance criterion and the percent of correct choices on the trials required to reach this criterion.

Results

Distraction task. E, PF and C rats all displayed significant decreases in the percent of correct choices when distractor objects were present [$F(1,18) = 46.6$, $p<0.001$](Figure 6). However, there were no significant differences among groups in the percent of correct choices on either the No Distractor or Distractor sessions.

Response perseveration. There were no significant differences among E, PF and C rats in object-discrimination (Figure 7) and object-discrimination reversal (Figure 8) in the number of trials required to learn the discrimination or in the percent of correct choices on the trials required to reach the criterion.

Experiment 2: Morris Water Maze Task

Methods

Subjects

The subjects were the same rats that had completed Experiment 1. Experiment 2 commenced 4 months following the termination of Experiment 1. Rats were maintained on *ad libitum* rat chow during this interval, and were tested at 16 to 17 months of age. One C rat was eliminated due to illness. The rats of the three groups were tested in pseudorandom order, and the experimenters were unaware of their prenatal dietary treatments.

Morris Water Maze Apparatus

The Morris water maze (Morris, 1981) is a circular pool (180 cm diameter x 60 cm high) filled to the 22 cm level with water made opaque with nontoxic powdered white paint. A circular platform (12 cm diameter x 18 cm high) was placed within the pool, submerged 3-4 cm below the level of the water. The pool was divided into quadrants, only apparent to the experimenters. The position of the platform was kept constant throughout the experiment in the center of one of the quadrants. The experimenters remained concealed behind a screen and observed the pool via a video monitor mounted above the pool. Each rat's search pattern was traced. Placed throughout the testing room were salient objects that the rats could use as landmarks to form the cognitive map. The orientation of the pool was kept constant over the test sessions.

Procedures

Each trial began with the rat being placed in the pool facing the side wall at one of four positions (the four boundaries of the quadrants), randomly predetermined. The rat was allowed to swim until it found the hidden platform, where it was allowed to remain for 10 s. If the platform was not found within 90 s, the rat was removed from the pool.

Each rat was tested on six trials on each of 10 consecutive daily sessions, for a total of 60 trials. For the first 58 trials, the platform was submerged so that the rats had to use distal cues in the testing room to orient themselves in locating the platform. On the 59th trial, the platform was made visible by raising it above the level of the water. This tested for differences among groups on factors other than spatial-navigational abilities, such as swimming ability and visual acuity. The 60th trial was the probe test in which the platform was removed from the pool and each rat's search pattern was recorded for 60 s.

The dependent measure on the first 59 trials was the latency to find the platform. On the probe test (60th trial), the number of annulus crossings (Goodlett et al., 1987) was measured. This is the number of times that the rat crossed the area where the platform had been located throughout the preceding trials.

Results

There were significant effects of group [$F(2,17) = 6.06$, $p<0.01$] and session [$F(9,153) = 14.44$, $p<0.001$] for the latencies to find the platform on the first trial of each of the 10 sessions (Figure 9; top panel). Post hoc tests revealed that the mean latency to find the platform over the 10 sessions was significantly greater for E rats than for PF and C rats (p's<0.05), and the latter did not differ significantly from each other (Figure 9; bottom panel). Latencies to find the platform significantly decreased over trials for all groups (p's<0.05). Furthermore, there were no significant differences among groups in the latencies to reach the visible elevated platform (Figure 10; top panel), or in the number of annulus crossing on the probe test (Figure 10; bottom panel).

Discussion

The hypothesis that prenatal ethanol exposure would produce performance deficits on both the object-recognition DNMS task and the spatial-navigation Morris water maze task was not supported. Prenatal ethanol exposure did not produce deficits in performance on the DNMS task (Experiment 1). There were no significant differences among E, PF and C rats in performance of the task at retention delays ranging from 4 to 300 s, or in reacquisition of the task following a 10 week rest period. Furthermore, prenatal ethanol exposure did not produce significant increases in distractibility or response perseveration in the DNMS apparatus. However, when the same

rats were tested on the water maze task, prenatal ethanol exposure produced performance deficit (Experiment 2). E rats took significantly longer than PF and C rats to find the submerged platform over the course of 10 daily sessions of training. Moreover, this performance deficit appeared to result from spatial-navigational impairments and not from extraneous factors such as swimming ability or visual acuity, because there were no significant differences among groups in latency to reach the platform when it was visible above the level of the water. The finding that there were no significant differences among groups in annulus crossings on the probe test may be due to overtraining, because the rats of all three groups had nearly reached asymptote in performance following the 10 sessions of training.

The results of Experiment 2 support those of previous studies that have demonstrated deficits in performance on the water maze task following prenatal ethanol exposure (Blanchard et al., 1987; 1990; Gianoulakis, 1990). Performance on a number of other spatial memory tasks such as the radial arm maze (Omoto et al., 1993; Reyes et al., 1989), T-maze (Lochry et al., 1985; Riley et al., 1979b; Zimmerberg et al., 1991), and spatial lever response (Zimmerberg et al., 1989) has been shown to be disrupted by prenatal ethanol exposure, although Abel (1979) reported no significant effect of prenatal ethanol exposure on T-maze performance. Moreover, postnatal ethanol exposure during the preweaning period has also been shown to produce performance deficits on the water maze task (Kelly et al., 1988; Goodlett et al., 1987). Thus, overall the detrimental effects of prenatal ethanol exposure on spatial memory tasks appear to be robust.

In Experiment 1, prenatal ethanol exposure produced no significant deficits in object-recognition memory, or increases in distractibility or response perseveration in the DNMS apparatus. These findings are consistent with the results of several studies that have found no

deficits on various nonspatial memory tasks such as active avoidance (Molina et al., 1984), taste discrimination (Becker et al., 1988), and runway learning (Cohen et al., 1985). These data are also consistent with the report of Means et al. (1986) that prenatal ethanol exposure does not increase distractibility and response perseveration on a visual-discrimination task. In contrast with the data of the present study, other studies have demonstrated deficits on various nonspatial memory tasks such as active avoidance (Abel, 1979; Abel et al., 1983; Blanchard & Riley, 1988; Bond & DiGiusto, 1978; Randall et al., 1986), passive avoidance (Becker & Randall, 1989; Driscoll et al., 1982; Molina et al., 1984; Riley et al., 1979a), odor aversion and odor association (Barron et al., 1988), taste aversion (Driscoll et al., 1985; Riley et al., 1984; Riley et al., 1979a), and operant lever response (Gentry & Middaugh, 1988; Viglieca et al., 1989). E animals also showed increases in response perseveration measured by impairments in tasks of reversal performance (Riley et al., 1979b; Wainwright et al., 1990) and spontaneous alterations (Abel, 1982; Riley et al., 1979b), as well as by impairments in passive avoidance (Becker & Randall, 1989; Driscoll et al., 1982; Molina et al., 1984; Riley et al., 1979a) and taste aversion (Driscoll et al., 1985; Riley et al., 1984; Riley et al., 1979a).

That deficits in performance were found on the spatial-navigation but not the object-recognition task can be explained in three ways, which are not necessarily mutually exclusive. One possibility is that prenatal ethanol exposure may produce greater damage to brain areas that underlie spatial cognitive abilities than those underlying object-recognition abilities. Evidence suggests that damage to the hippocampus may underlie the observed pattern of deficits on these two tasks. The hippocampus is thought to play a major role in spatial cognitive function (O'Keefe, 1993), and lesions to this site have been shown to produce deficits on spatial tasks such as the water maze (Morris et al., 1982) and radial arm maze (Olton, 1977). In contrast,

hippocampal lesions produced only very mild deficits in performance on the DNMS task (Mumby et al., 1992; 1995b). Interestingly, Duva et al. (in press) have shown that partial bilateral hippocampal lesions limited mainly to the CA1 area, with N-methyl-D-aspartate (NMDA), resulted in deficits only on the water maze task and not on the DNMS task. Furthermore, the hippocampus has been shown to be sensitive to the teratogenic effects of prenatal ethanol exposure: decreased pyramidal cells and dendritic spines, and alterations in dendritic and mossy fiber branchings have been reported (Hammer, 1986; Ward & West, 1992; West & Pierce, 1986). In particular, the hippocampal CA1 area seems to be especially vulnerable (Barnes & Walker, 1981). Moreover, prenatal ethanol exposure has been shown to produce many effects similar to those observed following hippocampal lesions, albeit of much less severity; these include deficits on spatial memory tasks (Blanchard et al., 1987; 1990; Gianoulakis, 1990; Omoto et al., 1993; Reyes et al., 1989) and behavioral abnormalities such as response perseveration (Riley, et al., 1986). Finally, the development of long-term potentiation (LTP) in the hippocampal CA1 region was impaired in E rats (Swartzwelder et al., 1988). LTP is thought to be a putative physiological correlate of memory (Bliss & Collingridge, 1993; Nicoll & Malenka, 1995; Rose & Dunwiddie, 1986; but see McEachern & Shaw, 1996).

Thus, although the present study did not directly measure hippocampal damage, indirect experimental evidence suggests that the cognitive impairments observed on the spatial-navigation task but not the object-recognition task may be due to hippocampal damage, especially to the CA1 area, following prenatal ethanol exposure. Clinical studies have also shown that the cognitive deficits in individuals with FAS/FAE are most apparent on spatial or complex tasks (Streissguth et al., 1991; 1994a). Indeed, performance deficits on tasks involving

spatial memory were shown to be particularly evident in individuals with FAS/FAE (Streissguth et al., 1994b).

A second possible explanation for the differences in performance on the two tasks in this study is that E animals may be differentially responsive to the stress involved in performance of these tasks. Prenatal ethanol exposure has been shown to result in HPA hyperresponsiveness to stressors. Increased levels of various stress hormones such as ACTH and CORT have been observed in E animals compared to controls following a variety of stressors (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1986; Taylor et al., 1982; Weinberg, 1992a; Weinberg et al., 1995; 1996). Although hormone levels were not measured in the present study, it is possible that exposure to the water maze task evoked a greater physiological stress response than did exposure to the DNMS task. Rats were trained on the DNMS apparatus for several weeks prior to the actual test trials, allowing for considerable habituation and therefore attenuation of the stress response. However, in the water maze, the rats were tested over only 10 days without any habituation or pretraining. Furthermore, this task may be stressful, as swim stress is know to be a potent stressor (Hannigan et al., 1987). Interestingly, E rats were shown to have greater levels of stress-induced behaviors compared to controls during a swim task (Hannigan et al., 1987); and early postnatal ethanol exposed female rats displayed a greater CORT response to forced swim compared to controls (Kelly et al., 1991). Furthermore, high stress responses have been shown to impair cognitive function. Although effects of CRF and ACTH have been examined (De Wied & Croiset, 1991), the evidence for the role of CORT in memory function is most compelling (Diamond & Rose, 1994; Kant, 1993; Lambert & Ven Murthy, 1986; Sharma & Panwar, 1987). High CORT levels have been shown to impair performance on memory tasks (McEwen & Sapolsky, 1995) and the induction of LTP and

primed-burst potentiation (Diamond & Rose, 1994; Diamond et al., 1992; 1994; Foy et al., 1987; Rey et al., 1994; Shors & Dryver, 1994; McEwen, 1994), which are putative physiological correlates of memory (Bliss & Collingridge, 1993; Nicoll & Malenka, 1995; Rose & Dunwiddie, 1986; but see McEachern & Shaw, 1996). Thus, it is possible that E animals had an increased hormonal stress response during testing compared to controls, a response that was further enhanced in the water maze compared to the DNMS task. The resulting increased stress response of E rats in the water maze task produced greater impairments of cognitive function, resulting in the observed deficits in the water maze task.

Finally, we must address the possibility that the different results on the object-recognition and spatial-navigation tasks may be due to E animals being differentially responsive to the effects of aging. There is a decrement in cognitive performance with aging (Lebrun et al., 1990; Wallace et al., 1980; Kubanis & Zornetzer, 1981). For example, deficits in performance on the radial arm maze with a delay imposed between the exploration of the first and last half of the arms, were observed in rats at 18 months of age compared to 6 and 12 months (Chrobak et al., 1995). Furthermore, Riley (1990) has argued that cognitive and behavioral abnormalities produced by prenatal ethanol exposure may be masked in young adult animals because of compensatory mechanisms or strategies. The deficits may be detected when these compensatory mechanisms break down, as is the case with old age, stressful situations or complex testing procedures. In the present study, deficits were not found on the DNMS task when rats were tested at 4 to 12 months of age, but were found on the water maze when tested at 16 to 17 months. Although the effects of aging cannot be conclusively rule out, it is unlikely because other studies have shown deficits in the water maze following prenatal ethanol exposure in young rats of 21 to 90 days of age (Blanchard et al., 1987; 1990; Gianoulakis, 1990).

In summary, prenatal ethanol exposure did not produce deficits in performance on the object-recognition DNMS task; this was the first use of the recently developed rat DNMS task in the study of prenatal ethanol exposure. However, consistent with previous research, prenatal ethanol exposure did produce performance deficits on the spatial-navigation Morris water maze task. There are two likely explanations for the differences in performance on these two task, that are not mutually exclusive. First, the brain areas underlying spatial cognitive abilities may be more vulnerable to the teratogenic effects of prenatal ethanol exposure than those underlying object-recognition abilities; this interpretation is supported by clinical evidence. Second, E animals, which display HPA hyperresponsiveness to stressors, may be differentially responsive to the stress involved in performance of these two tasks.

Table 3

Maternal Body Weights (g, Mean ± SEM) of E, PF and C Dams During Gestation (G) and Lactation (D)

Gestation	G1	G7	G14	G21
E	263.4 ± 2.5	261.0 ± 1.6*	287.2 ± 2.4*	353.7 ± 3.7+
PF	257.6 ± 2.0	253.1 ± 2.2*	276.4 ± 3.0*	343.6 ± 4.3+
C	259.6 ± 2.5	281.4 ± 2.5*	311.1 ± 3.2*	392.6 ± 4.2+

Lactation	D1	D8	D15	D22
E	292.1 ± 3.1	325.8 ± 2.9#	350.7 ± 3.4	329.9 ± 3.4
PF	282.8 ± 3.5^	313.7 ± 2.9#	338.3 ± 3.2	326.6 ± 3.5
C	300.0 ± 3.7^	324.4 ± 4.2	342.9 ± 4.3	324.5 ± 4.0

n = 21 per group during gestation and lactation

Gestation: *At G7 & G14, PF < E < C, p's<0.05

+At G21, E = PF < C, p's<0.05

Lactation: ^At D1, PF < C, $p<0.05$

#At D8, PF < E, $p<0.05$

Table 4

Male Offspring Body Weights (g, Mean ± SEM) of E, PF and C Pups From Birth to Weaning

	D1	D8	D15	D22
E	6.2 ± 0.1*	14.9 ± 0.3	30.5 ± 0.4	47.2 ± 0.7
PF	6.2 ± 0.1*	14.6 ± 0.5	29.8 ± 0.7	46.1 ± 1.1
C	6.8 ± 0.1*	15.9 ± 0.4	31.5 ± 0.7	48.4 ± 0.9

n = 20-21 per group

*At D1, E = PF < C, p's<0.05

Figure 1. Delayed-nonmatching-to-sample (DNMS) apparatus for rats.

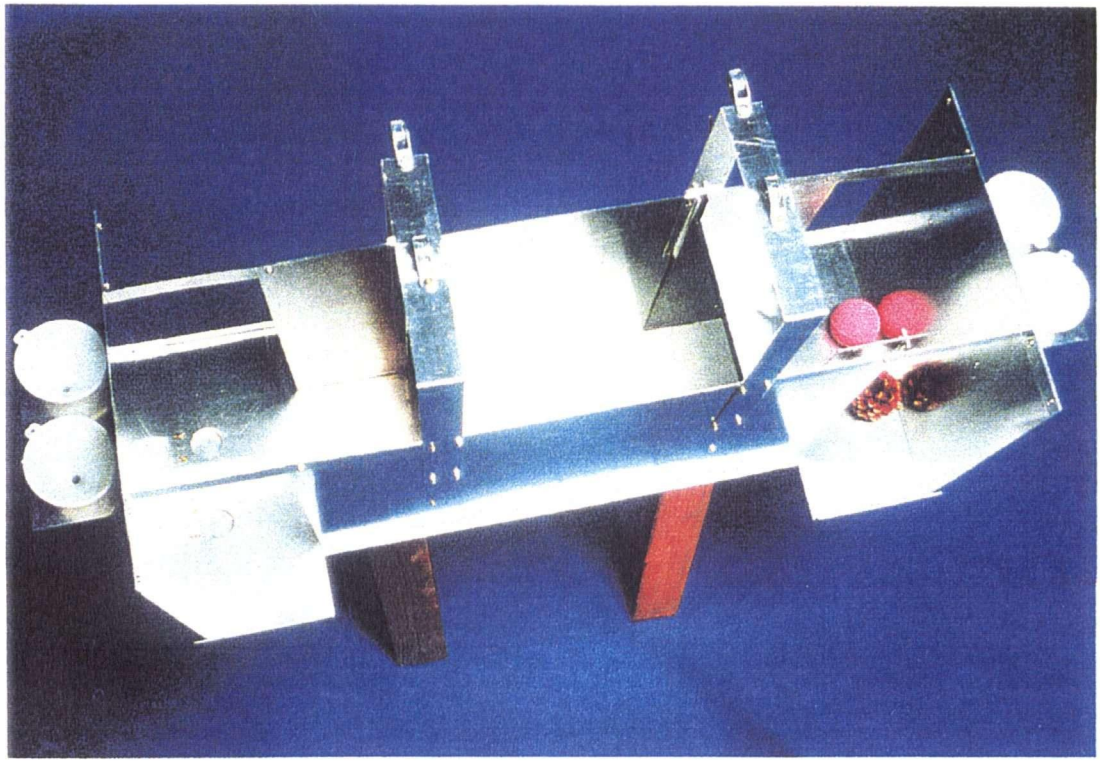


Figure 2. Diagrammatic representation of a typical DNMS trial. (A) With the rat enclosed in the central starting area, the sample object was placed over a food well baited with a food pellet, and the novel object was placed over one of the food wells at the other end of the apparatus. (B) The experimenter raised the first door to allow access to the sample object, which the rat approached and displaced from the food well. (C) The experimenter removed the sample object and positioned it over the vacant food well at the other end of the apparatus. (D) After the prescribed retention delay, the second door was raised and the rat was allowed to displace either the sample object or the novel object. If the novel object was displaced, it was considered a correct choice and a food pellet was delivered to the exposed food well. (E) The experimenter then removed both objects, and the rat was allowed to return to the central starting area. The doors were closed to confine the rat there until the next trial, which began after new sample and novel objects were positioned.

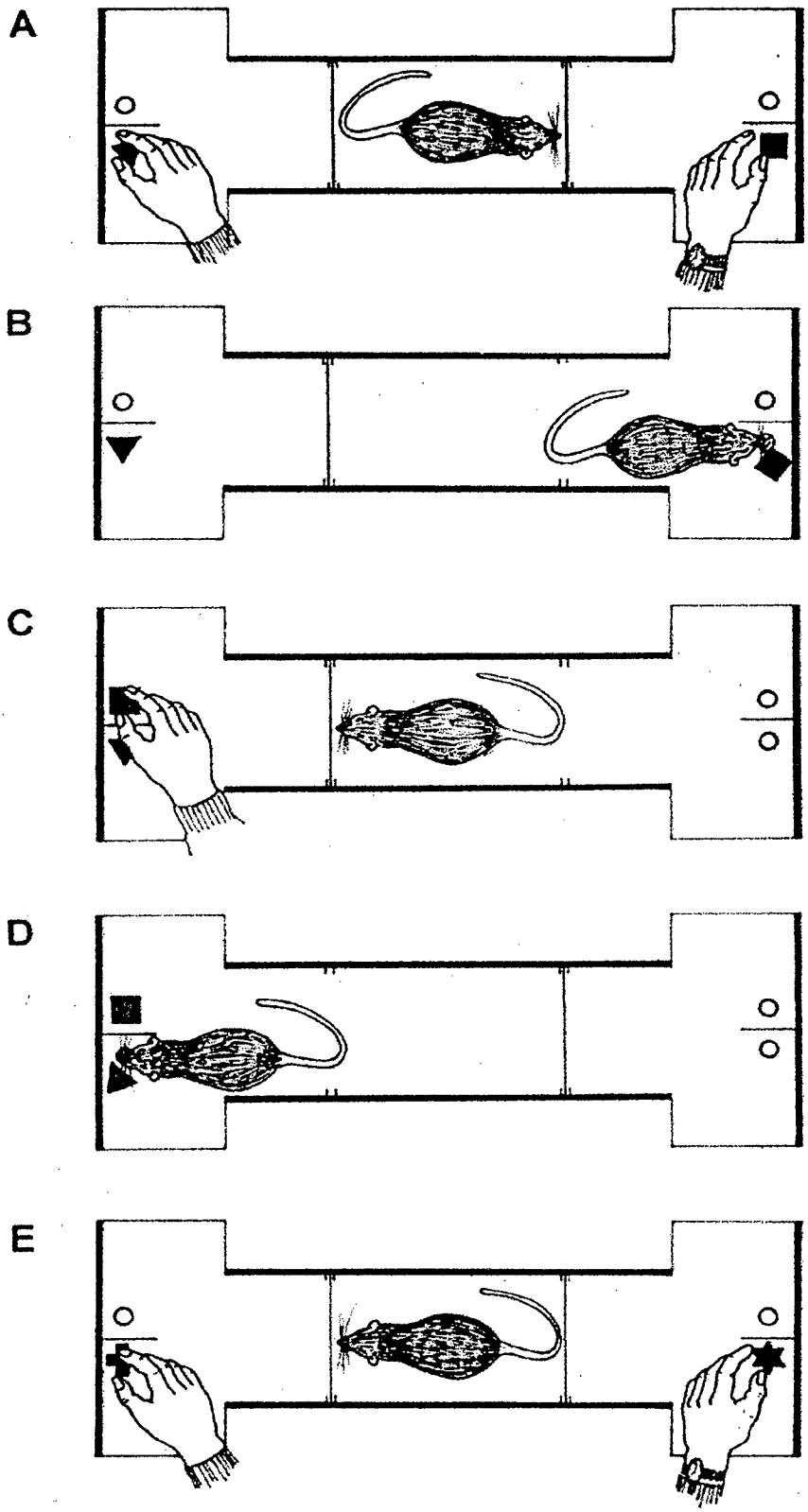

A
B
C
D
E

Figure 3. Acquisition of the DNMS task at a 4 s retention delay. There were no significant differences among E, PF and C rats in the number of trials required to reach the performance criterion of at least 17 of 20 correct trials on two consecutive sessions (top panel), or in the percent of correct choices on the trials required to reach this criterion (bottom panel).

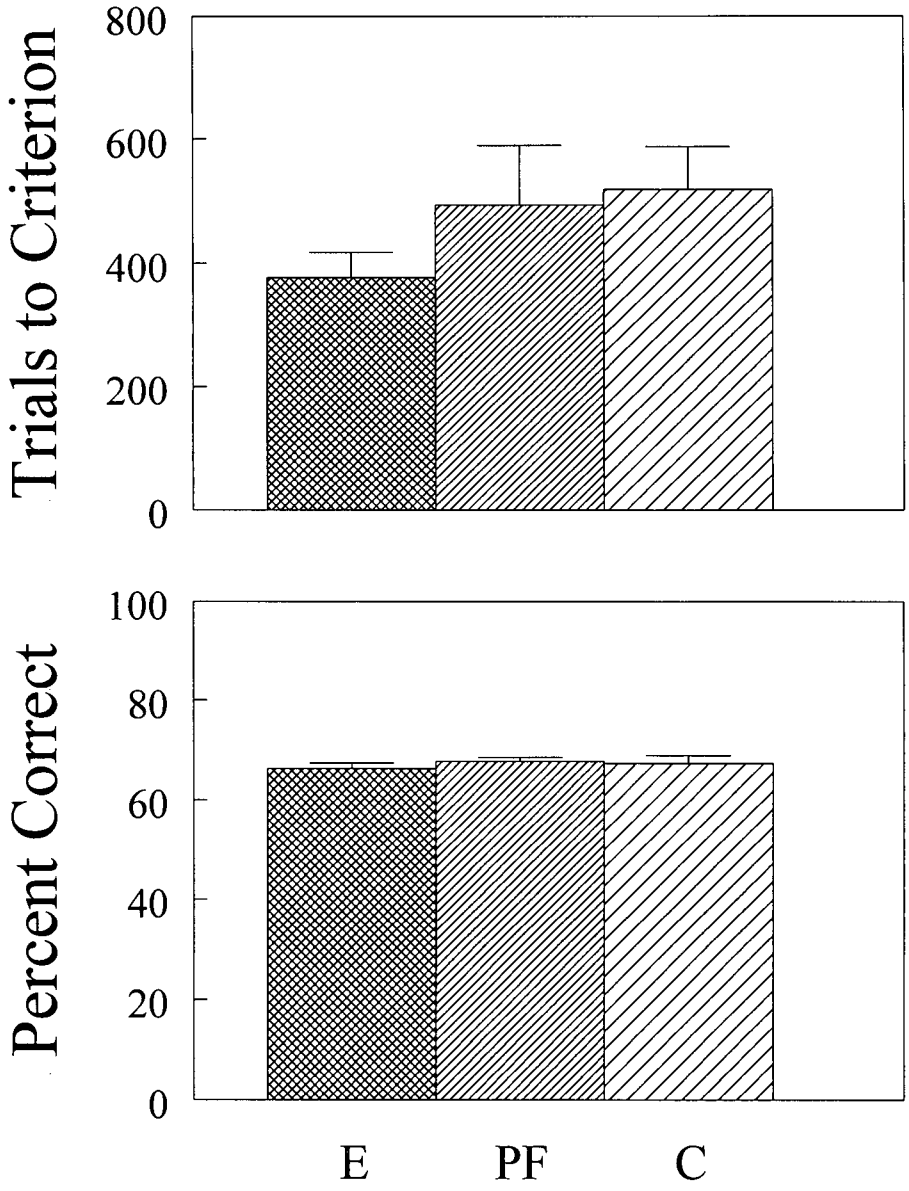

Trials to Criterion
800
600
400
200
0
Percent Correct
100
80
60
40
20
0
E
PF
C

Figure 4. DNMS task at retention delays of 15, 60 and 300 s. The percent of correct choices of E, PF and C rats significantly declined with progressively longer retention delays (15 s > 60 s > 300 s, p's<0.05). However, there were no significant differences among groups in the percent of correct choices at each retention delay.

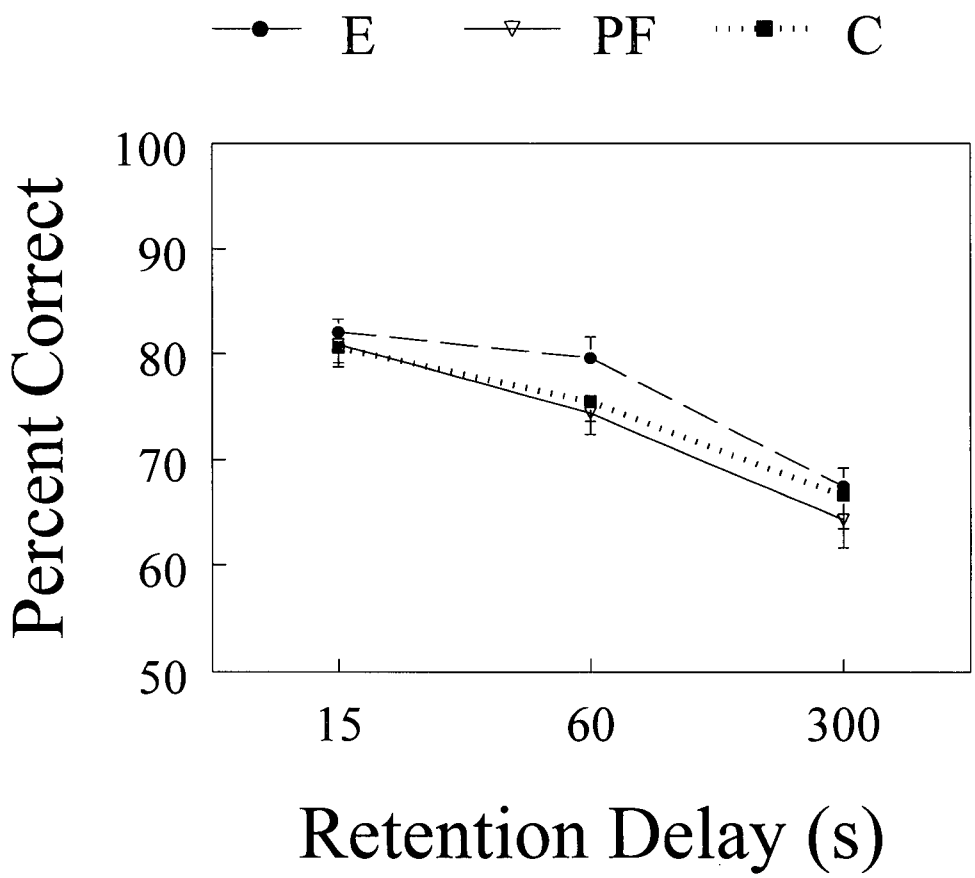

E
PF
C
Percent Correct
100
90
80
70
60
50
15
60
300
Retention Delay (s)

Figure 5. Reacquisition of the DNMS task at a 4 s retention delay. There were no significant differences among E, PF and C rats in the number of trials required to reach the performance criterion of at least 17 of 20 correct trials on two consecutive sessions (top panel), or in the percent of correct choices on the trials required to reach this criterion (bottom panel) following a 10 week rest period.

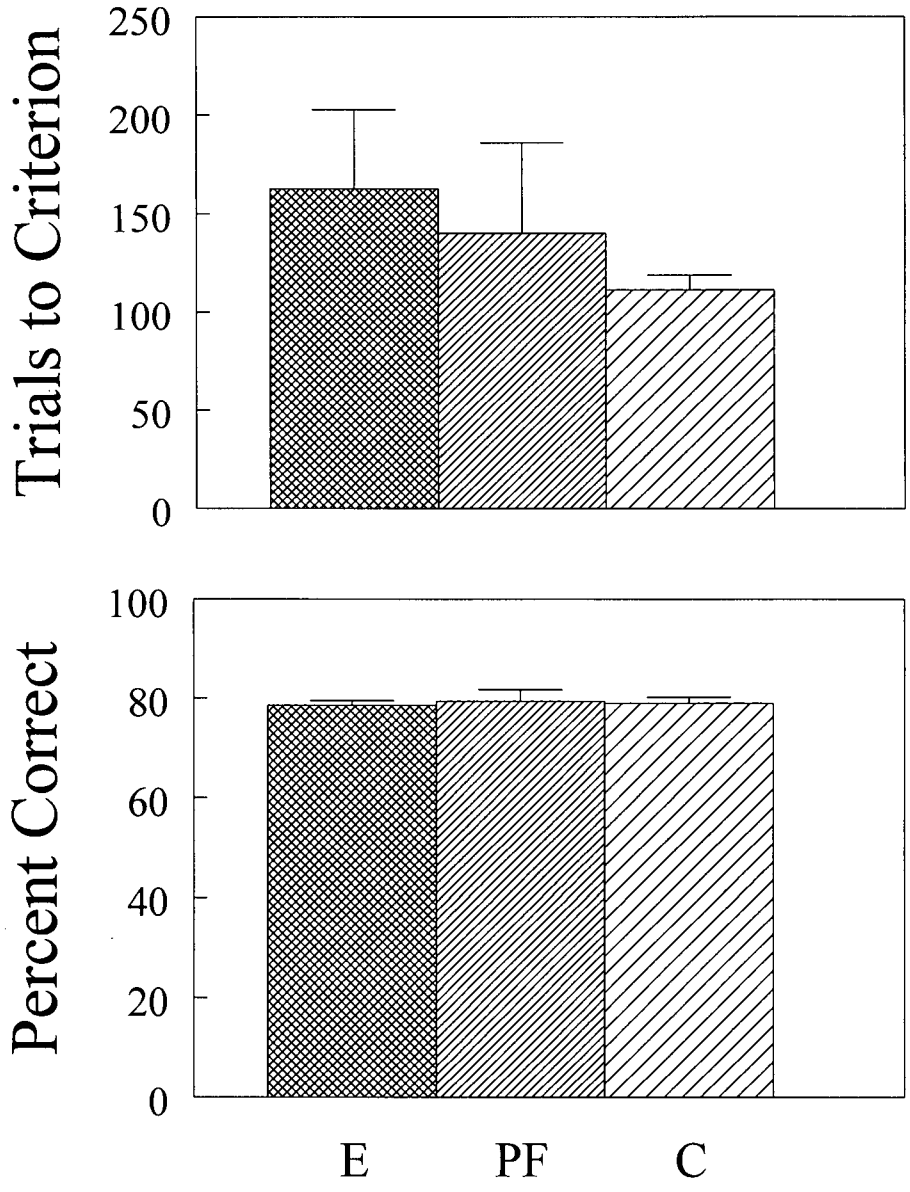

Trials to Criterion
250
200
150
100
50
0
Percent Correct
100
80
60
40
20
0
E
PF
C

Figure 6. Distraction task in the DNMS apparatus. E, PF and C rats all displayed significant decreases in the percent of correct choices when distractor objects were present ($p's<0.05$). However, there were no significant differences among groups in the percent of correct choices on either the No Distractor or Distractor sessions.

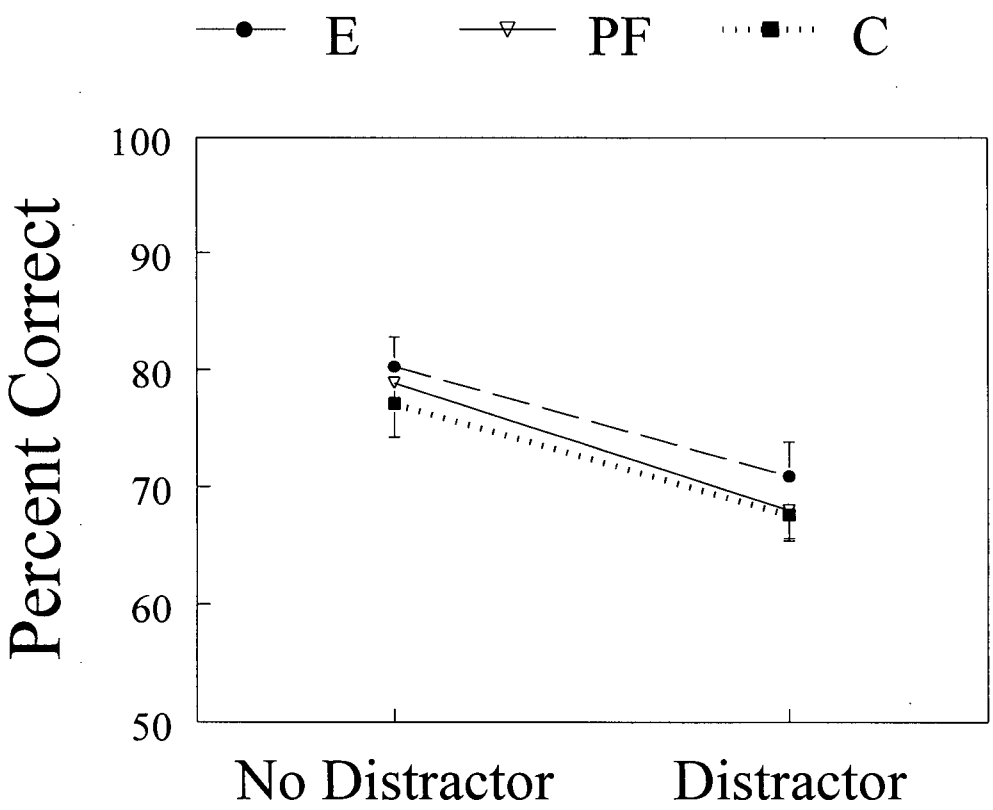

E
PF
C
Percent Correct
100
90
80
70
60
50
No Distractor
Distractor

Figure 7. Object-discrimination task in the DNMS apparatus. There were no significant differences among E, PF and C rats in the number of trials required to reach the performance criterion of at least 22 of 25 correct trials on two consecutive sessions (top panel), or in the percent of correct choices on the trials required to reach this criterion (bottom panel).

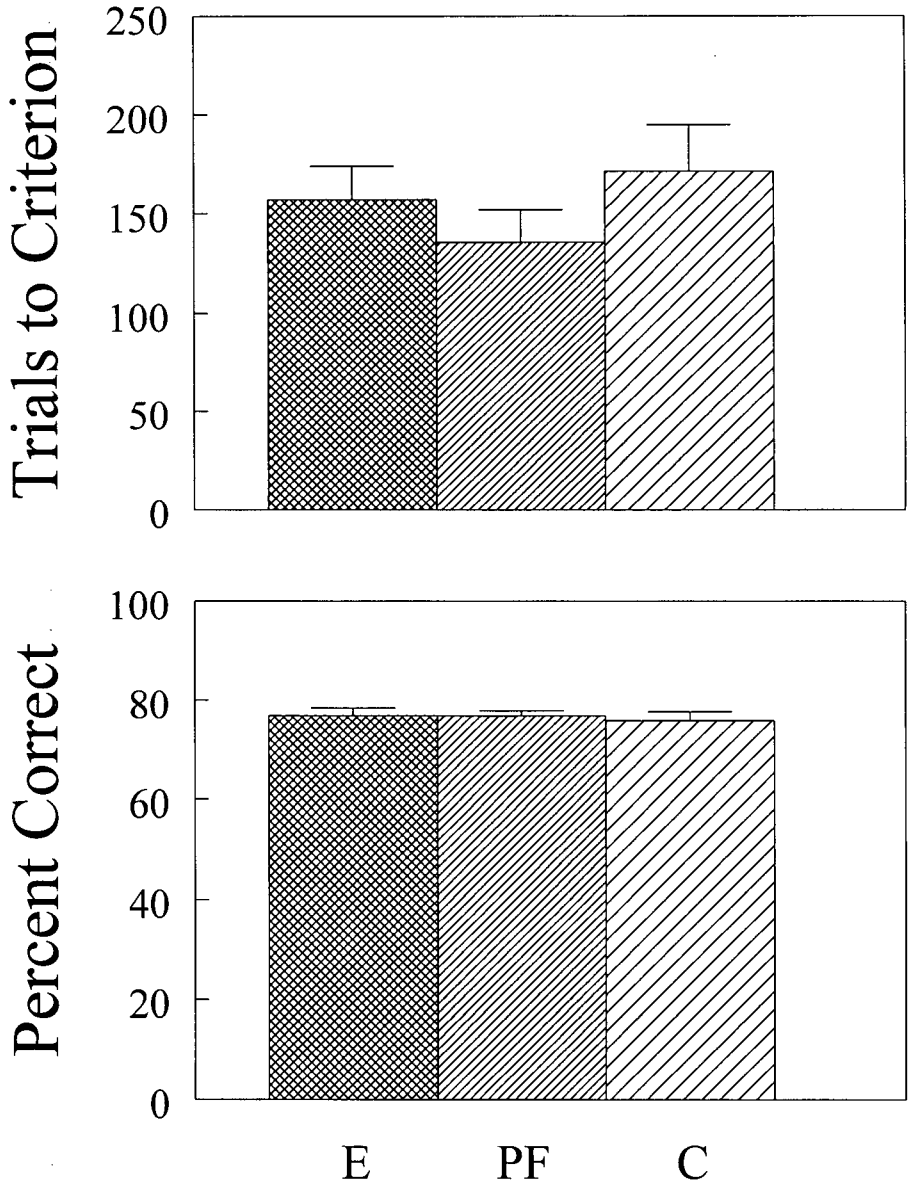

Trials to Criterion
250
200
150
100
50
0
Percent Correct
100
80
60
40
20
0
E
PF
C

Figure 8. Object-discrimination reversal task in the DNMS apparatus. There were no significant differences among E, PF and C rats in the number of trials required to reach the performance criterion of at least 22 of 25 correct trials on two consecutive sessions (top panel), or in the percent of correct choices on the trials required to reach this criterion (bottom panel).

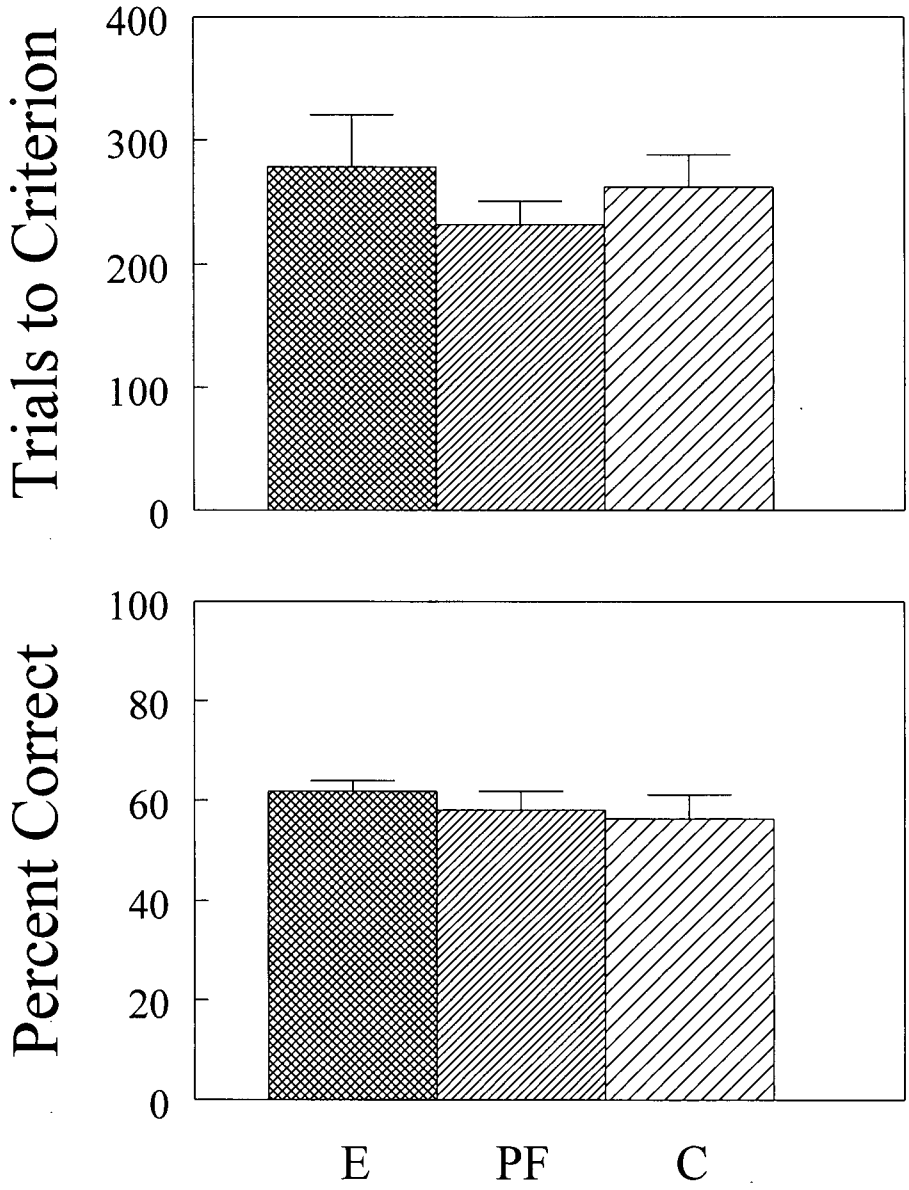

Trials to Criterion
400
300
200
100
0
Percent Correct
100
80
60
40
20
0
E
PF
C

Figure 9. Latency to find the hidden platform during training the sessions. The latency to find the hidden platform decreased over the 10 sessions for all rats (p's<0.05)(top panel). The mean latency to find the platform over all the sessions for E rats was significantly longer than for PF and C rats (*p's<0.05), and the latter did not differ significantly from each other (bottom panel).

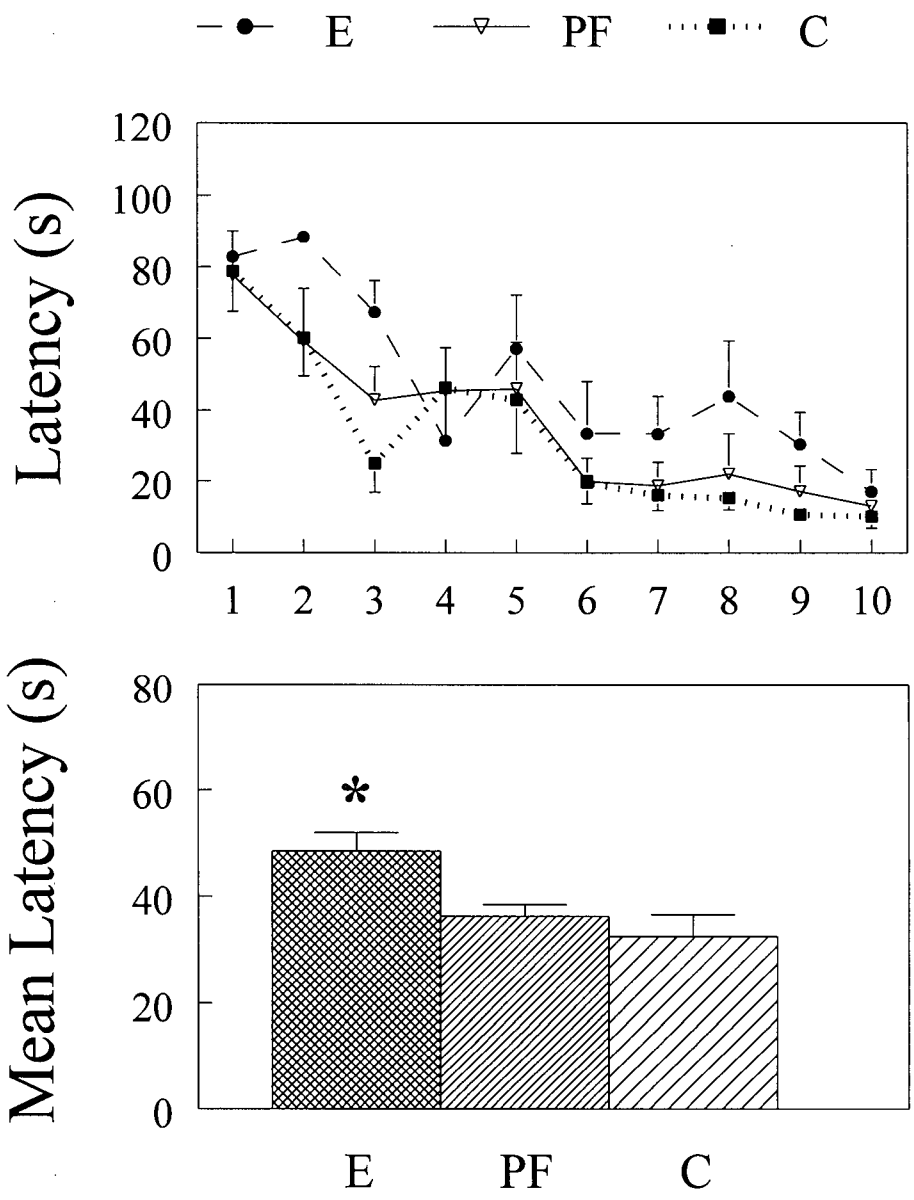
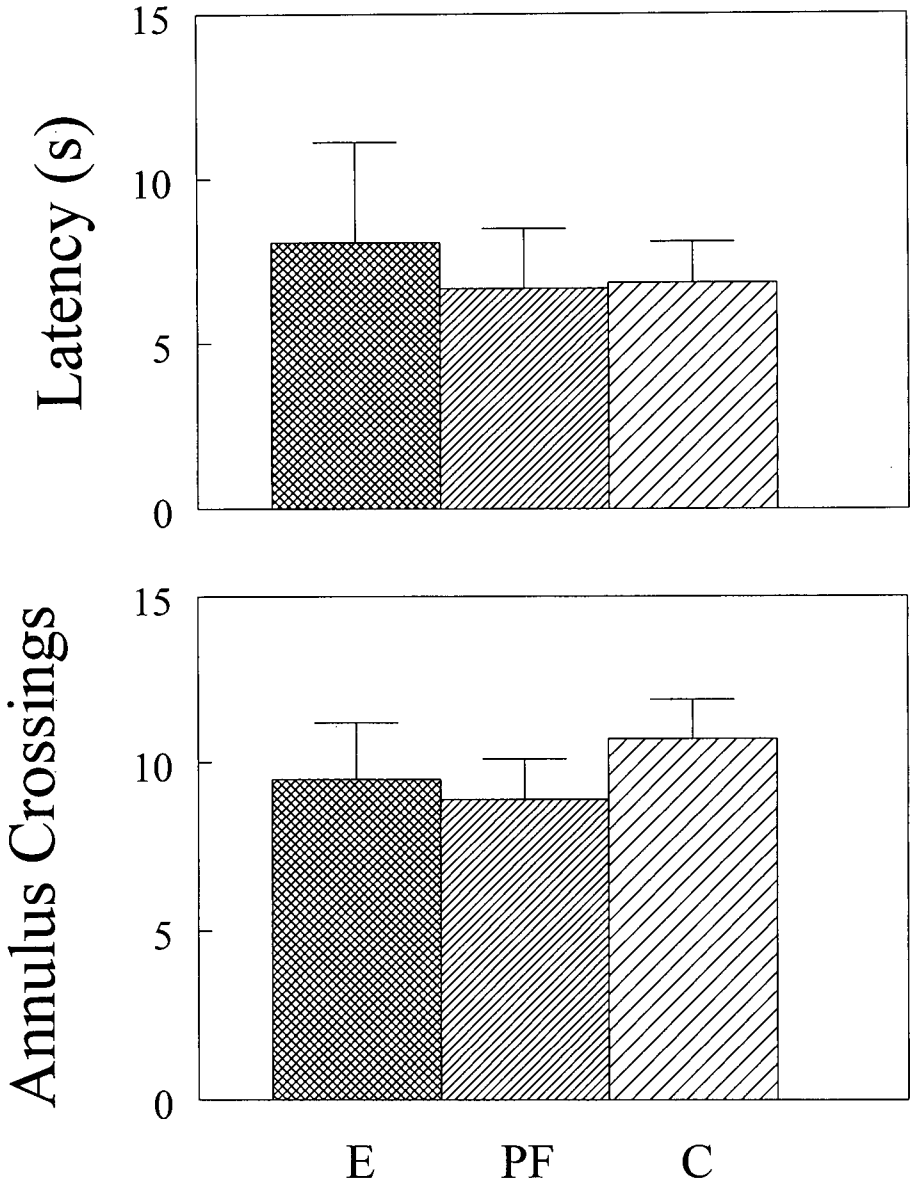

E
PF
C
Latency (s)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Mean Latency (s)
80
60
40
20
0
*
E
PF
C

Figure 10. Visible platform and annulus crossings. There were no significant differences among E, PF and C rats in the latency to reach the visible elevated platform (top panel). There were no significant differences among groups in the number of annulus crossing on the probe test, the number of passages into an area corresponding to where the platform has been throughout training (bottom panel).


Latency (s)
15
10
5
0
Annulus Crossings
15
10
5
0
E
PF
C

CHAPTER IV: EFFECTS OF PRENATAL ETHANOL EXPOSURE ON CORTICOSTEROID RECEPTORS

Introduction

The ability to respond to stress is an important basic adaptive mechanism, and HPA activation is known to be a central feature of this response. Following the termination of stress, there is a recovery process by which the endocrine, metabolic, immune and neural defensive reactions which were mobilized in response to stress are terminated (Chrousos & Gold, 1992; Johnson et al., 1992). Increased and/or prolonged HPA activation may have adverse physiological and behavioral consequences which could compromise health and possibly even survival of the organism. These adverse consequences include gastrointestinal ulceration, immunosuppression, weight loss, fatigue, myopathy, steroid diabetes, hypertension, psychogenic dwarfism, reproductive dysfunction and neuronal death (Sapolsky, 1992; Stratakis & Chrousos, 1995). Furthermore, dysregulation of the HPA axis has been implicated in a variety of psychiatric disorders such as depression, panic disorder, obsessive-compulsive disorder and anorexia nervosa (Chrousos & Gold, 1992; Johnson et al., 1992).

Although clinical studies have established that alcohol consumption markedly alters HPA function in chronic alcoholics (Merry & Marks, 1973), few clinical studies have investigated the effects of drinking during pregnancy on the HPA axis of the developing child. However, a recent study found that maternal drinking at conception and during pregnancy was associated with higher poststress cortisol levels in infants (Jacobson et al., 1993), indicating the importance of this issue for further study. Animals studies have shown that prenatal ethanol exposure produces HPA hyperresponsiveness to stressors (Angelogianni & Gianoulakis, 1989; Lee et al., 1990;

Nelson et al., 1985; 1986; Ogilvie & Rivier, 1996; Taylor et al., 1982; 1983; 1986; Weinberg, 1988; 1992a, Weinberg et al., 1995; 1996). Furthermore, it has been proposed that this HPA hyperresponsiveness may underlie some of the behavioral abnormalities (Kim et al., 1996) and immune deficits (Giberson et al., 1997; Giberson & Weinberg, 1995) that occur following prenatal ethanol exposure, both of which have been demonstrated in humans and animals.

HPA hyperresponsiveness in E animals has been shown to be mediated in part by deficits in the negative feedback regulation of the HPA axis (Nelson et al., 1985; Osborn et al., 1996). HPA feedback efficacy has been shown to be positively related to corticosteroid receptor densities at feedback sites such as the hippocampus, prefrontal cortex and hypothalamus. There is increased feedback efficacy with greater receptor density (De Kloet, 1991; Meaney et al., 1993; 1996; Sapolsky, 1992; Sapolsky et al., 1985). Thus, the purpose of this study was to test the hypothesis that the impaired negative feedback regulation of the HPA axis that occurs following prenatal ethanol exposure may be mediated, at least partially, by decreased levels of corticosteroid receptors at HPA feedback sites.

An earlier study in this laboratory (Weinberg & Peterson, 1991), demonstrated that under nonstressed or basal conditions, there were no significant effects of prenatal ethanol exposure on hippocampal GR and MR densities or binding affinities. However, it is possible that prenatal ethanol exposure may decrease densities of GRs and/or MRs at feedback sites other than the hippocampus (prefrontal cortex, hypothalamus and anterior pituitary). Furthermore, increased downregulation of these receptors may result following a period of chronic stress in E rats. That is, chronic stress may reveal an underlying vulnerability in the corticosteroid receptor system that may not be apparent under basal conditions. This is analogous to the finding that HPA hyperresponsiveness in E animals is typically evident only in stressful situations and not under

basal conditions. Thus, this study examined GR and MR densities at several feedback sites (hippocampus, prefrontal cortex, hypothalamus, and anterior pituitary) both in the nonstressed state and following chronic stress. To test the robustness of possible changes following stress, two different stress regimens were examined. In addition, hippocampal corticosteroid receptor densities were examined 7 days following ADX compared to that at the usual 24 h to determine if prenatal ethanol exposure results in differential receptor upregulation, a measure of receptor plasticity.

Methods

Blood Sampling

All blood sampling was by cardiac puncture, as discussed in the General Methods section, at 0830-0930 h.

Corticosteroid Receptor Assay

This cytosolic binding assay was an adaptation (Weinberg & Peterson, 1991) of the method of Luttge and colleagues (Emadian et al., 1986; Gray & Luttge, 1982; Luttge et al., 1984). Receptor binding affinities were not measured in this study because Weinberg and Peterson (1991) and others (Sapolsky et al., 1984; Reul et al., 1987a; 1987b) have shown that binding affinity is resistant to changes by experimental manipulations.

Animal surgery. ADX occurred 24 h prior to brain processing for the corticosteroid receptor assay to remove endogenous CORT, which would interfere with the assay. Bilateral ADX occurred at 1000-1200 h, under halothane (Halocarbon Laboratory, River Edge, NJ) anaesthesia, through small dorsolateral incisions below the rib cage. Previous studies have

determined that at 24 h following ADX, there is complete clearance of endogenous CORT and no changes in corticosteroid receptor levels (Luttge & Rupp, 1989; Reul et al., 1987a; 1987b).

Brain processing. Rats were anesthetized with ether (Fisher Scientific), and a blood sample was collected by cardiac puncture to assay for plasma CORT levels to ensure that the ADX was complete. Animals were then perfused through the heart with 60 ml ice-cold buffered saline [MR buffer (see below) diluted 1:9 in isotonic saline] to remove CBG and albumin which could bind the exogenous steroids, and thus interfere with the assay. Following decapitation, the brain was removed and the regions of interest (hippocampus, prefrontal cortex, hypothalamus, and anterior pituitary) were dissected quickly on a glass plate over ice. Tissue for the different brain regions was either assayed fresh or placed in individual aluminum foil packets, quickly frozen by immersion into liquid nitrogen, and stored at -80 °C until assayed. Pilot studies have shown that receptor loss with frozen tissue is negligible compared to fresh tissue. Within each experiment, all tissue from a specific site was assayed either fresh or frozen.

The brain dissections were made according to adaptations of Glowinski and Iversen (1966), McEwen and Pfaff (1970) and Sapolsky et al. (1984). The hippocampus is loosely attached to the rest of the brain and thus easily detached. The prefrontal cortex was isolated using the landmarks of the anterior boundary of optic chiasm on the ventral surface, the rhinal fissure and the corpus callosum. The hypothalamus was isolated using the landmarks of the optic chiasm on the ventral surface and the fornices for the dorsal and lateral boundaries. The pituitary detaches from the brain when the brain is removed from the skull and remains in the sella turcica, from which it can be easily removed.

Steroids. [1,2,4-^{3}H]-dexamethasone ([^{3}H]-DEX), 42 Ci/mmol, was from Amersham Ltd. (Oakville, ON); [1,2,6,7-^{3}H(N)]-aldosterone ([^{3}H]-ALD), 82 Ci/mmol, from DuPont; unlabelled DEX ([^{1}H]-DEX) and unlabelled ALD ([^{1}H]-ALD) from Sigma Chemical; and unlabelled

RU28362 was a gift from Roussel-UCLAF (Romainville, France). DEX and RU28362 are potent GR ligands, while ALD is a potent MR ligand.

Buffers. MR buffer consisted of 20 mM HEPES (Sigma Chemical) and 10% w/v glycerol (pH 7.6 at 4 °C)(Fisher Scientific). GR buffer consisted of MR buffer supplemented with 2 mM DL-dithiothreitol (DTT)(Sigma Chemical) and 20 mM sodium molybdate (BDH Inc., Vancouver, BC).

Gel Columns. Glass pipettes (5 ml capacity, 14 x 0.6 cm) were filled to the 4 ± 0.1 ml mark with Sephadex G-25 gel (Sigma Chemical), and were maintained hydrated at 4 °C. These gel columns were used to separate particles based on molecular size (Gel Filtration: Theory and Practice, 1982).

Assay method. Tissue from each brain site was placed into 1 ml of ice-cold MR buffer and homogenized in a glass tube with a Teflon pestle (1100 RPM). Homogenates were centrifuged at 100,000 x g for 20 min at 0 °C, and the resultant supernatant again centrifuged at 100,000 x g for 60 min at 0 °C to yield cytosol. An aliquot of cytosol was removed and stored at -20 °C for subsequent determination of protein concentration by the Lowry method (Lowry et al., 1951). The remaining cytosol was divided into two parts (for MR and GR) and diluted 9:1 with the appropriate MR or GR buffers. Aliquots (150 µl) were then added to pre-cooled tubes containing the desired steroid(s) dried under N_2. For MRs, cytosol was incubated with 20 nM [^{3}H]-ALD plus a 500-fold molar excess of RU28362, minus (total binding) or plus (nonspecific binding) a 200-fold molar excess of [^{1}H]-ALD. The specific GR ligand RU28362 was added to prevent the low affinity binding of [^{3}H]-ALD to GR receptors. For GRs, cytosol was incubated with 20 nM [^{3}H]-DEX, minus (total binding) or plus (nonspecific binding) a 200-fold molar excess of [^{1}H]-DEX. All tubes were incubated for 24 h at 0 °C. Duplicate 50 µl aliquots of

cytosol were then added to gel columns, preequilibrated and eluted with the appropriate MR or GR buffers, to separate bound and unbound steroids. Binding was determined by liquid scintillation counting of the bound fraction collected from the columns. Specific binding was calculated as the difference between total binding and nonspecific binding and expressed in fmol/mg protein. All columns had been preequilibrated with brain cytosol prior to their first use to prevent any loss of the bound fraction (see Chapter VI of thesis).

Chronic Stress Regimens

The chronic stress regimens were adapted from Sapolsky et al. (1984). Over 18 consecutive days, animals were exposed to two of six different stressors daily, in rotating order, to produce intermittent elevations in circulating CORT levels. Six different stressors, that previously have been shown to activate the HPA axis, were used in order to prevent habituation of the stress response. Stressors were presented at random times of day to prevent the animals from predicting their occurrence. Presentation of the stressors was separated by a minimum of 2 h to allow CORT to return to or decrease toward basal levels between stressors.

Stress Regimen I (Experiments 1 and 2). The six stressors included the following: (1) restraint in plastic tubes (19 x 7 cm for male, 15 x 6 cm for female) for 30 min with two presentations of four taps on the tubes applied randomly during the session; (2) forced swim (43 x 28 cm bucket filled to the 30 cm level) in room temperature water for 10 min; (3) restraint in a rectangular plastic container (11 x 12 x 9 cm) for 30 min with one presentation of six shakes and two rotations of the container applied randomly during the session; (4) exposure to ether vapors (20 x 21 cm glass jar) for 1 min; (5) brief restraint and an IP needle stick followed by exposure to ether vapors (as above); and (6) novel polycarbonate cage (20 x 27 x 15 cm) without bedding, food or water for 30 min with a loud startle noise presented randomly twice during the session.

In addition, water deprivation (1600-0800 hr) occurred twice weekly (total of six times) but never on consecutive nights or prior to ether stress. Rats were singly housed in suspended stainless-steel cages with mesh front and floor (24 x 18 x 18 cm) throughout the duration of the stress regimen.

Stress Regimen II (Experiment 3). The six stressors included: (1) restraint in plastic tubes for 45 min with three presentations of four taps and two rotations; (2) forced swim for 15 min; (3) restraint in a plastic rectangular container for 45 min with three presentations of four shakes and two rotations; (4) ether vapor exposure for 1 min; (5) drop, in which the animal was held by the base of the tail and rapidly lifted and lowered at arms length five times; and (6) novel cage for 45 min with a loud startle noise presented three times. Water deprivation occurred twice weekly (total of six times). In contrast to Stress Regimen I, rats were singly housed in opaque polycarbonate cages (20 x 27 x 15 cm) lined with bedding throughout the duration of the stress regimen.

CORT Pellet Implantation

CORT pellets were made according to Meyer et al. (1979) by melting powdered CORT and then pouring it into wax molds to harden. Pellets were implanted SC at the nape of the neck under halothane anesthesia. These pellets produced sustained elevations in circulating CORT levels that were at least 50% of the maximal stress levels for the duration of the implant.

Experiment 1

The purpose of this experiment was to establish a chronic stress regimen (Stress Regimen I) that would produce downregulation of corticosteroid receptors in the hippocampus.

Methods

Adult male and female rats from the C group only were randomly assigned to one of three treatment conditions. In all three conditions, body weights were measured immediately prior to ADX, adrenal weights were recorded, and rats were sacrificed and brains removed for receptor measurement 24 h after ADX.

(1) Stress condition rats were singly housed for two days in opaque polycarbonate cages (20 x 27 x 15 cm) lined with bedding, after which a pre-stress blood sample was collected. The following day, rats were transferred individually into suspended stainless-steel mesh cages and subjected to Stress Regimen I for 18 consecutive days. A post-stress blood sample was taken the day following the last stress day. The rats were subjected to ADX two days later.

(2) Implant condition rats were on the same schedule as the Stress rats. They were singly housed in polycarbonate cages and subjected to pre-implant blood sampling. The following day, rats were implanted with CORT pellets. Males were implanted with two pellets weighing a mean of 235.6 $\pm$ 1.6 mg and weighing 151.3 $\pm$ 3.5 mg upon removal; females were implanted with three pellets weighing a mean of 354.2 $\pm$ 1.6 mg and weighing 220.7 $\pm$ 8.2 mg upon removal. Females were implanted with pellets containing a higher concentration of CORT than males to account for the higher circulating CORT levels in intact females compared to males. Blood samples were taken at the mid-point (day 10) and end of the implantation period (day 19) prior to ADX to confirm that plasma CORT levels remained high. Pellets were removed at the time of ADX to be weighed.

(3) NoStress condition rats served as the nonstressed or basal controls. Rats remained group housed in polycarbonate cages (46 x 24 x 20 cm) until ADX.

Results

Corticosteroid Receptors

Males (Figure 11) displayed significant effects of treatment conditions for both GR [$F(2,34) = 6.36$, $p<0.005$] and MR [$F(2,34) = 7.45$, $p<0.002$] densities. NoStress males showed higher receptor densities than Stress and Implant males ($p<0.05$'s), and the latter did not differ from each other. Females (Figure 11) also displayed significant effects of treatment conditions for both GR [$F(2,36) = 4.88$, $p<0.013$] and MR [$F(2,36) = 3.29$, $p<0.049$] densities. NoStress females had higher receptor densities than Implant females ($p<0.05$'s), and Stress females displayed intermediate levels that did not differ from the other conditions. Males displayed significantly higher GR [$F(1,70) = 11.6$, $p<0.001$] and MR [$F(1,70) = 4.1$, $p<0.047$] densities than females.

Plasma CORT and CBG

Basal plasma CORT levels (Table 5) were significantly elevated following the chronic stress regimen in males [$F(1,15) = 6.81$, $p<0.02$], and showed a similar trend towards elevation in females that approached statistical significance [$F(1,14) = 3.70$, $p<0.075$]. The CORT pellets produced the expected elevations in circulating CORT levels in both males [$F(2,18) = 6.81$, $p<0.001$] and females [$F(2,20) = 6.81$, $p<0.001$]; CORT levels were higher on days 10 and 19 after implantation than prior to implantation ($p<0.05$'s). There were no significant differences in basal CORT levels among groups prior to stress or pellet implantation for either males and females. Females displayed significantly higher CORT levels than males in the Stress [$F(1,26) = 20.6$, $p<0.001$] and Implant [$F(1,19) = 67.7$, $p<0.001$] conditions.

Plasma CBG levels (Table 5) were not significantly different among groups prior to stress or pellet implantation, and there were no significant changes in CBG levels as a result of the chronic stress regimen for either males or females. However, CORT pellets produced significant decreases in CBG levels in both males [$F(2,18) = 10.96$, $p<0.001$] and females [$F(2,16) = 26.06$,

$p<0.001$] at days 10 and 19 post-implantation compared to pre-implantation levels ($p<0.05$'s). Females displayed significantly higher CBG levels than males in the Stress [$F(1,26) = 349.9$, $p<0.001$] and Implant [$F(1,17) = 53.2$, $p<0.001$] conditions.

Adrenal:Body Weight Ratio

The adrenal:body weight ratio (Table 6) differed among treatment conditions for both males [$F(2,32) = 95.04$, $p<0.001$] and females [$F(2,35) = 89.62$, $p<0.001$]. Ratios were increased in Stress compared to NoStress rats, and increased in both of these conditions compared to Implant rats ($p<0.05$'s). Females displayed significantly higher adrenal:body weight ratios than males [$F(1,67) = 348.2$, $p<0.001$].

Experiment 2

This experiment examined corticosteroid receptor densities in the hippocampus of adult E, PF and C female rats. Downregulation of receptors following exposure to Stress Regimen I as well as upregulation of receptors 7 days following ADX were examined. Both male and female rats were tested. However, due to technical difficulties male data were lost, and thus only female data are presented.

Methods

E, PF and C female rats were randomly assigned to Stress and NoStress treatment conditions identical to those of Experiment 1. Half the animals in each treatment condition were terminated at the usual 24 h post-ADX (1 Day condition) and half were terminated 7 day post-ADX (7 Day condition).

Results

Developmental Data

Ethanol intake. Ethanol intake by pregnant E rats (n = 14) was consistently high throughout gestation, averaging 9.8 ± 0.4, 11.3 ± 0.3 and 10.5 ± 0.2 g/kg body weight/day, respectively, for the 3 weeks of gestation. The overall mean for the 3 weeks was 10.5 ± 0.3 g/kg body weight/day. This laboratory has shown that intake at these levels result in maximal or near maximal BALs of 145-155 mg/dl (Weinberg, 1985; Osborn et al., 1996).

Maternal weights. Analysis of maternal body weights during gestation (Table 7) indicated significant effects of group [$F(2,40) = 16.3$, $p<0.001$] and day [$F(3,120) = 1211.6$, $p<0.001$], and a group x day interaction [$F(6,132) = 31.2$, $p<0.001$]. Post hoc tests revealed that all dams displayed the expected increase in body weights as gestation progressed (p's<0.05); and E and PF dams weighed less than C dams on G7, G14 and G21 (p's<0.05).

Analysis of maternal body weights during lactation (Table 7) indicated a significant effect of day [$F(3,126) = 150.3$, $p<0.001$], with weights of dams increasing and then stabilizing as lactation progressed (p's<0.05).

Pup data. There were no significant differences among E, PF and C groups in the number of live or stillborn offspring. Analysis of pup weights (male and female data collapsed) from birth to weaning (Table 8) revealed significant effects of group [$F(2,87) = 7.9$, $p<0.001$] and day [$F(3,261) = 12400.0$, $p<0.001$], and a group x day interaction [$F(6,261) = 6.1$, $p<0.001$]. Post hoc tests revealed that all pup weights progressively increased with age (p's<0.05); and E pups weighed less than C pups on D1 and D8, and less than both PF and C rats on D15 and D22 (p's<0.05).

Corticosteroid Receptors

Analysis of hippocampal GR densities (Figure 12) revealed significant effects of stress [$F(1,80) = 8.66$, $p<0.004$] and time following ADX [$F(1,80) = 9.36$, $p<0.003$]. In the 1 Day

condition, overall GR densities were higher in the NoStress than in the Stress condition ($p<0.05$) indicating receptor downregulation following stress. In addition, overall GR densities were upregulated in the 7 Day compared to the 1 Day condition, but this difference reached statistical significance only following Stress ($p<0.05$). There were no significant changes in hippocampal MR densities (Figure 12), and no significant differences among E, PF and C rats in hippocampal GRs and MRs under any of the experimental conditions.

Plasma CORT and CBG

Overall basal plasma CORT levels (Table 9) were significantly elevated following the stress regimen [$F(1,14) = 6.90$, $p<0.02$]. There were no significant differences in CBG levels (Table 9) between pre- and post-stress conditions. There were no significant differences among E, PF and C females in either CORT or CBG levels under any conditions.

Adrenal:Body Weight Ratio

Analysis of adrenal:body weight ratios (Table 10) indicated significant effects of group [$F(2,99) = 10.73$, $p<0.001$] and stress [$F(1,99) = 55.30$, $p<0.001$]. Animals in all prenatal treatment groups had higher adrenal:body weight ratios in the Stress than in the NoStress conditions ($p<0.05$); however, following chronic stress the ratio was greater in E compared to PF and C rats ($p<0.05$'s).

Experiment 3

This experiment examined corticosteroid receptor densities at the hippocampus, prefrontal cortex, hypothalamus and anterior pituitary in adult E, PF and C male and female rats under NoStress and Stress conditions. There were two aims of this experiment: (1) To examine receptor densities at the additional HPA feedback sites; (2) To determine the robustness of the

chronic intermittent stress paradigm. To this end, a second stress regimen (Stress Regimen II) was developed in which most individual stressors were 50% longer in duration and the housing conditions were changed from wire-mesh hanging cages to bedding lined polycarbonate cages.

Methods

Male and female rats from E, PF and C groups were randomly assigned to either the NoStress or Stress conditions. Rats in the Stress condition were single housed into polycarbonate cages and subjected to Stress Regimen II, as described above, followed by ADX and determination of receptor densities 24 h later. Pre- and post-stress blood samples were also obtained. Rats in the NoStress condition were singly housed into opaque polycarbonate cages and blood samples were obtained two days later. The following day, animals underwent ADX and receptor densities were determined 24 h later.

Results

Developmental Data

Ethanol intake. Ethanol intake by pregnant female E rats (n = 16) was consistently high throughout gestation, averaging 8.3 $\pm$ 0.3, 10.8 $\pm$ 0.3 and 10.9 $\pm$ 0.2 g/kg body weight/day, respectively, for the 3 weeks of gestation. The overall mean for the 3 weeks was 10.0 $\pm$ 0.3 g/kg body weight/day. This laboratory has shown that intake at these levels result in maximal or near maximal BALs of 145-155 mg/dl (Weinberg, 1985; Osborn et al., 1996).

Maternal weights. Analysis of maternal body weights during gestation (Table 11) indicated significant effects of group [$F(2,44) = 23.1$, $p<0.001$] and day [$F(3,132) = 815.5$, $p<0.001$], and a group x day interaction [$F(6,132) = 31.2$, $p<0.001$]. Post hoc tests revealed that all dams displayed the expected increase in body weight as gestation progressed (p's<0.05); and E and PF dams weighed less than C dams on G7, G14 and G21 (p's<0.05).

Analysis of maternal body weights during lactation (Table 11) indicated a significant effect of day [$F(3,129) = 256.8$, $p<0.001$] and a group x day interaction [$F(6,129) = 7.4$, $p<0.001$]. Post hoc tests showed that weights of dams increased and then stabilized as lactation progressed ($p's<0.05$); and E and PF dams weighed less than C dams on D1 ($p's<0.05$), but were no longer different in weight by D8.

Pup data. There were no significant differences among E, PF and C groups in the number of live or stillborn offspring. Analysis of pup weights (male and female data collapsed) from birth to weaning (Table 12) revealed signifcant effects of group [$F(2,89) = 24.4$, $p<0.001$] and day [$F(3,267) = 10400.0$, $p<0.001$], and a group x day interaction [$F(6,261) = 20.9$, $p<0.001$]. Post hoc tests revealed that all pup weights progressively increased with age ($p's<0.05$); and E and PF pups weighed less than C pups on D1, D8, D15 and D22 ($p's<0.05$).

Corticosteroid Receptors

In the NoStress condition, there were no significant differences in either GR or MR densities among E, PF and C males or females at the hippocampus (Figure 13), prefrontal cortex (Figure 14), hypothalamus (Figure 15) or anterior pituitary (Figure 16). However, following chronic stress, significant group x stress interactions for both males [$F(2,47) = 3.3$, $p<0.045$] and females [$F(2,53) = 3.6$, $p<0.035$] indicated subtle effects of prenatal treatment on hippocampal GRs. E males in the Stress condition had lower hippocampal GR densities than PF males ($p<0.05$), and C males did not differ from either group. C but not E and PF females displayed higher hippocampal GRs following stress ($p<0.05$). There were no significant differences in GR and MR densities among E, PF and C rats at any other site, and no significant changes in hippocampal MRs under any conditions for either males or females. Significant sex differences in hippocampal GRs [$F(1,100) = 24.9$, $p<0.001$] and MRs [$F(1,98) = 9.1$, $p<0.003$] as well as

prefrontal cortical GRs [$F(1,103) = 21.5$, $p<0.001$] and MRs [$F(1,103) = 4.1$, $p<0.046$] indicated that males displayed higher receptor densities than females.

Plasma CORT

There were no significant differences in basal CORT levels (Table 13) prior to or following the chronic stress regimen, or among E, PF and C males and females. In females, the increase in basal levels in the post-stress compared to the pre-stress condition is in the same direction as observed in Experiments 1 and 2, but did not reach statistical significance. Females displayed significantly higher CORT levels than males in the NoStress [$F(1,24) = 21.5$, $p<0.001$] and Stress [$F(1,44) = 18.4$, $p<0.001$] conditions. Plasma CBG levels were not measured in this experiment, because they were shown to be resistant to change by chronic stress (see Experiments 1 and 2).

Adrenal:Body Weight Ratio

Male adrenal:body weight ratios (Table 14) were higher in the Stress compared to the NoStress condition [$F(1,48) = 22.62$, $p<0.001$]. For females (Table 14), although there was a significant effect of stress [$F(1,52) = 12.80$, $p<0.001$], with only E females displaying a significant increase in adrenal:body weight ratio ($p<0.05$). Females had higher adrenal:body weight ratios than males [$F(1,98) = 448.2$, $p<0.001$].

Discussion

There were several major findings in this study. First, exposure to chronic intermittent stress (using Stress Regimen I) produced downregulation of hippocampal GR and MR densities in males, and downregulation of hippocampal GRs in females. This stress regimen also produced elevated basal plasma CORT levels without changes in plasma CBG levels, and

increased relative adrenal weights in both males and females. Second, small changes in the stress paradigm (in Stress Regimen II compared to Regimen I) eliminated the downregulation of hippocampal corticosteroid receptors and increase in basal CORT levels, and resulted in less robust increases in relative adrenal weights. Third, CORT pellet implants produced sustained high plasma CORT levels (equivalent to approximately 50-75% of the maximal stress response) that resulted in downregulation of hippocampal GRs and MRs, decreased plasma CBG levels, and decreased relative adrenal weights in both males and females. Fourth, upregulation of hippocampal GRs occurred 7 days following ADX compared to that at the usual 24 h in females. Finally, prenatal ethanol exposure produced greater increases in relative adrenal weights than controls following chronic stress. However, prenatal ethanol exposure did not produce changes in basal or nonstressed corticosteroid receptor densities; nor did it produce differential hippocampal GR downregulation under conditions of Stress Regimen I, or differential hippocampal GR upregulation following ADX. However, there were small differences in hippocampal GRs among E, PF and C rats under conditions of Stress Regimen II. Given the nature of these changes in receptor density, however, it would appear that these changes do not play a critical role in mediating the feedback deficits that have been observed in E animals. Thus, the hypothesis that decreased corticosteroid receptor densities underlie the deficits in feedback inhibition and resultant HPA hyperresponsiveness following prenatal ethanol exposure was not supported.

First, exposure to Stress Regimen I produced downregulation of hippocampal GR and MR densities in males, and downregulation of hippocampal GRs in females. The downregulation in females was less robust than that in males, achieving statistical significance in only one of two experiments (in Experiment 2). Importantly, this hippocampal receptor downregulation was

achieved with a much milder stress regimen than that previously reported. Sapolsky et al. (1984) demonstrated downregulation of hippocampal corticosteroid receptors (this study pre-dated the GR/MR distinction) using a stress regimen composed of three of seven different stressors applied daily for 3 weeks, and included such severe stressors as sham ADX under ether anesthesia and injections of histamine and lysine vasopressin. These investigators reported that their stress regimen produced such precarious postsurgical (following ADX) health that animals were sacrificed for receptor determination at 12 h rather than 24 h after ADX. Similarly, decreases in hippocampal GR densities were demonstrated following escape training using electrical footshock for 4 h daily, 5 days a week, for 6 months (Eldridge et al., 1989). However, downregulation of hippocampal GRs or MRs was not found following exposure to electrically induced seizures for 8 days (Young et al., 1990), restraint stress 2 h daily for 5 days (Lowy, 1991), daily sessions of restraint, shake and forced swim stressors for 3 weeks (Spencer & McEwen, unpublished, cited in Spencer & McEwen, 1990), nor was decreased GR immunoreactivity found in the hippocampal CA1 subfield following restraint stress administered 22 h daily for 14 days (Kitayama et al., 1989). Moreover, ethanol, which is a potent activator of the HPA axis, did not produce downregulation of hippocampal GRs or MRs when administered to adult animals for up to 19 days (Spencer & McEwen, 1990) or 20-24 weeks (Rachamin et al., 1989). Interestingly, no changes were found in GR and MR binding affinities when measured in any of the above studies. That the milder chronic stress regimen used in the present study, composed mainly of psychological rather than physical or painful stressors, was able to downregulate hippocampal corticosteroid receptors provides an important extension of previous work which used primarily severe or prolonged chronic stress regimens (Eldridge et al., 1989; Sapolsky et al., 1984). The mechanisms of corticosteroid receptor

downregulation by prolonged exposure to high CORT levels are not well understood, but is known to involve reduction of transcription rates of the receptor gene (Dong et al., 1988; Rosewicz et al., 1988) and shortened receptor half-life (McIntyre & Samuels, 1985).

Exposure to stress Regimen I also produced elevated basal plasma CORT levels without changes in plasma CBG levels, and increased relative adrenal weights in both males and females. The increased relative adrenal weights following chronic stress are consistent with other studies that have demonstrated adrenal gland hypertrophy, decreased body weights, and/or increased adrenal weights normalized to body weights following a variety of chronic stress regimens (Kitayama et al., 1989; Lowy, 1991; Sapolsky et al., 1984; Spencer & McEwen, 1990; Young et al., 1990). The fact that CBG binding capacity was not altered by chronic stress suggests that changes in CORT levels that were observed may be functionally important. Plasma levels of albumin were not measured in this study. However, since albumin accounts for only 10-15% of bound CORT (Berne & Levy, 1993), it is unlikely that possible changes in plasma albumin alone would have had a significant impact on free CORT levels. The observed increase in basal CORT levels and downregulation of corticosteroid receptors have also been reported following 3 weeks of chronic intermittent stress (Sapolsky et al., 1984). It has been suggested that this inverse relationship between receptor densities and basal CORT levels following prolonged CORT elevations reflect deficits in feedback regulation of the HPA axis (Sapolsky et al., 1985).

Second, it is noteworthy that seemingly small changes in the stress paradigm (in Stress Regimen II compared to Regimen I) produced marked changes in the measured stress effects. Stress Regimen II did not produce downregulation of hippocampal corticosteroid receptors nor increased basal CORT levels, and resulted in less robust increases in relative adrenal weights.

Despite the fact that the duration of most stressors was increased by 50% in Stress Regimen II, the change in housing conditions from suspended mesh cages to bedding lined polycarbonate cages appears to be a critical factor in attenuating the impact of the stress regimen on indices of HPA function. It is possible that the stress-induced increase in CORT levels was more sustained in animals returned to mesh cages compared to bedding lined polycarbonate cages.

The stress regimens used in the present study may be near the threshold for producing downregulation of corticosteroid receptors, with Regimen I just above the threshold and Regimen II just below the threshold for producing downregulation. Interestingly, under the conditions of Regimen II, there seems to be upregulation of hippocampal GRs in PF males and C females. Upregulation following chronic stress may be a form of physiological compensation. Receptors may be initially upregulated with stressful challenges in order to produce more efficient HPA negative feedback control. Then following more severe challenges, this compensatory mechanism may eventually break down and downregulation of receptors occurs. Interestingly, upregulation of MRs was previously observed in the hippocampus (Young et al., 1990; Spencer & McEwen, 1990), parietal cortex (Young et al., 1990) and basal forebrain plus septum (Spencer & McEwen, 1990) following exposure to electrically induced seizures (Young et al., 1990) and chronic ethanol administration (Spencer & McEwen, 1990) that was not severe enough to produce downregulation of GRs and MRs. This MR upregulation may be another example of compensatory adaptation to chronic stress since MRs are also thought to play a role in HPA feedback regulation (Bradbury et al., 1994; Ratka et al., 1989; Sapolsky et al., 1990). In this context, our data suggest that PF males and C females are better able to show this compensatory upregulation; significantly, neither E males or females displayed this upregulation.

Third, in contrast to the stress regimens, CORT pellet implants produced sustained high plasma CORT levels (equivalent to approximately 50-75% of the maximal stress response) that resulted in downregulation of hippocampal GRs and MRs, decreased plasma CBG levels, and decreased relative adrenal weights in both males and females. The downregulation of hippocampal GRs (Lowy, 1991; Reul et al., 1987a; Spencer et al., 1991) and MRs (Chao et al., 1989; Lowy, 1991; Spencer et al., 1991) following chronic exogenous CORT exposure is consistent with previous data, although not all studies have demonstrated this receptor downregulation (Chao et al., 1989; Reul et al., 1987a). The decrease in CBG levels following prolonged CORT exposure is consistent with data demonstrating that glucocorticoids decrease levels of circulating CBG by altering their rate of production and breakdown (Schlechte & Hamilton, 1987; Frairia et al., 1988). This decrease in CBG levels would result in increased circulating free CORT levels that would increase the impact of CORT on physiological responses. The decrease in relative adrenal weights resulted from adrenal gland atrophy due to the continual presence of exogenous CORT.

Fourth, upregulation of hippocampal GRs occurred 7 days following ADX compared to that at the usual 24 h. Upregulation is a measure of receptor plasticity that occurs in response to the removal of the CORT signal. The present data is in agreement with other studies that have demonstrated GR upregulation 3-10 days following ADX (Chao et al., 1989; Eldridge et al., 1989; Lowy, 1991; Luttge & Rupp, 1989; Luttge et al., 1989; Reul, et al., 1987a; 1987b; Spencer et al., 1991; Turner, 1986). It is interesting that statistically significant upregulation of GRs did not occur in the NoStress condition, but was apparent only in animals that had previously been exposed to chronic stress. It is possible that the initial downregulation of receptors resulting from chronic stress made the subsequent upregulation following ADX more

apparent. There was no upregulation of hippocampal MRs, which is consistent with most studies (Chao et al., 1989; Lowy, 1991; Reul, et al., 1987a; 1987b; 1989; Spencer et al., 1991; Turner, 1986). The studies that have demonstrated MR upregulation following ADX in the hippocampus (Luttge et al., 1989) or whole brain (Luttge & Rupp, 1989) did so in mice that had received gonadectomy in addition to ADX.

Finally, prenatal ethanol exposure did not produce changes on any measure of HPA activity in either males or females under basal or nonstressed conditions. There were no alterations in GR or MR densities at the hippocampus, prefrontal cortex, hypothalamus or anterior pituitary under nonstressed conditions. This supports and extends the data of Weinberg and Peterson (1991), who found no significant changes in hippocampal GRs and MRs under basal conditions. Furthermore, E rats did not differ from controls in basal plasma CORT levels, plasma CBG levels, or relative adrenal weights. These data are consistent with other studies that have demonstrated no alterations in basal HPA activity of E animals (Angelogianni & Gianoulakis, 1989, Lee et al., 1990; Nelson et al., 1986; Taylor et al., 1981; 1982; 1983; 1986; Weinberg, 1988; 1992a).

Following chronic intermittent stress, prenatal ethanol exposure did not differentially affect basal plasma CORT or CBG levels. However, E females displayed increased relative adrenal weights, a measure of HPA activity, in response to both stress regimens compared to controls. This finding is consistent with previous data demonstrating HPA hyperresponsiveness in E animals following stressors but not in the nonstressed state (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1985; 1986; Ogilvie & Rivier, 1996; Taylor et al., 1982; 1983; 1986; Weinberg, 1988; 1992a, Weinberg et al., 1995; 1996). Furthermore, prenatal ethanol exposure did not differentially downregulate hippocampal GRs in response to Stress

Regimen I, differentially upregulate hippocampal GRs following ADX, or alter MRs in response to Stress Regimen I or ADX. Moreover, there were no significant effects of prenatal ethanol exposure on GR or MR densities in the prefrontal cortex, hypothalamus and anterior pituitary, or MR densities in the hippocampus, of both males and females following Stress Regimen II. However, exposure to Stress Regimen II revealed subtle differential effects of chronic intermittent stress on hippocampal GRs among E, PF and C males and females. Given the nature and magnitude of these changes in receptor densities, it would appear that these changes do not play a role in mediating the feedback deficits and resultant HPA hyperresponsiveness of E animals.

Interestingly, Redei et al. (1993) reported that male rats exposed to ethanol *in utero* showed elevations in GR mRNA in the hypothalamus but not the anterior pituitary, while females displayed no changes in GR mRNA levels at either site. However, following 5 weeks of chronic ethanol administration, E males no longer displayed increases in GR mRNA at the hypothalamus but did show increases at the anterior pituitary, whereas E females showed decreases at the anterior pituitary. In the context of the present finding that GR protein levels were not increased, the functional significance of these changes in GR mRNA signal remain to be determined. It is important to note that although changes in GR protein and its mRNA can occur together (Burnstein et al., 1990; Dong et al., 1988), changes do not always occur in parallel (Chao et al., 1989). Indeed, mRNA levels, determined as a parameter of biosynthesis, do not always reflect protein levels, since factors such as posttranslational processing can modify changes in gene expression (Owens & Nemeroff, 1991; Rondeel & Jackson, 1993).

Several sex differences were observed in the present study. Females displayed higher plasma CORT and CBG levels and higher relative adrenal weights compared to males. This

confirms the sexual dimorphism of the HPA axis that has previously been observed. Typically, females display heavier adrenal glands, higher ACTH, CORT and CBG levels, and higher diurnal variation in CORT levels (Chisari et al., 1995; Critchlow et al., 1963; Kitay, 1961; 1963). These sex differences are due to prenatal/postnatal exposure to sex hormones during critical developmental periods, and to circulating levels of sex hormones as adults. Estrogen seems to be responsible for the increased HPA activity in females (Kitay, 1961; 1963; Ramaley, 1976), progesterone appears to inhibit many of the facilitory effects of estrogen in females (Viau & Meaney, 1991), and testosterone has been shown to decrease HPA activity in males (Kitay, 1963). Furthermore, in the present study males displayed higher corticosteroid receptor densities than females. Previous studies have shown either higher receptor densities in males, higher densities in females or no differences between the sexes (Ferrini et al., 1991; Turner, 1992; Turner & Weaver, 1985; Weinberg & Peterson, 1991). The present study is in agreement with the suggestion that estrogen decreases corticosteroid receptor densities (Turner, 1990). Moreover, E females but not E males displayed differential increases in relative adrenal weights following chronic intermittent stress compared to controls. Previous studies have similarly shown that prenatal ethanol exposure differentially affects HPA function in males and females (Halasz et al., 1993; Osborn et al., 1996; Redei et al., 1993; Weinberg, 1988; 1992a; Weinberg et al., 1996). Although E males and females may both exhibit increased HPA responsiveness, it appears that the nature and intensity of the stressor, the time course, and the hormonal endpoint measured may influence the outcome observed and may reveal differential effects in males and females (Weinberg et al., 1996).

In summary, prenatal ethanol exposure has been shown to produce robust HPA hyperresponsiveness to stressors, which is mediated in part by deficits in feedback inhibition of

the HPA axis. The results of the present study support and extend previous data demonstrating HPA hyperresponsiveness in E animals. However, the data suggest that decreased corticosteroid receptor densities, at least as measured by the total cytosolic binding assay, do not underlie the deficits in feedback inhibition and resultant HPA hyperresponsiveness following prenatal ethanol exposure.

Figure 11. Hippocampal GR and MR densities for the NoStress, Stress and Implant conditions for both males and females in Experiment 1 (n = 11-15 per condition). For males, NoStress rats displayed significantly higher densities compared to Stress and Implant rats (p's<0.05; *GR and +MR), and the latter did not differ from each other. For females, the NoStress rats displayed significantly higher densities compared to Implant rats (p's<0.05; ^GR and #MR), and Stress rats displayed intermediate levels that did not differ from the other conditions.

Hippocampus

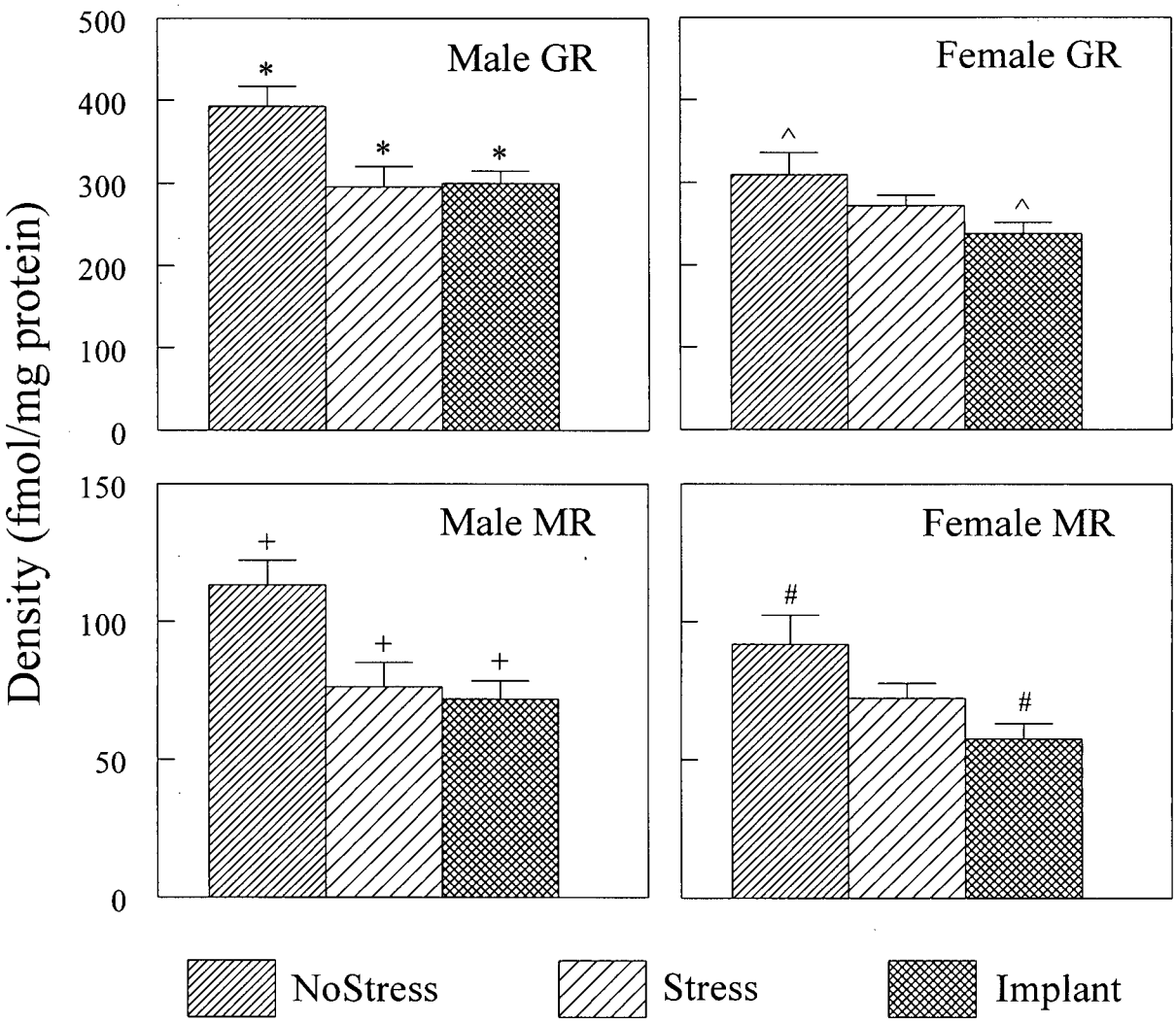


Table 5

Basal Plasma CORT and CBG Binding Capacity (Mean ± SEM, μg/100 ml) in the Stress and Implant Conditions for Males and Females in Experiment 1

Male	Stress		Implant		
	Pre-stress	**Post-stress**	**Pre-implant**	**Day 10**	**Day 19**
CORT	1.2 ± 0.1*	2.0 ± 0.3*	1.1 ± 0.1^	15.9 ± 0.5^	12.0 ± 1.0^
CBG	30.5 ± 1.2	29.7 ± 1.8	30.2 ± 1.7+	18.2 ± 2.9+	21.7 ± 2.2+

Female	Stress		Implant		
	Pre-stress	**Post-stress**	**Pre-implant**	**Day 10**	**Day 19**
CORT	3.7 ± 0.7#	7.2 ± 1.6#	2.5 ± 0.7♦	31.3 ± 2.2♦	25.0 ± 1.7♦
CBG	83.3 ± 3.0	76.8 ± 3.8	72.7 ± 6.3¶	36.9 ± 3.2¶	31.8 ± 3.5¶

n = 10-16 per condition for males and females

Male CORT: *Pre-stress < Post-stress, $p<0.05$

^Pre-implant < Day 19 < Day 10, p's<0.05

Male CBG: +Pre-implant > Day 10 = Day 19, p's<0.05

Female CORT: #Pre-stress < Post-stress, $p<0.075$

♦Pre-implant < Day 19 < Day 10, p's<0.05

Female CBG: ¶Pre-implant > Day 10 = Day 19, p's<0.05

Table 6

Adrenal:Body Weight Ratios ($x10^5$, Mean ± SEM) of NoStress, Stress and Implant Males and Females in Experiment 1

	NoStress	Stress	Implant
Males	10.5 ± 0.6*	12.9 ± 0.4*	3.8 ± 0.2*
Females	22.5 ± 0.8^	25.6 ± 1.0^	10.4 ± 0.5^

n = 10-14 per condition for males and females

Males: *Stress > NoStress > Implant, p's<0.05

Females: ^Stress > NoStress > Implant, p's<0.05

Table 7

Maternal Body Weights (g, Mean ± SEM) of E, PF and C Dams During Gestation (G) and Lactation (D) for Experiment 2

Gestation	G1	G7	G14	G21
E	275.1 ± 3.2	275.2 ± 3.3*	295.9 ± 3.9*	350.9 ± 7.3*
PF	269.6 ± 2.9	266.2 ± 2.9*	285.7 ± 3.2*	349.7 ± 4.6*
C	265.0 ± 3.2	292.0 ± 4.6*	322.2 ± 5.0*	403.8 ± 3.0*

Lactation	D1	D8	D15	D22
E	298.2 ± 8.5	334.8 ± 5.2	356.8 ± 6.0	334.2 ± 5.0
PF	299.2 ± 4.2	334.7 ± 5.6	356.8 ± 4.5	334.7 ± 4.2
C	307.3 ± 4.4	332.9 ± 5.2	350.6 ± 4.6	329.8 ± 4.7

n = 14-16 per group during gestation and lactation

Gestation: *At G7, G14 & G21, E = PF < C, p's<0.05

Table 8

Body Weights (g, Mean ± SEM) of E, PF and C Pups (Male and Female Data Collapsed) From Birth to Weaning for Experiment 2

	D1	D8	D15	D22
E	5.8 ± 0.1^	16.3 ± 0.3^	31.3 ± 0.5*	49.5 ± 0.8*
PF	6.0 ± 0.1	16.7 ± 0.3	33.4 ± 0.4*	52.7 ± 0.6*
C	6.2 ± 0.1^	17.2 ± 0.2^	33.5 ± 0.3*	52.7 ± 0.6*

n = 14-16 litters per group

^At D1 & D8, E < C, p's<0.05

*At D15 & D22, E < PF = C, p's<0.05

Figure 12. Hippocampal GR and MR densities of E, PF and C females for the NoStress versus Stress conditions and 1 Day versus 7 Day post-ADX conditions in Experiment 2 (n = 6-10 per condition). For GRs, in the 1 Day condition, GR densities were significantly higher in the NoStress than in the Stress condition ($p<0.05$) indicating receptor downregulation following stress. GR densities were upregulated in the 7 Day compared to the 1 Day condition, but this difference reached statistical significance only following Stress ($p<0.05$). There were no significant changes in MR densities, and no significant differences among E, PF and C rats in GRs and MRs under any of the experimental conditions.

Hippocampus - Female

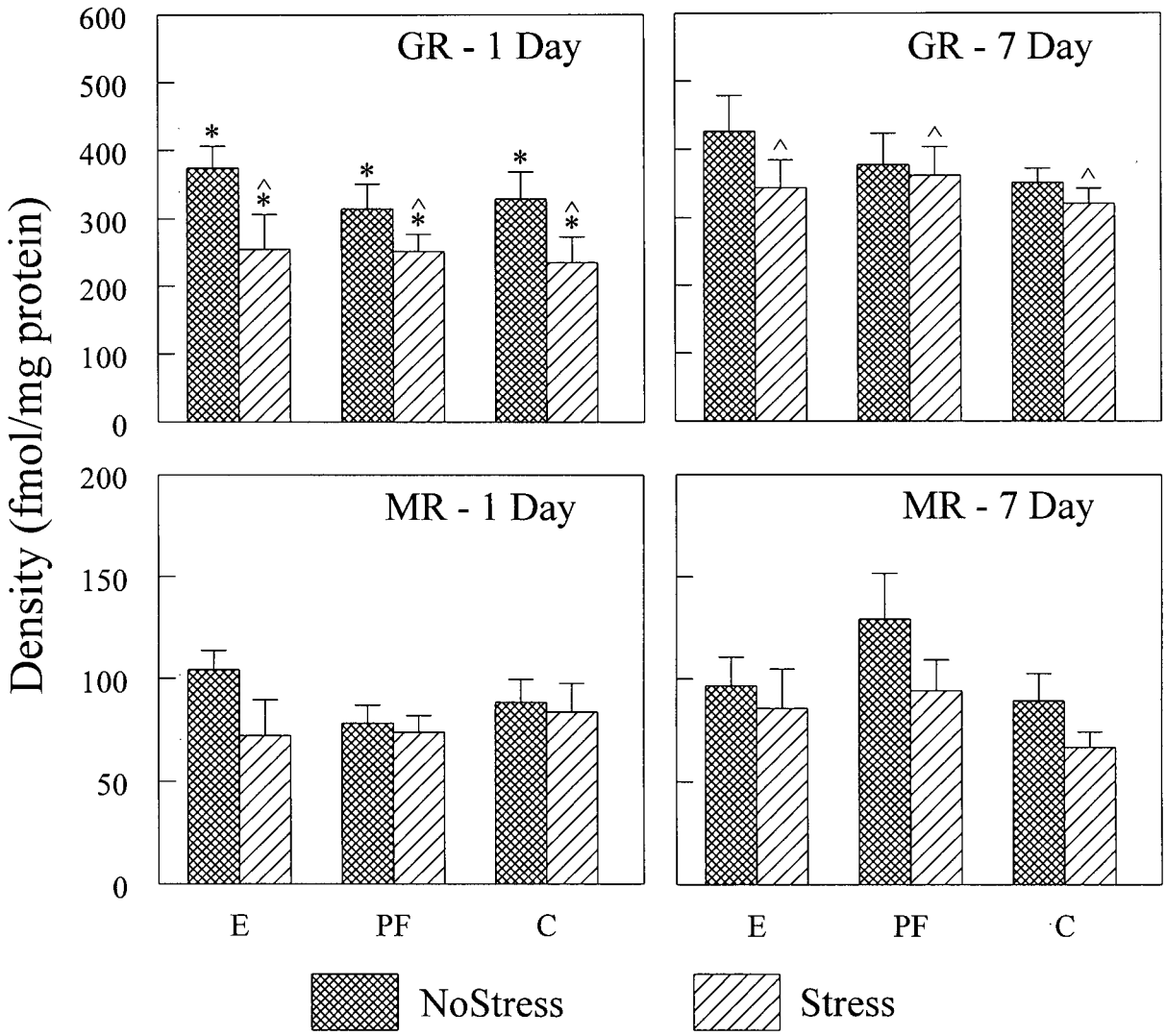


Table 9

Basal Plasma CORT and CBG Levels (μg/100 ml, mean ± SEM) in E, PF and C Females in the Stress Condition in Experiment 2

CORT	E	PF	C
Pre-stress	2.1 ± 0.8	2.2 ± 0.7	2.5 ± 0.5
Post-stress	4.9 ± 2.1	3.1 ± 1.4	6.5 ± 1.4

CBG	E	PF	C
Pre-stress	85.0 ± 3.0	71.4 ± 5.6	75.4 ± 4.8
Post-stress	79.9 ± 3.4	74.1 ± 4.9	79.7 ± 5.1

n = 5-8 per condition for CORT and CBG

CORT: Pre-stress < Post-stress, $p<0.02$

Table 10

Adrenal:Body Weight Ratios ($x10^5$, Mean ± SEM) of E, PF and C Females for the NoStress and Stress Conditions in Experiment 2

	E	PF	C
NoStress	24.6 ± 0.6*	21.8 ± 0.8*	22.3 ± 0.6*
Stress	31.6 ± 1.3*^	26.5 ± 1.2*^	27.4 ± 1.0*^

n = 15-22 per condition.

*For E, PF & C, NoStress < Stress, p's<0.05

^For Stress, E > PF = C, p's<0.05

Table 11

Maternal Body Weights (g, Mean ± SEM) of E, PF and C Dams During Gestation (G) and Lactation (L) for Experiment 3

Gestation	G1	G7	G14	G21
E	275.1 ± 3.2	268.1 ± 2.7*	289.2 ± 4.1*	350.9 ± 7.3*
PF	269.6 ± 2.9	266.0 ± 3.7*	287.0 ± 4.8*	349.7 ± 4.6*
C	265.0 ± 3.2	296.0 ± 3.0*	323.8 ± 4.8*	403.8 ± 3.0*

Lactation	D1	D8	D15	D22
E	298.2 ± 8.5+	335.2 ± 4.9	356.9 ± 4.5	334.2 ± 5.0
PF	299.2 ± 4.2+	330.7 ± 5.2	357.7 ± 5.8	334.7 ± 4.2
C	307.3 ± 4.4+	341.4 ± 5.6	358.7 ± 5.2	329.8 ± 4.7

n = 15-16 per group during gestation and lactation

Gestation: *At G7, G14 & G21, E = PF < C, p's<0.05

Lactation: +At D1, E = PF < C, p's<0.05

Table 12

Body Weights (g, Mean ± SEM) of E, PF and C Pups (Male and Female Data Collapsed) From Birth to Weaning for Experiment 3

	D1	D8	D15	D22
E	5.4 ± 0.1*	14.4 ± 0.3*	30.0 ± 0.4*	45.9 ± 0.6*
PF	5.6 ± 0.1*	14.1 ± 0.5*	30.1 ± 0.7*	47.3 ± 0.9*
C	6.5 ± 0.1*	17.3 ± 0.3*	33.5 ± 0.5*	53.5 ± 0.8*

n = 14-16 litters per group

*At D1, D8, D15 & D22, E = PF < C, p's<0.05

Figure 13. Hippocampal GR and MR densities of E, PF and C rats in the NoStress and Stress conditions for males and females in Experiment 3 (n = 7-11 per condition). In the NoStress condition, there were no significant differences among E, PF and C rats in GR and MR densities for both males and females. However, E males in the Stress condition had lower GR densities than PF males (*$p<0.05$), and C males did not differ from either group. Furthermore, C but not E and PF females showed higher GR densities following stress (^$p<0.05$). There were no significant changes in MR densities under any conditions for males and females.

Hippocampus

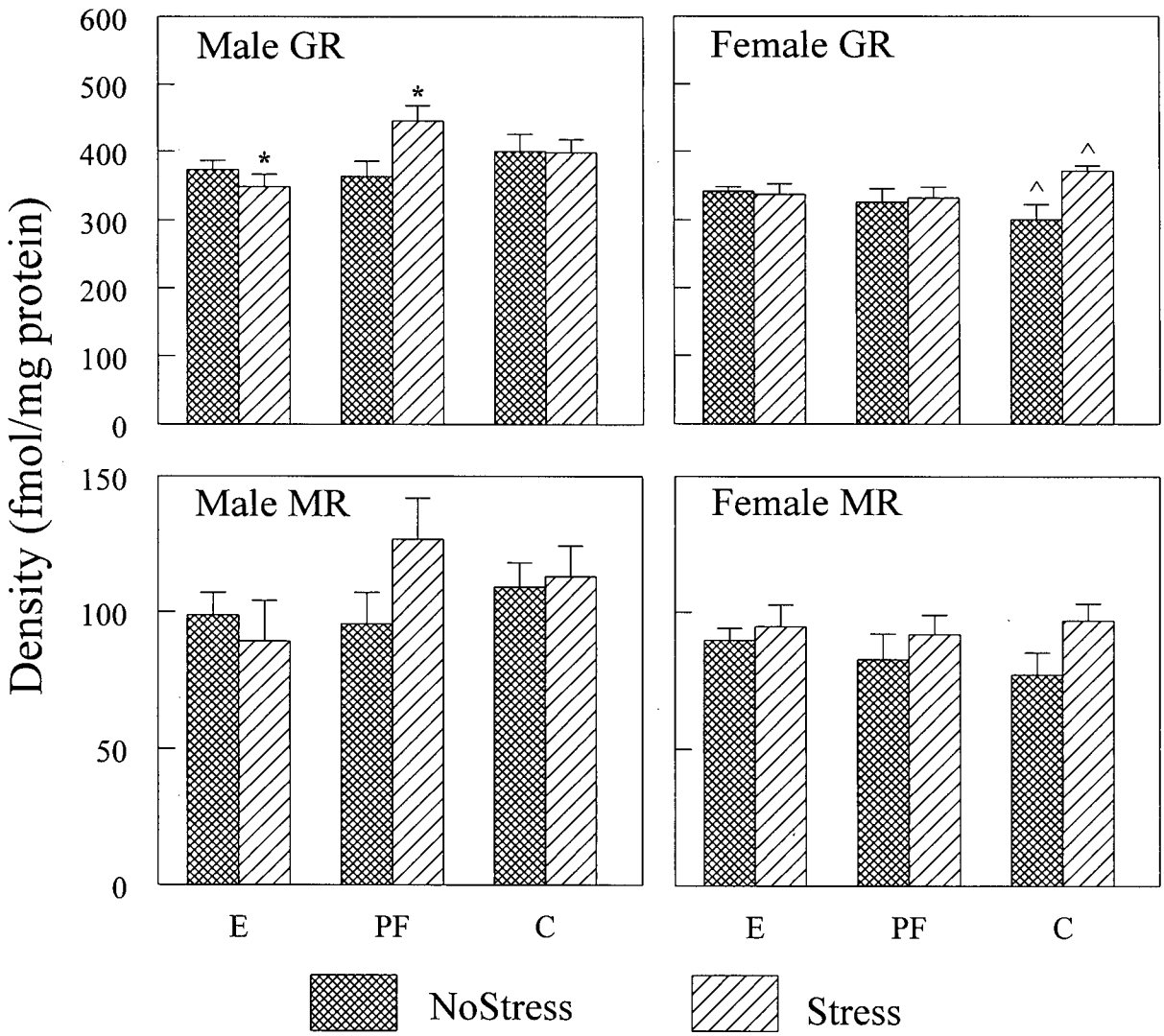


Figure 14. Prefrontal cortical GR and MR densities of E, PF and C rats in the NoStress and Stress conditions for males and females in Experiment 3 (n = 8-12 per condition). There were no significant changes in GR or MR densities under any conditions for males and females.

Prefrontal Cortex

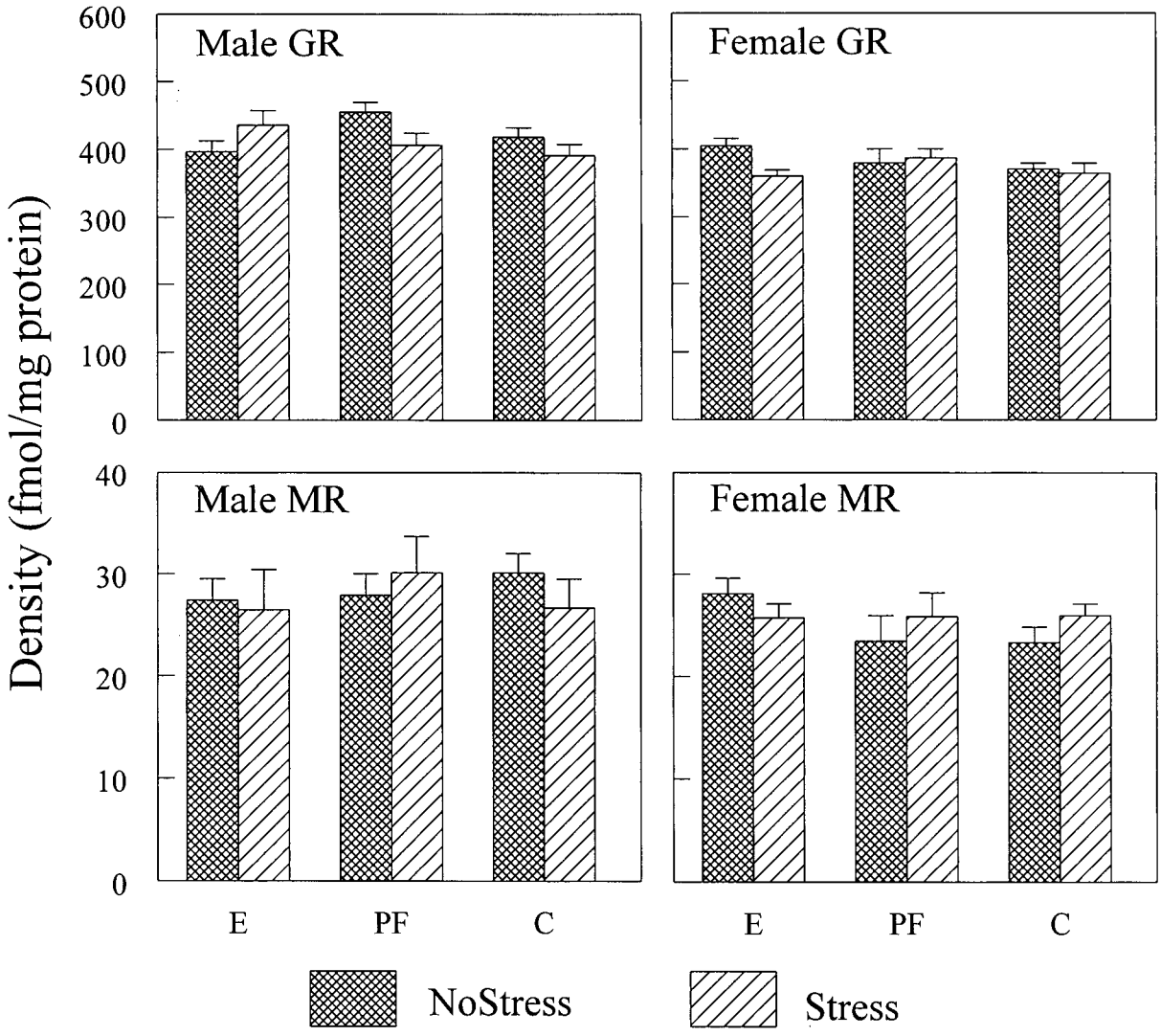


Figure 15. Hypothalamic GR and MR densities of E, PF and C rats in the NoStress and Stress conditions for males and females in Experiment 3 (n = 6-11 per condition). There were no significant changes in GR or MR densities under any conditions for males and females.

Hypothalamus

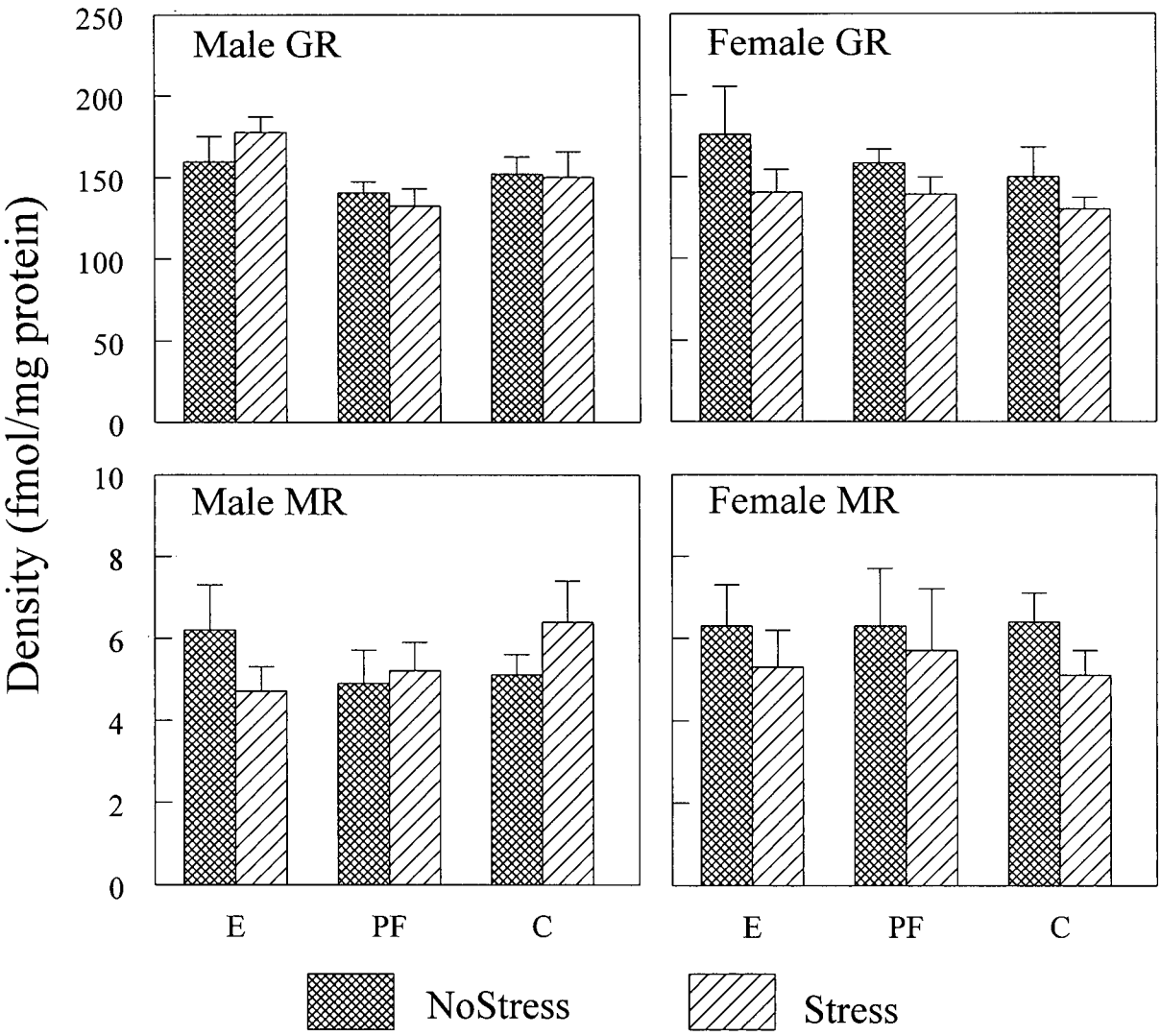


Figure 16. Anterior pituitary GR and MR densities of E, PF and C rats in the NoStress and Stress conditions for males and females in Experiment 3 (n = 7-12 per condition). There were no significant changes in GR or MR densities under any conditions for males and females.

Anterior Pituitary

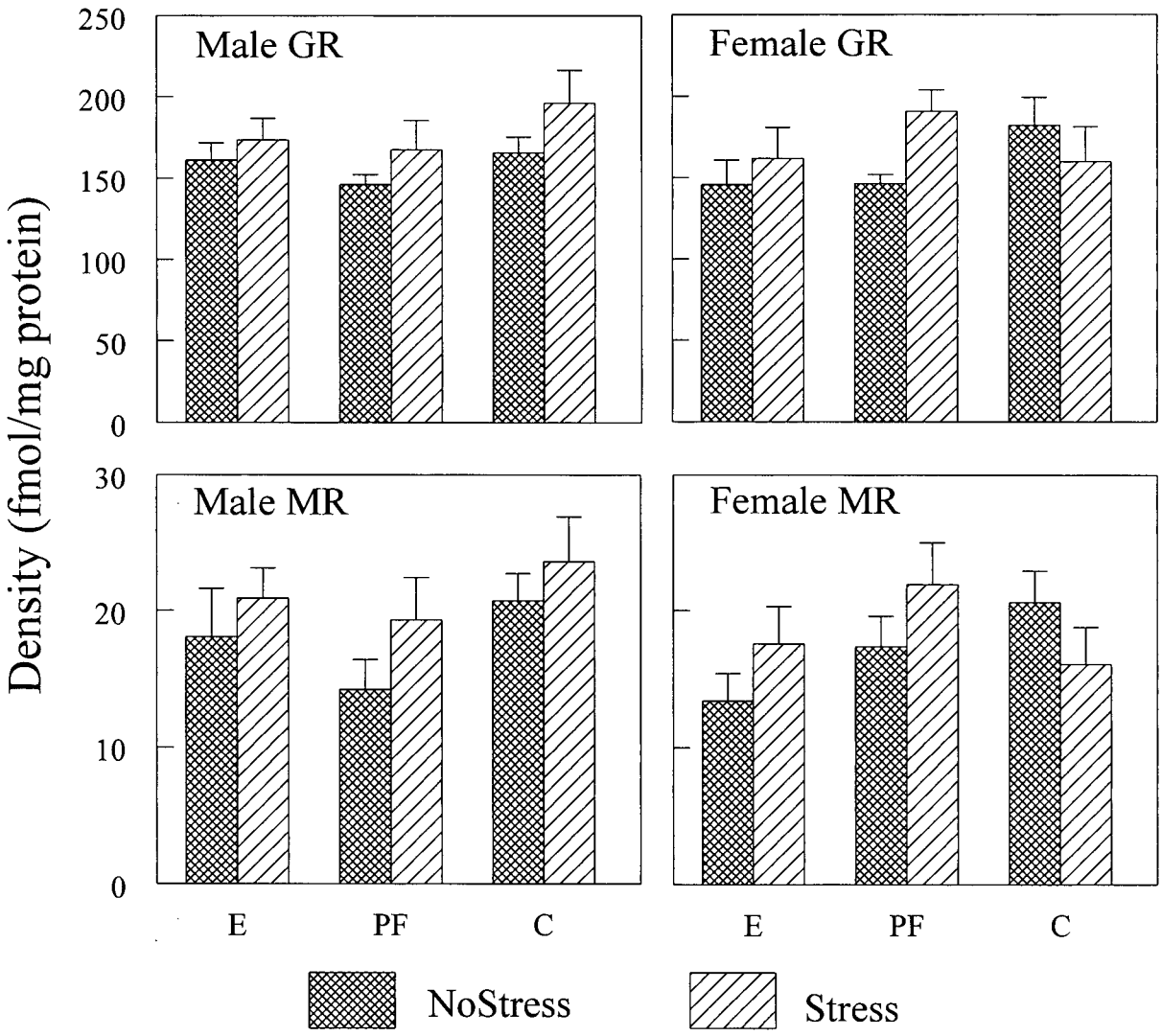


Table 13

Basal Plasma CORT Levels (μg/100 ml, Mean ± SEM) in E, PF and C Groups for the NoStress and Stress (Pre-stress and Post-stress) Conditions for Both Males and Females in Experiment 3

Male	**E**	**PF**	**C**
NoStress	1.3 ± 0.2	0.9 ± 0.5	1.1 ± 0.1
Pre-stress	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1
Post-stress	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.2

Female	**E**	**PF**	**C**
NoStress	3.2 ± 0.7	2.2 ± 0.6	3.1 ± 1.1
Pre-stress	2.8 ± 0.8	2.0 ± 0.4	2.7 ± 0.9
Post-stress	5.5 ± 2.9	3.6 ± 1.5	5.1 ± 2.0

n = 5-11 per condition for males and females

Table 14

Adrenal:Body Weight Ratios ($x10^5$, Mean ± SEM) in E, PF and C Males and Females for the NoStress and Stress Conditions in Experiment 3

Male	**E**	**PF**	**C**
NoStress	4.0 ± 0.3*	3.9 ± 0.4*	4.0 ± 0.2*
Stress	4.9 ± 0.3*	5.2 ± 0.3*	5.3 ± 0.2*

Female	**E**	**PF**	**C**
NoStress	11.2 ± 0.6^	10.4 ± 0.4	11.1 ± 0.7
Stress	13.7 ± 0.7^	11.9 ± 0.4	12.8 ± 0.7

n = 7-12 per condition for males and females

Male: *At E, PF & C, NoStress < Stress, $p<0.05$

Female: ^At E, NoStress < Stress, $p<0.05$

CHAPTER V: EFFECTS OF PRENATAL ETHANOL EXPOSURE ON HPA RESPONSE TO CHRONIC COLD STRESS

Introduction

Prenatal ethanol exposure produces HPA hyperresponsiveness to a wide variety of psychological and physical stressors. Greater increases in circulating ACTH and CORT levels have been demonstrated to acute stressors such as footshock, noise and shake, cardiac puncture, restraint, ether and cold in E compared to control animals (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1992a; Weinberg et al., 1995; 1996). A limited number of studies have utilized repeated episodes of restraint stress over several days, and have found similar HPA hyperresponsiveness (Weinberg, 1992a; Weinberg et al., 1996).

The present study investigated the effects of prenatal ethanol exposure on HPA responses to chronic cold stress. Cold stress typically refers to exposure to temperatures ranging from 4-6^{o} C. "Acute" stress generally refers to periods of cold exposure ranging from 5 min to several hours and "chronic" refers to periods of at least 24 h. Chronic cold stress is qualitatively different from other stressors previously tested following prenatal ethanol exposure in that it is a continuous stressor and has both psychological and physical (metabolic) components. Cold stress is not only a potent activator of the HPA axis, producing elevations in plasma levels of CORT and ACTH, as well as increases in corticotropin releasing factor (CRF) and vasopressin (VP) mRNA levels in the parvocellular nucleus (PVN) of the hypothalamus (Angulo et al., 1991; Donnerer & Lembeck, 1990; Gibbs, 1984; Hauger & Aguilera, 1992; Jobin et al., 1975; 1976; Lembeck & Amann, 1986; Sasaki et al., 1990; Wu & Childs, 1990; 1991), but also

activates the hypothalamic-pituitary-thyroid (HPT) axis (ArÑncibia et al., 1996). The thyroid hormones are critical for normal growth and development of the fetus and young organism, as well as the regulation of the body's metabolic rate (Aräncibia et al., 1996; Berne & Levy, 1993). In response to cold stress, thyrotropin-releasing hormone (TRH) containing neurons of the PVN of the hypothalamus are stimulated, producing elevations in TRH mRNA, and subsequent elevation in TRH, thyrotropin (TSH), thyroxine (T4) and triiodothyronine (T3) levels (Zoeller & Rudeen, 1992; Zoeller et al., 1990).

During chronic cold exposure, the initial activation of the HPA axis is often followed by what appears to be adaptation. That is, elevated levels of plasma ACTH and/or CORT return to basal levels despite the continuous exposure to the cold stress (Bligh-Tynan et al., 1993; Daniels-Severs et al., 1973; Fakuhara et al., 1996; Sakellaris & Vernikos-Danellis, 1975; Uehara et al., 1989). However, although normalization of basal hormone levels may occur, when a novel acute stressor is superimposed during the period of chronic cold stress, sensitization of the response to the acute stressor may occur. That is, ACTH and/or CORT responses to the acute stressor appear to be increased compared to that in animals not exposed to cold stress (Daniels-Severs et al., 1973; Sakellaris & Vernikos-Danellis, 1975; Vernikos et al., 1982).

The present study is the first to document the effects of chronic cold stress on the HPA axis in animals prenatally exposed to ethanol. Multiple indices of HPA activity were measured following 0, 1 or 3 days of cold stress, including plasma ACTH, CORT and CBG levels as well as CRF and VP mRNA levels in the PVN. PVN TRH mRNA levels were also measured as one index of HPT activity. In addition, ACTH and CORT responses were measured following exposure to a novel acute stressor (isotonic saline injection, IP) superimposed during chronic cold exposure, to determine whether sensitization of the acute stress response would be

increased in fetal ethanol exposed animals compared to controls. It was hypothesized that prenatal ethanol exposure would produce HPA hyperresponsiveness to cold stress, reflected in increased plasma hormone responses, increased sensitization to acute stress, and increased peptide mRNA levels in the PVN.

Methods

Experimental Procedure

Subjects were adult male and female rats from E, PF and C groups. The warm (21 °C) and cold (4 °C) rooms were maintained on the same light:dark cycle (lights on 0700-1900 h). Three days prior to sampling at 0930-1030 h, each rat was weighed and singly housed into opaque polycarbonate cages (20 x 27 x 15 cm) with free access to rat chow and water. Rats were assigned randomly to one of three groups: 0 day rats were placed into the warm room for 3 days; 1 day rats were placed in the warm room for 2 days then moved to the cold room for 1 day; and 3 day rats were placed into the cold room for 3 days. All rats were moved between warm and cold rooms at 0930-1030 h. All rats were weighed again at 1830 h the evening before sampling.

Testing occurred at 0830-1030 h. Half the rats in each experimental condition remained undisturbed in their home cages until termination (0 min), and half were subjected to a novel acute stressor of IP isotonic (0.9%) saline injection (0.5 ml for males, 0.3 ml for females) 15 min before decapitation. Following injection, animals were returned to their home cages, water bottles were removed, and cages were placed back into their respective rooms until decapitation. Sampling occurred 15 min following IP injection in order to capture the maximal or near maximal stress response levels of both ACTH and CORT (Weinberg et al., 1996). Trunk blood was collected for ACTH, CORT and CBG determination. CBG was analyzed only for the 0 min

group, since no change in its levels was expected between 0 and 15 min (Sandberg & Slaunwhite, 1959). At termination, the right adrenal gland was removed and weighed. Brains from 0 min rats were prepared (described below) for measurement of mRNAs for CRF, VP and TRH via *in situ* hybridization; again, no change in mRNA levels was expected between 0 and 15 min.

***In situ* Hybridization**

Brain preparation. Following decapitation, brains were quickly removed and frozen in powdered dry ice, then wrapped in parafilm and aluminum foil, and stored at -80 °C. Brains were transferred to a cryostat 1 h before slicing and warmed to -18 °C. Coronal sections (12 μm thick) were taken through the PVN of the hypothalamus (-1.4 from bregma; coordinates from Paxinos & Watson, 1986); this site was identified by staining preceeding sections with 5% toludine blue. Two brain sections were mounted onto each slide, that had been twice-coated with gelatin; 12 adjacent slides were taken from each rat. The slides were stored in a sealed container at -80 °C until processing.

Probes. Oligonucleotide sequences for CRF, VP and TRH are given in Table 15. They were synthesized using an Applied Biosystems model 380A DNA synthesizer and purified by electroporesis on an 8% polyacrylamide 8 M urea TBE gel, followed by electrophoresis into 1% LMP agarose, and then phenol extracted.

Probes were labelled at the 3'-end by incubating 50 units of terminal oligodeoxynucleotidyl transferase (Boehringer-Mannheim, Indianapolis, IN) with 5 pmol oligomer and 50 pmol [^{35}S]-dATP (Dupont) in the presence of 200 mM potassium cacodylate, 25 mM-Tris HCl, 0.25 mg/ml bovine serum albumin, and 1.5 mM $CoCl_2$ (pH 6.6) for 15 min at 37 °C. This was followed by phenol-chloroform extraction and ethanol precipitation.

Hybridization. This method was according to Zoeller et al. (1990; 1992). Sections were warmed to room temperature, and prehybridization treatments were as follows: 4% formaldehyde/phosphate buffered saline (PBS) for 5 min, PBS rinse, 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% NaCl (pH 8.0) for 10 min, 2x 300 mM NaCl/30 mM sodium citrate (SSC) rinse, dehydration by a graded series of ethanol baths, delipidation in chloroform, rehydration in 95% ethanol, and then air dried.

Hybridization buffer (50 μl) was applied to each slide, slides were covered with parafilm cover slips, and incubated at 37 °C for 20 h in a humid chamber. The hybridization buffer contained 50% formamide, 4x SSC, transfer RNA (250 μg/ml), sheared single-stranded salmon sperm DNA (100 μg/ml), 1x Denhardt's soluton (0.02% each of BSA, Fiscoll, and polyvinylpyrrolidone), 10% (w/v) dextran sulfate (MW 500,000), 100 mM DTT and the appropriate probe.

Posthybridization treatments were as follows: coverslips were floated off in 1x SSC, 2x SSC wash twice, 2x SSC/50% formamide for 15 min at 55 °C four times, 1x SSC for 30 min twice, deionized water rinse, 70% ethanol, and then air dried.

Autoradiography and signal quantitation. Four adjacent sections (two slides) were used to measure CRF and VP mRNAs, and two sections (one slide) to measure TRH mRNA. Slides were held against film (Kodak Biomax MR; Eastman Kodak Co., Rochester, NY) with ^{14}C-standards (American Radiolabelled Chemical Inc., St. Louis, MO) in sealed cassettes for 10 days for CRF, 5 h for VP, and 5.5 days for TRH. ^{14}C-standards were used because it closely approximates the β energy emission of ^{35}S, but with a substantially longer half-life. Microdensitometry was performed on the signal over the PVN using a BioQuant MEG System IV interfaced with a Dage-MTI 70 series video camera equipped with a 2x Nikon objective

mounted onto a bellows system over a light box. Signal density was used as an index of mRNA levels and measured with the NIH Image program (version 1.58). The standards were used to ensure that films were not saturated.

The hybridization signal was evaluated for each probe as follows: average grey-level was measured for signal and background to provide a numerical value of average density over the specified region, background density was subtracted from signal density, and then averaged over the four (CRF and VP) or two (TRH) brain sections measured for each animal. This value was specified as optical density (OD). Integrated Optical Density (IOD) was obtained by multiplying the signal density by the area of the signal; this measure took into account the area of the signal. Both OD and IOD were expressed in arbitrary units.

Results

Developmental Data

Ethanol Intake. Ethanol intake by pregnant female E rats (n = 21) was consistently high throughout gestation, averaging 8.4 $\pm$ 0.3, 11.3 $\pm$ 0.3 and 11.6 $\pm$ 0.3 g/kg body weight/day, respectively, for the 3 weeks of gestation. The overall mean for the 3 weeks was 10.5 $\pm$ 0.2 g/kg body weight/day. This laboratory has shown that intake at these levels results in maximal or near maximal BALs of 145-155 mg/dl (Weinberg, 1985; Osborn et al., 1996).

Maternal Weights. Analysis of maternal body weights during gestation (Table 16) indicated significant effects of group [$F(2,43) = 29.6$, $p<0.001$] and day [$F(3,129) = 1376.6$, $p<0.001$], and a group x day interaction [$F(6,129) = 38.4$, $p<0.001$]. Post hoc tests revealed that all dams displayed the expected increase in body weights as gestation progressed (p's<0.05). On

G7, G14 and G21, E and PF dams weighed less than C dams; and on G14, PF dams also weighed less than E dams (p's<0.05).

Analysis of maternal body weights during lactation (Table 16) indicated a significant effect of day [$F(3,123) = 204.0$, $p<0.001$] and a group x day interaction [$F(6,123) = 2.4$, $p<0.033$]. Post hoc tests revealed that weights of all dams increased and then stabilized as lactation progressed (p's<0.05); and PF dams weighed less than C dams on D8 ($p<0.05$).

Pup Data. There were no significant differences among E, PF and C rats in the number of live or stillborn offspring. Analysis of pup weights (male and female data collapsed) from birth to weaning (Table 17) revealed significant effects of group [$F(2,40) = 8.1$, $p<0.001$] and day [$F(3,120) = 4189.3$, $p<0.001$], and a group x day interaction [$F(6,120) = 8.0$, $p<0.001$]. Post hoc tests revealed that all pup weights progressively increased with age (p's<0.05). On D1, D15 and D22, weights of E pups were less than those of C pups (p's<0.05); and on D15 and D22 weights of PF pups were also lower than those of C pups (p's<0.05).

Plasma CORT

Males (Figure 17) displayed significant main effects of cold stress [$F(2,108) = 11.8$, $p<0.001$] and acute stress [$F(1,108) = 128.4$, $p<0.001$], and a cold stress x acute stress interaction [$F(2,108) = 5.2$, $p<0.007$] in CORT responses. CORT levels in animals exposed to cold stress alone (0 min condition) were elevated at 1 and 3 d compared to 0 d in E, but not PF and C males (p's<0.05). IP injection increased CORT levels in E, PF and C males on all test days (0 min < 15 min; p's<0.05). Furthermore, sensitization of the CORT response to IP injection was observed in males from all prenatal treatment groups; CORT levels following this acute stressor (15 min condition) were higher at 1 and 3 d compared to 0 d (p's<0.05).

Females (Figure 18) displayed significant main effects of cold stress [$F(2,106) = 6.4$, $p<0.002$] and acute stress [$F(1,106) = 177.4$, $p<0.001$] in CORT responses. CORT levels in animals exposed to cold stress alone (0 min) were elevated at 1 and 3 d compared to 0 d in females from all prenatal treatment groups (p's<0.05). IP injection increased CORT levels in females from all prenatal treatment groups on all test days (0 min < 15 min; p's<0.05). Sensitization of the CORT response to this acute stressor was not observed, and there were no significant differences among E, PF and C females.

Females displayed higher CORT levels than males [$F(1,214) = 190.3$, $p<0.001$].

Plasma CBG

There were no significant effects of prenatal treatment or chronic cold stress on plasma CBG levels for either males or females (Table 18). However, females displayed higher CBG levels than males [$F(1,112) = 835.9$, $p<0.001$].

Plasma ACTH

Males (Figure 19) displayed significant main effects of group [$F(2,104) = 5.8$, $p<0.004$] and acute stress [$F(1,104) = 7.9$, $p<0.006$] in ACTH responses. With cold exposure alone (0 min), there were no significant changes in ACTH levels over days. However, ACTH levels, averaged across days, were higher in E compared to PF and C males (p's<0.05). IP injection produced a significant increase in ACTH levels (0 min < 15 min, $p<0.05$), but this increase did not occur in all animals on all test days. In addition, ACTH levels, averaged across days, were higher in E compared to C males at 15 min after acute stress ($p<0.05$); PF males did not differ from either group.

Females (Figure 20) displayed significant main effects of group [$F(2,103) = 6.2$, $p<0.003$] and acute stress [$F(1,103) = 8.4$, $p<0.005$], as well as group x cold stress [$F(4,103) = 2.5$,

$p<0.045$] and cold stress x acute stress [$F(2,103) = 3.8$, $p<0.025$] interactions in ACTH responses. ACTH levels following cold stress alone (0 min) were increased in females at 1 d compared to 0 d cold stress ($p<0.05$) but were no longer elevated by 3 d. Furthermore, ACTH levels averaged across days were higher in E compared to PF and C females (p's<0.05). Similar to the data from males, IP injection produced a signficant increase in ACTH levels (0 min < 15 min, $p<0.05$), but this increase did not occur in all animals on all test days. There were no significant differences among E, PF and C females in stress (15 min) ACTH levels.

There were no significant difference in ACTH levels between males and females.

CRF, VP and TRH mRNA

OD and IOD measures produced similar results for all mRNA data; only the OD measures are presented. Levels of mRNA for CRF (Figure 21) and VP (Figure 22) in the PVN of the hypothalamus were not significantly affected by prenatal treatment or chronic cold stress for either males or females. However, males displayed significantly higher mRNA levels of both CRF [$F(1,111) = 4.9$, $p<0.028$] and VP [$F(1,115) = 5.7$, $p<0.019$] than females.

TRH mRNA levels in the PVN of the hypothalamus (Figure 23) were significantly affected by chronic cold stress in both males [$F(2,58) = 16.2$, $p<0.001$] and females [$F(2,54) = 13.4$, $p<0.001$] across all prenatal treatment groups. Males displayed an increase in TRH mRNA levels at 3 d compared to 0 and 1 d cold stress (p's<0.05). Similarly, females displayed an increase in TRH mRNA levels at 3 d compared to 0 d (p's<0.05). There was no significant difference between males and females in TRH mRNA levels.

Body Weight Change

Chronic cold stress produced significant body weight loss (Table 19) in both males [$F(2,117) = 27.8$, $p<0.001$] and females [$F(1,115) = 4.15$, $p<0.018$]. E males showed significant

weight loss at 1 and 3 days compared to 0 day (p's<0.05), whereas PF and C males displayed significant weight loss only at 3 day compared to 0 day (p's<0.05). Females displayed significant weight loss at 3 day compared to 0 day (p<0.05); there were no significant differences among E, PF and C females. The weight loss was greater in males compared to females [$F(1,214) = 19.6$, $p<0.001$].

Adrenal:Body Weight Ratio

Chronic cold stress produced significant increases in adrenal:body weight ratios (Table 20) in males [$F(2,113) = 4.5$, $p<0.013$] but not females. Males displayed a higher overall ratio at 3 days compared to 0 day ($p<0.05$). There were no significant differences among E, PF and C males and females. Females displayed significantly greater adrenal:body weight ratios than males [$F(1,210) = 1685.1$, $p<0.001$].

Discussion

This is the first demonstration of the effects of prenatal ethanol exposure on the HPA and HPT responses to chronic cold stress, and one of the few studies to examine responses to cold stress in females. The hypothesis that prenatal ethanol exposure would result in HPA hyperresponsiveness was confirmed; hyperresponsiveness in E animals was observed in CORT and ACTH responses to cold exposure. Furthermore, patterns of response differed in E males and females. The data suggest that E males may be more responsive than E females to this type of chronic stressor.

In the present study, we found that basal levels of CORT and ACTH did not differ among E, PF and C males and females maintained in the warm (21° C) room (0 d cold stress). This is consistent with previous reports demonstrating no differences in basal hormone levels between

E and control animals under nonstressed conditions (Angelogianni & Gianoulakis, 1989, Lee et al., 1990; Nelson et al., 1986; Taylor et al., 1983; 1986; Weinberg, 1988; 1992a). However, exposure to chronic cold stress resulted in elevated CORT levels in E males, and elevations in overall ACTH levels in both E males and females compared to controls. In addition, overall ACTH levels were higher in E compared to C males following acute stress. E males also showed greater weight loss than PF and C males following cold stress, another indicator of increased responsiveness to cold exposure in E compared to control animals. The increased hormonal responsiveness to cold stress in E animals supports and extends previous reports demonstrating HPA hyperresponsiveness in E animals following a variety of acute stressors and following repeated exposures to restraint stress (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1992a; Weinberg et al., 1995; Weinberg et al., 1996). HPA hyperresponsiveness following a single episode of acute cold stress (4^{o} C) in E animals has been assessed previously; however the data have been inconsistent. Angelogianni and Gianoulakis (1989) demonstrated increased CORT and β-EP responses in 22 day old E rat pups (male and female data combined) following 5 min of cold stress compared to controls, whereas, Taylor et al. (1982) showed no significant differential effects of prenatal ethanol exposure in adult female rats after 60 min of cold stress. The present data suggest that exposure to chronic cold stress may produce significantly greater HPA activation in E compared to control animals, and that E males may be more vulnerable to the effects of chronic cold stress than E females.

The finding that CBG binding capacity was not changed by prenatal treatment suggests that the changes in CORT levels that were observed may be functionally important. Plasma levels of albumin were not measured in this study. However, since albumin accounts for only

10-15% of bound CORT, it is unlikely that possible changes in plasma albumin alone would have had a significant impact on free CORT levels.

Dissociations between ACTH and CORT levels were observed in E animals following chronic cold stress. E males displayed changes in both CORT and ACTH whereas E females displayed changes only in ACTH levels compared to controls. Although ACTH directly stimulates CORT release, changes in ACTH and CORT levels are not always directly proportional to each other. The amount of CORT that is released is proportional to the logarithm of the ACTH concentration (Keller-Wood et al., 1983), and maximal CORT levels can be achieved with less than maximal levels of ACTH. Furthermore, increasing the level of ACTH may not lead to further increases in CORT levels, although the duration of CORT release may be prolonged. Thus, in the present study, although E, PF and C animals may show differences in ACTH levels, maximal CORT levels may occur in all groups, eliminating group differences in the CORT levels observed. Previous studies have also reported a dissociation between ACTH and CORT following prenatal ethanol exposure. Both Lee et al. (1990) and Weinberg et al. (1996) found that E animals had increased stress induced ACTH levels but not CORT levels.

Sex differences in the effects of prenatal ethanol exposure on the HPA response to chronic cold stress were also observed in the present study. Chronic cold stress appears to have greater effects on E males than on E females. Previous studies have similarly shown that prenatal ethanol exposure differentially affects HPA function in males and females (Halasz et al., 1993; Osborn et al., 1996; Redei et al., 1993; Weinberg, 1988; 1992a; Weinberg et al., 1996). Although E males and females may both exhibit increased HPA responsiveness, it appears that the nature and intensity of the stressor, as well as the time course, and hormonal endpoint measured may all influence the outcome observed and may reveal differential effects in males

and females (Weinberg et al., 1996). Rats have been shown to exhibit sexual dimorphism of the HPA axis. Typically, females have heavier adrenals glands, higher ACTH, CORT and CBG levels, and higher diurnal variations in CORT levels than males (Chisari et al., 1995; Crtichlow et al., 1963; Kitay, 1961; 1963). These sex differences are due to both organizational and activational effects of the sex hormones (Kitay, 1961, 1963; Ramaley, 1976; Viau & Meaney, 1991). The sex differences in responsiveness observed in the present study may be due to interactions of prenatal ethanol, exposure to stressors, and the naturally occurring sexual dimorphism of HPA activity.

Prenatal ethanol exposure did not produce changes in CRF or VP mRNA levels in the PVN of the hypothalamus in either males or females maintained at normal room temperature. The present data replicate those of Osborn et al. (1995) in which no signficant effects of prenatal ethanol exposure were found in basal CRF or VP mRNA levels in the PVN of adult males and females. In contrast, Redei et al. (1993) found increased basal levels of CRF mRNA in the whole hypothalamus of adult E males but not females, and Lee et al. (1990) reported increased basal CRF mRNA levels in the PVN of 20 day old E rats (male and female data combined). It does not appear that methodological differences in measurement of mRNA levels can account for the differences among these studies, since Redei et al. (1993) used Northern blot analysis, and Lee et al. (1990), Osborn et al. (1995) and the present study used in situ hybridization techniques. Further studies are needed to identify the reasons for these differential results.

Interestingly, CRF and VP mRNA levels were not elevated in E animals following chronic cold exposure despite elevations in basal ACTH and/or CORT, the downstream products. In this context, it is noteworthy that Osborn et al. (1995) found that both E males and females treated with dexamethasone (DEX), a synthetic glucocorticoid, to suppress HPA activity, showed

marginally higher CRF mRNA levels compared to their PF and/or C counterparts following ether stress. In the present study, without data on levels of CRF and VP, it is not known if there are dissociations between the mRNAs and their respective peptides, or between these peptide secretagogues and ACTH. CRF and VP mRNAs are typically thought to reflect the activity of CRF and VP neurons and release of their respective peptides (Antoni, 1993; Lightman et al., 1993; Lightman & Harbuz, 1993), and direct correspondence between mRNA levels and biosynthesis has been reported (Herman & Morrison, 1996). However, mRNA levels, determined as a parameter of biosynthesis, do not always reflect peptide levels or release, since widely diverse mechanisms such as posttranslational processing, axonal transport and tissue or plasma degradation can all modify changes in gene expression (Owens & Nemeroff, 1991; Rondeel & Jackson, 1993).

One possible explanation for the increased ACTH levels in E animals following chronic cold stress despite the lack of change in CRF mRNA levels is that E animals may show an increased pituitary response to CRF. Support for this possibility comes from data of Yu et al. (1996) who demonstrated that E animals exhibit increased ACTH responses to CRF administration compared to controls following DEX suppression. The increased sensitivity of E animals may be due to increased CRF receptor density or changes in processes that occur following CRF binding to its receptors. An alternative and not incompatible possibility is that E animals may exhibit decreased corticosteroid feedback inhibition compared to controls. Data from Osborn et al (1996) indicating that E animals exhibit higher post-stress CORT levels following DEX blockade provide support for a deficit in feedback regulation in E animals, occurring primarily in the intermediate time domain. Data from Taylor et al (1988) demonstrating increased ACTH responses in E compared to control animals to repeated

footshock stress presented over a 15 min interval suggest that feedback deficits in E animals may also occur in the fast feedback time domain. The mechanisms underlying these feedback deficits remain to be determined. Both glucocorticoid and mineralocorticoid receptor densities at the anterior pituitary, hippocampus, and other feedback sites were found to be unaffected by prenatal ethanol exposure, both under basal conditions (Weinberg & Petersen, 1991) and following chronic intermittent stress (Kim et al., submitted). However, it is possible that chronic cold stress, which is qualitatively a very different stressor from chronic intermittent stress, could produce changes in corticosteroid receptor densities. Effects of prenatal ethanol exposure on ACTH levels could also be mediated through long term effects on events occurring between the binding of CORT to its receptors and the eventual feedback inhibition that is observed, as well as on the ultra-short feedback loop by which ACTH inhibits its own release (Boscaro et al., 1988).

There were no significant differences among E, PF and C males and females in TRH mRNA levels following chronic cold stress. Clinical studies have established that chronic alcoholism has marked effects on the HPT axis, affecting levels of TSH, T3, T4 and thyroxine-binding globulin (TBG)(Baumgartner et al., 1994). However, very little research on the effects of prenatal alcohol exposure on the HPT axis of the offspring has been done to date in humans. One study found that 6 of 8 children diagnosed with FAS had T4 levels below the median for the standard normal range; however, all 8 children had TSH, T4 and T3 levels considered within the normal range (Castells et al., 1981), confirming another report (Spiegel et al., 1979). A recent report using data bases to correlate measures of maternal alcohol use during pregnancy with infant blood T4 levels found that alcohol was not significantly associated with variations in T4 levels (Hannigan et al., 1995). In contrast, animal studies (mostly rodent) have found that

prenatal ethanol exposure produces disturbances in HPT function. Retarded growth of the fetal thyroid gland (Yamamoto et al., 1989), reduced fetal and neonatal levels of T3 and T4 (Hannigan & Bellisario, 1990; Kornguth et al., 1979; Lee & Wakabayashi, 1986; Portolés et al., 1988; Rose et al., 1981), and reduced neonatal TSH, T4 and T3 responses to TRH infusion (Premachandra et al., 1980; Rose et al., 1981) have been reported. Furthermore, the effects of prenatal ethanol exposure on the HPT axis have been shown to persist past the neonatal period. Decreased levels of T4, but not T3, were noted in rats up to 50 days of age (Portolés et al., 1988). Decreased T4 and T3 levels were found in 60 day old rats following prenatal and postnatal ethanol exposure; however, a nutritional control was not included in this study (Premachandra et al., 1980). The present study demonstrates that despite the reported changes in various indices of the HPT axis in animals prenatally exposed to ethanol, TRH mRNA levels were not shown to be affected by prenatal ethanol exposure.

In addition to the effects of prenatal ethanol exposure on the HPA axis, exposure to chronic cold stress resulted in several major stress-related effects across prenatal treatment groups. The observed increases in CORT and ACTH levels following chronic cold stress are consistent with other reports demonstrating HPA activation with cold exposure periods ranging from 1-14 days (Bligh-Tynan et al., 1993; Daniels-Severs et al., 1973; Hauger & Aguilera, 1992; Vernikos et al., 1982). The decrease in body weights and increase in relative adrenal weights are further indicators of HPA activation, and are also consistent with data from previous studies examining chronic cold stress (Daniels-Severs et al., 1973; Vernikos et al., 1982). Furthermore, cold stress produced significant elevations of TRH mRNA in the PVN in animals in all prenatal treatment groups, as demonstrated previously (Zoeller & Rudeen, 1992; Zoeller et

al., 1990). The decreased body weights may thus, in part, reflect an increase in metabolic rate resulting from activation of the HPT axis by cold stress (Berne & Levy, 1993).

Females showed adaptation of ACTH but not CORT responses to chronic cold exposure. That is, cold stress produced an initial elevation of ACTH levels at 1 day but levels were normalized by 3 days, whereas CORT levels remained elevated throughout the duration of cold exposure. Although adaptation of ACTH and/or CORT responses typically has not been reported with periods of cold exposure shorter than 1 week (Bligh-Tynan et al., 1993; Sakellaris & Vernikos-Danellis, 1975; Uehara et al., 1989), Fakuhara et al. (1996) found adaptation in ACTH but not CORT after 1 day and adaptation in both ACTH and CORT by 5 days of cold exposure. These results are similar to the present data and again emphasize the fact that changes in ACTH and CORT levels may not be directly proportional (Keller-Wood et al., 1983b). The mechanisms responsible for the regulation of HPA responses during adaptation to chronic stress are not well understood. These may involve relative changes in secretion of CRF, VP and/or other hypothalamic regulators, changes in pituitary CRF and/or VP receptors, and alterations in sensitivity of the glucocorticoid feedback mechanisms (Aguilera, 1994).

Males but not females displayed sensitization of the CORT response to an IP isotonic saline injection superimposed during the chronic cold stress. Sensitization in ACTH and/or CORT responses to IP saline injection and to ether stress has previously been demonstrated following 3 days to 8 weeks of cold exposure, and was observed whether or not HPA adaptation had occurred (Daniels-Severs et al., 1973; Sakellaris & Vernikos-Danellis, 1975; Vernikos et al., 1982). Interestingly, Daniels-Severs et al. (1973) showed that sensitization of ACTH and CORT responses following 1-8 weeks of cold stress was only apparent at specific times following the acute stressor, and that sensitization of both ACTH and CORT responses did not always occur at

the same time points. Thus, in the present study, sensitization of the CORT response may have been observed only for males because only a single time point, 15 min following the acute stressor, was examined. The mechanism of this sensitization, like that for adaptation, is not well understood. However, a possible mechanism may involve the VP system. Following chronic cold stress, there may be increased activation of VP producing neurons in the PVN, such that the VP:CRF ratio is increased. In addition, cold stress may increase the density of VP receptors at the pituitary (Aguilera, 1994). Pituitary CRF receptors are probably not involved since previous studies reported no changes in CRF receptor densities after 60 h of cold stress (Hauger & Aguilera, 1992).

The finding that CRF and VP mRNA levels were not elevated in either males or females from any prenatal treatment group following chronic cold stress was somewhat surprising. Zoeller et al. (1990) demonstrated increased CRF mRNA levels in the PVN after 30 and 48 h of cold exposure; and Angulo et al. (1991) demonstrated increased VP mRNA levels in the PVN following cold stress for 3 h per day for 4 days. A possible explanation for these conflicting results relates to the method of measurement of mRNA levels. In the present study, OD/IOD were measured from autoradiographic films, whereas Zoeller et al. (1990) and Angulo et al. (1990) measured the number or density of grains from emulsion-dipped slides. Although the measurement of OD/IOD from films is a widely accepted method of quantifying mRNA levels (Harbuz et al., 1991a; 1991b; Zoeller et al., 1992), measuring number/density of grains following emulsion treatment may provide a more sensitive measure of mRNA levels in that distinctions between the parvocellular and magnocellular divisions of the PVN can be made. These distinction may be important for VP since there are distinct pools of VP neurons in the PVN; parvocellular neurons are primarily responsive to nonosmotic stressors while

magnocellular neurons are primarily responsive to osmotic stimulation (Antoni, 1993). In contrast, the CRF neurons that are activated by stressors are located in the parvocellular region of the PVN (Owens & Nemeroff, 1991).

In summary, data from the present study provide the first demonstration of HPA hyperresponsiveness to chronic cold exposure in animals prenatally exposed to ethanol. These data support and extend previous work demonstrating differential effects of prenatal ethanol exposure on HPA responsiveness of male and female offspring, and suggest that E males may be more vulnerable to the effects of chronic cold stress than E females.

Table 15

Synthetic Oligonucleotides Sequences are Listed from 5′ to 3′ and are Complementary to the Base Number or mRNA-coding Region Listed

Probe	Sequence (5′-3′)	Base No.	Reference
CRF	CAGTTTCCTGTTGCTGTGAGCTTGCTG AGCTAACTGCTCTGCCCTGGC	496-543	Jingami et al., 1985
VP	TAGACCCGGGGCTTGGCAGAATCCAC GGACTCTTGTGTCCCAGCCAGC	Glycopeptide	Ivell & Richter, 1984
TRH	GTCTTTTTCCTCCTCCTCCCTTTTGCC TGGATGCTGGCGTTTTGTGAT	319-366	Lechan et al., 1986

Table 16

Maternal Body Weights (g, Mean ± SEM) of E, PF and C Dams During Gestation (G) and Lactation (D)

Gestation	G1	G7	G14	G21
E	270.5 ± 3.3	271.6 ± 3.3*	295.8 ± 3.8^	354.9 ± 5.7*
PF	264.0 ± 4.1	262.3 ± 3.5*	281.3 ± 4.2^	349.9 ± 6.5*
C	269.2 ± 1.9	291.3 ± 2.2*	320.4 ± 2.4^	408.8 ± 3.6*

Lactation	D1	D8	D15	D22
E	307.8 ± 6.2	339.6 ± 4.9	365.1 ± 5.7	332.1 ± 5.7
PF	297.7 ± 4.6	322.5 ± 5.9+	352.5 ± 4.3	328.6 ± 3.9
C	308.9 ± 2.8	342.4 ± 4.0+	359.0 ± 3.6	340.4 ± 3.9

n = 14-18 per group during gestation and lactation

Gestation: *At G7 & G21, E = PF < C, p's<0.05

^At G14, PF < E < C, p's<0.05

Lactation: +At D8, PF < C, p<0.05

Table 17

Body Weights (g, Mean ± SEM) of E, PF and C Pups (Male and Female Data Collapsed) From Birth to Weaning

	D1	D8	D15	D22
E	5.9 ± 0.2*	14.5 ± 0.4	29.0 ± 0.7+	46.9 ± 1.2+
PF	6.0 ± 0.2	15.3 ± 0.6	30.1 ± 0.8+	49.8 ± 1.6+
C	6.4 ± 0.1*	15.9 ± 0.3	32.3 ± 0.5+	53.6 ± 0.8+

n = 14-16 litters per group

*At D1, E < C, $p<0.05$

+At D15 & D22, E = PF < C, p's<0.05

Figure 17. Plasma CORT levels (mean ± SEM) in males from E, PF and C groups following 0, 1 or 3 d cold stress, measured prior to (0 min) or 15 min (15 min) following exposure to a novel acute stressor (IP isotonic saline injection) (n = 5-9 per condition). CORT levels following cold stress alone (0 min) were elevated selectively in E males at 1 and 3 d compared to 0 d (*p's<0.05). Acute stress increased CORT levels in all males at 0, 1 and 3 d (0 min < 15 min; p's<0.05). Sensitization of the CORT response to acute stress was observed in all males (15 min condition, 1 d = 3 d > 0 d, p's<0.05).

Male Corticosterone

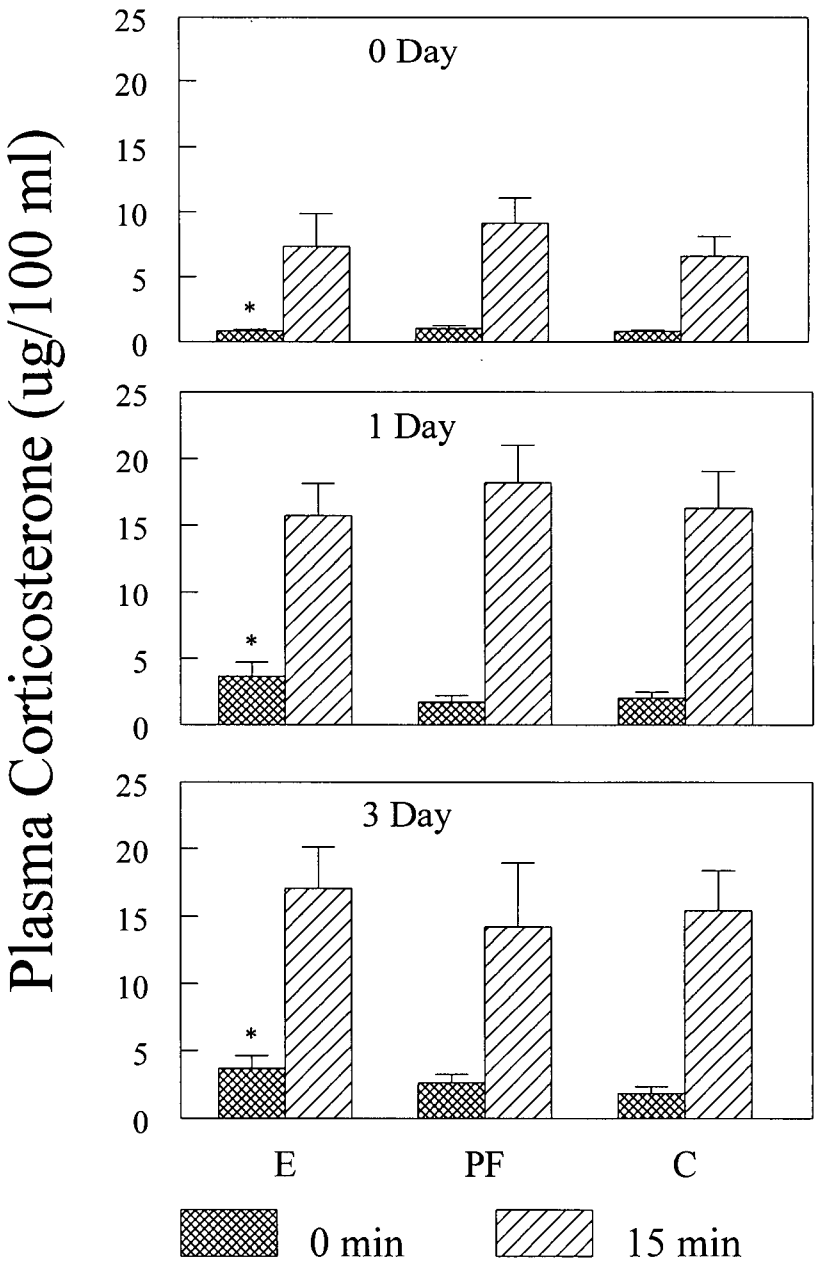


Figure 18. Plasma CORT levels (mean ± SEM) in females from prenatal E, PF and C groups following 0, 1 or 3 d cold stress, measured prior to (0 min) or 15 min (15 min) following exposure to a novel acute stressor (IP isotonic saline injection) (n = 5-9 per condition). CORT levels following cold stress alone (0 min) were elevated at 1 and 3 d compared to 0 d in all females (+p's<0.05). Acute stress increased CORT levels in all females at 0, 1 and 3 d (0 min < 15 min; p's<0.05). Sensitization of the CORT response to IP injection did not occur.

Female Corticosterone

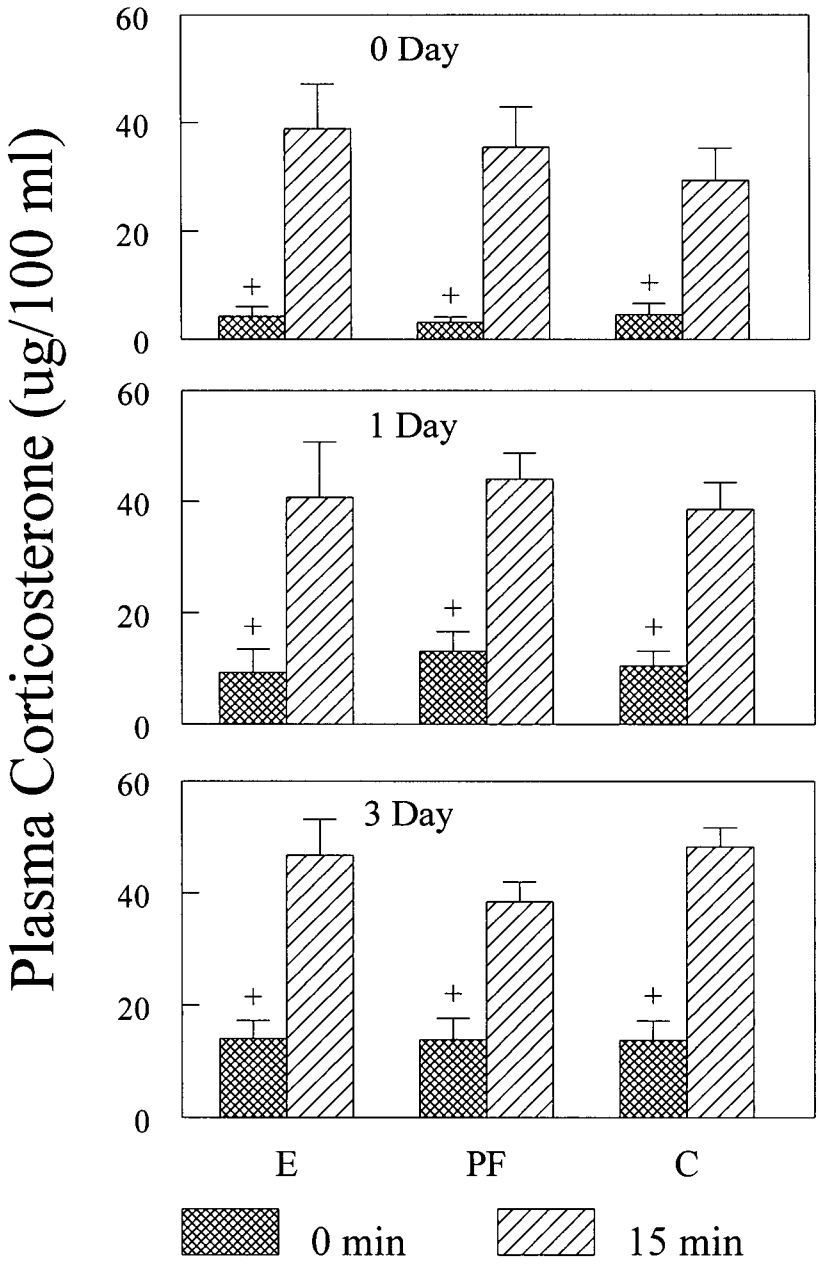


Table 18

Basal Plasma CBG Levels (μg/100 ml, Mean ± SEM) in E, PF and C Males and Females Following 0, 1 or 3 Days of Cold Stress

Male	0 Day	1 Day	3 Day
E	24.8 ± 2.8	24.8 ± 1.2	29.6 ± 3.4
PF	27.2 ± 2.7	25.8 ± 4.0	28.0 ± 2.1
C	23.5 ± 2.3	28.5 ± 2.1	31.0 ± 2.9

Female	0 Day	1 Day	3 Day
E	74.3 ± 3.4	76.5 ± 5.2	82.4 ± 5.5
PF	71.6 ± 3.2	89.5 ± 2.8	81.9 ± 6.8
C	83.8 ± 3.8	83.1 ± 4.7	72.5 ± 4.3

n = 6-9 per condition for males and females

Figure 19. Plasma ACTH levels (mean ± SEM) in males from prenatal E, PF and C groups following 0, 1 or 3 d cold stress, measured prior to (0 min) or 15 min (15 min) following exposure to a novel acute stressor (IP isotonic saline injection) (n = 5-9 per condition). ACTH levels, averaged over days, were significantly elevated in E compared to PF and C males at 0 min (p's<0.05) and in E compared to C males at 15 min ($p<0.05$).Acute stress produced significant increases in overall ACTH levels (0 min < 15 min; $p<0.05$).

Male ACTH

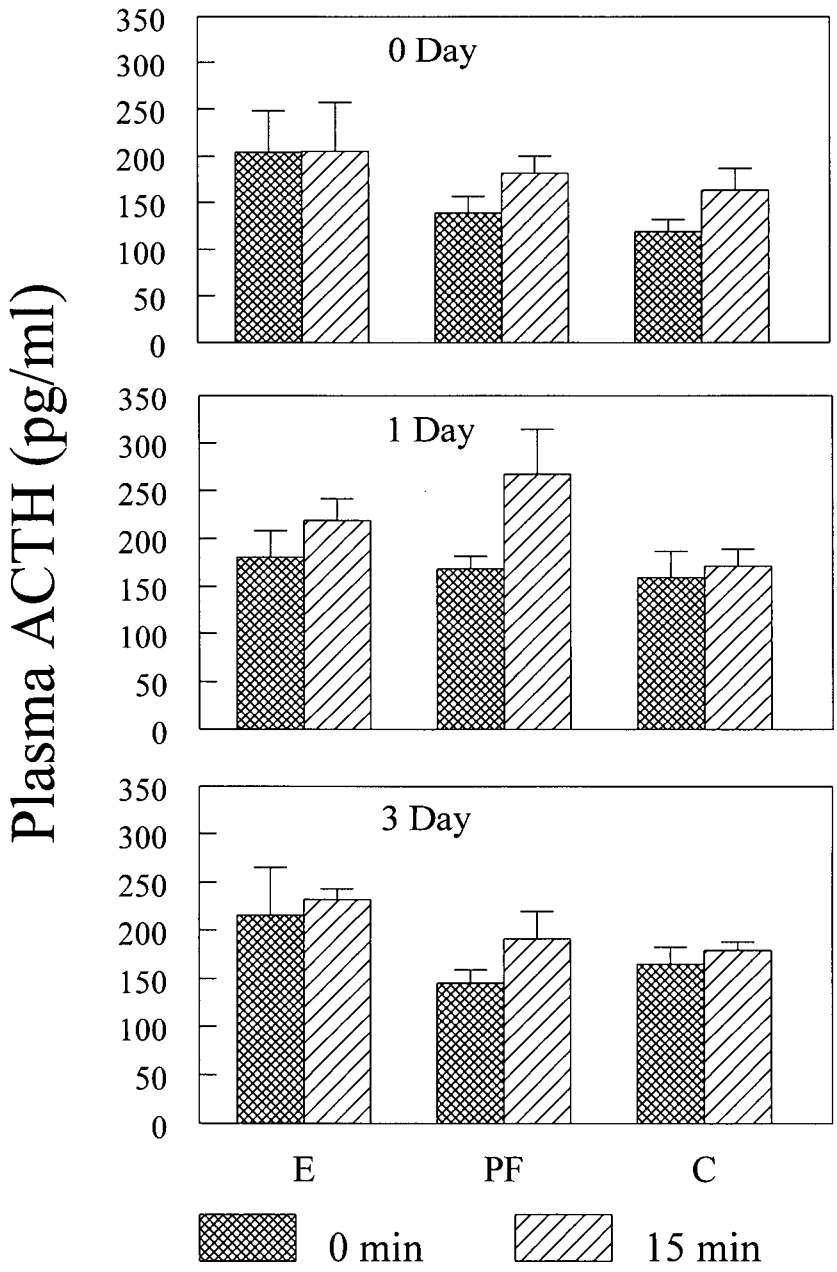

Plasma ACTH (pg/ml)
0 Day
1 Day
3 Day
350
300
250
200
150
100
50
0
E
PF
C
0 min
15 min

Figure 20. Plasma ACTH levels (mean ± SEM) in females from E, PF and C groups following 0, 1 or 3 d cold stress, measured prior to (0 min) or 15 min (15 min) following exposure to a novel acute stressor (IP isotonic saline injection) (n = 5-9 per condition). ACTH levels following cold alone (0 min) were elevated in all females at 1 d compared to 0 d ($p<0.05$), but were no longer elevated by 3 d. ACTH levels, averaged over days, were elevated in E compared to PF and C rats ($p's<0.05$) at 0 min. Acute stress produced significant increases in overall ACTH levels (0 min < 15 min; $p<0.05$).

Female ACTH

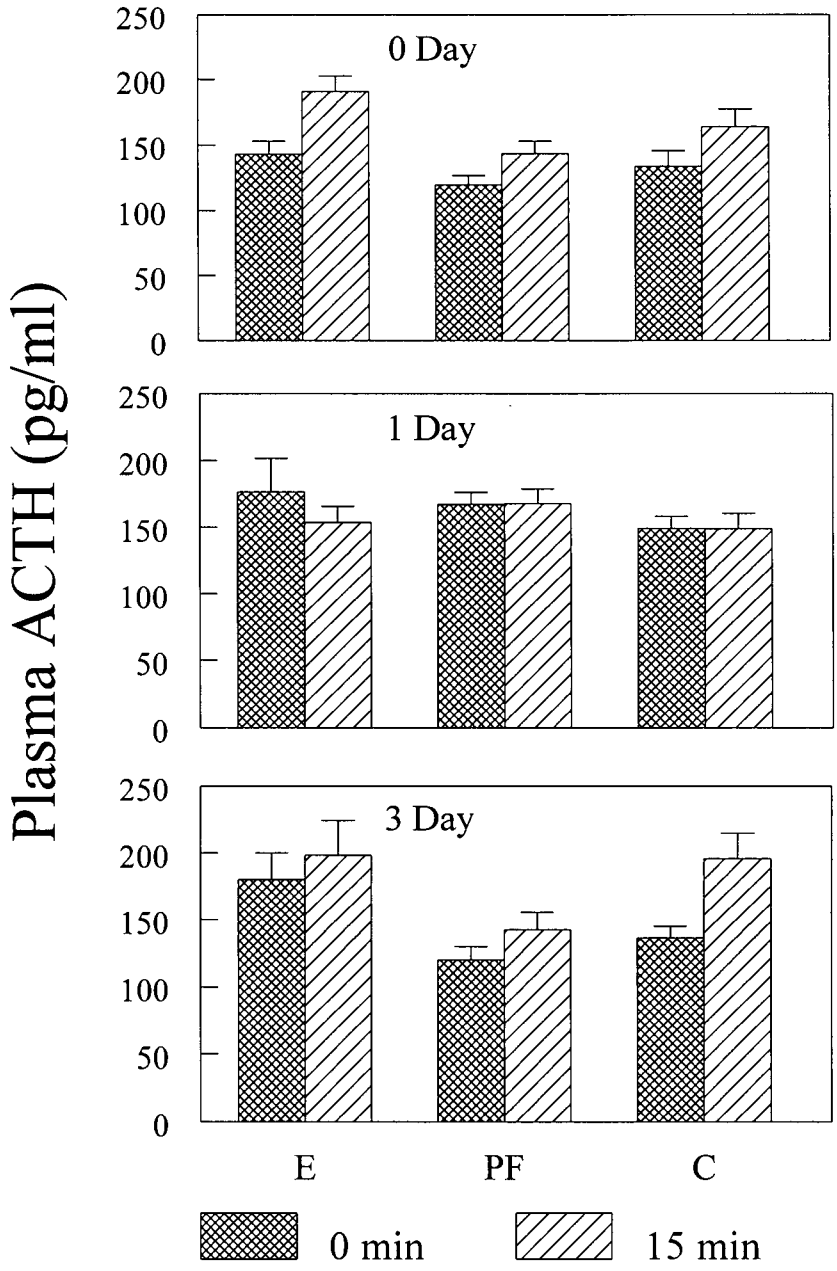

Plasma ACTH (pg/ml)
250
200
150
100
50
0
0 Day
1 Day
3 Day
E
PF
C
0 min
15 min

Figure 21. CRF mRNA levels (OD in arbituary units) in the PVN of the hypothalamus for males and females from E, PF and C groups following 0, 1 or 3 d cold stress at 0 min (n = 6-9 per condition). There were no signifcant differences among E, PF and C males or females in CRF mRNA levels.

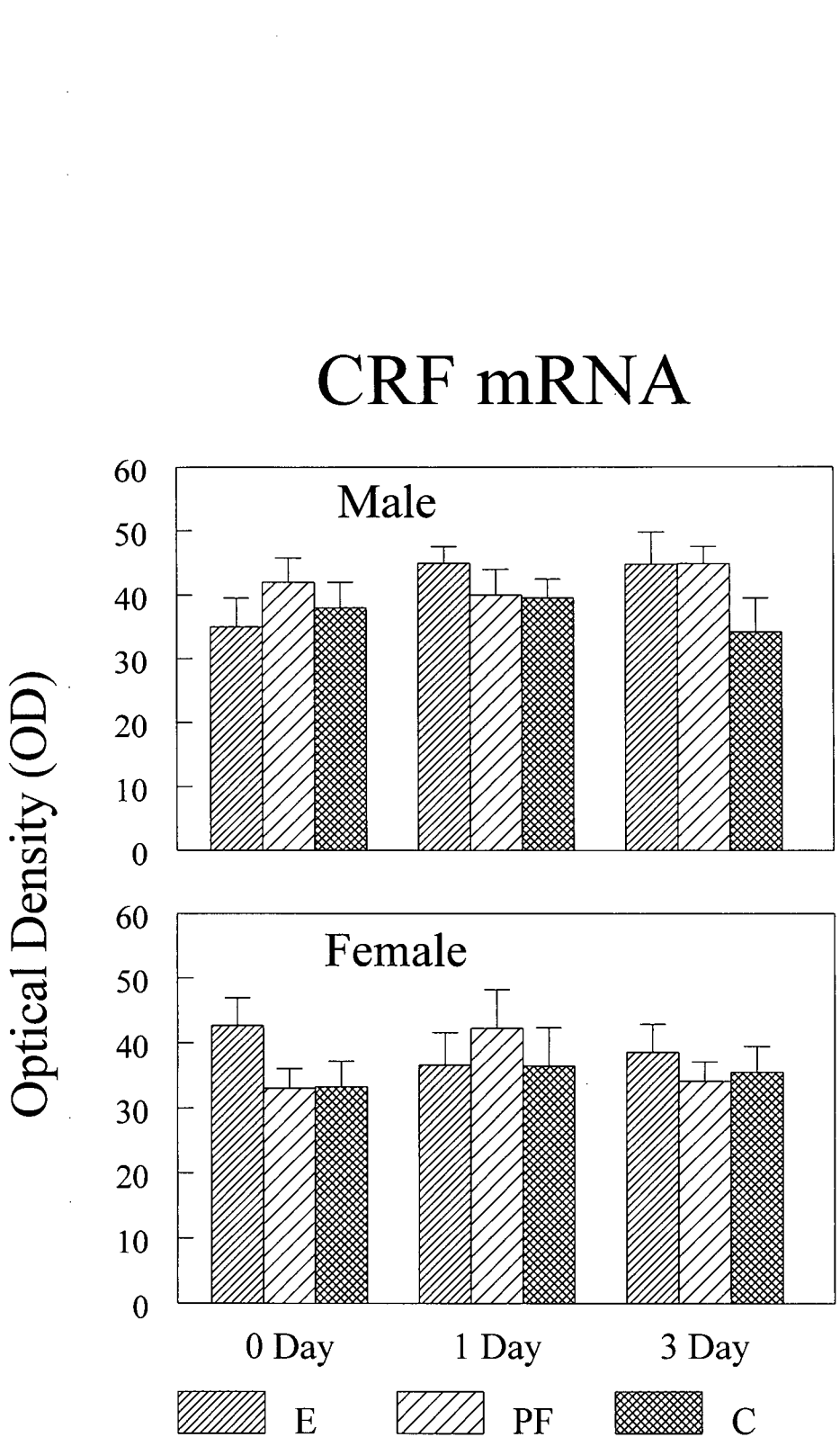

CRF mRNA
Optical Density (OD)
Male
Female
60
50
40
30
20
10
0
0 Day
1 Day
3 Day
E
PF
C

Figure 22. VP mRNA levels (OD in arbituary units) in the PVN of the hypothalamus for males and females from prenatal E, PF and C groups following 0, 1 or 3 d cold stress at 0 min (n = 6-9 per condition).There were no signifcant differences among E, PF and C males or females in VP mRNA levels.

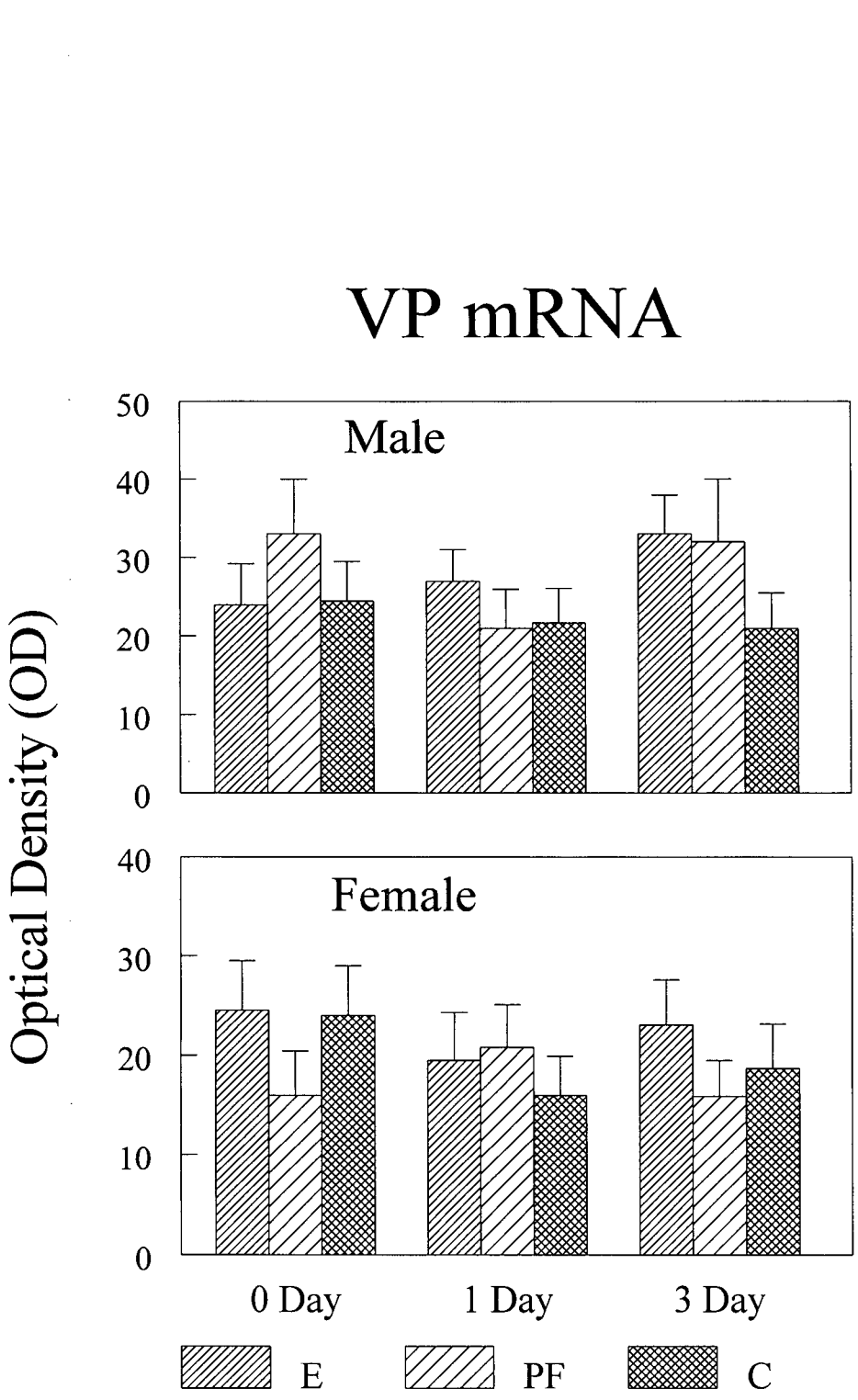

VP mRNA
Optical Density (OD)
Male
Female
50
40
30
20
10
0
0 Day
1 Day
3 Day
E
PF
C

Figure 23. TRH mRNA levels (OD in arbituary units) in the PVN of the hypothalamus for males and females from prenatal ethanol (E), pair-fed (PF) and ad lib fed control (C) groups following 0, 1 or 3 d cold stress at 0 min (n = 6-9 per condition). Males displayed greater TRH mRNA levels at 3 d compared to 0 and 1 d (p's<0.05). Females displayed greater TRH mRNA levels at 3 d compared to 0 d, (p's<0.05).

TRH mRNA

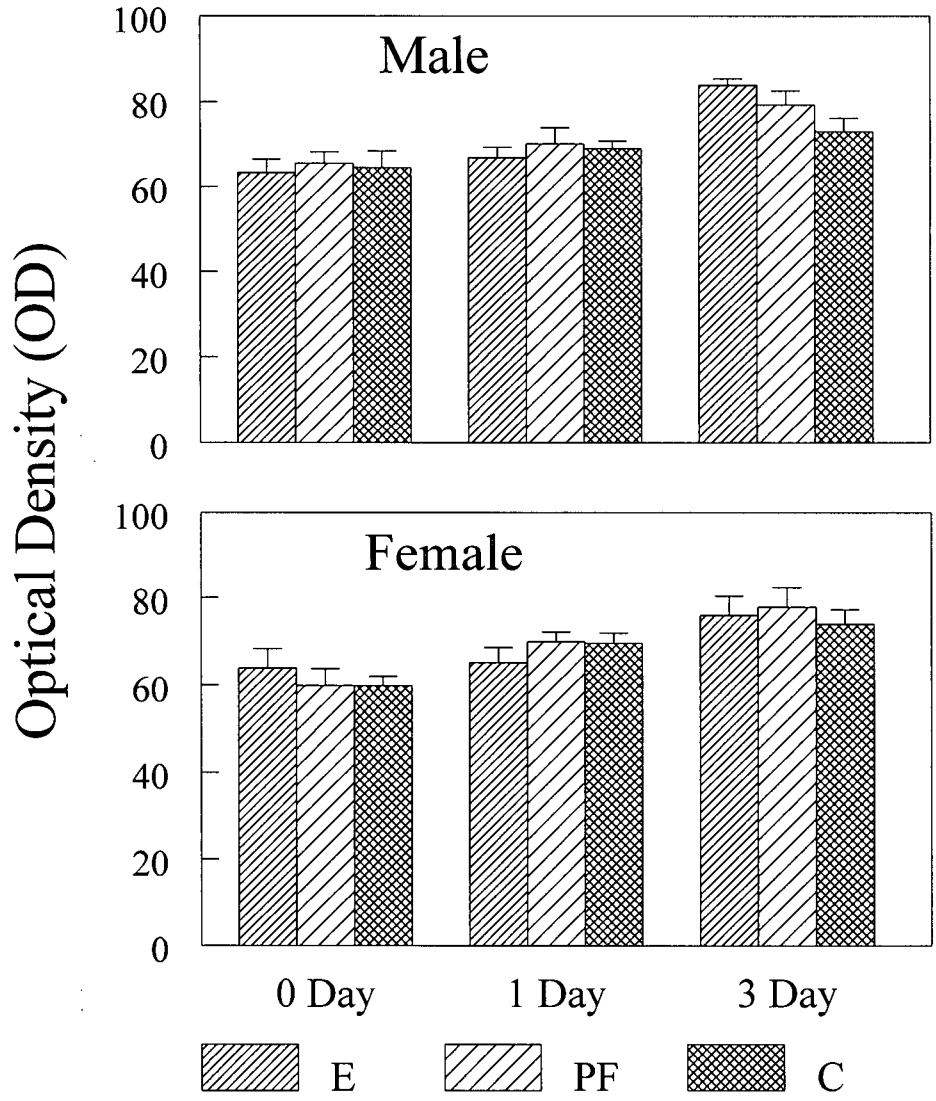

Optical Density (OD)
Male
Female
100
80
60
40
20
0
0 Day
1 Day
3 Day
E
PF
C

Table 19

Decrease in Body Weights (g, Mean ± SEM) of E, PF and C Males and Females Following 0, 1 or 3 Days of Cold Stress

Male	0 Day	1 Day	3 Day
E	9.8 ± 2.4*	19.8 ± 2.1*	23.3 ± 2.6*
PF	9.8 ± 1.6^	14.8 ± 1.3	22.9 ± 1.8^
C	10.2 ± 1.3^	16.4 ± 2.2	21.0 ± 2.3^

Female	0 Day	1 Day	3 Day
E	11.8 ± 2.1	15.7 ± 1.7	14.5 ± 0.9
PF	10.0 ± 1.8	13.5 ± 1.7	13.2 ± 1.2
C	10.3 ± 1.5	9.9 ± 1.2	15.2 ± 1.4

n = 12-18 per condition for males and females

Male: *For E, 0 day < 1 day = 3 day, p's<0.05

^For PF & C, 0 day < 3 day, p's<0.05

Female: 0 day < 3 day, p's<0.05

Table 20

Adrenal:Body Weight Ratios ($x10^5$, Mean ± SEM) in E, PF and C Males and Females Following 0, 1 or 3 Days of Cold Stress

Male	0 Day	1 Day	3 Day
E	9.2 ± 0.6	10.2 ± 0.5	10.7 ± 0.4
PF	10.1± 0.5	10.2 ± 0.3	10.9 ± 0.5
C	9.0 ± 0.2	9.8 ± 0.3	9.9 ± 0.4

Female	0 Day	1 Day	3 Day
E	25.2 ± 1.0	25.1 ± 1.0	26.4 ± 0.9
PF	23.1 ± 1.0	25.9 ± 1.0	25.4 ± 1.1
C	24.2 ± 0.8	24.4 ± 1.1	24.2 ± 0.8

n = 12-18 per condition for males and females

Males: 0 day < 3 day, $p<0.05$

CHAPTER VI: CORTICOSTEROID RECEPTOR ASSAY

Introduction

Gel filtration is a commonly used method to separate particles based on molecular size (Gel Filtration: Theory and Practice, 1982). Gel columns are typically glass or plastic pipettes that are packed with gel filtration beads. These gel beads contain specific sized pores that allow only certain sized particles to enter. These pores contain the stationary phase that slows the downward progress of any particle that enters it. Larger particles move around the beads and never enter the stationary phase, thus they move through the gel columns more quickly. In this way, large and small particles can be physically separated.

In the experiments of this thesis that examined corticosteroid receptor densities (Chapter IV), glass columns packed with Sephadex gel beads (Sigma Chemical) were used to separate the radioisotope-labelled steroids bound to the corticosteroid receptors (larger particles; bound fraction) from the excess unbound steroids (smaller particles; unbound fraction). The density of receptors were subsequently determined by the measurement of the bound fraction (receptor plus steroid) recovered from the gel columns. The present study examined the effect of the number of times the gel columns had previously been used on the amount of bound fraction that was recovered.

Methods

The cytosolic binding assay method is as described in the methods section of Chapter IV of this thesis. Twenty-four h following ADX, brains were processed and the hypothalamus of each rat was isolated and homogenized. Aliquots of cytosol from each rat (n = 6) were added to new gel columns and to gel columns that had been used 2-4 times, and direct comparisons were

made in GR densities measured by the new and used gel columns. A similar comparison was made between gel columns that had been used once with those that had been used 3-5 times (n = 4). Statistical analysis was by paired t-test, with $p<0.05$, 2-tailed.

Results

As can be seen in Table 21, there were significantly higher GR density values (of 37%) generated by gel columns that had been used 2-4 times compared to new gel columns ($t = 6.3$, $p<0.002$). However, there were no significant differences in measured GR densities between gel columns that had been used once and those that had been used 3-5 times.

Discussion

The measured GR densities were significantly higher with gel columns that had previously been used compared to new columns. Furthermore, the measured GR densities became stable after just one use of the columns. The change in the measured bound fraction between new versus used gel columns (of 37%) is sizable enough to produce serious confounding of experimental results if attention is not given to whether new or used gel columns are used. The decrease in the bound fraction that is recovered with new gel columns may result from the binding of receptor proteins to the gel beads or glass columns. To the best of the author's knowledge, there is no other written documentation of differences in the measured bound fraction between new and previously used gel columns. Thus, this study makes an important methodological point regarding gel filtration using Sephadex gel beads that may extend to other types of gel filtration systems. It should be noted that in all the reported experiments of this thesis that used the gel filtration technique (Chapter IV), the gel columns had been preequilibrated with brain cytosol prior to their first use.

Table 21

GR Densities (Mean ± SEM; fmol/mg protein) Measured with Sephadex Gel Packed Columns That Have Been Used 0 Versus 2-4 Times, and 1 Versus 3-5 Times

0 Time	2-4 Times
109.6 ± 22.5*	150.0 ± 20.1*

1 Time	3-5 Times
104.9 ± 22.6	106.7 ± 23.6

n = 6 for 0 versus 2-4 times

n = 4 for 1 versus 3-5 times

*0 Time < 2-4 Times, $p<0.002$

CHAPTER VIII: GENERAL DISCUSSION

The main purpose of this thesis was to investigate the effects of prenatal ethanol exposure on (1) cognitive and behavioral function and (2) HPA stress response. The first study of this thesis (Chapter III) examined the cognitive deficits and behavioral abnormalities produced by prenatal ethanol exposure, the second and third studies (Chapters IV and V) examined the HPA hyperresponsiveness produced by prenatal ethanol exposure, and the fourth study (Chapter VI) made an important methodological point regarding the gel filtration technique that was used in the second study of this thesis. Accordingly, the General Discussion of this thesis is divided into four sections: Section A discusses the studies of this thesis; Section B provides future directions of this research; Section C presents possible clinical implications of the present research; and finally Section D closes with conclusions to the thesis.

A. General Discussion of Studies

The maternal consumption of alcohol during pregnancy has serious adverse effects on the developing fetus. It affects virtually every system of the organism, producing a wide range of abnormalities that impacts the individual's health and ability to function in society. The teratogenic potential of maternal alcohol consumption has been appreciated for thousands of years (Colangelo & Jones, 1982; Warner & Rosett, 1975). However, it was not until Lemoine et al. (1968) and Jones et al. (1973a; 1973b) independently documented a set of abnormalities in children of mothers who had chronically consumed high doses of alcohol during pregnancy that there was widespread acceptance of alcohol's teratogenic potential. The term FAS was initially used to describe this set of abnormalities resulting from prenatal alcohol exposure (Jones &

Smith, 1973a). Since then, other terms such as FAE, partial FAS, ARBD and ARND have been used to describe cases where all diagnostic criteria of FAS are not met, yet there are still symptoms of prenatal alcohol exposure (Sokol & Clarren, 1989; Stratton et al., 1996).

The purpose of the first study (Chapter III) of this thesis was to assess the cognitive and behavioral abnormalities produced by prenatal ethanol exposure. Two memory tasks that placed different cognitive demands on the subject were used: the object-recognition nonrecurring-items DNMS task and the spatial-navigation Morris water maze task. The object-recognition DNMS task was different from other tasks that have been used to study memory function in E animals because it is a task to which there are direct human and well as monkey equivalents. This task has recently been adapted for use in rats (Mumby et al., 1990), and this is the first time that it has been used in the study of prenatal ethanol exposure in rats. The well established spatial-navigation water maze task is sensitive to damage to the hippocampus (Morris, 1981), an area that has been shown to be affected by prenatal ethanol exposure (Hammer, 1986; Ward & West, 1992; West & Pierce, 1986). This study demonstrated that there were no significant effects of prenatal ethanol exposure in performance of the DNMS task at retention delays ranging from 4 to 300 s, or in reacquisition of the task following a 10 week rest period. Furthermore, prenatal ethanol exposure did not produce significant increases in distractibility or response perseveration in the DNMS apparatus. However, when the same rats were tested on the water maze task, prenatal ethanol exposure produced performance deficits. E rats took significantly longer than PF and C rats to find the hidden platform over the course of the training sessions. There are two likely explanations for the differences in performance on these two tasks, that are not mutually exclusive. First, the brain areas underlying spatial cognitive abilities may be more vulnerable to the teratogenic effects of prenatal ethanol exposure than those underlying object-

recognition abilities. Second, E animals, which display HPA hyperresponsiveness to stressors, may be differentially responsive to the stress involved in performance of these two tasks.

The second and third studies (Chapters IV and V) of this thesis examined HPA hyperresponsiveness produced by prenatal ethanol exposure. The main purpose of the second study (Chapter IV) was to determine the mechanism of the HPA hyperresponsiveness produced by prenatal ethanol exposure. It tested the hypothesis that decreased corticosteroid receptor densities at HPA feedback sites mediate, at least partially, the feedback deficits and resultant HPA hyperresponsiveness. Both GR and MR densities were measured at the hippocampus, prefrontal cortex, hypothalamus, and anterior pituitary under basal or nonstressed conditions. Furthermore, differential downregulation of corticosteroid receptors following one of two intermittent chronic stress regimens (two of six stressors daily for 18 days), and differential upregulation of receptors 7 days following ADX compared to that at the usual 24 h were examined. This study demonstrated that prenatal ethanol exposure did not produce changes in basal corticosteroid receptor densities, nor differentially downregulate hippocampal GRs under conditions of the more stressful Stress Regimen I, or differentially upregulate hippocampal GRs 7 days following ADX. However, there were small changes in hippocampal GRs among E, PF and C rats under the conditions of the milder Stress Regimen II. Given the nature of these changes in receptor densities, however, it would appear that these changes do not play a critical role in mediating the feedback deficits. Thus, the hypothesis that decreased corticosteroid receptor densities, as measured by the whole site cytosolic binding assay, underlie the deficits in feedback inhibition and resultant HPA hyperresponsiveness following prenatal ethanol exposure was not supported. However, prenatal ethanol exposure produced increased adrenal weights normalized to body weights following chronic stress, supporting and extending previous data

demonstrating HPA hyperresponsiveness of E animals only following stress and not under basal conditions. But no changes were noted in basal plasma CORT or CBG levels among E, PF and C rats under any of the experimental conditions of this study.

The main purpose of the third study (Chapter V) of this thesis was to test the hypothesis that prenatal ethanol exposure would produce HPA hyperresponsiveness to chronic cold stress, and increased sensitization to a novel acute stressor (IP isotonic saline injection) superimposed during the chronic cold stress. Exposure to chronic cold stress resulted in elevated CORT levels in E males, and elevated ACTH levels in E males and females. However, prenatal ethanol exposure did not increase basal levels of ACTH and CORT, increase hormonal sensitization to the acute stressor, nor produce changes in mRNA levels for CRF and VP in the PVN under basal conditions or following chronic cold stress. Thus, the hypothesis that prenatal ethanol exposure would produce HPA hyperresponsiveness to cold stress was confirmed. However, the hyperresponsiveness of E animals was not observed at all levels of the HPA axis examined or under all experimental conditions. This study was also a preliminary exploration of the effects of prenatal ethanol exposure on the HPT response to chronic cold stress. Cold stress increased TRH mRNA levels in both males and females, but there were no signifcant differences among the E, PF and C groups.

A fourth study (Chapter VI) of this thesis made an important methodological point regarding the use of the gel filtration technique using Sephadex gel beads. Gel filtration is a commonly used method to separate particles based on molecular size, and was used in the corticosteroid receptor assay (Chapter IV of this thesis). The measured receptor densities were significantly higher with previously used gel columns compared to new gel columns, and the measured densities became stable after just one use of the columns. The change in measured

densities between new versus used gel columns is sizable enough to produce serious confounding of experimental results if attention is not given to whether new or used gel columns are used. It is possible that this feature of Sephadex gel filtration beads, may generalize to other types of gel filtration systems.

B. Future Directions

Despite two and a half decades of research on prenatal alcohol exposure, many questions remain unanswered. As is typical of scientific inquiry, the pursuit of an answer to a question yields many more questions. The data of the present thesis lead to many questions and provide future directions for research.

(1) Cognitive deficits were observed on the spatial-navigation Morris water maze task but not on the object-recognition DNMS task following prenatal ethanol exposure. The interpretation that prenatal ethanol exposure produces greater damage to brain areas that underlie spatial-navigation abilities than those that underlie object-recognition abilities may be tested. Evidence suggests that damage to the hippocampus may underlie the observed pattern of deficits. The hippocampus is thought to play a major role in spatial cognitive function (O'Keefe, 1993), and lesions to this site have been shown to produce deficits on spatial tasks such as the water maze (Morris et al., 1982), but only very mild deficits on the DNMS task (Mumby et al., 1992; 1995b). Interestingly, Duva et al. (1997a) have shown that partial bilateral hippocampal lesions limited mainly to the CA1 area resulted in deficits only on the water maze task and not on the DNMS task. Furthermore, the hippocampus, especially the CA1 region, has been shown to be sensitive to the teratogenic effects of prenatal ethanol exposure (Hammer, 1986; Ward & West, 1992; West & Pierce, 1986). Moreover, prenatal ethanol exposure has been shown to

produce many effects similar to those observed following hippocampal lesions, albeit of much less severity; these include deficits on spatial memory tasks (Blanchard et al., 1987; 1990; Gianoulakis, 1990; Omoto et al., 1993; Reyes et al., 1989) and behavioral abnormalities such as response perseveration (Riley, et al., 1986). Finally, the development of long-term potentiation (LTP) in the hippocampal CA1 region was inhibited in E rats (Swartzwelder et al., 1988). Thus, hippocampal damage, especially in the CA1 area, could be compared with spatial task performance deficits in E animals. A positive correlation would support the interpretation that prenatal ethanol exposure produces greater damage to brain areas underlying spatial-navigation abilities than those underlying object-recognition abilities.

(2) The interpretation that prenatal ethanol exposure produces differential responsiveness to the stress involved in performance of the spatial-navigation and object-recognition tasks may be tested. Prenatal ethanol exposure has been shown to result in HPA hyperresponsiveness to stressors (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1992a; Weinberg et al., 1995; 1996). Furthermore, the water maze task may have evoked a greater physiological stress response than did the DNMS task. Moreover, high stress responses have been shown to impair cognitive function. In particular, high CORT levels have been shown to impair performance on memory tasks (McEwen & Sapolsky, 1995), and induction of LTP and primed-burst potentiation (Diamond & Rose, 1994; Diamond et al., 1992; 1994; Foy et al., 1987; Rey et al., 1994; Shors & Dryver, 1994; McEwen, 1994). Thus, it is possible that E animals had an increased hormonal stress response during testing compared to controls, a response that was further enhanced on the water maze compared to the DNMS task. The resulting increased stress response of E rats in the water maze task produced greater impairments of cognitive function, resulting in the observed deficits

in the water maze task. Thus, spatial task performance could be compared with stress hormone levels, especially CORT, measured after testing on the task. A positive correlation between performance deficits and stress hormone levels in E rats would support the interpretation that prenatal ethanol exposure produces differential responsiveness to the stress involved in performance of the spatial-navigation and object-recognition tasks.

(3) Corticosteroid receptor densities at HPA feedback sites, as measured by the total cytosolic binding assay, were not shown to be affected by prenatal ethanol exposure. Although the cytosolic binding method is a powerful technique, it cannot make regional distinctions within a site of interest since it involves homogenization of the whole site. The hippocampus is a structure in which there are several subfields (CA1-CA4, dentate gyrus), with regional differences in levels of GR and MR protein and mRNA (De Kloet, 1991). Thus, measures of GR and MR proteins and their mRNAs in hippocampal subfields using autoradiography and *in situ* hybridization, respectively, may yield regional differences that were not apparent with the whole hippocampal preparation following prenatal ethanol exposure.

(4) Total corticosteroid receptor densities at HPA feedback sites were not shown to be affected by prenatal ethanol exposure. However, there are many subsequent events following the binding of CORT to its receptor and eventual negative feedback regulation of the HPA axis that could be affected by prenatal ethanol exposure.

(5) One possible mechanism of HPA hyperresponsiveness resulting from prenatal ethanol exposure was examined, that of negative feedback deficits. There is evidence to support other possible mechanisms, each of which could be explored. (a) There may be increased sensitivity of the anterior pituitary and adrenal cortex to their respective secretagogues in E animals. Increased ACTH response to CRF in DEX suppressed E rats (Yu et al., 1996), and increased

basal levels of POMC mRNA in the anterior pituitary of E rats (Redei et al., 1993) have been reported. (b) It is possible that altered neurotransmitter systems may be involved in mediating the HPA hyperresponsiveness of E animals. NE content in the cortex and hypothalamus was found to be lower in E animals compared to controls following restraint stress (Rudeen & Weinberg, 1993). NE has been shown to stimulate CRF release in a dose dependent manner (Plotsky, 1987). If lower hypothalamic NE levels in E animals is indicative of increased NE turnover, it is possible that prenatal ethanol effects on NE regulation of CRF secretion may play a role in HPA hyperactivity. The GABA system also appears to be altered in E animals, as demonstrated by altered responsiveness to the anxiolytic effects of benzodiazepines (Yu et al., 1995). CORT can modulate $GABA_A$ receptor activity and in turn GABA has a role in regulating HPA activity (Jones et al., 1984; Majewska et al., 1985). Thus, prenatal ethanol exposure may affect the HPA axis via alterations in the GABA system.

(6) Chronic cold stress was shown to elevate basal plasma CORT levels in E animals. The elevated basal CORT levels may result from an impairment in negative feedback regulation of the HPA axis. Thus, the effects of prenatal ethanol exposure on corticosteroid receptor density at HPA feedback sites following chronic cold stress could be assessed.

C. Clinical Implications

The present research may have important clinical implications. However, caution must be used in extrapolating data obtained from rodents to that of humans, given the large phylogenic gap between the two species. Nevertheless, as discussed in the General Introduction to this thesis, many similarities have been demonstrated in prenatal alcohol effects between humans

and rodents. In fact, the rodent model has proven to be very valuable in the study of prenatal alcohol exposure.

Cognitive deficits and behavior abnormalities have been demonstrated in both clinical populations and animal models. Cognitive deficits and behavioral abnormalities are among the most detrimental consequences in individuals diagnosed with FAS/FAE, resulting in a compromised ability to function in society (Conry, 1990; Coles, 1992; Streissguth, 1986; Streissguth & LaDue, 1987; Streissguth et al., 1980; 1991). Animals studies have replicated many of the cognitive deficits (Abel, 1979; Becker & Randall, 1989; Driscoll et al., 1985; Gentry & Middaugh, 1988; Gianoulakis, 1990; Reyes et al., 1989; Zimmerberg et al., 1991) and behavioral abnormalities (Anandam et al., 1980; Abel, 1982; Becker & Randall, 1989; Bond, 1986; Martin et al., 1978; Riley et al., 1979a; 1979b; 1979c) that have been found in humans. The present study demonstrated that E rats displayed performance deficits on the spatial-navigation water maze task but not on the object-recognition DNMS task. Clinical studies have shown that the cognitive deficits in individuals with FAS/FAE are most apparent on spatial and complex tasks (Streissguth et al., 1991; 1994a). To the best of the author's knowledge, no study has assessed the performance of individuals diagnosed with FAS/FAE on an object-recognition DNMS task. Thus, the present study performed on rodents, provides a prediction to be tested clinically.

HPA hyperresponsiveness has also been demonstrated in both humans and animals. Although clinical studies have established that alcohol consumption markedly alters HPA function in chronic alcoholics (Merry & Marks, 1973), few clinical studies have investigated the effects of drinking during pregnancy on the HPA axis of the developing child. However, a recent study found that maternal drinking at conception and during pregnancy was associated with

higher poststress cortisol levels in infants (Jacobson et al., 1993), indicating the importance of this issue for further study. Many animals studies have shown that prenatal ethanol exposure produces HPA hyperresponsiveness (Angelogianni & Gianoulakis, 1989; Lee et al., 1990; Nelson et al., 1986; Redei et al., 1993; Taylor et al., 1982; Weinberg, 1992; Weinberg et al., 1995; 1996). The present research confirms that prenatal ethanol exposure produces HPA hyperresponsiveness to stressors, and extends this finding to chronic stressors--intermittent stress regimen and continuous cold stress. Given that HPA hyperresponsiveness has been shown to have adverse physiological and behavioral consequences which could compromise health and possibly even survival of the organism, the importance of understanding the HPA hyperresponsiveness produced by prenatal alcohol exposure is emphasized. The adverse consequences of HPA hyperresponsiveness include gastrointestinal ulceration, immunosuppression, weight loss, fatigue, myopathy, steroid diabetes, hypertension, psychogenic dwarfism, reproductive dysfunction and neuronal death (Sapolsky, 1992; Stratakis & Chrousos, 1995). Furthermore, dysregulation of the HPA axis has been implicated in a variety of psychiatric disorders such as depression, panic disorder, obsessive-compulsive disorder and anorexia nervosa (Chrousos & Gold, 1992; Johnson et al., 1992). It has been proposed that HPA hyperresponsiveness following prenatal alcohol exposure may underlie some of the behavioral abnormalities (Kim et al., 1996) and immune deficits (Giberson et al., 1997; Giberson & Weinberg, 1995) that have been observed in both humans and animals. The data of this thesis further suggest that the HPA hyperresponsiveness produced by prenatal ethanol exposure may also underlie the observed cognitive impairments found in both humans and animals.

D. Conclusions

In conclusion, the experiments of this thesis demonstrate that prenatal ethanol exposure has long-term effects on cognitive function and the HPA stress response. Prenatal ethanol exposure produced cognitive deficits on a spatial-navigation task but not on a recently developed object-recognition DNMS task for rats. Prenatal ethanol exposure also produced HPA hyperresponsiveness to chronic stressors--intermittent stress regimen and continuous cold stress. The mechanism of the HPA hyperresponsiveness following prenatal ethanol exposure was not due to changes in total corticosteroid receptor densities at the HPA feedback sites.

FAS/FAE are preventable disorders, by simple abstinence of alcohol consumption during pregnancy. Despite the awareness of the teratogenic potential of alcohol on the developing fetus, FAS/FAE remain very prevalent societal problems. Emphases on prevention should be foremost; however, given that this problem is currently prevalent and may always exist, research on the effects of *in utero* alcohol exposure is important. It is hoped that a better understanding of the effects of prenatal alcohol exposure and its underlying mechanisms may lead to treatments for affected individuals, whether it be pharmacological or behavioral.

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