HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FEEDBACK REGULATION

IN RATS PRENATALLY EXPOSED TO ETHANOL

by

Candace Erica Hofmann

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Department of __Psychology__

The University of British Columbia
Vancouver, Canada

Date __July 28/98__

ABSTRACT

Animals exposed to ethanol *in utero* to ethanol exhibit hormonal hyperresponsiveness to stressors in adulthood. One possible mechanism for this hyperresponsiveness is a deficit in negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The present study tested the hypothesis that a deficit in the fast feedback time domain may play a role in the hormonal hyperresponsiveness in ethanol-exposed rats.

Sprague-Dawley offspring from prenatal ethanol (E), pair-fed (PF) and ad lib-fed control (C) groups were tested in two experiments. Expt 1 utilized a swim stress paradigm and tested animals at the trough of the corticosterone (CORT) circadian rhythm; Expt 2 utilized ether stress and tested animals at the peak of the circadian rhythm. Animals were injected sc with CORT or saline and were immediately subjected to either a 5 min swim stress or a 1 min ether stress. Half the animals were terminated immediately after stress (5 min post injection) and the rest were terminated 25 min later. Plasma levels of CORT and adrenocorticotropin (ACTH) were assayed to determine whether E animals differed from control animals in showing a CORT induced blunting of the ACTH response to the stressor, indicating alterations in fast feedback regulation.

Injection of CORT significantly blunted the ACTH response to swim stress (Expt 1) in E, PF and C females and males compared to their saline injected counterparts. There were no significant differences among groups. Similarly, CORT injected males in E, PF and C groups all exhibited a significantly blunted ACTH response to ether stress (Expt 2). CORT injected C females also exhibited a significantly blunted ACTH response to

ii

ether stress, while E females showed a clear decrease in plasma CORT that approached significance, indicating functional fast feedback in E and C females. However, PF females showed a clear deficit in fast feedback regulation.

Together, these data suggest that: 1) CORT injection can serve as a fast feedback signal that can blunt the ACTH response to a stressor; 2) prenatal ethanol exposure does not produce a deficit in HPA feedback regulation in the fast feedback time domain.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER I: INTRODUCTION

Fetal Alcohol Syndrome (FAS) results from excessive, chronic alcohol consumption during pregnancy. Children who are diagnosed with FAS have distinct craniofacial anomalies, growth retardation and CNS neurodevelopmental abnormalities. In addition to these diagnostic characteristics, other characteristics such as hyperactivity, learning difficulties, and poor impulse control are also frequently present. Animals exposed prenatally to ethanol (E animals) have deficits similar to children prenatally exposed to ethanol. E animals show low birth weight, altered brain development, learning and behavioral deficits, and neuroendocrine abnormalities. The neuroendocrine abnormalities observed after prenatal ethanol exposure in E animals are the focus of the present study.

Data have shown that prenatal ethanol exposure results in long-term effects on the organism's ability to respond and adapt to stress, as measured by alterations of hypothalamic-pituitary-adrenal (HPA) function. In rats, the effects of prenatal ethanol exposure on HPA activity can be observed through several stages of maturation. Immediately after parturition E animals show elevated basal brain, plasma and adrenal corticosterone (CORT) and elevated stress plasma CORT levels in comparison to control animals (Weinberg, 1989; Taylor, Branch, Cooley-Matthews, & Poland, 1982; Kakihana, Brutte, & Moore, 1980). During the preweaning period, the first three weeks of life, normal animals exhibit a period of reduced adrenocortical activity to stress or hyporesponsiveness, referred to as the stress nonresponsive period (Sapolsky & Meaney, 1986). E animals show an even greater HPA hyporesponsiveness to stressors compared to

1

control animals during this period (Angelogianni & Gianoulakis, 1989; Taylor, Branch, Nelson, Lane, & Poland, 1986; Weinberg, 1989). Reduced responsiveness in E compared to control animals has been observed following stressors such as ether, novelty, saline injection, cold stress, and alcohol and morphine challenge (Weinberg, 1989; Taylor, et al, 1982; Kakihana, *et al*, 1980). In contrast to the hyporesponsiveness observed during the preweaning period, in adulthood E animals exhibit HPA hyperresponsiveness. Increased HPA activation following acute stressors, including cardiac puncture, noise/shake, and continuous or intermittent footshock stress has been observed (Taylor, Branch, Liu, & Kokka, 1982; Nelson, Taylor, Redei, Branch, & Lewis, 1984). In addition, following chronic or repeated stress, E animals have been shown to exhibit delayed habiutation and/or delayed recovery to basal levels as reflected in increased or prolonged plasma elevations of CORT, ACTH and/or β-EP (Weinberg, Taylor, Gianoulakis, 1996; Taylor, Branch, Van Zuylen, & Redei, 1986).

A possible mechanism for the hormonal hyperresponsiveness and/or deficits in recovery following exposure to stress in E animals is a deficit in glucocorticoid negative feedback regulation of the HPA axis. Glucocorticoids exert negative feedback in three time domains and through multiple sites in the brain (Keller-Wood & Dallman, 1984). CORT fast feedback inhibition occurs within seconds to 1 hr of an initial stress induced rise in CORT and is sensitive to the rate increase of glucocorticoids. Fast feedback prevents further release of CORT by inhibiting release of ACTH and corticotropin releasing hormone (CRH). Intermediate feedback occurs between 2 - 10 hrs after an initial stress induced rise in CORT. In addition to inhibition of release of ACTH and CRH, synthesis of these hormones is also inhibited during intermediate feedback. Slow

feedback occurs after 12 or more hours of prolonged CORT exposure and as in intermediate feedback, CRH and ACTH synthesis and release are both inhibited. However, unlike fast and intermediate feedback, slow feedback occurs most often in pathological conditions (Keller-Wood, & Dallman, 1984). Previous research indicates that E animals may have a feedback deficit in one or more of these time domains. Nelson, Redei, Liebeskind, Branch, and Taylor (1985) found elevated basal CORT levels in E females 4 hr after dexamethasone (DEX, a synthetic glucocorticoid) administration, suggesting a possible deficit in intermediate feedback. Research in our laboratory supports and extends these findings that E animals show a deficit in the intermediate feedback time domain (Osborn, Kim, Yu, Herbert, & Weinberg, 1996). We demonstrated that at the trough of the CORT circadian rhythm, E females showed increased stress CORT levels at 3 and 6 hrs post DEX injection. At the peak of the CORT circadian rhythm, E males showed increased stress CORT levels 3 hrs following DEX injection while E females showed increased levels of both CORT and ACTH (Osborn et al, 1996). In addition to a deficit in intermediate feedback, other research suggests a deficit in fast feedback in E animals. Taylor et al (1986; 1988) found that E females have elevated ACTH levels 10 min after a 1 min intermittent footshock stress compared to controls. Although Taylor et al (1986; 1988) found that hormonal hyperresponsiveness in E animals exists during the fast feedback time domain, it does not conclusively show that E animals have a deficit in fast feedback. Further experiments using paradigms of fast feedback are required to confirm and extend this suggestion to other stressors, time points in the corticoid circadian rhythm, and to males.

The purpose of the present study was to examine whether a deficit in fast feedback regulation underlies the observed hormonal hyperresponsiveness and/or delays in recovery from stress in E animals. Two fast feedback paradigms, one utilizing swim stress (Expt 1) and one utilizing ether stress (Expt 2) were employed. In both paradigms animals were injected with either CORT or saline, subjected to a stressor, and blood samples were collected at 5 min or 30 min following initiation of stress for analysis of CORT and ACTH. CORT injection was designed to produce a rapid rise in plasma CORT which served as a negative feedback signal to the hypothalamus, pituitary and higher brain centers, capable of inhibiting ACTH secretion to the subsequent stressor. This procedure was based on a validated paradigm of fast feedback developed by Young, Akana and Dallman (1990) who found that CORT injection resulted in a significantly blunted ACTH response to swim stress at both 5 and 30 min post injection, i.e., saline injected animals showed a significantly greater ACTH response to the swim stress than CORT injected animals.

In the present study, Expt 1 utilized a 5 min swim stress during the trough of the corticoid circadian rhythm (AM, 2-4 hrs after lights on) and Expt 2 utilized a brief ether stress during the peak of the corticoid circadian rhythm (PM, 1-2 hrs prior to lights off). The use of two different stressors allowed examination of the robustness of the effects of prenatal ethanol exposure on fast feedback. Two time points in the corticoid circadian rhythm were chosen for examination because it has previously been shown that HPA sensitivity to feedback differs during the trough and peak of the circadian corticoid rhythm. The deficit in intermediate feedback in E animals was more pronounced in the PM compared to the AM (Osborn et al, 1996). If a deficit in fast feedback does underly

4

HPA hyperresponsiveness and/or delays in recovery from stress, CORT injected E animals should not exhibit a reduced ACTH response, compared to saline injected animals, in response to either the swim or ether stress.

CHAPTER II: METHODS

I. Animals and Mating:

Sprague-Dawley rats (females, 200-250g, n = 45; males, 250-275g, n = 20) were obtained from the Animal Care Centre, University of British Columbia, Vancouver, B.C., Canada. After a 1 - 2 week adaptation period, females were placed singly in hanging stainless steel cages (25 x 18 x 18cm), with mesh front and floor, with a male. Cage papers were checked daily for the presence of a vaginal plug, indicating day 1 of gestation (G1). A 24 hr light/dark cycle was maintained with lights on from 0600 - 1800 hr. Temperature was controlled at 21°C. During mating animals were allowed *ad libitum* access to standard laboratory chow (Jamieson's Pet Food Distributors Ltd., Delta, B.C.) and water. Two replicate breedings were done, each with 45 females. All animal use procedures were in accordance with the Canadian Council on Animal Care and the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of British Columbia Animal Care Committee.

II. Diets and Feeding:

On G1 females were housed in polycarbonate cages (24 x 16 x 46 cm) and randomly assigned to one of three prenatal treatment groups: 1) Ethanol-fed (E) females received a liquid diet containing 36% ethanol-derived calories; 2) Pair-fed (PF) females received a liquid diet identical in composition to that of E females, with maltose-dextrin isocalorically substituted for ethanol, in an amount equal to that consumed by an E

female, per kg body weight/ per day of gestation; 3) Ad lib-fed control (C) females received ad libitum access to standard laboratory rat chow and water.

Diets were formulated to meet the nutritional requirements of pregnant dams (Weinberg & Bezio, 1987) and were prepared by Bio-Serv, Inc (Frenchtown, NJ). Animals were maintained on experimental diets until G21. Bottles were weighed and replaced daily at approximately 1600 hr. This feeding schedule was designed to eliminate the previously observed shift in the corticoid rhythm that occurs in animals on a restricted feeding schedule, such as that of the PF females (Gallo & Weinberg, 1981).

On day 1 of birth, designated postnatal day 1 (PN1), dam and pups were weighed and litters culled to 5 females and 5 males. Pregnant females and pups remained undisturbed except for weighing and cage changing on G1, 7, 14, and 21 and PN1, 8, 15, and 22. On PN22 pups were weaned and rehoused and remained group housed until testing, at 70-90 days of age.

III.   Testing Procedures and Blood Sampling:

Animals were randomly selected from each litter; no more than one female and one male from any one litter were chosen for each test condition to ensure that no condition was biased by litter effects. Two fast feedback paradigms, one utilizing swim stress (Expt 1) and one utilizing ether stress (Expt 2) were employed. In both paradigms animals were injected with either CORT or saline, subjected to a stressor, and blood samples were collected at 5 min or 30 min following injection. CORT injection was designed to produce a rapid rise in plasma CORT which served as a negative feedback signal capable of inhibiting ACTH secretion to the subsequent stressor. In the interest of

decreasing the number of animals sacrificed, and based on the results of Expt 1, Expt 2 utilized fewer animals at the 5 min time point. Corticosterone was obtained from Sigma Chemical Co., St. Louis, MO, USA, and dissolved in 10% ethanol to form a stock solution of 500 µg/ml. Working solutions were made to provide an injection volume of approximately 1 ml for each animal, with CORT concentrations of 30-90 µg/100 g body weight. In both experiments, blood samples were obtained by decapitation. Blood was collected on ice in 12 x 75mm polystyrene tubes containing 7.5 mg EDTA and 1000 KIU aprotinin. Blood was centrifuged at 3600 x g for 10 min at 4°C and stored in polypropylene microcentrifuge tubes at -70°C until the time of assay.

A.   Experiment 1

Methods of testing followed those described by Young *et al* (1990). All testing occurred within the trough of the CORT circadian rhythm, 2 - 4 hrs after lights on (AM). On the day of testing, animals were removed from the colony room to an adjacent laboratory and injected sc with either CORT (males, 30µg/100g body weight, females, 60µg/100g body weight) or physiological saline (0.9% NaCl). Immediately after injection animals were subjected to a 5 min swim stress in a 28.5 x 28.5 x 44 cm container. Immediately after the swim half the animals from each experimental condition were terminated (time = 5 min post injection); the remaining animals were returned to their home cages and placed in a quiet holding room for 25 min, and then terminated (time = 30 min post injection). The containers were cleaned and refilled daily. Water was allowed to equilibrate to room temperature for approximately 20 hr before testing occurred.

A.   Experiment 2

Methods of testing were similar to Expt 1, however, exposure to ether vapors was used as the stressor. All testing occurred within the peak of the CORT circadian rhythm, 1 - 2 hr prior to lights off (PM). On the day of testing, animals were removed from the colony room to an adjacent laboratory and injected sc with either CORT (males, 30μg/100g body weight, females, 90 μg/100g body weight) or physiological saline (0.9% NaCl). The higher dose of CORT for females in this PM study was chosen based on pilot studies and previous research in our laboratory and by others indicating that HPA regulation in the PM requires higher levels of glucocorticoids than in the AM (Osborn *et al*, 1996; Akana, Cascio, Du, Levin, & Dallman, 1986). Immediately after injection animals were returned to their home cages and at approximately 4.5 min post injection were subjected to ether vapors in an enclosed container (20 x 20 x 20 cm.). Immediately after the ether stress half the animals from each experimental condition were terminated (time = 5 min post injection); the remaining animals were returned to their home cages and placed in a quiet holding room for 25 min and then terminated (time = 30 min post injection).

IV.   Assays

Total CORT (bound plus free) was measured by radioimmunoassay (RIA) using our adaptation of the method of Kaneko *et al* (1981) (Weinberg & Bezio, 1987). Antiserum was obtained from Immunocorp, Montreal, PQ, Canada and tracer was obtained from Mandel Scientific, Guelph, ON, Canada. Dextran coated charcoal was

used to absorb and precipitate free steroids after incubation. The intra and interassay coefficients of variation were 1.55% and 4.26%, respectively.

ACTH was assayed using a modification of the Incstar ACTH Equilibrium RIA kit (Incstar Inc, Stillwater, MN, USA). All reagent volumes were halved and 50 μl of plasma per sample was used. The mid-range intra and interassay coefficients of variation were 3.9% and 6.5%, respectively.

V.   Statistical Analysis

An overall analysis of variance (ANOVA) for the factors of sex, prenatal treatment, injection type and time was performed. Significant main effects were further analyzed by one way ANOVA, for the factor of injection type, for each sex and at each time point, to assess differences among prenatal treatment groups. Significant main and interaction effects were further analyzed by Tukey's post hoc analysis.

CHAPTER III: RESULTS

I.   Developmental Data

A.   Experiment 1.

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 11.6 ± 1.4, 13.0 ± 1.6 and 12.4 ± 1.1 g/ kg body weight for week 1, 2 and 3 of gestation, respectively.

A repeated measures ANOVA on maternal weight gain during pregnancy revealed significant main effects of Day, $F(3,114) = 1081.40$, $p<0.001$, and Group, $F(3,117) = 89.49$, $p<0.001$ (Table 1, Panel A). Tukey's post hoc tests revealed that all females increased significantly in weight from G7 to G21, p's<0.001. In addition E and PF females weighed less than C females, p's<0.005. Analysis of maternal weights during lactation again revealed a main effect of Day, $F(3,105) = 71.63$, $p<0.001$, and a Group x Day interaction, $F(6,105) = 3.74$, $p<0.005$ (Table 1, Panel B). PF females weighed significantly less than C females on PN1, $p<0.0005$. E females did not differ in weight from PF or C females. By PN8 there were no significant differences among groups.

There were no significant differences among groups for litter size or number of liveborn or stillborn pups. Analysis of pup body weights indicated main effects of Day, $F(3,180) = 555.92.49$, $p<0.001$, and Group, $F(5,60) = 8.62$, $p<0.001$ (Table 2). All pups showed a significant increase in weight over the 4 week pre-weaning period. In addition, E animals weighed significantly less than C animals, p's<0.001, throughout the preweaning period.

B.   Experiment 2.

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 8.9 ± 1.1, 11.4 ± 0.8 and 11.0 ± 0.8 g/ kg body weight for week 1, 2 and 3 of gestation, respectively.

A repeated measures ANOVA on maternal weight gain during pregnancy revealed a significant main effect of Day, $F(3,105) = 947.00$, $p<0.001$, and a Group x Day interaction, $F(6,105) = 9.08$, $p<0.001$ (Table 3, Panel A). E and PF females weighed less than C females on G7, G14, G21, p's<0.0005. Analysis of maternal weights during lactation again revealed a main effect of Day, $F(3,108) = 26.17$, 0.001, and a Group x Day interaction, $F(6,105) = 3.74$, $p<0.005$, (Table 3, Panel B). E and PF females weighed significantly less than C females on PN22, p's $= 0.001$.

There were no significant differences among groups for litter size or number of liveborn or stillborn pups. Analysis of pup body weights indicated main effects of Day, $F(3,204) = 2104.62$, $p<0.0001$, and Group, $F(5,68) = 8.68$, $p<0.001$, and a Group x Day interaction, $F(15,204) = 5.50$, $p<0.0001$ (Table 4). E and PF animals weighed significantly less than C animals on PN22, p's<0.005.

II.   CORT and ACTH

A.   Experiment 1 (AM Swim Stress)

A.1. CORT.

5 min blood sampling time point

At 5 min post injection there was a significant main effect of injection type (saline, CORT) for both females, $F(1,50) = 140.34$, $p<0.001$ (Fig 1, Panel A), and males,

F(1,54) = 50.15, p<0.001 (Fig 1, Panel B). Injection of CORT resulted in significant increases in plasma CORT at 5 min compared to that in saline injected animals, suggesting a rise in plasma CORT large enough to provide a negative fast feedback signal.

30 min blood sampling time point

At 30 min post injection the main effect of injection type (saline, CORT) persisted for both females, F(1,50) = 17.76, p<0.001 (Fig 1, Panel C), and males, F(1,54) = 27.16, p<0.001 (Fig 1, Panel D). Animals that received an injection of CORT had significantly elevated plasma CORT levels compared to animals that received an injection of saline. Among females there was also a main effect of prenatal treatment, F (2,50) = 5.921, p<0.01. Overall, E females showed a small but significant elevation of plasma CORT levels at 30 min compared to control animals. CORT injection did not result in a significant increase in plasma CORT levels from 5 to 30 min in either females or males, suggesting that plasma CORT levels peaked approximately 5 min following injection. In contrast, in saline injected animals there was a significant increase in plasma CORT levels from 5 to 30 min in both females, F(1,50) = 78.06, p<0.001, and males, F(1,54) = 27.16, p<0.001.

A.2. ACTH.

5 min blood sampling time point

At 5 min there were no significant effects of prenatal treatment or injection type (saline, CORT) on plasma ACTH levels. The absence of a decrease in ACTH levels in CORT injected compared to saline injected animals suggests that 5 min may be too early to observe fast feedback.

30 min blood sampling time point

At 30 min, plasma ACTH levels were significantly lower in CORT injected compared to saline injected females, $F_{(1,49)} = 24.226$, $p<0.01$ (Fig 2, Panel C). Similarly, CORT injected males showed a small but significant decrease in plasma ACTH levels compared to saline injected males, $F_{(1,54)} = 7.643$, $p<0.01$ (Fig 2, Panel D). These results indicate functional fast feedback in all animals. In addition, overall plasma ACTH levels were significantly decreased at 30 min compared to 5 min in saline injected males, $F_{(1,55)} =47.15$, $p<0.001$, as well as in CORT injected males, $F_{(1,53)} = 88.92$, $p<0.001$ and females, $F_{(1,50)} = 68.98$, $p<0.001$. Saline injected females, however, had similar plasma ACTH levels at 30 min compared to 5 min. There were no effects of prenatal treatment at 30 min for either sex.

B.   Experiment 2 (PM Ether Stress)

B.1. CORT.

5 min blood sampling time point

Plasma CORT levels at 5 min post injection were similar to those reported in Expt 1. There was a significant main effect of injection type (saline, CORT) for both females, $F_{(1,22)} = 81.39$, $p<0.001$ (Fig 3, Panel A) and males, $F_{(1,23)} = 44.12$, $p<0.01$ (Fig 3, Panel B). CORT injection resulted in significant increases in plasma CORT at 5 min compared to that in saline injected animals, replicating our data indicating a rise in plasma CORT large enough to provide a negative fast feedback signal.

30 min blood sampling time point

At 30 min post injection, the main effect of injection type (saline, CORT) persisted for both females, $F_{(1,50)} = 55.16$, $p<0.001$ (Fig 3, Panel C), and males, $F_{(1,51)}$

= 27.62, p<0.001 (Fig 3, Panel D). Elevated levels of plasma CORT were still present in CORT injected compared to saline injected animals at 30 min. CORT injection did not result in a significant increase in plasma CORT levels from 5 to 30 min in males, suggesting that plasma CORT levels peaked approximately 5 min following injection. In contrast, CORT injected females showed a significant CORT increase from 5 to 30 min, $F(1,36) = 9.70$, p<0.001, suggesting a different time course of response in females compared to males. As in Expt 1, saline injected males, $F(1,37) = 17.07$, p<0.001, and females, $F(1,36) = 77.42$, p<0.001, showed significantly higher plasma CORT levels at 30 min compared to 5 min. There was no effect of prenatal treatment at 5 or 30 min for either sex.

B.2. ACTH.

5 min blood sampling time point

As in Expt 1, at 5 min there were no significant effects of prenatal treatment or injection type (saline, CORT) on plasma ACTH levels in either females or males (Fig 4, Panel A and B). Similarly, the absence of a decrease in ACTH levels in CORT injected compared to saline injected animals suggests that 5 min may be too early to observe fast feedback.

30 min blood sampling time point

At 30 min there was a prenatal treatment x injection type (saline, CORT) interaction for females, $F(2,46) = 3.42$, p<0.05 (Fig 4, Panel C) and a main effect of injection type (saline, CORT) for males, $F(1,51) = 21.47$, p<0.001 (Fig 4, Panel D). CORT injected C females showed a significant decrease in ACTH levels, p<0.05, and CORT injected E females had a decrease that approached significance, p = 0.08, PF

15

females injected with CORT showed no change in ACTH and were similar to their saline injected counterparts. In contrast, all males, regardless of prenatal treatment, had lower ACTH levels if they received CORT compared to saline, and this decrease was greater than the decrease observed for males in Expt 1. These data indicate that fast feedback was functional in all animals except for PF females. Finally, there was a significant drop in plasma ACTH from 5 to 30 min in CORT injected males, $F(1,37) = 41.38$, $p<0.001$, and an increase in plasma ACTH in saline injected females, $F(1,35) = 16.09$, $p<0.001$. Saline injected males and CORT injected females did not show changes in ACTH levels from 5 to 30 min.

Table 1:
Maternal weights during gestation (A) and lactation (B) (g, mean ± SEM) for experiment 1.

(A)

Prenatal Treatment	Gestation			
	G1	G7	G14	G21
E n=13	*221.0 ± 3.3	*237.8 ± 3.5	*271.8 ± 3.8	*350.0 ± 6.4
PF n=14	*223.9 ± 4.4	*230.8 ± 5.2	*260.7 ± 6.4	*333.9 ± 8.8
C n=13	235.5 ± 7.1	270.6 ± 7.3	312.4 ± 8.8	397.3 ± 10.7

(E: ethanol, PF: pair-fed, C: ad lib-fed)

- Main effect of Day: F(3,114) = 1081.4, p<0.001, G7<G14<G21, p's<0.001
- Main effect of Group: F(3,117) = 89.49, p<0.001, E = PF<C, (*) p's<0.005

(B)

Prenatal Treatment	Lactation			
	PN1	PN8	PN15	PN22
E n=13	284.8 ± 3.5	317.3 ± 4.8	335.1 ± 3.2	318.8 ± 5.1
PF n=14	†276.0 ± 8.4	302.5 ± 8.4	323.7 ± 6.3	306.9 ± 5.8
C n=13	304.5 ± 8.1	313.4 ± 5.1	327.3 ± 8.2	315.5 ± 5.6

(E: ethanol, PF: pair-fed, C: ad lib-fed)

- Main effect of Day: F(3,105) = 71.63, p<0.001
- Group x Day interaction: F(6,105) = 3.74, p<0.005
- For PN1: PF<C, (†) p<0.0005

Table 2: Mean pup weights (g, mean $\pm$ SEM) for experiment 1.

	PN1 Male	Female	PN8 Male	Female	PN15 Male	Female	PN22 Male	Female
E n=12	*5.7 $\pm$ 0.1	*5.4 $\pm$ 0.1	*14.3 $\pm$ 0.5	*13.6 $\pm$ 0.5	*28.5 $\pm$ 0.9	*27.4 $\pm$ 0.7	*42.7 $\pm$ 1.3	*41.2 $\pm$ 1.2
PF n=12	5.9 $\pm$ 0.2	5.5 $\pm$ 0.2	15.2 $\pm$ 0.5	14.3 $\pm$ 0.5	30.7 $\pm$ 0.9	29.2 $\pm$ 0.8	47.6 $\pm$ 1.3	44.9 $\pm$ 1.2
C n=12	6.5 $\pm$ 0.2	6.2 $\pm$ 0.1	19.1 $\pm$ 1.5	18.1 $\pm$ 1.4	35.0 $\pm$ 1.9	33.6 $\pm$ 1.9	52.3 $\pm$ 1.5	50.3 $\pm$ 1.0

(E: ethanol, PF: pair-fed, C: ad-lib fed)

- Main effect of Day, $F_{(3, 180)} = 555.92$, $p < 0.0001$, PN1<PN8<PN15<PN22, p's<0.00001
- Main effect of Group, $F_{(5,60)} = 8.62$, $p < 0.0001$, E<C, (*) p's<0.001

Table 3:
Maternal weights during gestation (A) and lactation (B) (g, mean $\pm$ SEM) for experiment 2.

(A)

Prenatal Treatment	Gestation			
	G1	G7	G14	G21
E n=14	266.2 $\pm$ 6.0	*270.4 $\pm$ 7.0	*302.1 $\pm$ 8.0	*375.3 $\pm$ 9.7
PF n=13	267.7 $\pm$ 5.3	*266.8 $\pm$ 5.6	*293.1 $\pm$ 6.3	*366.5 $\pm$ 8.5
C n=13	273.9 $\pm$ 4.9	299.0 $\pm$ 5.6	325.1 $\pm$ 7.5	418.5 $\pm$ 10.5

(E: ethanol, PF: pair-fed, C: ad lib-fed)

- Main effect of Day: $F(3,105) = 947.00$, $p<0.001$
- Group x Day interaction: $F(6, 105) = 9.08$, $p<0.001$
- For G7, G14 and G21: E = PF<C, (*) p's<0.0005

(B)

Prenatal Treatment	Lactation			
	PN1	PN8	PN15	PN22
E n=13	307.3 $\pm$ 8.0	338.0 $\pm$ 8.6	357.5 $\pm$ 8.4	†336.8 $\pm$ 8.2
PF n=12	302.6 $\pm$ 8.4	319.3 $\pm$ 8.1	344.6 $\pm$ 7.2	345.8 $\pm$ 10.1
C n=13	321.4 $\pm$ 6.0	339.7 $\pm$ 6.4	349.7 $\pm$ 14.4	389.6 $\pm$ 17.0

(E: ethanol, PF: pair-fed, C: ad lib-fed)

- Main effect of Day: $F(3,108) = 26.17$, $p<0.001$,
- Group x Day interaction: $F(6,105) = 3.74$, $p<0.005$
- For PN22: E<PF=C, (†) p's<0.001

Table 2: Mean pup weights (g, mean ± SEM) for experiment 1.

	PN1 Male	Female	PN8 Male	Female	PN15 Male	Female	PN22 Male	Female
E n=12	*5.7 ± 0.1	*5.4 ± 0.1	*14.3 ± 0.5	*13.6 ± 0.5	*28.5 ± 0.9	*27.4 ± 0.7	*42.7 ± 1.3	*41.2 ± 1.2
PF n=12	5.9 ± 0.2	5.5 ± 0.2	15.2 ± 0.5	14.3 ± 0.5	30.7 ± 0.9	29.2 ± 0.8	47.6 ± 1.3	44.9 ± 1.2
C n=12	6.5 ± 0.2	6.2 ± 0.1	19.1 ± 1.5	18.1 ± 1.4	35.0 ± 1.9	33.6 ± 1.9	52.3 ± 1.5	50.3 ± 1.0

(E: ethanol, PF: pair-fed, C: ad-lib fed)

- Main effect of Day, $F_{(3, 180)} = 555.92$, $p < 0.0001$, PN1<PN8<PN15<PN22, p's<0.00001
- Main effect of Group, $F_{(5, 60)} = 8.62$, $p < 0.0001$, E<C, (*) p's<0.001

Figure 1. Plasma CORT Levels (mean $\pm$ SEM) at 5 and 30 Minutes Post-Injection for Females and Males (n's = 8-10 per group).

At 5 min * main effect of CORT for females A) [$F(1, 50) = 140.34$, $p<0.001$] and males B) [$F(1, 54) = 50.15$, $p<0.001$]; CORT > SAL. At 30 min * main effect of CORT for females C) [$F(1, 50) = 17.76$, $p<0.001$] and males D) [$F(1, 54) = 27.16$, $p<0.001$]; CORT > SAL. # Main effect of prenatal treatment C) [$F(2, 50) = 5.291$, $p<0.01$]; E>C, $p<0.01$

CORT Levels Following AM Swim Stress

| | Ethanol | Pair-fed | Ad lib-fed |

5 min   30 min

Plasma CORT (ug/dl)

A. Females   C. Females

B. Males   D. Males

SAL CORT   SAL CORT   SAL CORT   SAL CORT   SAL CORT   SAL CORT

Figure 2. Plasma ACTH Levels (mean $\pm$ SEM) at 5 and 30 Minutes Post-Injection for Females and Males (n's = 8 - 10 per group).

At 5 min, no main effects of CORT or prenatal treatment. At 30 min * main effect of CORT for females C) [$F(1,49) = 24.226$, $p<0.01$] and males D) [$F(1, 54) = 7.643$, $p<0.01$]; CORT < SAL.

ACTH Levels Following AM Swim Stress

Figure legend: Ethanol / Pair-fed / Ad lib-fed

5 min   30 min

Plasma ACTH (pg/ml)

A. Females
B. Males
C. Females
D. Males

Y-axis values: 0, 110, 220, 330, 440, 550

X-axis: SAL CORT   SAL CORT   SAL CORT

24

Figure 3. Plasma CORT Levels (mean $\pm$ SEM) at 5 and 30 Minutes Post-Injection for

Females and Males (5 min: n's = 4 - 5 per group; 30 min: n's = 8 - 10).

At 5 min * main effect of CORT for females C) [$F_{(1,22)}$ = 81.39, p<0.001] and males B)

[$F_{(1,23)}$ = 44.12, p<0.01]; CORT > SAL. At 30 min * main effect of CORT for females

C) [$F_{(1,50)}$ = 55.16, p<0.001] and males D) [$F_{(1,51)}$ = 27.62, p<0.001]; CORT > SAL.

CORT Levels After PM Ether Stress

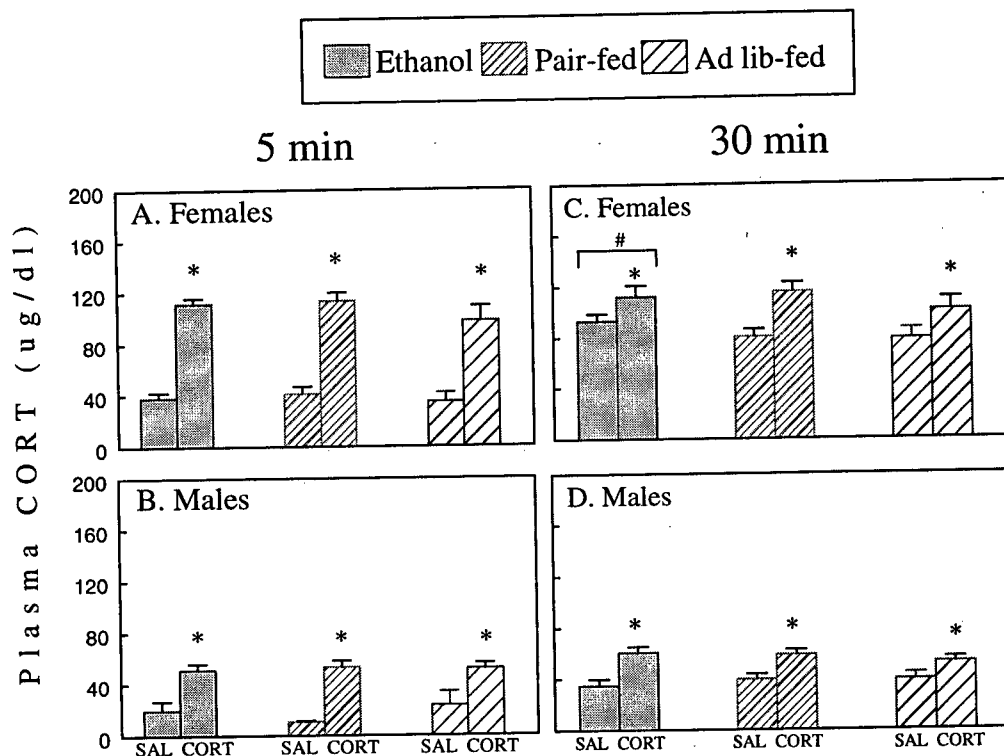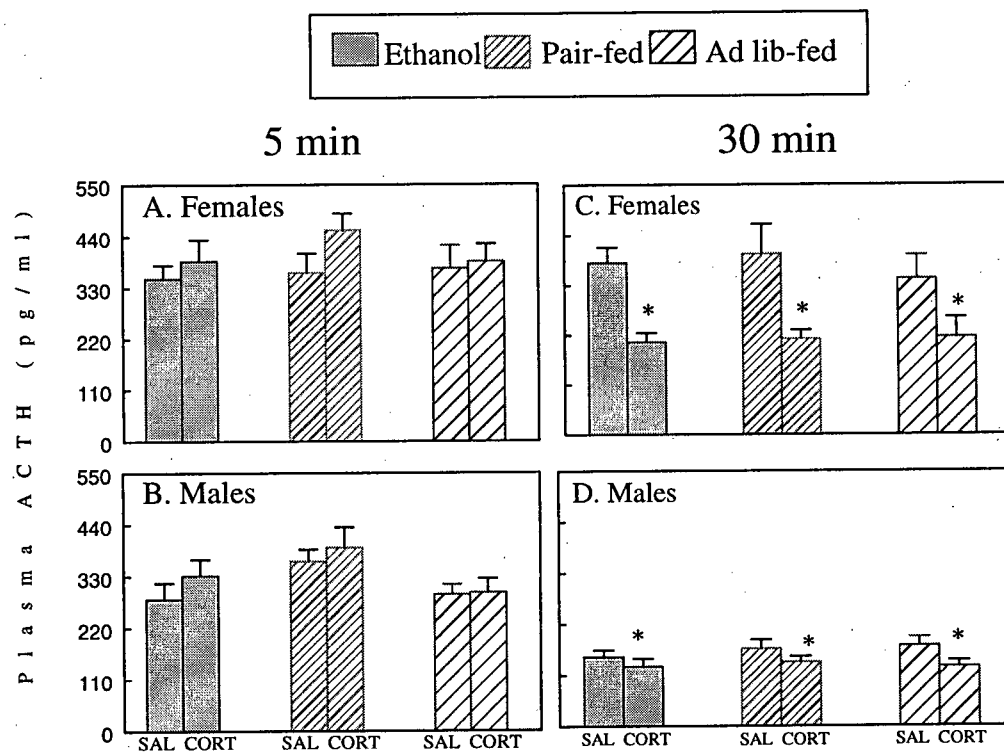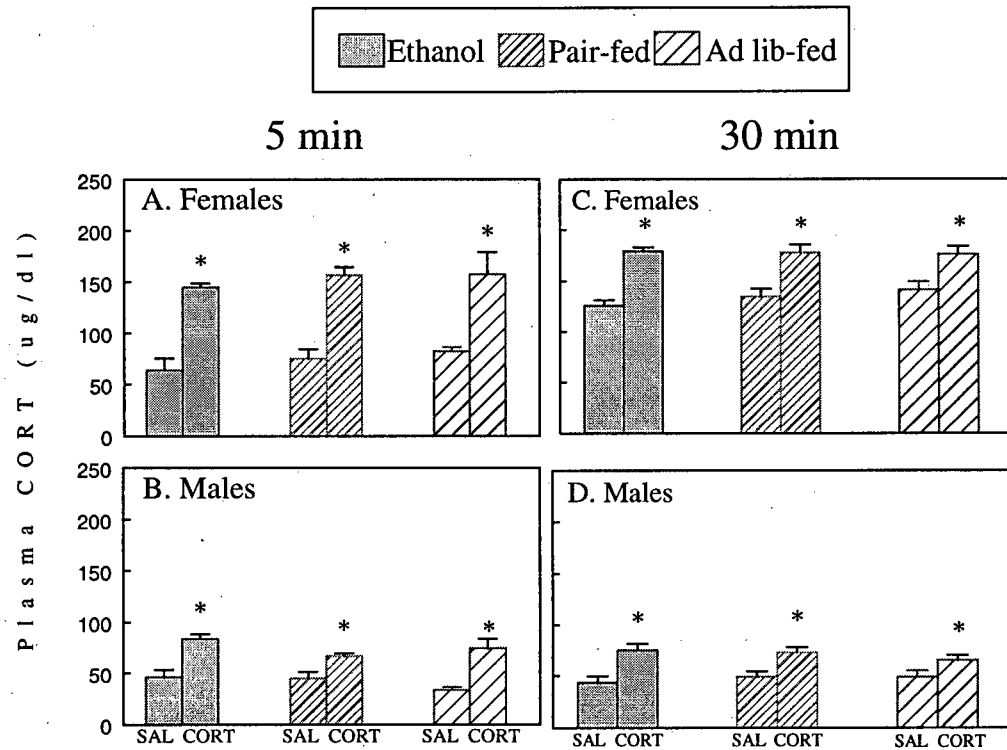Figure 4. Plasma ACTH Levels (mean $\pm$ SEM) at 5 and 30 Minutes Post-Injection for

Females and Males (5 min: n's = 4 - 5 per group; 30 min: n's = 8-10 per group).

At 5 min no main effects of CORT or prenatal treatment. At 30 min * CORT by prenatal

treatment interaction for females C)[$F(2, 46) = 3.42$, $p<0.05$]. * Main effect of CORT for

males D) [$F(1, 51) = 21.47$, $p<0.001$]; CORT < SAL.

ACTH Levels After PM Ether Stress

Legend: Ethanol | Pair-fed | Ad lib-fed

5 min | 30 min

A. Females
B. Males
C. Females
D. Males

y-axis: Plasma ACTH (p g / m l)

x-axis labels: SAL CORT SAL CORT SAL CORT
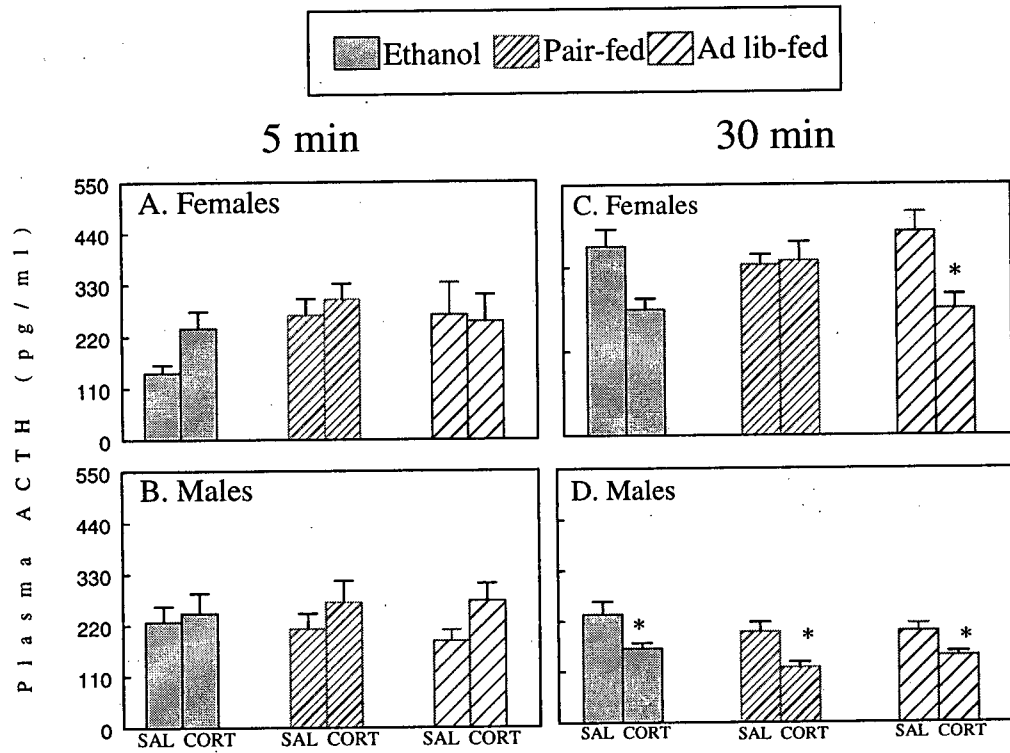
CHAPTER IV. DISCUSSION

The results from these experiments suggest that a deficit in the glucocorticoid fast feedback time domain does not mediate the hyperresponsiveness and/or delays in recovery from stressors that occur in E animals. Administration of CORT resulted in a similar blunting of the ACTH response to swim stress in females and males from all prenatal treatment groups in the AM, at the trough of the CORT circadian rhythm. Similarly, in the PM, at the circadian peak, E, PF and C males as well as E and C females showed blunting of the ACTH response to ether stress after CORT injection indicating a functional fast feedback response. Interestingly, although PF females showed significant blunting of their ACTH response to swim stress, in the AM, they did not exhibit a blunted ACTH response to ether stress in the PM after CORT injection, suggesting that PF females may have some deficit in fast feedback inhibition, at least when measured at the circadian peak. In addition, E females, but not E males, exhibited HPA hyperresponsiveness after AM swim stress as evidenced by their elevated CORT levels compared to C females at 30 min post injection.

Although no effects of prenatal ethanol exposure on fast feedback regulation of the HPA axis were observed in this study, E pups weighed less than C pups for one or all days measured during the preweaning period. This finding is consistent with previous findings in our laboratory and others (Weinberg, 1988; Weinberg, Taylor, & Gianoulakis, 1996; Taylor *et al*, 1982; Ogilvie, & Rivier, 1997), indicating that prenatal ethanol exposure adversely affects offspring growth.

The possibility that E animals exhibit a deficit in fast feedback was suggested by the data of Taylor et al (1986). They found elevated plasma ACTH levels 10 min after a 1

29

min intermittent footshock stress, suggesting, but not directly implicating, a possible deficit in the fast feedback domain. Interestingly, Taylor *et al* (1986) found hyperresponsiveness in E animals to footshock at only one time point measured, 10 min. ACTH levels were not elevated at 5 or 15 min post stress. It is possible that a deficit in fast feedback regulation does exist in E animals, but only during a short period within the fast feedback domain that was not sampled in the present study. It is also possible that the hyperresponsiveness observed during the fast feedback time domain is not a robust finding, but occurs only under specific stress conditions. For example, Ogilvie & Rivier (1997) did not find hyperresponsiveness 10 min after 10 min of mild footshock in non handled E females or males, in either fostered or non-fostered conditions.

In the present study, injection with CORT did result in increased plasma levels of CORT capable of providing a negative fast feedback signal across all prenatal treatment groups. The present study used two similar fast feedback paradigms, one utilizing swim stress (Expt 1) and one utilizing ether stress (Expt 2). These two paradigms were based on a paradigm developed by Young *et al* (1990) and data from our study were consistent with their findings. CORT injection in both the AM and the PM resulted in a significant increase in plasma CORT levels at 5 min, suggesting a rapid rate of increase in CORT levels after injection, which was capable of producing a negative feedback signal. Indeed, a blunted ACTH response to both swim and ether stress was observed at 30 min post injection, indicating effective fast feedback. However, in contrast to Young *et al* (1990), the present data do not indicate fast feedback regulation at 5 min post CORT injection. In addition, Young *et al* (1990) found a decrease in plasma CORT levels from 5 to 30 min while the present study found either no difference or an increase in CORT levels from 5

to 30 min. The discrepancy between the findings of this study and the findings reported by Young *et al* (1990) may be due to age and weight differences in the animals used in the two studies which could affect distribution and metabolism of CORT. The weight of the male rats used by Young *et al* (1990) was 100 – 150g, approximately 30 days of age, while the present study used animals with mean weights at time of testing of 250g (females) and 450g (males), at approximately 70 - 90 days of age. It is also possible that a small difference in water temperature may have contributed to differences in the results of the two studies. Young *et al* (1990) reported water temperature of 25° C while the present study allowed water to equilibrate to room temperature over a 20 hr period.. A temperature difference of only 5° C has previously been shown to result in differences in swim stress induced analgesia (Mogil, Sternberg, Kest, Marek, & Liebeskind, 1993). However, at present there is no strong evidence supporting effects of water temperature on hormonal responses to stress and this hypothesis remains speculative.

Although, the present data did not support the hypothesis that a deficit in glucocorticoid fast feedback underlies hormonal hyperresponsiveness and/or delays in recovery following stress observed in E animals, our previous data demonstrates that E animals do show a deficit in HPA feedback regulation during the intermediate feedback time domain. In a previous study in this laboratory, E females showed higher stress levels of plasma CORT at 3 and 6 hr post DEX injection when tested in the circadian trough. At the circadian peak both E females and E males showed increased stress levels of plasma CORT, while E females also showed increased plasma levels of ACTH compared to PF and control animals (Osborn *et al* 1996). Since fast and intermediate feedback inhibition occur through different mechanisms, the finding of a deficit in E animals in one time

domain but not another is not surprising. First, fast feedback inhibition occurs while plasma CORT levels are rising, and inhibition depends on the rate of increase of plasma CORT. A CORT increase of at least 1.3ug/100ml/min is required. In contrast, intermediate feedback depends on the total dose of CORT and the duration of CORT exposure (Keller-Wood, & Dallman, 1984). Second, in fast feedback, CRH and ACTH release are inhibited, while both release and synthesis of these hormones are inhibited in intermediate feedback (Keller-Wood & Dallman, 1984). Third, fast feedback may exert inhibitory effects through interactions with catecholaminergic neurons, while intermediate feedback may act through serotonergic neurons (Kaneko & Hiroshige, 1978).

Our previous study demonstrating a deficit in intermediate feedback in E animals used DEX to provide a negative feedback signal whereas the present study used CORT. While the actions of DEX are similar to CORT, there are important differences. One dissimilarity is that DEX binds preferentially to the anterior pituitary, while CORT binds throughout the brain (De Kloet, Wallach, & McEwen, 1975). Another difference is that the two glucocorticoids differ with respect to binding affinities. DEX has a higher affinity for Type II (glucocorticoid) receptors; conversely, CORT has a higher affinity for Type I (mineralcorticoid) receptors (Reul & DeKloet, 1985). Therefore, it is possible, that these differences account for the finding of a deficit in the intermediate feedback time domain but not the fast feedback time domain.

The present data also demonstrate a sex difference. In the AM after swim stress, E females show a slight but significant increase in plasma CORT levels at 30 min post injection compared to C females. Our previous data indicate that hyperresponsiveness to

stressors may be exhibited differentially in E females and E males depending on the type of stressor, time of blood sampling, and the hormonal endpoint examined (Osborn et al, 1996; Weinberg, Taylor, & Gianoulakis, 1996; Weinberg, 1988; Weinberg, 1992). While both E females and E males exhibit HPA hyperresponsiveness to stressors (Lee, Imaki, Vale, & Rivier, 1990; Weinberg, 1992, Weinberg, Taylor, & Gianoulakis, 1996), females may show this heightened response more frequently and under a greater variety of situations than males (Osborn et al, 1996; Kelly, Mahoney, Randich, & West, 1991; Weinberg, 1988). There is a normal sexual dimorphism in HPA activity (Kitay, 1961), and in adulthood females typically show greater HPA responsivity than males (Lesniewska, Miskowiak, Nowak, & Malendowicz, 1990; Rivier, 1994). Female rats also release more ACTH and CORT in response to ethanol administration than males (Rivier, 1993). If increased responsivity is also present in female fetuses, prenatal ethanol exposure may differentially affect females and males *in utero*, thus resulting in sex differences in HPA activity in adulthood. Clearly, further investigation is needed to examine the differential HPA responsiveness to stressors in females compared to males prenatally exposed to ethanol.

In addition to differential HPA responsiveness of E females and males, sex differences were found across all prenatal treatment groups possibly reflecting the sexual dimorphism in the HPA axis. Increased plasma levels of CORT following CORT injection resulted in decreased ACTH levels at 30 min compared to 5 min after AM swim stress for both females and males, whereas injection of saline resulted in decreased ACTH levels at 30 min compared to 5 min in males, but not in females. Sex differences were also found after PM ether stress. Both saline injected females and males showed

significantly increased plasma levels of CORT at 30 compared to 5 min, however, injection of CORT resulted in peak plasma CORT levels at approximately 5 min post injection in males. In contrast, CORT injected females showed higher plasma CORT levels at 30 compared to 5 min, suggesting that peak levels in females follow a different time course than in males. This latter finding is consistent with data of Kitay (1961) who reported that ether stress produced higher and more prolonged plasma CORT elevations in females than in males. Kitay (1961) interpreted this finding as an increased sensitivity of the female adrenal gland to stimulation by ACTH, or that the pituitary gland releases more ACTH in response to ether. The sex differences observed at both the AM and PM may be due to an interaction between sex steroid hormones, the HPA axis and the time of day. The increased responsiveness of the HPA axis of female rats to ether may depend on stimulatory actions of estradiol (Lesniewska, Miskowiak, Nowak & Molendowicz, 1990). Estrogen elevates and prolongs activation of the HPA axis after stress and interferes with Type II receptor down regulation in the hippocampus after 4 days of RU 28362 (Type II receptor agonist) administration (Burgess & Handa, 1992).

The slightly different time course for CORT and ACTH levels over the 30 min test in Expt 1 compared to Expt 2 could be due to a number of factors including the change in sampling time from the trough to the peak of the circadian rhythm or the difference in stressor type. The stressor used in the AM (swim) may involve both psychological and physical or physiological stress, in contrast to the PM stressor (ether vapors) which is likely to involve primarily physiological stress. Indeed, repeated swim stress is used as paradigm of behavioral despair (Porsolt, 1978). Another possible explanation for the slightly different time course is the timing of sampling. There is a

diurnal variation in stress-induced HPA hormone release (Bradbury, Cascio, Scribner, & Dallman, 1991; Kant, Mougey, & Meyerhoff, 1986). CORT, ACTH, β-EP and β-lipotropin release are greater in the AM than in the PM following a variety of stressors (Bradbury *et al*, 1991, Kant *et al*, 1986). Therefore, differential sensitivity of the HPA axis to stressors may underly observed differences between the AM and the PM.

Finally, although no effects of prenatal ethanol exposure on fast feedback were observed, PF females had a clear deficit in fast feedback following ether stress in the PM. Although pair-feeding provides a necessary nutritional control group, PF dams are fed on a restricted meal feeding schedule. That is, PF dams receive an amount of diet matched to an E dam in g/kg body weight for the same day of gestation, with maltose-dextrin isocalorically substituted for ethanol. Although E females have *ad-libitum* access to food, they consume less than they would if their diets did not contain ethanol. This may be partly due to aversive or appetite suppressant effects or the high caloric value of ethanol . Thus, PF dams receive a ration that is less than they would consume *ad libitum*. They typically consume this ration within a few hours after it is presented and are then food deprived until the next day's feeding. Therefore, although both groups are receiving the same number of calories, PF dams experience deprivation, while E dams do not. Indeed, previous studies from our laboratory support the finding of altered HPA activity in PF dams. PF dams may show prolonged corticoid levels following stress and decreased CBG binding capacity on day 21 of gestation, compared to E and C dams (Weinberg & Gallo, 1981; Weinberg & Bezio, 1987). In this respect, pair-feeding may be a type of prenatal stressor. Prenatal stress is believed to cause effects on offspring through an elevation of maternal corticoids which then pass through the placenta to the fetus (Joffe, 1978).

Prenatally stressed offspring have been shown to exhibit elevated plasma CORT and ACTH levels following restraint stress, open field testing and saline injection (McCormick, Smythe, Sharma, & Meaney, 1995; Weinstock, Matlina, Maor, Rosen, & McEwen, 1992; Peters, 1982). Delayed habituation of the adrenocortical response to repeated stress exposure has also been observed in prenatally stressed offspring (Fride, Dan, Feldon, Hale, & Weinstock, 1986). Weinstock (1997) suggests that the elevated levels of plasma CORT and ACTH following stress may reflect a feedback deficit in prenatally stressed animals. The results of the present study support this suggestion with the observed finding of a deficit in the fast feedback time domain in offspring of PF dams at the peak of the CORT circadian rhythm. At present, there is no evidence of a feedback deficit in other time domains or at the trough of the circadian rhythm. For example, neither PF females or males show a feedback deficit in the intermediate time domain in either the AM or the PM (Osborn *et al*, 1996). Importantly, the hyperresponsiveness observed in E and PF animals likely occur through different mechanisms. PF animals are exposed to elevated maternal corticoids, which may suppress fetal HPA activity and thus alter the development of HPA regulation. However, the HPA axis of an E fetus is exposed to both the inhibitory actions of ethanol induced elevations of maternal corticoids that cross the placenta and direct HPA stimulatory effects of ethanol that itself crosses the placenta. Thus the effects of prenatal ethanol exposure on the offspring HPA axis result from an interaction of both inhibitory and stimulatory effects.

In summary, although previous research in our laboratory has found a deficit in the intermediate time domain in E animals, the present study suggests that a deficit does not occur in the fast feedback time domain. Previous work in our laboratory also suggests

that in addition to a deficit in intermediate feedback, increased sensitivity of the anterior pituitary to CRH stimulation may also mediate hyperresponsiveness. During the trough of the circadian rhythm, E females showed increased ACTH and CORT release compared to PF and C females in response to CRH administration under DEX suppression (Yu, 1996). In addition, research in our laboratory suggests that altered feedback inhibition of neurotransmitter stimulated CRH release may be involved in the hyperresponsiveness observed in E animals. Norepinephrine, which stimulates CRH release (Plotsky, 1987), is decreased in cortical areas and the hypothalamus following restraint stress in E animals compared to control animals (Rudeen & Weinberg, 1993). 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 CRH secretion may play a role in HPA hyperactivity.

Finally, the present data may have important clinical implications. Children prenatally exposed to ethanol are hyperactive, uninhibited, impulsive and have attentional deficits that may reflect an inability to inhibit responses (Streissguth, Clarren & Jones, 1985; Streissguth, Barr & Martin, 1983). Poorer habituation to repeated stimuli and increased arousal were also reported in infants exposed to alcohol prenatally (Streissguth et al, 1983). These deficits become especially apparent in stressful situations (Streissguth, 1986). Other research in infants prenatally exposed to alcohol, show that heavy alcohol drinking during conception resulted in higher post stress salivary cortisol levels (Jacobson, Bihun & Chiodo, submitted). Our previous research suggests that deficits in the pituitary-adrenal response inhibition or recovery following stress could accompany these behavioral deficits and affects a child's responding in challenging situations (Weinberg, 1988). ACTH, CRH, and glucocorticoids are known to influence behavioral

responses to stressful stimuli (McEwen, De Kloet & Rostene, 1986). Therefore, prolonged or elevated levels of HPA hormones may play a role in the behavior of children prenatally exposed to alcohol while in stressful situations (Weinberg, 1988).

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