2004

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0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(8):3778–3787

doi: 10.1210/en.2003-1759

Sexually Dimorphic Effects of Prenatal Stress on Cognition, Hormonal Responses, and Central Neurotransmitters

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Exposure to stress during gestation results in physiological and behavioral alterations that persist into adulthood. This study examined the effects of prenatal stress on the postnatal expression of sexually differentiated cognitive, hormonal, and neurochemical profiles in male and female rats. Pregnant dams were subjected to restraint stress three times daily for 45 min during d 14–21 of pregnancy. The offspring of control and prenatally stressed dams were tested for anxiety-related and cognitive behaviors, stress and gonadal steroid hormone levels, as well as monoamines and metabolite levels in selected brain regions. Postnatal testosterone levels (measured at 1 and 5 d) did not differ between controls and prenatally stressed animals. In adulthood, the serum corticosterone response to stress was attenuated in prenatally stressed females, eliminating the sex difference normally observed in this parameter. Prenatally stressed females exhibited higher anxiety levels, evidenced by longer open field entry latencies. Prenatal stress had no effect on object recognition memory, but eliminated the advantage normally seen in the male performance of a spatial memory task. Neurochemical profiles of prenatally stressed females were altered toward the masculine phenotype in the prefrontal cortex, amygdala, and hippocampus. Thus, prenatal stress altered subsequent cognitive, endocrine, and neurochemical responses in a sex-specific manner. These data reinforce the view that prenatal stress affects multiple aspects of brain development, interfering with the expression of normal behavioral, neuroendocrine, and neurochemical sex differences. These data have implications for the effects of prenatal stress on the development of sexually dimorphic endocrine and neurological disorders. (*Endocrinology* 145: 3778–3787, 2004)

A SUBSTANTIAL BODY of evidence indicates that prenatal and/or early postnatal stress adversely affects human development, increasing the susceptibility to diseases later in life (1, 2) as well as altering behavioral and cognitive development (3–5). These effects have been linked to persistent alterations in the hormonal and metabolic responses to stress associated with changes in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (5, 6). The mechanisms underlying these lasting developmental responses are not fully understood.

Effects of prenatal stress (PS) have also been observed in a number of laboratory animal models, including primates (7) and sheep (8) as well as rodents and guinea pigs (9, 10). In rats, the offspring of dams subjected to various gestational stressors (*e.g.* immobilization, foot shock, or overcrowding) display physiological and behavioral alterations that persist into adulthood. In males, PS alters a number of sexually dimorphic behaviors including sex behavior, partner preference, analgesia sensitivity, and taste preference (for reviews, see Refs. 11, 12). PS is believed to demasculinize and feminize many sexually dimorphic behaviors through its disruption of the late pregnancy testosterone (T) surge in male rats (*e.g.* Ref. 13; reviewed in Ref. 14). Early sex differences in catecholamine levels, aromatase activity, and androgen 5α-reductase activity in the preoptic area and mediobasal hypothalamus are prevented by PS (15). Furthermore, T's prenatal effect, through its conversion to estradiol, appears to modulate the development of catecholamine activity in the frontal cortex continuing into the neonatal period (16).

A growing body of evidence, however, indicates that the effects of PS cannot be explained entirely by disruption of the normal late gestational increase in T levels in the male, but also include significant effects in the female. Many studies of PS have shown effects on the HPA axis in adult rats, such as elevated basal or stress-induced plasma ACTH and corticosterone (CORT) levels (17–19). In males, PS elevated stress-induced CORT levels through decreased feedback inhibition mediated by a reduction in the number of hippocampal type I and type II CORT receptors (18). A closer analysis of the effects of PS on HPA function reveals that the stress response in adult PS females (F-PS) is more sensitive than that observed in PS males (M-PS). Plasma ACTH and/or CORT stress-induced levels were elevated in female, but not male,

Abbreviations: 5-HIAA, 5-Hydroxyindole acetic acid; 5-HT, serotonin; CORT, corticosterone; DA, dopamine; DG, dentate gyrus; DOPAC, 3,4-dihydroxyphenylalanine; F-CON, female control; F-PS, prenatally stressed female; GC,glucocorticoid; HPA, hypothalamic-pituitary-adrenal; HVA, homovanillic acid; LSD, least significant difference; M-CON, male control; M-PS, prenatally stressed male; NE, norepinephrine; PS, prenatal stress; PFC, prefrontal cortex; RAM, radial arm maze; T, testosterone.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

PS offspring (20–24). Additional evidence for an increased vulnerability to PS in females in comparison with males is observed in prenatal ethanol exposure (25), prenatal ACTH exposure (26), and stress-induced analgesia (27).

Sex-specific neuroendocrine alterations in response to PS may also indirectly impact the expression of anxiety and cognitive behaviors. Behavioral reactivity (*e.g.* exposure to novel environment, open field activity, and maze activity) is altered by PS exposure, with many reports indicating that both PS males and females exhibit increased anxiety/emotionality responses to the open field (24) and a decreased tendency to explore (17, 28). The potential effect of PS on cognition has received limited attention, but evidence for sex differences is emerging. For example, whereas PS reduced hippocampal weight in both sexes, performance on the Morris water maze was impaired in PS males but not females (22, 29). However, PS has also been shown to moderately enhance male performance on the eight-arm radial arm maze, another test of spatial ability (30).

In sum, previous work suggests that PS does not exert the same neuroendocrine effects in males and females. However, these previous studies have used a range of PS paradigms and have not consistently compared males and females in sexually differentiated behavioral tasks. The goal of the present study, therefore, was to examine the effects of a defined PS on the postnatal expression of sexually differentiated anxiety-related and cognitive behavior patterns. The modified stress paradigm chosen has previously been shown to affect sexually differentiated reproductive behavior. Potential effects of PS on anxiety-related and cognitive behaviors were examined using a set of behavioral measures that consistently reveal gender differences (under stressed and nonstressed conditions). Because of the possible contribution from altered T levels in the male during the postnatal critical period for sexual differentiation, we measured T in male and female pups in the neonatal period. In addition, monoamines and metabolites were measured in brain areas known to contribute to the behaviors. The results of the study support the hypothesis that higher CNS function is affected by mild PS in a sex-dependent manner.

Materials and Methods

Subjects

Adult, male (n = 3) and female (n = 8), Sprague Dawley rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All animals were maintained on a 14/10-h light/dark cycle (lights on 0500 h). All experimental procedures were approved by the Hunter College institutional animal care and use committee and in accordance with the National Institutes of Health Guide for the Care and Use of Animals. All animals were individually housed and had free access to rat chow and water. All females received daily vaginal smears (1000 h) to determine estrus cycle day. When screened females were found to be in proestrus, they were housed overnight with a male, who was then removed in the morning. Vaginal samples were microscopically checked to determine the presence of sperm, and this was considered d 1 of pregnancy.

On d 14–21 of pregnancy, half of the dams were randomly assigned to a stress group (n = 4) and the other half to a control (CON) group (n = 4). Stressed dams were placed in Plexiglas animal holders (13 × 58.3 cm; Harvard Apparatus, Holliston, MA) three times daily (0900 h, 1300 h, and 1600 h) for 45 min, according to a modified protocol of Ward and colleagues (31). Control dams remained in their home cages during the stress period.

Upon delivery, approximately half of the pups in each litter [19 CON males (M-CONs), 14 M-PSs, 14 CON females (F-CONs), and 13 F-PSs] were assigned as experimental subjects for examination of the postnatal T surge (see below for details). The remaining male and female pups were earmarked as to prenatal treatment (16 M-CONs, 17 M-PSs, 14 F-CONs, and 15 F-PSs), and the culled litters consisted of eight pups per dam (four male and four female offspring to each of four CON dams and four stressed dams). Pups were weaned at 21 d, weighed weekly, and group housed. Animals were allowed to mature to 2 months of age, at which point behavioral testing began (see below). We were unable to prevent litter effects by taking one representative pup from each litter (it was not feasible to use 152 litters). However, counterbalancing was used in the random assignment of male pups to either the postnatal T surge experiment or the behavioral experiments. Additionally, cursory analysis revealed no differences in litter sizes, ratio of male to female offspring, or gestational length.

Postnatal T assessment

Trunk blood was obtained from male and female pups on postnatal d 1 and 5 after rapid decapitation. Blood was allowed to separate at room temperature; serum was collected after centrifugation in a clinical centrifuge and frozen in sealed tubes at −80 C until assay. Serum T levels were measured by RIA using commercial kits (Coat-A-Count kits catalog item TKTT1, Diagnostic Products Corp., Los Angeles, CA) according to kit protocol instructions. To correct for possible blank effects of rat serum, a volume of serum equal to the volume of the samples was added to the kit standards from a serum pool obtained from castrate adult rats. The volumes in the samples were counterbalanced by adding the kit zero calibrator. For comparison purposes, all assays also included serum samples from intact adult male rats as internal reference standards.

Behavioral measures

Remaining pups born to PS and CON dams were allowed to mature to 2 months of age, at which point behavioral testing began. Open field was conducted first followed by object recognition testing and then radial arm maze testing.

Open field. Rats were placed in a 15 × 15-cm small box with a sliding door opening to the corner of a 117 × 70 × 45-cm black, Plexiglas, open-top box, with the floor marked into 9-in. square grids (5 × 3). The sliding door was opened and latency to enter the field scored. Activity was scored for 6 min, divided into two 3-min intervals. Behaviors recorded included outer sector visits (movements across squares), inner sector visits, rears (rearing up on haunches with forelimbs 3–4 cm off the floor), wall climbs, grooms, and defecations.

Object recognition. Object recognition memory was assessed using the object recognition task. Trials consisted of a sample trial (T1) and a recognition trial (T2). The two trials were separated by an intertrial interval of varying lengths. In T1, two identical objects were placed at one end of the open field and amount of time spent exploring the two objects was recorded for 3 min. For T2, or the recognition trial, a new object replaced one of the previous objects. In T2, the time spent exploring the old (familiar object) and the new (novel) object was recorded for 3 min. Exploration was defined by the subject sniffing at, whisking at, or looking at the object from no more than 2 cm away. The objects used for trials were various bottles, cans, and containers (such as soda cans and candlesticks). The position of the objects and which object was replaced was counterbalanced across both sex and treatment. All animals received acclimation sessions with intertrial delays of 1, 10, 60, and 120 min and were then tested for object recognition with a 4-h delay.

Radial arm maze (RAM). Spatial learning was assessed using the eight-arm RAM as previously described (32). Animals, both PS and CON, were placed on food restriction (90% of baseline body weight) to induce motivation to perform the task. Briefly, subjects received 10 training trials (two training trials per day). During training, the rats' behavior was gradually shaped so that the animal goes to the end of the arms for a food reinforcer (1/4 peanut). After training, subjects received five regular trials (one trial per day). Rats were placed in the center of the maze and given a maximum of 10 min to visit all eight arms and receive the peanut reward at the end of each arm. An arm was counted as being

visited if the rat traversed two thirds of the arm's length, if the arm was entered and the peanut eaten, or if the arm was entered but the peanut was not eaten. Performance was scored by three choice accuracy measures: number of correct choices in first eight arm visits, choice where the first mistake was made, and total number of choices to complete the task.

Assessment of stress-induced CORT release

Two weeks after the end of behavioral testing, basal and stress-induced CORT levels were measured. PS and CON subjects were placed in Plexiglas restrainer tubes (described above), and blood was immediately collected from the tail to provide initial levels. Briefly, the tail was soaked in warm water and the tip quickly removed via a straight-blade razor. The tail was palpated to collect the blood sample and then treated with antibiotic spray. Subjects remained restrained and had blood collected at 1 and 2 h to determine the rise and, possibly, habituation of stress-induced CORT levels. Total CORT levels were measured by RIA using the Coat-A-Count assay kit, according to kit protocol (Diagnostic Products). Samples were analyzed in duplicate. All samples were run in one assay, and the detection limit was 5.7 ng/ml, as defined by the 95% confidence limits of the zero standard. CORT levels are expressed as ng/ml.

Neurochemical analysis

Two days after the stress test, all rats were killed by rapid decapitation and the brains rapidly frozen and stored at –70 C. A microtome cryostat at −8 C was used to obtain 300-μm-thick serial sections of the brain. Using a 500-μm inner-diameter cannula, tissue samples from various brain regions were obtained from the frozen sections maintained at approximately −11 C and placed in 1.4-ml Eppendorf tubes. From 4–12 punches were taken, dependent upon the area and neurotransmitter being sampled. The atlas of Palkovits and Brownstein (33) served as reference. Monoamine and metabolite neurotransmitters levels were measured by dissolving the punches in 75 μl of acetic acid buffer (pH 6.5), and a process of freezing and thawing was used to disrupt cellular structures and release cellular components including neurotransmitters of interest. 3,4-Dihydroxybenzylamine hydrobromide was added as an internal standard, and samples were centrifuged at 12,000 rpm for 10 min. The supernatant was removed, and the pellet was resuspended in 100 μl of 1.0 N NaOH for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA).

HPLC with electrochemical analysis was used to quantify neurotransmitter levels. The supernatant (60 μl) was used in the detection of monoamines, including dopamine (DA) and its metabolites, 3,4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-methoxy-4-hydroxyphenylglycol; and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). Monoamines were measured in a Waters Associates (Chicago, IL) chromatographic system (Waters 2690) consisting of an Alliance module containing an automated refrigerated injector, pump, C-18 reverse-phase column (Novapak 3 micron), and an ESA coulometric detector (+0.48–+0.50 V potential). The mobile phase, described elsewhere (34), contained 3% acetonitrile, and peak sharpness was increased by the addition of 100% methanol (99.5% mobile phase: 0.5% methanol).

Millenium software (Waters Associates) was used to run the chromatography system, and concentrations of transmitters and metabolites were calculated by reference to standards and internal standards using peak integration. Monoamine levels were measured in prefrontal cortex (PFC), hippocampus (CA1, CA3, and dentate gyrus, DG), and amygdala brain regions.

Statistical analyses

Serum levels of postnatal T and CORT were analyzed using three-way ANOVAs (sex × prenatal treatment × time). Three-factor (sex × prenatal treatment × time) ANOVAs were used to test for statistical differences among the groups on the open field task and the RAM. For object recognition data, an ANOVA (sex × prenatal treatment) was used to test differences in exploration times during T1, ANOVAs with repeated measures were used to test differences in time spent with the objects (old and new) during T2 (sex × prenatal treatment × object), and paired *t* tests on each group tested whether time spent with the old object was less than time spent with the new object. A two-factor ANOVA (sex × prenatal treatment) was used to test for statistical differences among the groups in monoamine and metabolite levels. Type I error rate was set at 0.05 for determining statistical significance. Where appropriate, Fisher's least significant difference test (LSD) was used for *post hoc* testing.

Results

Weight gain

Body weights for all the animals studied, from weaning onwards, are shown in Fig. 1. The growth curves were fitted to a four-parameter logistical function and analyzed by constrained curve fitting and analysis of residual variance (35). Although a significant difference was observed between males and females, with the males growing faster than the females ($F_{1,72} = 64.1$; $P < 0.0001$), no significant effect of PS was observed ($F_{1,69} = 0.64$; $P = 0.76$).

Postnatal T surge

Serum levels of T in the pups are shown in Fig. 2, together with data for reference 80- to 100-d-old adult male rats that were assayed alongside the neonatal serum samples. Levels of T were higher in males than in females and were approx-

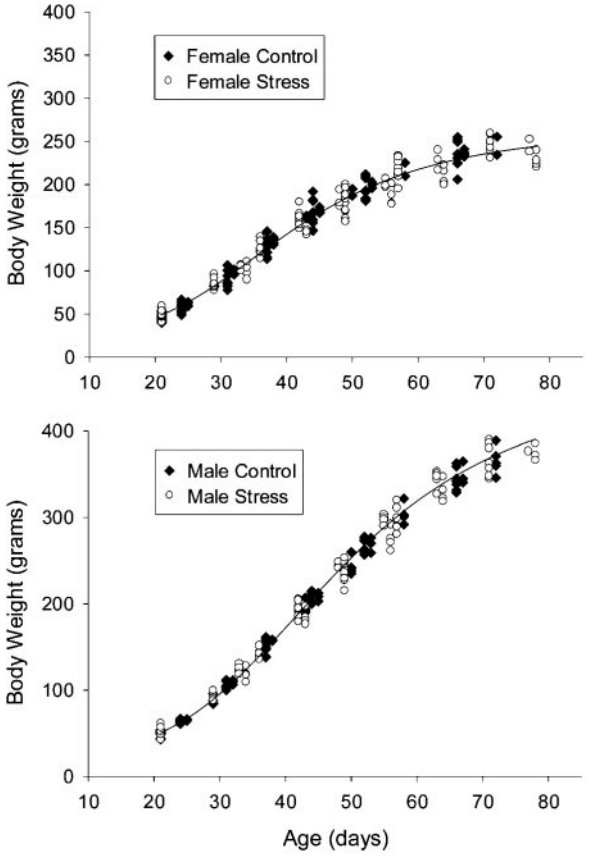

FIG. 1. Body weights for the CON and PS rats used in this study, from weaning to adulthood. Males and females are plotted separately, reflecting the more rapid weight gain in the males ($F_{1,72} = 64.1$; $P < 0.0001$). In both sexes, the growth curves for CON and PS animals were superimposable ($F_{1,69} = 0.64$; $P = 0.76$). *Lines* indicate the best-fit curves for the data for each sex, obtained by computer-assisted least-squares regression analysis.

imately 3-fold higher in males on the day of birth than at postnatal d 5. On the day of birth, mean T concentrations in the serum from male pups (1.16 ± 0.24 ng/ml) approached the levels observed in intact adult males (1.97 ± 0.57 ng/ml). Three-way ANOVA on the data for serum T levels at d 1 and 5 revealed significant effects of both age ($F_{1,50} = 5.11$; $P < 0.03$) and sex ($F_{1,50} = 23.1$; $P < 0.0001$). However, there was no significant effect of PS; levels of T were not different in PS compared with CON pups ($F_{1,50} = 0.47$; $P = 0.5$), and there was no significant prenatal treatment by sex interaction ($F_{1,50} = 1.77$; $P = 0.24$).

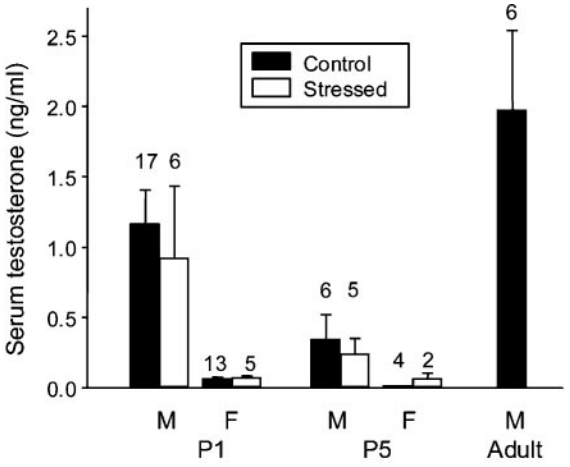

FIG. 2. T levels measured by RIA in the serum from male (M) and female (F) rat pups, from CON (*black bars*) and PS (*white bars*) dams at postnatal d 1 (P1) and 5 (P5). For comparison purposes, data are also shown for normal, intact adult males. Results represent means ± SEM of the number of observations indicated above each histogram bar.

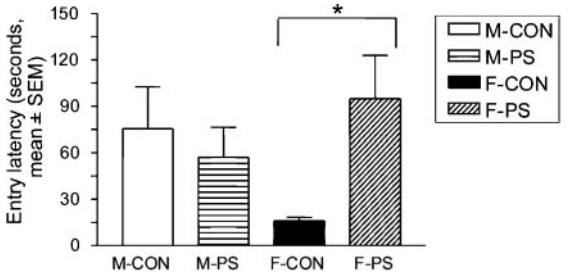

FIG. 3. Sex-specific effects of PS on entry anxiety. Entries are the mean ± SEM for each group (M-CON = 16; M-PS = 17; F-CON = 14; F-PS = 15). There was a sex × treatment interaction on the latency to enter the open field ($F_{1,58} = 4.78$; $P < 0.03$). *Post hoc* testing showed that entry latencies did not differ between M-CON and M-PS but was increased in F-PS. Significant group differences: *, $P < 0.05$ (Fisher's LSD *post hoc* test).

Behavioral measures

Open field. A three-factor ANOVA with sex and prenatal treatment as between-subjects design factors and time as within-subjects or repeated design factor was used to test for statistical differences among the groups on the open field task. There was a sex × prenatal treatment interaction with regards to the latency to enter the open field ($F_{1,58} = 4.78$; $P < 0.03$); see Fig. 3 with PS increasing female but not affecting male entry latencies. Open field measurements were quantified for the first 3 min and second 3 min of the task (Table 1). All animals, regardless of sex or prenatal treatment, made fewer outer sector crossings ($F_{1,43} = 33.88$; $P < 0.0001$) and fewer inside visits ($F_{1,43} = 12.80$; $P < 0.0008$) during the second 3 min of the task. All treatment groups made more rears ($F_{1,43} = 6.46$; $P < 0.01$) and grooms ($F_{1,43} = 11.68$; $P < 0.001$) during the second 3 min. Overall, males made fewer wall climbs than females ($F_{1,43} = 5.25$; $P < 0.03$). There were no significant group differences in defecations ($P > 0.05$, data not shown).

Object recognition. There were no significant group differences during the acclimation sessions (1-min, 10-min, 1-h, and 2-h delays) with regard to time spent exploring objects during T1, T2, or in discrimination between old and new objects during T2. During the 4-h delay test trial, a two-way ANOVA (sex × treatment) revealed no significant differences between the groups on the amount of time spent exploring objects during the sample trial T1: M-CON, 14.6 + 2.5 sec; M-PS, 19.1 + 1.1 sec; F-CON, 19.6 + 1.9 sec; F-PS, 17.6 + 4.3 ($P > 0.05$). After the 4-h delay, all groups spent significantly more time exploring the new than the old object, suggesting they remembered the old object and that the PS did not affect performance of this task [three-way ANOVA, sex × prenatal treatment × object (old *vs.* new)] (Fig. 4).

RAM. There were significant differences in RAM performance among the groups as measured by the visit during which the first mistake occurred. Although there were no main effects of either sex or prenatal treatment, there was a significant sex × prenatal treatment interaction ($F_{1,112} = 4.43$;

TABLE 1. Summary of behavioral measures on the open field in PS and CON male and female rats

Group	Outer crossings		Inner visits		Wall climbs		Rears		Grooms	
	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
M-CON	53.2 ± 4.0	42.9 ± 3.7[a]	4.6 ± 1.2	2.8 ± 0.9[a]	6.4 ± 1.7	5.1 ± 0.8	3.1 ± 1.3	2.3 ± 0.9[b]	0.3 ± 0.2	0.7 ± 0.2[b]
F-CON	61.6 ± 3.9	42.2 ± 2.8[a]	5.9 ± 1.1	4.3 ± 1.2[a]	9.2 ± 1.4	7.2 ± 1.1	2.4 ± 0.5	4.0 ± 1.0[b]	0.4 ± 0.2	0.7 ± 0.2[b]
M-PS	51.6 ± 4.0	45.3 ± 3.1[a]	4.8 ± 1.0	2.7 ± 0.5[a]	6.2 ± 1.0	6.2 ± 0.8	2.2 ± 0.6	3.9 ± 0.9[b]	0.4 ± 0.2	0.9 ± 0.3[b]
F-PS	51.9 ± 4.3	38.7 ± 2.2[a]	4.3 ± 1.1	3.1 ± 0.7[a]	7.9 ± 1.2	7.7 ± 0.7	1.2 ± 0.3	2.8 ± 1.1[b]	0.1 ± 0.01	0.7 ± 0.2[b]

Entries are the mean ± SEM. Time 1 refers to the first 3 min on the field and time 2 the second 3 min. A three-factor mixed ANOVA (sex × treatment × time) with Fisher's LSD *post hoc* testing was used to test for significant differences among the groups. All groups showed habituation to the open field with a decrease in outer sector crossing ($F_{1,43} = 33.88$; $P < 0.001$) and inner sector visits ($F_{1,43} = 12.8$; $P < 0.001$) across time. All groups made more rears ($F_{1,43} = 6.46$; $P < 0.01$) and grooms ($F_{1,43} = 11.68$; $P < 0.001$) during the second 3 min. Overall, males made fewer wall climbs than females ($F_{1,43} = 5.25$; $P < 0.03$). There were minimal incidences of defecations, and no significant differences among the groups were observed ($P > 0.05$; data not shown).

[a] Decreases across time.

[b] Increases across time.

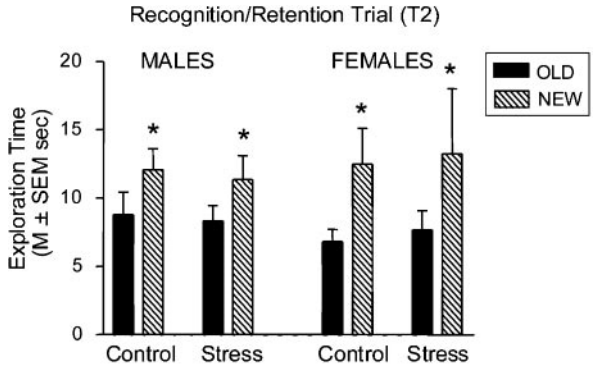

FIG. 4. Object recognition memory is unaffected by either PS or sex. Entries are the mean + SEM for each group (n = 8 per group). After a 4-h delay object recognition trial, all groups spent significantly more time exploring the new than the old object, suggesting they remembered the old object and that the PS does not affect performance of this task ($P > 0.05$).

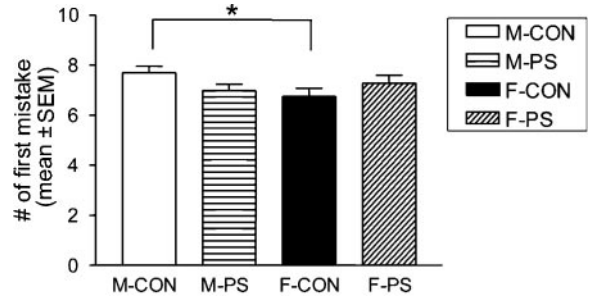

FIG. 5. Sex and prenatal treatment effects on RAM performance. Data shown are the mean ± SEM of the visit during which the first mistake occurred for all groups (n = 8 per group). There was a significant sex × treatment interaction ($P < 0.04$), with F-CONs performing significantly worse than M-CONs. Prenatal stress treatment ameliorated the standard sex difference in RAM performance.

$P < 0.04$). *Post hoc* testing revealed that M-CONs performed significantly better than F-CONs (Fig. 5). PS groups were not different from other groups, suggesting that PS did not affect performance of this spatial memory task; however, PS eliminates the sex difference normally observed in RAM performance. There was a similar sex × prenatal treatment interaction with regard to the number of correct choices in the first eight visits, but this trend failed to reach statistical significance ($P = 08$). There were no significant differences among any of the groups with regard to the total number of visits required to complete the task ($P > 0.05$).

Stress-induced CORT response

A three-way (sex × prenatal treatment × time) repeated-measures ANOVA was used to test for differences in serum CORT levels in response to stress challenge. Stress-induced CORT levels were significantly affected by sex ($F_{1,41} = 7.44$; $P < 0.01$), prenatal treatment ($F_{1,41} = 4.73$; $P < 0.04$), and time ($F_{1,41} = 15.39$; $P < 0.0001$). Although the sex × prenatal treatment interaction failed to reach significance ($P = 0.08$), there was a significant sex × time interaction ($F_{1,82} = 3.21$; $P < 0.04$) (Fig. 6). *Post hoc* testing revealed that F-CONs failed to show serum CORT habituation to the stress challenge and had sustained CORT levels compared with all other groups.

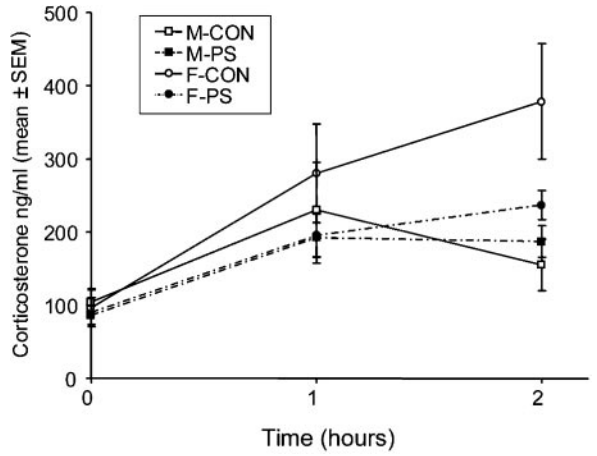

FIG. 6. Stress-induced CORT release is attenuated in F-PSs. Data shown are group-averaged serum CORT levels (mean ± SEM) expressed as ng/ml (n = 16 M-CONs; n = 17 M-PSs; n = 13 F-CONs; n = 14 F-PSs). CORT levels in response to stress challenge were significantly affected by sex ($P < 0.01$), treatment ($P < 0.04$), and time ($P < 0.0001$). A significant sex × time interaction with Fisher's LSD *post hoc* test showed that F-CONs failed to show serum CORT habituation to the stress challenge.

Neurochemical analyses

A summary of all central monoamine and metabolite levels in measured brain areas may be seen in Table 2. Both sex and prenatal treatment altered monoamine and metabolite levels in the PFC (Fig. 7). PFC levels of NE ($F_{1,26} = 8.84$; $P < 0.006$) and 5-HT ($F_{1,26} = 4.35$; $P < 0.04$) were elevated in females, regardless of prenatal treatment, compared with males (Fig. 7A). The 5-HT metabolite 5-HIAA was altered by sex ($F_{1,25} = 13.29$; $P < 0.001$), with higher levels observed in females, and the sex × prenatal treatment interaction ($F_{1,25} = 5.75$; $P < 0.024$), with lowest levels observed in the M-PSs (Fig. 7A). There was a sex × prenatal treatment effect on the PFC levels of the DA metabolite HVA, with the lowest levels observed in the M-PSs ($F_{1,25} = 5.10$; $P < 0.033$) (Fig. 7B). A significant sex × prenatal treatment interaction effect on PFC DOPAC levels was observed where DOPAC levels decreased in M-PSs and increased in F-PSs ($F_{1,25} = 5.0$; $P < 0.035$) (Fig. 7B). Both sex and prenatal treatment influenced neurochemical activity in the CA3 hippocampal region (Fig. 8). DA levels in the CA3 region were elevated in males compared with females ($F_{1,19} = 4.55$; $P < 0.04$). Both sex and prenatal treatment influenced CA3 HVA levels, with lower levels observed in females ($F_{1,22} = 4.55$; $P < 0.04$) and PS animals ($F_{1,22} = 7.17$; $P < 0.014$). Additionally, there was a significant effect of sex on 5-HIAA levels in the amygdala ($F_{1,26} = 4.47$; $P < 0.04$), with higher levels observed in females (Fig. 9). There were no significant effects of either sex or prenatal treatment in the DG.

Discussion

These results corroborate the growing body of literature showing that the prenatal environment has important consequences for expression of adult neural function (22, 29, 30). We have extended previous observations by simultaneously evaluating effects on higher order neural functions, recognition and spatial memory, in male and female rats. Taken

TABLE 2. Effect of sex and prenatal treatment on brain monoamine and metabolite levels

Area	Neurotransmitter					
	NE	DA	HVA	DOPAC	5-HT	5-HIAA
PFC						
M-CON	2.12 ± 0.14	0.47 ± 0.09	1.73 ± 0.44	0.75 ± 0.17	1.37 ± 0.19	1.37 ± 0.15
M-PS	2.09 ± 0.24	0.36 ± 0.05	1.09 ± 0.14	0.40 ± 0.06	1.52 ± 0.18	0.87 ± 0.15[a]
F-CON	2.82 ± 0.29[b]	0.46 ± 0.05	1.59 ± 0.20	0.41 ± 0.09[a]	1.76 ± 0.21[b]	1.57 ± 0.1[b]
F-PS	2.99 ± 0.37[b]	0.36 ± 0.07	2.29 ± 0.33[a]	0.68 ± 0.18	1.91 ± 0.15[b]	1.18 ± 0.21[b]
CA1						
M-CON	2.51 ± 0.23	ND	ND	ND	1.48 ± 0.27	1.49 ± 0.0
M-PS	3.45 ± 0.31	ND	ND	ND	1.47 ± 0.18	1.85 ± 0.25
F-CON	2.76 ± 0.26	ND	ND	ND	1.20 ± 0.19	1.70 ± 0.11
F-PS	2.85 ± 0.52	ND	ND	ND	1.75 ± 0.38	1.88 ± 0.43
CA3						
M-CON	3.86 ± 0.86	4.21 ± 2.15	0.62 ± 0.16	0.38 ± 0.21	1.20 ± 0.24	2.43 ± 0.42
M-PS	2.64 ± 0.38	2.05 ± 1.01	0.21 ± 0.07[c]	0.40 ± 0.27	1.05 ± 0.32	2.39 ± 0.30
F-CON	2.75 ± 0.36	0.61 ± 0.19[b]	0.26 ± 0.05[b]	0.13 ± 0.04	0.8 ± 0.56	1.79 ± 0.22
F-PS	2.79 ± 0.49	0.29 ± 0.07[b]	0.14 ± 0.02[b,c]	0.36 ± 0.04	0.73 ± 0.05	2.17 ± 0.18
DG						
M-CON	3.87 ± 0.64	ND	0.62 ± 0.1	ND	0.95 ± 0.23	4.79 ± 0.5
M-PS	4.19 ± 0.42	ND	0.71 ± 0.17	ND	1.20 ± 0.21	4.84 ± 0.69
F-CON	3.49 ± 0.23	ND	0.61 ± 0.06	ND	1.07 ± 0.14	5.60 ± 0.56
F-PS	4.52 ± 0.28	ND	0.76 ± 0.1	ND	1.33 ± 0.22	4.47 ± 0.33
Amygclala						
M-CON	2.41 ± 0.46	2.79 ± 0.69	0.68 ± 0.14	0.89 ± 0.33	3.37 ± 0.79	2.23 ± 0.19
M-PS	3.25 ± 0.52	2.64 ± 0.68	0.41 ± 0.08	0.45 ± 0.1	2.31 ± 0.29	2.05 ± 0.08
F-CON	3.68 ± 0.6	2.19 ± 0.49	0.49 ± 0.08	0.34 ± 0.06	3.79 ± 0.77	2.69 ± 0.42[b]
F-PS	3.60 ± 0.69	2.89 ± 0.17	0.62 ± 0.2	0.41 ± 0.16	3.28 ± 0.86	3.03 ± 0.44[b]

Entries are mean + SEM (pg/μg). Monoamine and metabolite levels were measured by standard HPLC techniques in the PFC, hippocampus (CA1, CA3, and DG), and the amygdala. Significant effects of sex and prenatal treatment in the PFC, CA3 region of the hippocampus, and amygdala may be seen in Figs. 7, 8, and 9, respectively. There were no significant group differences observed in either the CA1 or DG regions of the hippocampus. ND, Not detected. Significant group differences: [a] sex × prenatal treatment interaction; [b] main effect of sex; [c] main effect of prenatal treatment.

together with results of previous studies, the data suggest that PS reduces the magnitude of normal sex differences in some brain functions by feminizing males and masculinizing females (current results and Refs. 27, 36, 37). Although current and previous changes are generally small, PS clearly alters the pattern of adult sex differences in rats.

The male and female offspring of both CON and stressed dams did not differ in their weight gain across the experimental time period. This suggests that there were no gross effects of the maternal stress paradigm on the care and development of the offspring. These observations do not exclude the possibility that subtle differences in maternal care may have influenced the subsequent endocrine and behavioral development patterns in the PS animals. Previous studies have provided ample precedent for the hypothesis that small differences in postnatal care may have lasting developmental effects (*e.g.* Refs. 38, 39). The aim of the present studies, however, was not to determine whether pre- or postnatal factors were the primary determinant of sexually differentiated nonreproductive effects of PS, but rather to determine first whether such effects could be consistently observed. Future studies, incorporating cross-fostering of offspring from CON and stressed dams, will be necessary to determine the relative contributions from PS *vs.* postnatal maternal effects.

Postnatal T levels

The development of sexually differentiated behaviors and neuroendocrine function is profoundly affected by PS (40–44). The most dramatic effects are seen in terms of the differentiation of the male; M-PSs show diminished masculinization and impaired defeminization, compared with nonstressed CONs. The mechanism of this effect appears to involve transmission of stress from mother to fetus, resulting in suppression of late prenatal testicular androgen secretion. Ward and collaborators (13, 45) found that prenatal restraint stress, using an experimental paradigm essentially identical with that used here, decreases circulating T levels as well as aromatase activity in the brains of the male fetuses late in gestation. How this effect is translated into permanent impairment of sexual differentiation remains unclear. Previous work has shown that, in the rat, masculine sexual differentiation is critically dependent on postnatal androgen, particularly the surge in circulating T levels that occurs during the first 24 h after birth (46–48). In studies published while the present experiments were under way, Ward and colleagues (49, 50) found that PS male rats exhibit a normal postparturitional T surge. The present data confirm and extend these observations; prenatal restraint stress does not significantly affect mean circulating T levels in male rats at either postnatal d 1 or postnatal d 5. Moreover, T levels in females are also not altered by PS. These results are consistent with the view that prenatal factors are critical for sexual differentiation of neural functions that are affected by PS. The immediate postnatal T surge is important for the normal development of sex differences in reproductive behavior and neuroendocrine function, but postnatal changes in T do not appear to contribute to PS effects (present results and Ref. 49).

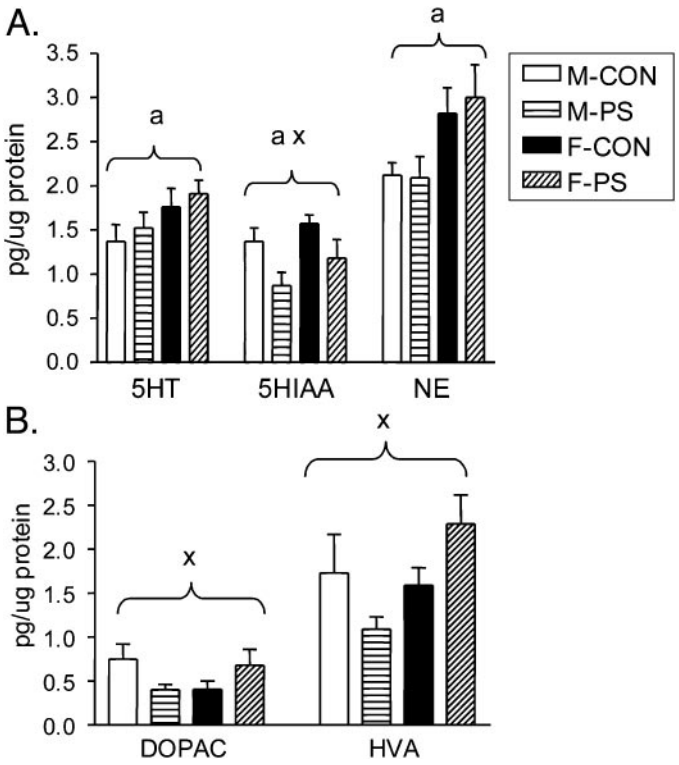

FIG. 7. Sex and stress alterations in PFC monoaminergic activity. Data are expressed as pg/μg protein (mean $\pm$ SEM; n = 8 per group). Group differences are identified by main effect of sex (a) and the sex $\times$ prenatal treatment interaction (x). Females had higher levels of NE ($P < 0.006$) and 5-HT ($P < 0.05$) than males, regardless of prenatal treatment. 5-HIAA levels were influenced both by sex (higher in females; $P < 0.001$) and the sex $\times$ treatment interaction (lowest in M-PSs; $P < 0.02$). DA activity, as indexed by HVA ($P < 0.03$) and DOPAC ($P < 0.03$) metabolite levels, were influenced by the sex $\times$ treatment interaction, with lowest levels observed in M-PSs and highest levels in F-PSs.

Behavioral measures

Anxiety. Females were less anxious than males as indicated by the latency to enter the field, which is consistent with previous reports (51, 52). Prenatal stress influenced this measure of anxiety in a sex-specific manner; PS increased female latency to enter but did not alter male entry latency. More specifically, PS masculinizes female latency to enter, inasmuch as the entry latencies of F-PSs approximate those of M-CONs (*post hoc* testing revealed no differences in entry latency of M-CONs and F-PSs). This PS influence on subsequent measurements of anxiety is similar to previous reports showing that PS increases anxiety-based behavior (53, 54). Interestingly, this stress-induced anxiousness was transient, as there is no effect of stress on subsequent inner sector visits, another index of anxiety. Notably, PS rats did not experience impairments in habituating to the novel environment, and thus, PS-induced anxiety should not be a long-term factor in other observed cognitive changes.

PS and cognition. PS did not globally affect cognitive performance but rather exerted differential influences on cognitive function, dependent upon the specific task. PS did not affect the object recognition task but did influence sex-specific performance on the spatial memory-dependent task, the RAM.

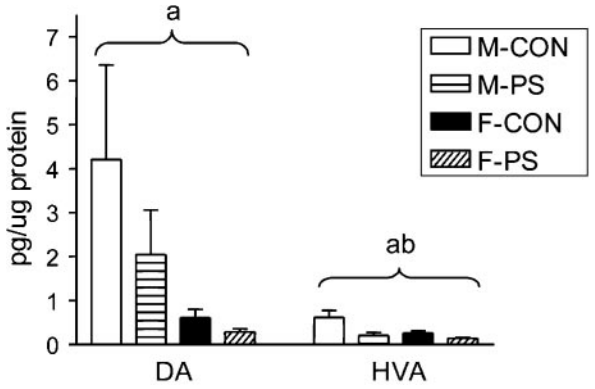

FIG. 8. Sex and prenatal treatment induced alterations in DA and metabolite levels in CA3. Data are expressed as pg/μg protein (mean $\pm$ SEM; n = 8 per group). Group differences are identified by main effects of sex (a) and prenatal treatment (b). DA levels were influenced by sex (higher in males than females; $P < 0.05$). HVA levels were altered both by sex (higher in males; $P < 0.04$) and prenatal treatment (lower in PS animals; $P < 0.02$).

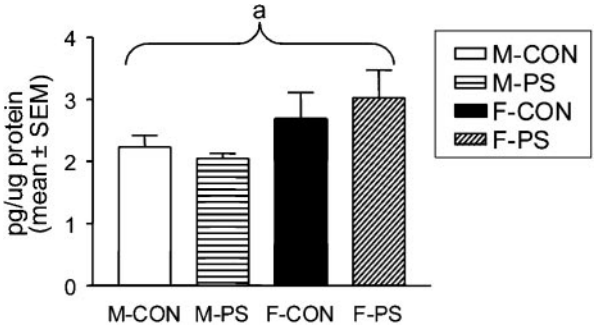

FIG. 9. Elevated 5-HIAA levels in amygdala in females. Data are expressed as pg/μg protein (mean $\pm$ SEM; n = 8 per group). Group differences are identified by main effect of sex (a), with females, regardless of prenatal treatment, having elevated 5-HIAA levels ($P < 0.04$).

Object recognition. Object recognition is a working, nonspatial memory-dependent task that uses both cortical and hippocampal input (55). To date, few studies have examined performance of this task in females, but it appears that there is no sex difference in performance; *i.e.* females and males both significantly discriminate between known and new objects at short and long intertrial delays. PS, as given here, did not affect performance in either sex. These current results are different from stress effects during adulthood on object recognition. Specifically, chronic stress during adulthood impairs object recognition in males but does not affect female performance (51). Thus, whereas female recognition memory appears resistant to stress effects both prenatally and during adulthood, males appear less sensitive to stress effects, on this specific cognitive task, only during development.

RAM. RAM is a working, spatial memory task that uses spatial cues and is dependent on hippocampal function. On the RAM, F-CONs made their first mistake sooner than M-CONs, and this small, but significant, sex difference in performance is consistent with previous reports for other spatial memory tasks (for reviews, see Refs. 56, 57). Of particular interest is the finding that PS attenuated this standard sex difference. PS impaired male performance while enhancing

female performance to the extent that the sex difference in favor of male RAM performance was not apparent in the PS animals. PS appears to have masculinized the female performance on the RAM, and this finding would be consistent with others who have observed PS-induced masculinization of the female offspring (27, 36). For example, in guinea pigs, social stress during pregnancy masculinizes many female behaviors and endocrine responses [*e.g.* daughters of mothers exposed to social stress during pregnancy have higher serum T levels than CON daughters (36, 37)]. Furthermore, these masculinized daughters also displayed an up-regulation of androgen receptors and α-estrogen receptor in brain regions including the hypothalamus, thalamus, and CA1 region of the hippocampus (37). In the guinea pig, it is hypothesized that the masculinization of daughters after PS is due to an increase in HPA axis activity, which increases both glucocorticoid (GC) and androgen secretion from the adrenal glands, which then cross the placenta and masculinizes vulnerable brain regions (36). It seems feasible that a similar mechanism may mediate the apparent masculinizing PS effects on female spatial performance in the current study.

Additional support for the notion that PS is masculinizing female behavior comes from the open field data. Although not statistically significant, there is a consistent pattern of masculinized behavior of the F-PS animals across several of the dependent measures. For example, with regard to outer visits and wall climbs during the first 3 min, as well as inner visits during both the first and second 3 min, the females exposed to PS clearly behave in a more masculine fashion than their F-CON counterparts. More studies are necessary to elucidate the exact cascade of events underlying these effects.

Neurochemical effects

The sex-specific cognitive changes in response to PS may be mediated through subsequent neurochemical alterations, particularly in brain regions such as the prefrontal cortex and hippocampus. Prefrontal cortex dopaminergic activity is critical for both spatial and nonspatial rodent memory and in general, an inverted U relationship exists between PFC DA levels and memory function. In general, inadequate or excessive amounts of DA interfere with working memory (58, 59). In the current study, stress-induced behavioral changes were accompanied by changes in DA activity in the PFC. Specifically, DA activity, as reflected by HVA and DOPAC levels, was decreased in males by PS, whereas stressed females had elevated HVA and DOPAC levels in the PFC compared with CONs (Fig. 7). It seems reasonable that elevated DA turnover in the PFC of the stressed females may have been responsible for the enhancement of spatial memory and elimination of the sex difference normally observed in RAM performance (Fig. 5). Further support for the role of DA in mediating the PS-induced behavior changes is evidenced by the interaction of elevated PFC DA activity and the HPA axis (60, 61). It has been shown that the medial PFC is a target site for the negative-feedback effects of GC on restraint stress-induced HPA activity (60), and thus, it is possible that PS has differential effects on this interaction in males and females. The effects of PS on DA activity were also observed in the CA3 region, which plays a critical role in the transmission of information from the mossy fibers of the DG to the CA1 regions. Overall, males had higher levels of DA than females, and this seems to be reflected in the overall male advantage on RAM performance. DA activity, as indexed by HVA levels, was higher in males than females; however, both M-PSs and females had lower levels of HVA in the CA3. The lower DA metabolite levels in the PS animals may be related to the observed behavioral changes.

In the current study, PFC 5-HT turnover was influenced by sex and prenatal treatment. Overall, 5-HIAA levels were higher in females than males, and PS decreased male levels but did not affect females. It is possible that the increased activity in females, regardless of PS, is contributing to their enhanced cognitive performance. Other forms of early environmental stimuli, such as neonatal handling, have been shown to increase 5-HT activity in both the PFC and hippocampus, and this effect appears to mediate increased GC receptor gene expression in these areas (38). As such, PS could be exerting differential effects on the development and expression of GC receptors in brain sites critical to cognition and thus altering behavioral changes in a sex-specific manner.

Stress-induced CORT response

Consistent with previous studies (32), there were significant group differences in serum CORT levels in response to stress challenge in adulthood. In particular, the F-CONs did not appear to habituate to the stress test, as their serum levels remain elevated across the restraint period. However, the CORT release pattern of F-PSs closely resembled that of males. This PS-dependent masculinization effect on female stress-induced CORT levels is of particular interest because it appears to parallel the observed cognitive effects. We regard the current data as indicative of failure to habituate (*vs.* a prolonged CORT release response) because other studies, in which serum CORT samples are obtained after the termination of the stress challenge, report similar findings. Specifically, adult females continued to show elevated CORT responses 90 min after the termination of restraint stress compared with males (62).

Conclusion

Prenatal stress appears to alter endocrine, cognitive, and neurochemical profiles in adulthood in a sex-specific manner. Prenatal stress exposure alters CORT secretion patterns in females after a stress-challenge test and transiently increases anxiety in a sex-dependent fashion. Furthermore, PS differentially influences cognitive function in a task-specific manner with changes noted in spatial memory but not visual memory. These effects of PS are most profound in the female offspring, masculinizing some female responses, including both neuroendocrine responses to stress and spatial memory. The PS effects may be mediated through effects on central neurotransmitter levels, particularly DA activity.

These results have important implications for work on the effects of developmental stress in both humans and animals. Tests of cognitive function in animals are a vital component of the criteria used to evaluate the neurotoxicological risks of

prenatal chemical exposure (63). Because toxicant exposure can also induce nonspecific metabolic responses in the mother that indirectly elicit stress in the fetus [*e.g.* suppression of food intake (9, 10)], the potential exists for developmental neurotoxicology studies to be confounded by sex-dependent effects of stress. In human beings, sexual differentiation occurs *in utero*, and sex hormones have been implicated in the development of learning disorders such as autism and dyslexia that occur at different rates in males and females (64, 65). Maternal stress influences the fetal autonomic nervous system, as well as central nervous system function (*i.e.* recognition, memory, and habituation; for review see Ref. 66). Stress during pregnancy has also been linked to an increase in attention deficit hyperactivity disorder (67), long-term changes in the regulation of the HPA axis (5), as well as affects on sexual orientation (3). Although it remains to be determined whether these effects reflect mechanisms analogous to those observed in the present study, gender clearly represents one of the factors that should be considered in evaluating the long-term outcome of human PS.

Acknowledgments

Received December 29, 2003. Accepted May 3, 2004.

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This work was supported by NIH Grants GM60654, DA12136, and GI2RR3037 to V.N.L.

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