

Exposure to Repetitive Versus Varied Stress during Prenatal Development Generates Two Distinct Anxiogenic and Neuroendocrine Profiles in Adulthood

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Early life experiences can shape brain function and behavior in adulthood. The present study sought to elucidate the effects of repetitive, predictable vs. varied, unpredictable prenatal stress on sexually dichotomous neuroendocrine and anxiety-related behavioral responses in adult offspring. Rat dams were exposed repeatedly during the last week of pregnancy to no stress, only restraint stress [prenatal stress (PS)-restraint], or a randomized sequence of varied stressors (PS-random), and several behavioral and endocrine measures were assessed in adult male and female offspring. Repeated exposure to the same stressor (restraint) generated the most robust changes, including increased anxiety-related behaviors (both passive, measured on the elevated plus maze, and active, measured using defensive burying tests), a delayed and prolonged hypothalamic-pituitary-adrenal (HPA) axis response to stress in female offspring. Conversely, PS-restraint males showed no

changes in anxiety-like behavior and had elevated basal ACTH and a blunted HPA response to stress; consistent with attenuated HPA responsivity was an increase in glucocorticoid receptor immunoreactivity in the hippocampus, suggesting increased negative feedback on the HPA axis in these animals. Prenatal exposure to a varied, unpredictable pattern of stressors did not have as much effect on HPA function, with most neuroendocrine measures residing intermediate to PS-restraint and control animals within each sex. Gonadal steroids were altered independent of the type of prenatal stress, but changes were measurable only in males (lower testosterone). The present data exemplify the differential sensitivity of the developing nervous and endocrine systems to stress, depending on not only gender but also the nature of the stressful experience endured by the mother during pregnancy. (*Endocrinology* 147: 2506–2517, 2006)

MOOD DISORDERS such as anxiety and depression have considerable economic and health impact on society (1, 2). Clinical and epidemiological studies indicate a causal role for early adversity in these disorders (3, 4). Critical to advancement in understanding the pathophysiology and treatment of mood disorders is the development of animal models of early adversity that also reflect the increased prevalence of these disorders in women (5, 6).

In rodents, the hypothalamic-pituitary-adrenal (HPA) axis and emotional behavior are particularly sensitive to perinatal environmental manipulations (7–9). Daily restraint of pregnant rats increases HPA responses to stress (10, 11) and anxiety-like behavior (11–13) in adult offspring. Whereas gestational stress increases anxiety-like behavior, there are many forms of anxiety, and ignoring their distinctions may limit our understanding of empirical findings. The term anxiety may be used to describe an emotional state, a predisposition to respond with fear to a particular stimulus, or a personality trait (14). Anxious behaviors in humans range from incapacitating freezing to directed avoidance to compulsive attending to anxiety-provoking stimuli. Prenatal

stress elicits behavioral changes in the elevated plus maze (EPM) (11–13), Y-maze (11), and open field tests (11), which reflect passive anxiety-like behavior (15). How prenatal stress shapes other aspects of anxiety-like behavior is unknown. The present study used tests assessing passive (EPM) (15) and active anxiety-like behavior (shock-elicited defensive burying) (16) along with conditioned aversion or phobia (persistence of defensive burying without shock) (17) in prenatally stressed adult male and female offspring to address this question.

The predictability of a stressor, in terms of time and type, is an essential dimension of its behavioral and neuroendocrine consequences (18). HPA hormones can adapt to a predictable, homotypic stressor over time (19–22). However, chronic exposure to the same, predictable stressor repeatedly differentially affects hormones and affective behaviors, compared with chronic exposure to varied, unpredictable stressors (23–25). We hypothesized that prenatal exposure to repeated restraint would yield different neuroendocrine and behavioral profiles in adulthood, compared with prenatal exposure to a randomized sequence of varied stressors, yielding two distinct developmental models of anxiety.

In summary, the present study investigated the effects of repeated restraint, randomized stress, or no stress during pregnancy on HPA hormones before, during, and after stress, and distinct anxiety-like behaviors in adult offspring. Our main goal was to fully characterize the hormonal and behavioral profiles of prenatally stressed offspring, which could then be used as models of anxiety in future studies. We

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Abbreviations: CA, Cornus ammonis; DG, dentate gyrus; EPM, elevated plus maze; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; PS-random, prenatal stress-randomized stressors; PS-restraint, prenatal stress-repeated restraint; PVN, paraventricular nucleus of the hypothalamus.

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also sought to elucidate whether females were more susceptible to the different stressing paradigms and whether neuroendocrine and behavioral changes were associated with changes in gonadal steroid levels because testosterone and estradiol, respectively, have suppressive and facilitatory effects on HPA hormones (26, 27) and glucocorticoid receptor expression in the hippocampus, a site implicated in negative feedback regulation of the HPA axis (28). We report here notable differences in sensitivity of the developing nervous system to stress dependent on not only gender but also the nature of the stressful experience endured by the mother during pregnancy.

Materials and Methods

Pregnant dams

Female CD rats were obtained from a commercial vendor (Charles River, Wilmington, MA). Dams arrived on the fifth day of gestation and were individually housed and maintained under controlled lighting (12-h light, 12-h dark, lights on at 0600 h) with food and water available *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of The Salk Institute.

Prenatal treatment

Pregnant dams were assigned to one of the following three treatment conditions for the last week of pregnancy (gestational d 14–21, Table 1): 1) control ($n = 9$); dams were left alone in the animal housing room; 2) prenatal stress-repeated restraint (PS-restraint, $n = 10$); dams were exposed to three daily 45-min sessions at 0900, 1200, and 1700 h under illumination by two 150-W bulbs (29); or 3) prenatal stress-randomized stressors (PS-random, $n = 10$); each day, dams were exposed to one of three types of stress: restraint stress (three 45-min sessions, as described above), foot shock stress [one 30-min session between 09 and 1100 h (two randomized 0.4 mA AC foot shocks/min; Coulbourn HO2-08 grid floor controlled by a Macintosh computer, Coulbourn Instruments, Allentown, PA (30)) or injection stress (one 0.5 cc sc injection of sterile isotonic saline outside the animal housing room under bright light, between 0900 and 1100 h). The type of stress each dam received was randomly determined for that animal each day. The cages of all dams were changed twice per week.

Litters

Pups from all dams were born on gestation d 22. Dams and litters were left undisturbed until 5 d after birth to reduce the risk of infanticide and stress. On postnatal d 5, total litter size averaged (\pm SEM) 12.3 ± 0.6 (control), 13.2 ± 0.7 (PS-restraint), and 10.9 ± 0.7 (PS-random) pups. Litter sizes were within the normal range observed in this strain (http://www.criver.com/flex_content_area/documents/rm_rm_r_tox_studies_crlcd_sd_br_rat.pdf). Because litters of PS-random dams were significantly

smaller 5 d postparturition than litters of control ($P = 0.04$) and PS-restraint ($P = 0.006$) dams, litters were culled to a maximum of 14 pups with minimal disturbance of the nest, reducing two PS-restraint litters and one control litter. The number of pups in each litter was not different among the prenatal treatment groups after culling.

Litters were left undisturbed after culling until pups were weaned at 21 d, when females and males were separated from their mothers and group housed with same-sex littermates until adulthood. There was no effect of prenatal stress on sex ratio. The mean number of pups per litter (\pm SEM) was 6.2 ± 0.5 (control males), 6.0 ± 0.5 (control females), 5.7 ± 0.5 (PS-restraint males), 6.9 ± 0.3 (PS-restraint females), 5.5 ± 0.7 (PS-random males), and 4.9 ± 0.8 (PS-random females). To avoid litter effects, which can result in a variety of problems with data interpretation (31), only one to two animals (of each sex) per litter were used for experiment 1 and only one to three animals (of each sex) per litter were used for experiment 2. Animals were not weighed early in development to minimize stress and handling; in adulthood, body weight was not different among the three prenatal groups within each sex (control males = 307.77 ± 8.27 g; PS-restraint males = 294.70 ± 5.14 g; PS-random males = 316.80 ± 7.07 g; control females = 249.55 ± 8.81 g; PS-restraint females = 255.40 ± 5.54 g; PS-random females = 230.75 ± 7.83 g, all $P > 0.05$).

Experiment 1: effect of prenatal stress on HPA function, gonadal steroid levels, and glucocorticoid receptor immunoreactivity in the hippocampus in adult male and female offspring

Animals. Experiment 1 sought to examine the effects of the above-described prenatal stress models on the HPA function and gonadal steroid hormone levels of adult male and female offspring ($n = 7$ – 10 per sex per prenatal treatment). Because gonadal steroids can influence hormones of the stress axis (26), estrous cycles were synchronized in females by giving two doses of $2 \mu\text{g}$ of the potent GnRH agonist [DTrp⁶, Pro⁹, Net]GnRH (sc) synthesized by solid-phase methodology (32) (generously provided by Dr. Jean Rivier, The Salk Institute, La Jolla, CA) at 0900 and 1400 h 3 d before testing (33). Injections were timed so that females were in diestrus on the day of sampling, as confirmed by vaginal smears. Females not in diestrus on the day of sampling were eliminated from the study.

Surgery. Under isoflurane anesthesia, iv catheters were aseptically inserted in the right jugular vein as previously described (34). After surgery, animals were singly housed to prevent chewing of the catheter and left undisturbed for 2 d until the experiment.

Blood sampling. On the morning of the experiment (0700 h), catheters were connected to tubing attached to a syringe containing heparinized saline. Rats were placed in individual buckets, where they remained awake and freely moving. This allowed for the collection of blood without disturbing the animals. Animals rested for 3 h after connection to allow stress-responsive hormones to return to basal levels (Rivier, C., unpublished data). Four blood samples were taken via the catheter (0.3

TABLE 1. Schedule of stress exposure of dams exposed to no treatment (control), PS-restraint, or PS-random during embryonic day (E) 14–21

	Animal	E14	E15	E16	E17	E18	E19	E20	E21
Control	All	No stress							
PS-restraint	All	R,R,R							
PS-random	1	I	S	R,R,R	I	R,R,R	R,R,R	S	S
	2	R,R,R	R,R,R	R,R,R	S	R,R,R	S	I	I
	3	R,R,R	R,R,R	I	I	S	R,R,R	S	I
	4	I	I	S	S	R,R,R	I	S	I
	5	S	S	S	R,R,R	I	R,R,R	S	R,R,R
	6	S	S	I	I	S	I	I	R,R,R
	7	S	S	I	I	S	R,R,R	S	S
	8	S	S	R,R,R	S	I	R,R,R	R,R,R	I
	9	I	S	I	I	R,R,R	S	R,R,R	S
	10	S	I	S	S	I	S	S	R,R,R

R, Restraint; S, shock; I, injection.

ml each sample, 0, 15, 30, and 45 min after the start of shock), and a fifth trunk blood sample was obtained immediately after decapitation (2 h after the start of shock). All animals were sampled from between 1000 and 1330 h to avoid the influence of circadian rhythms on stress responses.

Foot shock stress. After the basal (0 min) blood sample was obtained, animals were placed in custom-built shock boxes (Coulbourn HO2–08 grid floor controlled by a Macintosh computer, Coulbourn Instruments (30)) and given mild electrofootshocks (two 0.25 mA AC shocks/min, 1 sec duration per shock) for 45 min. After shocks, animals were quickly placed back in the sampling buckets and left alone until decapitation.

ACTH and corticosterone levels. Plasma ACTH levels were determined using a commercially available two-site immunoradiometric assay kit (Allegro kit, Nichols Institute, San Juan Capistrano, CA). Data are expressed in picograms per milliliter plasma, and the lowest limit of detectability was 5 pg/ml. This assay has been validated for rats in our laboratory (35) and has intra- and interassay coefficients of variation of 3.2 and 6.8%, respectively. Plasma corticosterone levels were determined using a commercially available RIA kit (MP Biomedicals, Inc., Orangeburg, NY). Data are expressed in nanograms per milliliter plasma, and the lowest limit of detectability was 5 ng/ml. Intra- and interassay coefficients of variation are 7.3 and 13.2%, respectively.

Gonadal steroid levels. To determine whether gonadal steroids were altered by prenatal stress, plasma testosterone was measured in adult males and estradiol and progesterone was measured in adult diestrous females exposed prenatally to no treatment, repeated restraint, or randomized stress. Because plasma from subjects in experiment 1 was needed for ACTH and corticosterone immunoassays, gonadal hormone levels were determined in plasma from subjects of experiment 2. Four days after the last behavioral test, animals in experiment 2 were killed by deep anesthesia with 35% chloral hydrate, and blood samples were obtained via cardiac puncture.

Plasma concentrations of testosterone, estradiol, and progesterone were measured using the Coat-A-Count kits for total testosterone, estradiol, and progesterone, respectively (Diagnostic Products Corp., Los Angeles, CA). The lower limits of detectability were 0.1 ng/ml, 5 pg/ml, and 0.1 ng/ml, respectively. Intra- and interassay coefficients of variation for testosterone, estradiol, and progesterone are less than 7% and less than 11%, less than 7% and less than 8%, and less than 6% and less than 10%, respectively. Each assay has been validated in our laboratory for the measurement of plasma gonadal hormone levels in the rat.

Glucocorticoid receptor immunohistochemistry. To investigate whether prenatal stress altered glucocorticoid receptor (GR) expression in the hippocampus, a region important for corticosterone-negative feedback regulation of the HPA axis (28), animals from experiment 2 were intracardially perfused with 4% paraformaldehyde immediately after the cardiac puncture procedure described above. Brains were postfixed for 4 h, snap frozen in isopentane, and stored at -80°C until they were later cut on a microtome into 30- μm sections. Tissue was then processed for immunohistochemical labeling of glucocorticoid receptor using a polyclonal antibody generated against the c terminus of human GR (rabbit antihuman GR) that was generously donated by Drs. Ron Evans and Wylie Vale (The Salk Institute). Briefly, every eighteenth section through the brain was slide mounted and dried overnight before immunohistochemistry. Slides were coded before immunohistochemistry and the code was not broken until after analysis was complete. All incubations were performed at room temperature unless otherwise indicated. Slide-mounted sections were subjected to three pretreatment steps as described previously (36). Slides were incubated with 0.3% H_2O_2 for 30 min to remove any endogenous peroxidase activity. Nonspecific binding was then blocked with 3% serum and 0.3% Triton X-100 in 1 \times PBS for 30 min and incubated with the primary antibody (in 3% serum and 0.3% Tween 20) for 18–20 h. After washing with 1 \times PBS, the sections were exposed to biotin-tagged secondary antibody (goat antirabbit; catalog no. BA-1000; Vector Laboratories, Burlingame, CA; 1:200) for 60 min. After secondary antibody incubation, slides were incubated in ABC for 1 h (catalog no. PK-6100; Vector Laboratories), and then staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride (catalog no. 34065; Pierce Laboratories, Rockford, IL). Specificity of GR immunoreactivity was determined using two controls: elimination of the primary

or secondary antiserum. No staining occurred under either of these conditions, and staining was consistent with what has been previously reported (37).

After immunohistochemistry, the staining was visualized and specific staining was seen in the dentate gyrus (DG) and cornu ammonis (CA)1/CA2 of the hippocampus as reported previously (37). Sections containing either the left or right hippocampal DG and CA1/CA2 regions were captured with a Axiophot photomicroscope (Zeiss, New York, NY) fitted with a Zeiss ZVS video camera. Captured sections were sorted into DG and CA1/CA2 sections and each were separately analyzed by National Institutes of Health Scion Image 4.03 software to determine qualitative changes in GR immunoreactivity. The DG was further divided into the superior and inferior portions to determine changes in the rostral and caudal regions of the hippocampus.

Experiment 2: effect of prenatal stress on behavioral measures of anxiety in adult male and female offspring

Animals. Experiment 2 sought to examine the effects of the prenatal stress models on the anxiety-like behavior of a separate group of adult male and female offspring ($n = 10\text{--}13$ per sex per prenatal treatment). In females, estrous cycles were synchronized by giving two doses of 2 μg [DTrp⁶, Pro⁹, Net]GnRH (sc) at 0900 and 1400 h 7 d before testing (33). Injections were timed so that females were in diestrus on the testing day of the EPM, metestrus for the first defensive burying test, and diestrus for the second defensive burying test. Estrous cycle status was confirmed by vaginal smears taken 4 d (one complete cycle) after behavioral testing was completed. Females not in diestrus on this day were eliminated from the study.

EPM. The EPM is a widely used test of anxiety-like behavior and was used to assess passive anxiety-like behavioral responses (15). This test is sensitive to putative anxiogenic and anxiolytic drugs (38–40). It is designed to present the animal with a conflict between its natural tendency to explore a novel environment and its reluctance to move away from the sheltering walls and into the open environment in which the risk of falling or exposure to predators is much higher. The maze was made of black Plexiglas and consisted of four arms (50 cm long \times 10 cm wide); two arms had 40-cm-high dark walls (closed arms), and two arms had 0.5-cm-high ledges (open arms). The floor of the apparatus was 50 cm high. Open arms received 3–4 lux of illumination. Animals were handled daily for 1 wk before testing to adapt them to handling and introduce them to ledges. They were allowed to rest in the anteroom for at least 2 h before testing and were tested during their dark cycle between 1900 and 2200 h.

The EPM was performed in dark when baseline percent open arm time is high (due to increased exploration in the dark cycle) to more easily allow anxiogenic-like effects to be detected. White noise (70 dB) was present throughout habituation and testing. Previous experience can significantly diminish the validity of the EPM test to measure anxiety-like behavior (41); therefore, animals were not given prior exposure to the testing apparatus, allowing for the assessment of unconditioned fear of open spaces. For testing, rats were placed individually onto the center of the maze facing a closed arm and removed after a 5-min period. The session was video recorded so that the animal was undisturbed during this time. Behavior was recorded and scored by one experimenter naive to the treatment condition of the animals. The apparatus was wiped clean with water and dried between subjects. The primary measures were the percent of total arm time and entries directed toward the open arms [*i.e.* $100 \times \text{open arm}/(\text{open arm} + \text{closed arm})$], which are validated indices of anxiety-related behavior or unprotected exploration (42). The number of entries into the closed arms and total arm entries are validated indices of locomotor activity based as documented in factor analysis studies (42, 43). Thus, we measured these variables to assure that reductions in open arm entries were due to an anxious-like state in these animals rather than decreased general locomotor activity. It should be noted that locomotor activity in the EPM, as measured in the current study, may be a different construct than what is measured by locomotor activity as defined by photocell interruptions in a test cage.

Defensive burying test d 1 (with shocks). Defensive burying tests were used to assess active anxiety-like behavior (defensive burying test 1) and conditioned fear response [or avoidance retention, defensive burying

test 2 (16, 17)]. Anxiolytic and anxiogenic compounds decrease and increase defensive burying behavior, respectively (17, 44). Animals were allowed at least 1 wk of rest after being tested on the EPM before being tested for defensive burying behavior. For 2 consecutive days before defensive burying testing, animals were acclimated to the testing apparatus by placing them for 45 min in the testing cage (a polycarbonate rat housing cage with 2 cm of bedding covering the floor and a small hole centered on a long dimension of the cage 1 in. above the bedding to accommodate the shock probe on the subsequent test day). On the day of testing, animals were brought into the anteroom at least 1 h before testing began. They were then placed individually in the test cage, and a shock probe connected to a Coulbourn precision shocker (model E13–01) delivered one 1.5 mA (lasting < 1 sec) shock on contact. As soon as the animal was shocked (verified by a startle response), the shock current was deactivated and the 10-min test began. Contact with the shock probe typically results in the rat displacing bedding material with treading-like movements of the forepaws and shoveling movements of the head, often directed toward the noxious stimulus posing a threat (*i.e.* shock probe). Latency to first display burying behavior and time spent burying (in five 2-min bins throughout the 10 min test) were assessed (44). All defensive burying testing occurred in the light cycle (between 1100 and 1700 h), when baseline levels of burying are low, allowing for anxiogenic-like effects (increases in burying) to be detected. The test was recorded, and an experimenter naive to the treatment conditions of the animals scored the behavior.

Defensive burying test d 2 (without shock). Twenty-four hours after the first defensive burying test, animals were tested again using a protocol similar to the first test except the probe current was turned off so that the animal was not shocked on contact with the probe. This test was used to assess the conditioned defensive response to a noxious stimulus (the probe) (16). The 10-min test was recorded, and burying behavior (latency to first bury and burying time) was determined by an experimenter naive to the treatment conditions.

Statistics

Analyses of endocrine and brain data. In a few cases, there was not enough plasma to measure ACTH (six of 200 samples) or corticosterone (five of 245 samples) at single time points after shock. To prevent the loss of all data from these animals in within-subject analyses due to a single lost time point, missing values of ACTH and corticosterone responses to stress were imputed for five observations using the Gibbs sampler algorithm for the multivariate linear mixed model with incomplete data, as previously described (45, 46). The multiple imputation was done only in cases in which no more than one sample was missing; thus, six samples were imputed for ACTH and five samples were imputed for corticosterone. Imputed data sets were then analyzed using PROC MIANALYZE of SAS 9.1 (SAS Institute, Cary, NC) after the procedures outlined elsewhere (47). It should be noted that effects that were significant in the imputed model also were significant in a repeated-measures ANOVA using listwise deletion of subjects with missing values. ACTH and corticosterone levels at the 0 min time point were also analyzed separately as an index of basal HPA activity, given that the animals were allowed 3 h of rest after hook-up before this sample was obtained (Rivier, C., unpublished findings).

To further characterize functional changes in the HPA axis in prenatally stressed males and females, area under the curve, simple regression, and time to peak analyses were conducted on ACTH and/or corticosterone data. ACTH and corticosterone area under the curve (relative to basal levels) was calculated using the trapezoid rule, and data were analyzed using a 3 (prenatal treatment) \times 2 (sex) between-factors ANOVA. Simple regression analyses were used to determine whether prenatal treatment altered the nature (slope) or regularity (correlation) of the relation between ACTH and corticosterone levels (collapsing across the 0- to 45-min time points) in males and females. Peaks were analyzed by comparing the average latency to peak using ANOVA and the proportion of animals that peaked as late as 45 min using χ^2 analyses. The time point at which ACTH was highest for each animal was used for the time peak value. Gonadal steroid levels and GR immunoreactivity in the hippocampus were analyzed using ANOVAs comparing the three prenatal treatment groups. Unless stated otherwise, all analyses

were followed by Fisher's protected least significant differences *post hoc* tests. Differences were considered significant when $P \leq 0.05$.

Analyses of behavioral data. All behavioral measures on the EPM were analyzed using a 3 (prenatal treatment) \times 2 (sex) between-factors ANOVA. For defensive burying tests 1 and 2, time spent burying was analyzed using a 3 (prenatal treatment) \times 2 (sex) \times 5 (within subject: test bin time) mixed-design ANOVA. Total bury time data were analyzed using a 3 (prenatal treatment) \times 2 (sex) between-factors ANOVA. Because latency data had skewed distributions and unequal variance between the comparison groups, a logarithmic transformation was used to normalize distributions and homogenize variance to permit parametric pairwise comparisons between groups.

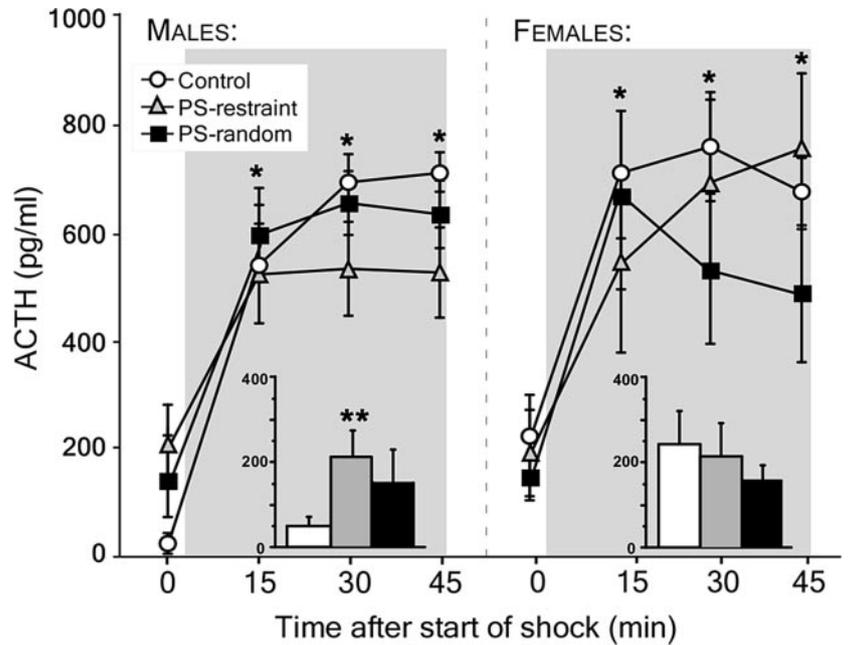
Results

Experiment 1: effect of prenatal stress on HPA function, gonadal steroid levels, and glucocorticoid receptor immunoreactivity in the hippocampus in adult male and female offspring

ACTH and corticosterone levels. Basal ACTH (time 0 min, *inset bar graphs*) and ACTH responses to shock are shown in Fig. 1. Main effects of prenatal treatment or sex on basal ACTH were not significant, although pairwise comparisons indicated elevated basal ACTH in PS-restraint males, compared with controls ($P = 0.05$). Shocks elicited a significant increase in ACTH levels [$F(3, 129) = 61.4752, P \leq 0.0001$]. Area under the curve analyses indicated PS-restraint males had a reduced area under the curve of ACTH relative to basal levels, compared with control males ($11,328 \pm 4,381$ pg/min·ml *vs.* $21,861 \pm 1,580$ pg/min·ml, respectively, $P = 0.03$). Basal corticosterone (time 0, *inset bar graphs*) and corticosterone responses to shocks are shown in Fig. 2. Basal levels were not different among the groups, but females had significantly higher basal corticosterone than males [$F(1, 43) = 22.60, P < 0.0001$] independent of prenatal treatment. In all groups, corticosterone was significantly elevated aftershock [$F(4, 172) = 26.36, P < 0.0001$]. There was a significant interaction between time after shocks and sex on corticosterone levels [$F(8, 172) = 2.32, P < .0001$]. *Post hoc* analyses indicated that PS-restraint males had a blunted corticosterone response, compared with control males, 30 and 45 min after shock onset ($P_s = 0.04$). Decreased responsiveness to shock stress is consistent with a trend of a smaller area under the curve of corticosterone relative to basal levels in PS-restraint males, compared with controls, although this was not significant ($7,374 \pm 3,116$ ng/min·ml *vs.* $15,539 \pm 2,212$ ng/min·ml, $P = 0.08$). *Post hoc* analyses indicated that corticosterone remained elevated 120 min after shock onset in PS-restraint females, compared with controls ($P = 0.002$), signifying inadequate return to basal levels in these animals (a delayed ACTH response may also contribute to elevated levels at this time point, as noted below). PS-random females had corticosterone levels intermediate between PS-restraint and control females at the 120-min time point, but they were not significantly different from either group. Prenatal treatment did not alter the area under the curve relative to basal for ACTH or corticosterone in females.

To characterize the temporal responsiveness of the HPA axis to stress, the time that elapsed after shocks to the peak level of ACTH was compared between the different prenatal stress groups in males and females. The average time to reach

FIG. 1. Basal plasma levels of ACTH (picograms per milliliter, *bar graph insets*) and time course of plasma ACTH levels in response to foot shock stress in adult males and females exposed prenatally to no treatment (control), PS-restraint, or PS-random. *Shading* indicates the presence of shock. *Asterisks* (*) indicate significant increases in ACTH after shock, compared with the 0 min time point for each prenatal group ($P < 0.0001$). *Double asterisks* (**) indicate higher basal ACTH in PS-restraint males, compared with controls ($P = 0.05$). Data are shown as mean \pm SEM ($n = 8$ –10 per treatment group and sex).

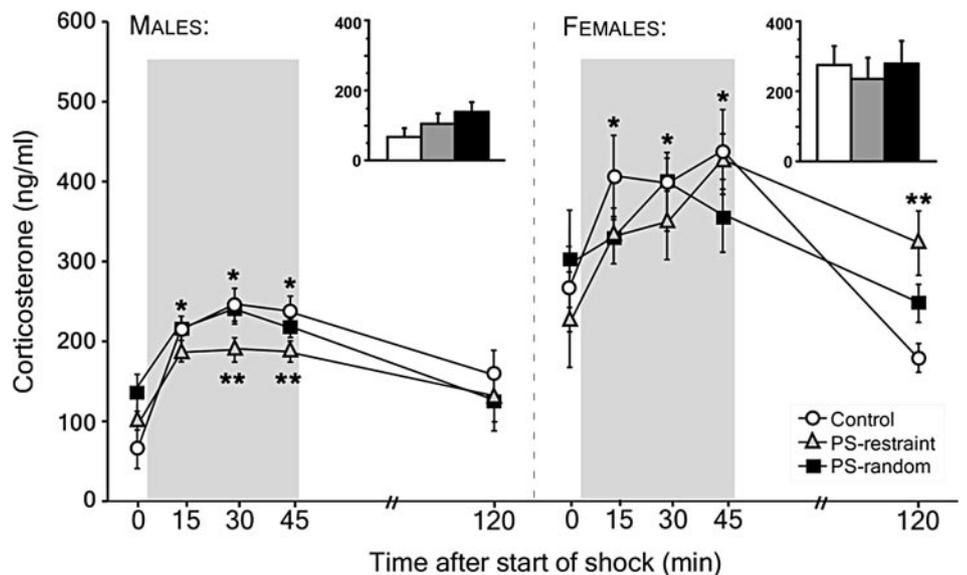


peak ACTH levels occurred later in PS-restraint females (37 ± 4 min), compared with control (26 ± 4 min) and PS-random (24 ± 4 min) females ($F_{2,20} = 3.34, P = 0.05$). Consistent with this, χ^2 analyses indicated that a significantly higher proportion of PS-restraint females (62.5%) had a later ACTH response to stress (levels peaking at 45 min) than control (12.5%) and PS-random (14.3%) females ($\chi^2 = 5.96, P = 0.05$). The delayed ACTH response to stress in PS-restraint females may contribute to the prolonged corticosterone response at 120 min in addition to a possible failure of recovery to baseline in these animals. There were no significant differences in time to peak in males (data not shown).

Simple regression analyses are shown in Fig. 3. ACTH and corticosterone levels were positively correlated in males and females of the different prenatal treatment groups (PS-control males: $r^2 = 0.7, P < 0.0001$; PS-restraint males: $r^2 = 0.49, P < 0.0001$; PS-random males: $r^2 = 0.37, P = 0.0002$; PS-

control females: $r^2 = 0.42, P = 0.0001$; PS-restraint females: $r^2 = 0.29, P = 0.001$; PS-random females: $r^2 = 0.19, P = 0.02$). This suggests that differences in corticosterone responses to stress (attenuated in males and prolonged in females) caused by prenatal exposure to repeated restraint are occurring, at least in part, by mechanisms upstream of the adrenal gland (*e.g.* changes in CRH, vasopressin, and/or ACTH release). With that said, subtle changes in the HPA axis may also be occurring at the level of the adrenals because the slope of the correlation between ACTH and corticosterone was reduced in PS-restraint ($0.15 \pm 0.026, P = 0.01$) and PS-random ($0.14 \pm 0.03, P = 0.007$) males, compared with controls (0.26 ± 0.031), indicating that adrenal output of corticosterone in response to ACTH may be slightly attenuated in these animals. Prenatally stressed females had a similar trend, but it was not significant (control = 0.31 ± 0.06 vs. PS-restraint = $0.18 \pm 0.05, P = 0.10$ and PS-random = $0.164 \pm 0.06, P = 0.09$).

FIG. 2. Basal plasma levels of corticosterone (nanograms per milliliter, *bar graph insets*) and time course of plasma corticosterone levels in response to foot shock stress in control, PS-restraint, and PS-random adult males and females. *Shading* indicates the presence of shock. *Single asterisks* (*) indicate significant increases in corticosterone aftershock, compared with the 0 min time point for each prenatal group ($P < 0.0001$). Females had higher corticosterone than males ($P < 0.0001$, no indicator). *Double asterisks* (**) indicate higher corticosterone at the 120-min time point in PS-restraint females, compared with controls ($P = 0.002$) and lower corticosterone at the 30- and 45-min time points in PS-restraint males, compared with controls ($P = 0.04$). Data are indicated as mean \pm SEM ($n = 7$ –10 per treatment group and sex).



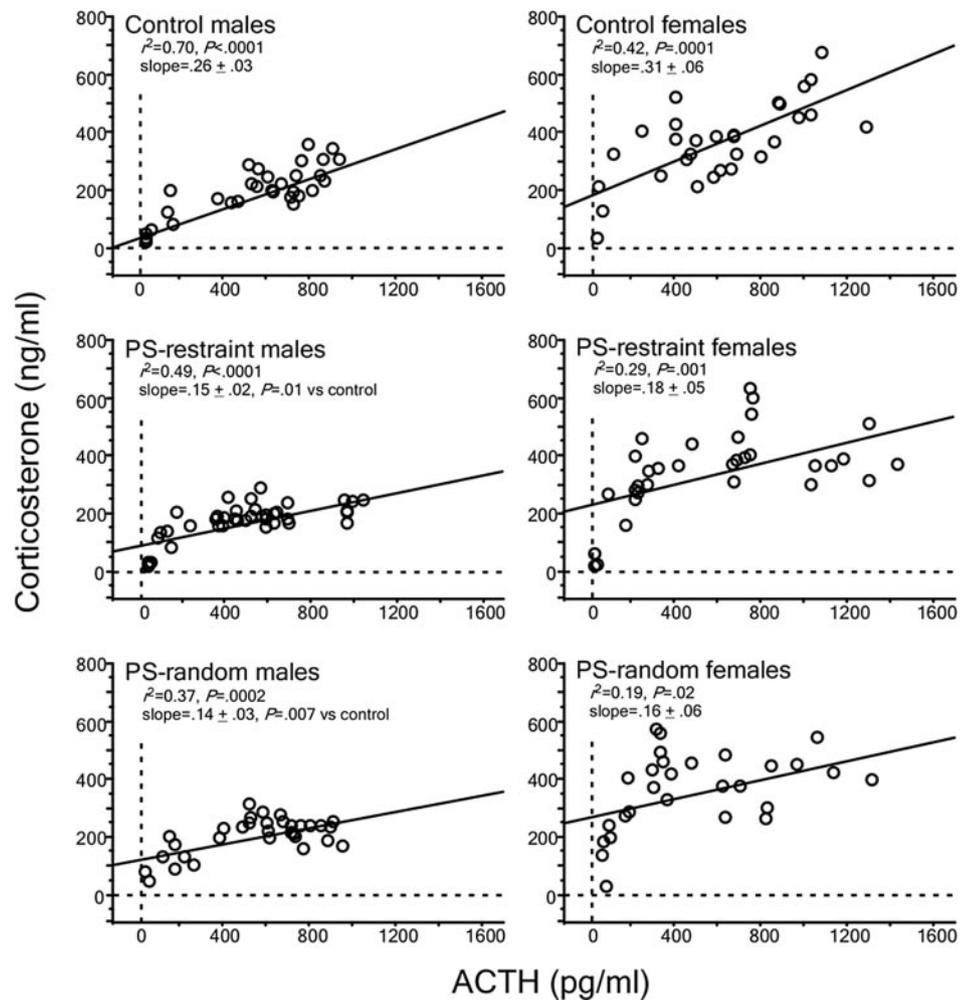


FIG. 3. Simple regression scatter plots of ACTH and corticosterone correlations in adult males and females exposed prenatally to no treatment (control), PS-restraint, or PS-random. In all groups, ACTH and corticosterone were positively correlated (all P s < 0.05). However, the slope of the relation was reduced in PS-restraint ($P = 0.01$) and PS-random ($P = 0.007$) males, compared with controls.

Gonadal steroid levels. To investigate whether adult gonadal hormones, which influence HPA activity (48), differed between prenatal treatment groups, testosterone was measured in males and estradiol and progesterone were measured in diestrous females of the three prenatal treatment groups. Data are shown in Fig. 4. There was a main effect of prenatal treatment on plasma concentrations of testosterone [$F(2, 32) = 3.67, P = 0.03$] such that PS-restraint ($P = 0.03$) and PS-random ($P = 0.01$) males had reduced levels, compared with control males. Although there was a trend of an increase in estradiol and a decrease in progesterone plasma levels in females of both prenatal stress groups, these levels were not significantly different.

Glucocorticoid receptor immunolabeling in the hippocampus. To investigate whether prenatal stress altered GR expression in the hippocampus, a region important for corticosterone-negative feedback (28), GR immunoreactivity in the dentate gyrus and CA1/2 region of the hippocampus was compared in males and females of the three prenatal treatment groups (Fig. 5). There was a significant effect of sex ($F_{2,29} = 4.83, P = 0.03$) and a sex by prenatal treatment interaction ($F_{2,29} = 3.12, P = 0.05$) of GR immunoreactivity in the inferior limb of the dentate gyrus. *Post hoc* analyses indicated that inferior DG GR immunoreactivity was higher in control females, com-

pared with control males ($P = 0.005$), and prenatal exposure to repeated restraint caused a slight elevation in GR immunoreactivity in the inferior DG in males ($P = 0.02$ vs. PS-random males and $P = 0.18$ vs. control males) and in the DG as a whole (PS-restraint vs. PS-random males, $P = 0.02$). A similar pattern of prenatal stress was seen in the CA1/2 region of the hippocampus. Whereas the interaction between sex and prenatal treatment was not significant ($F_{2,29} = 2.54, P = 0.09$), *a posteriori* comparisons indicated elevated GR immunoreactivity in the CA1/2 region in PS-restraint males, compared with random ($P = 0.004$) and control ($P = 0.01$) males and compared with PS-restraint females ($P = 0.01$). Prenatal restraint stress appeared to have the opposite effect in females than males (*i.e.* a reduction rather than an elevation) in GR immunoreactivity in the inferior DG, although this trend was not significant ($P = 0.07$, control vs. PS-random females). GR immunoreactivity was not significantly altered in the superior limb of the DG by prenatal treatment in either males or females.

Experiment 2: effect of prenatal stress on behavioral measures of anxiety in adult male and female offspring

EPM. There was a main effect of prenatal treatment on the percentage of open-arm entries ($F_{2,61} = 4.72, P = 0.01$, Fig.

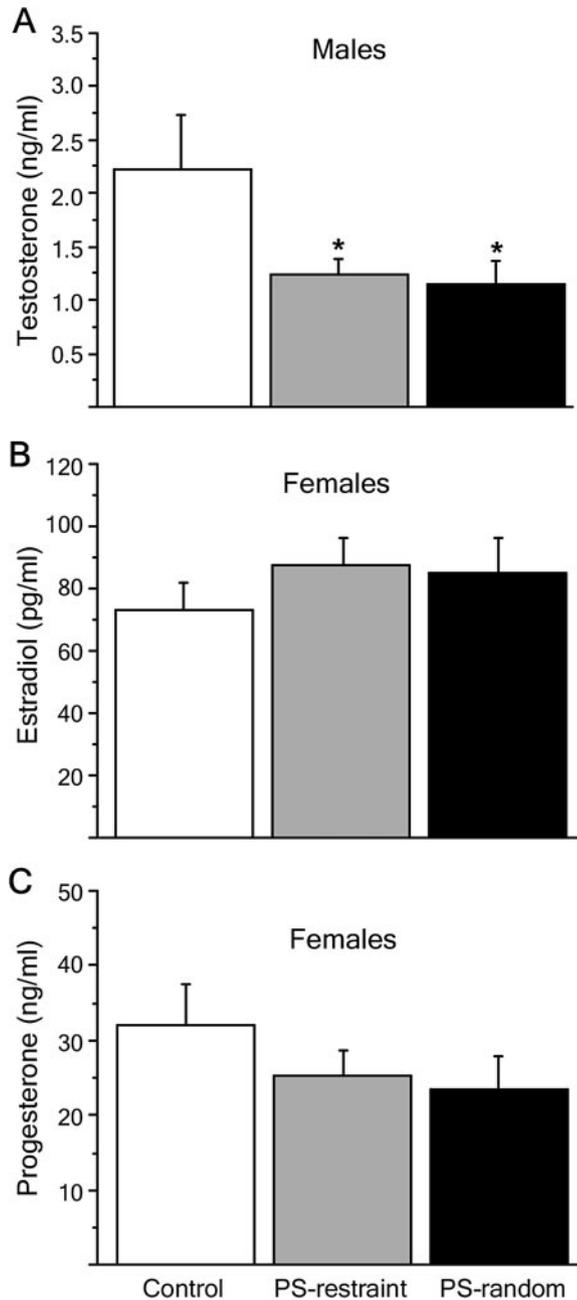


FIG. 4. Plasma levels of testosterone from adult males (A) and estradiol (B) and progesterone (C) from adult diestrous females exposed prenatally to no treatment (control), PS-restraint, or PS-random. Asterisks (*) indicate that PS-restraint and PS-random males had significantly lower testosterone levels than control males ($P = 0.03$, PS-restraint; $P = 0.01$, PS-random). Data are indicated as mean \pm SEM (for each gonadal steroid, $n = 10$ – 13 per treatment group and sex).

6A). There was not a significant main effect of sex or an interaction between sex and prenatal treatment. However, inspection of Fig. 6A suggested that the prenatal stress effect did not appear to be attributable to males but rather due to differences between the female groups. *A posteriori* comparisons confirmed that female offspring of prenatally restrained dams engaged in proportionally fewer entries to the open arms than did offspring of control ($P = 0.03$) and PS-

random females ($P = 0.005$), indicating an increase in anxiety-like behavior, whereas there were no significant difference in this measure in males. There was a similar trend for an effect of prenatal treatment on the percentage of time spent in the open arms, which reflected the tendency for PS-restraint females to have reduced percent open-arm time, compared with control and PS-random females ($F_{2,61} = 2.92$, $P = 0.06$, Fig. 6B).

Closed-arm and total-arm entries were assessed to determine whether the reduction in open-arm entries in PS-restraint females was due to a general reduction in locomotor activity rather than increased anxiety-like behavior in these animals (Fig. 6, C and D) (42, 43). There were no significant main effects of prenatal stress or gender on the number of closed-arm or total-arm entries [prenatal stress (all $P > 0.05$)], which verifies that the anxiogenic-like behavior seen in the PS-restraint females is not due to a general reduction in locomotor activity. It should be noted although that *a posteriori* comparisons indicated a lower number of closed-arm ($P = 0.04$) and total-arm entries in PS-restraint males ($P = 0.02$), compared with controls, signifying reduced locomotor activity on the EPM in this group of animals. The number of total-arm entries reported here is consistent with low levels of activity on the EPM in the Sprague Dawley strain as reported by others (49).

Defensive burying tests

Test d 1 (with shocks). Defensive burying data (expressed in back-transformed log) are shown in Fig. 7. Latency to first display burying behavior on test 1 was slightly reduced in PS-random females (and to a lesser extent PS-random males), but these differences were not statistically significant (all $P > 0.05$, Fig. 7A, left graph). In both males ($F_{4,124} = 2.55$, $P = 0.04$) and females ($F_{4,104} = 4.51$, $P = 0.002$), there was a main effect of test bin time, which reflected that most burying behavior occurred within the first 4–6 min of the test. In females, prenatal treatment influenced the duration of defensive burying, as reflected by a prenatal treatment by test bin time interaction ($F_{8,104} = 2.32$, $P = 0.02$) and a main effect of prenatal treatment ($F_{2,26} = 3.35$, $P = 0.05$). *Post hoc* analyses indicated that both PS-restraint ($P = 0.01$) and PS-random ($P = 0.004$) females spent more time burying in the 3- to 4-min bin than controls. PS-restraint females also buried more than controls during the 5- to 6-min bin ($P = 0.004$) and cumulatively across the 10-min test period ($P = 0.04$) (Fig. 7, bar graph insets). PS-random males also spent more time burying in the first 2 min, compared with control males ($P = 0.04$).

Test d 2 (without shocks). PS-restraint females were quicker to bury, compared with controls, on the second defensive burying test day, 24 h after they were briefly shocked by the probe ($P = 0.02$, Fig. 7A, right graph). There were no significant differences in latency to bury in PS-restraint males or in PS-random males or females, compared with controls. There were no differences in the total duration of burying on the second defensive burying test when data were analyzed in separate 2-min bins or collapsed across the entire 10-min test (data not shown).

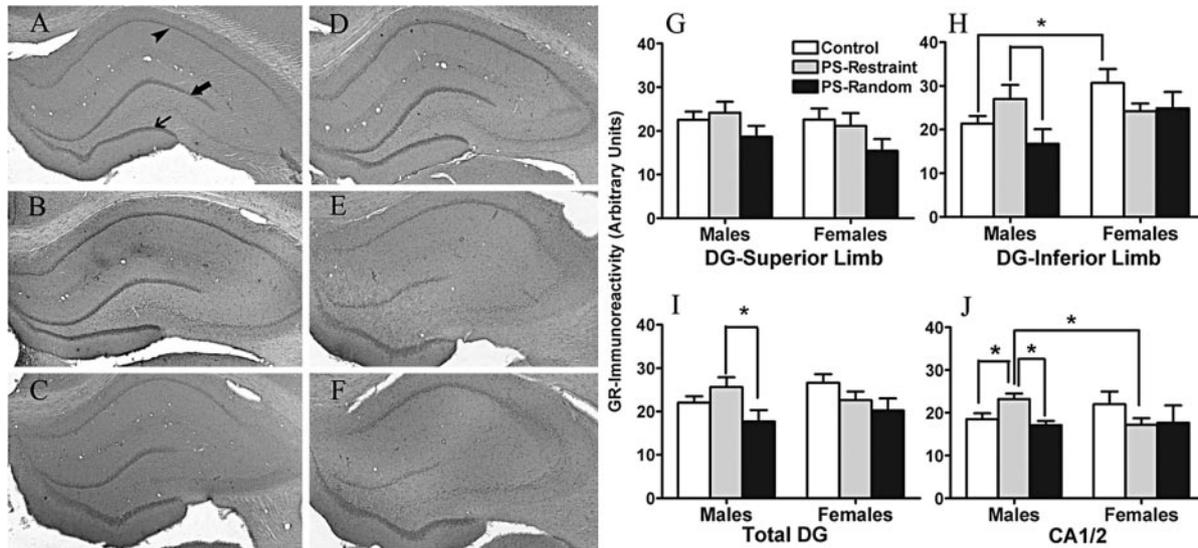


FIG. 5. GR immunoreactivity (IR) in the superior (*thick arrow*) and inferior (*thin arrow*) DG and CA1/2 region (*arrowhead*) of the hippocampus of adult males and females exposed prenatally to no treatment (control), PS-restraint, or PS-random. A–C, Qualitative representations of GR-IR in the males (control, A; PS-restraint, B; PS-random, C), D–F, Qualitative representation of GR-IR in the females (control, D; PS-restraint, E; PS-random, F). G–J, Densitometric analyses of GR-IR in the hippocampus; superior DG (G), inferior DG (H), entire DG (I), and entire CA1/2 (J) regions. Data are expressed as arbitrary units of GR-IR. Asterisks (*) indicate differences between groups (all P s < 0.05). Data are indicated as mean + SEM ($n = 5–6$ per treatment group and sex).

Discussion

The present findings illustrate the profound impact that gestational stress has on the functional status of endocrine and behavioral systems in adulthood. The pattern and/or type of stress exposure along with the gender of the offspring dictate susceptibility to the effects of prenatal stress. Daily

exposure to repeated, predictable stress (restraint) during prenatal development generated the most robust changes in adult offspring, and the effects were more extensive in females. This treatment increased anxiety-related behaviors (both passive and active), including avoidance retention, and resulted in a prolonged corticosterone response to stress in

FIG. 6. Behavioral measures of males and females exposed prenatally to no treatment (control), PS-restraint, or PS-random and tested on the EPM in adulthood. A, Asterisk (*) indicates a significant reduction in percent open-arm entries in PS-restraint females, compared with control ($P = 0.03$) and PS-random ($P = 0.005$) females. The percentage of time spent in the open arms (B), the number of closed-arm entries (C), and the number of total arm entries (D) are shown. D, Asterisks (*) indicate that PS-restraint males had significantly fewer closed- and total-arm entries, compared with control males ($P = 0.02$). Data are indicated as mean \pm SEM ($n = 10–12$ per treatment group and sex).

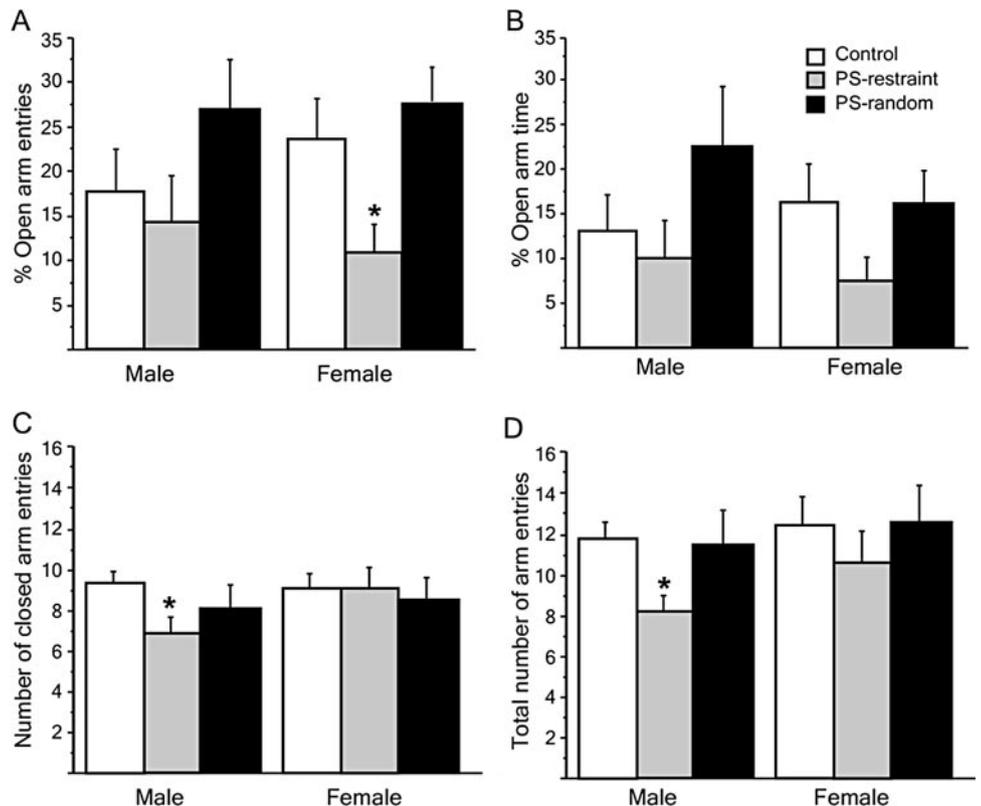
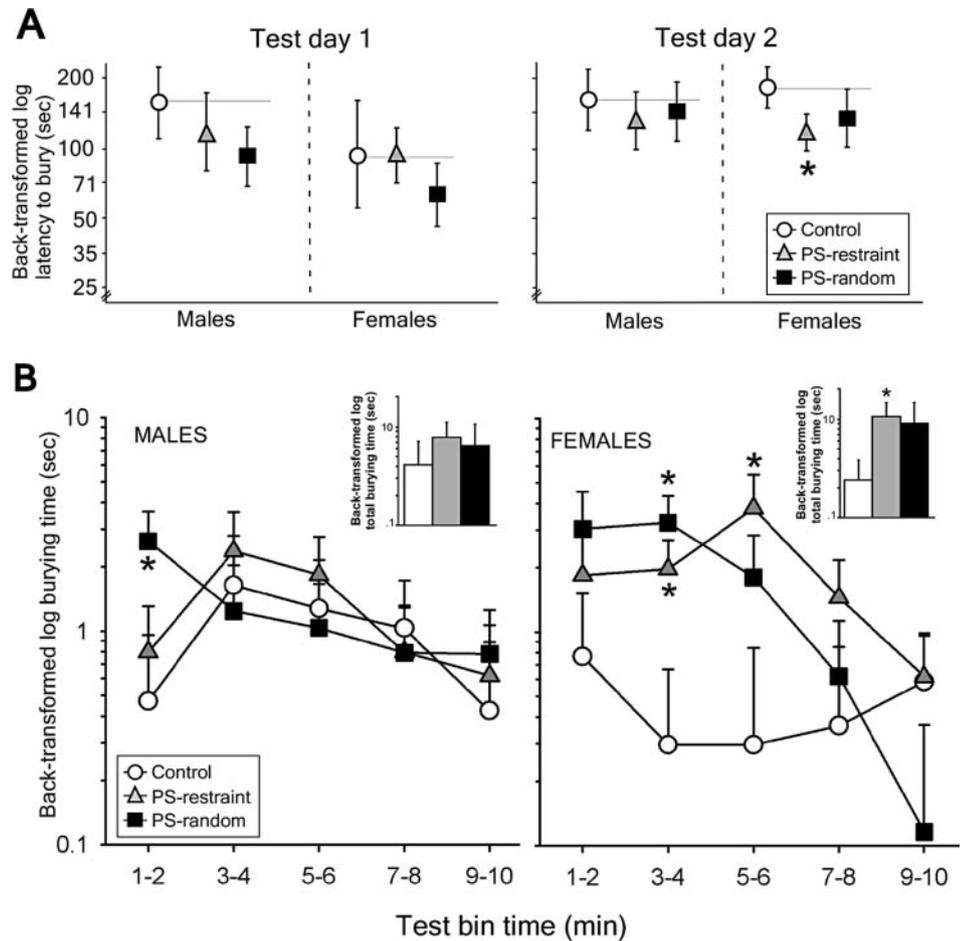


FIG. 7. Defensive burying behavior of adult males and females exposed prenatally to no treatment (control), PS-restraint, or PS-random. A, Latency to first engage in burying behavior on test d 1 (with shock, A, *left graph*) and test d 2 (without shock, A, *right graph*) are shown. Asterisk (*) indicates a significant reduction in the latency to bury in PS-restraint females, compared with controls on test d 2 ($P = 0.02$). Gray lines highlight the mean latency of control animals. B, Time spent burying within five 2-min bins and total burying time (*insert bar graphs*) during the 10-min test are shown. In males and females, there was a main effect of test bin time, which reflected that most burying behavior occurred within the first 4–6 min of the test ($P < 0.05$, no indicator). Asterisks (*) indicate specific time points at which prenatally stressed animals buried more than controls (PS-random males: $P = 0.04$ for 1–2 min; PS-random females: $P = 0.004$ for 3–4 min; PS-restraint females: $P = 0.01$ for 3–4 min, $P = 0.004$ for 5–6 min, and $P = 0.04$ for increased cumulative burying across the 10-min test period, *inset bar graph*). Because of unequal variance and non-Gaussian distribution of burying behavior, all data are indicated as back-transformed means \pm SEM after logarithmic transformation ($n = 8$ –13 per treatment group and gender).



females (due to a delayed ACTH response to stress and/or a failure of recovery). PS-restraint males did not show changes in anxiety-related behaviors but did have elevated basal ACTH and a blunted HPA response to stress. Increased GR immunoreactivity in the hippocampus provides one means by which the HPA responsivity could be blunted in these animals. Prenatal exposure to a varied, unpredictable pattern of stressors did not have as much effect on HPA function, with most neuroendocrine measures residing intermediate to PS-restraint and control animals within each sex. Behaviorally, these animals displayed increased active, possibly coping (17), anxiogenic-like behavior, an effect most evident in females. Finally, prenatal stress-induced changes in gonadal steroids occurred independent of the stressing paradigm and were measurable only in males, with almost a 40% decrease in testosterone in both prenatal stress groups. These findings exemplify the considerable difference in sensitivity of the developing nervous and endocrine systems to stress, which depends on gender and the nature of the stressful experience to which the mother is exposed during pregnancy.

Prenatal stress: gender differences, HPA function, and gonadal hormones

Males and females differ significantly with respect to HPA function (50). Under normal prenatal conditions, females

have heightened HPA activity, compared with males in adulthood (51). We extended these findings and demonstrated that corticosterone, but not ACTH, was higher in control females, compared with control males, under basal conditions and in response to foot-shock stress and found that gender differences in HPA function were modified by prenatal stress. It should be noted that the basal levels of ACTH and corticosterone were slightly elevated in control animals, especially females, even after 3 h of rest after hook-up in the present study. However, if these animals were experiencing a low baseline level of stress, it does not appear to have had ample impact on ACTH or corticosterone responses to shock (4- to 7-fold increases in ACTH and 2- to 5-fold increases in corticosterone).

Prenatal stress had differential effects on HPA responses to stress, depending on gender and the stress paradigm. Whereas PS-random did not significantly alter stress-induced HPA responses in either gender, PS-restraint caused opposing effects in males and females. In agreement with other studies using this gestational stress model (10, 52), PS-restraint extended corticosterone responses in females, possibly due to both a delayed ACTH response and failure of recovery in females. Hippocampal GR immunoreactivity was not significantly altered in PS-restraint females, but there was a trend of a reduction, consistent with a possible reduction in negative feedback in these animals. This same treat-

ment resulted in blunted ACTH and corticosterone responses to stress in males, supporting an earlier report [(52); see also Ref. 11]. Hippocampal GR immunoreactivity was increased in PS-restraint males, suggesting that enhanced negative feedback inhibitory tone could contribute to the attenuated HPA responses to stress characteristic of these animals. Additional studies using more quantitative measures could help determine whether changes in GR immunoreactivity reflect functional changes in negative feedback. Neuroendocrine responses to stress were not significantly altered in PS-random offspring with one exception. Adrenal sensitivity to ACTH may be altered in both PS-restraint and PS-random males as evidenced by lower regression slopes between the two measures, but more extensive studies (*e.g.* ACTH challenge, adrenal histology) would need to be done to confirm this.

Gonadal steroids have considerable impact on hormones of the HPA axis (48). Testosterone suppresses, and estrogen stimulates, HPA activity (26, 27). Testosterone was reduced in PS-restraint and PS-random adult males in the present study. Lower testosterone could be contributing to the significant elevation of basal ACTH in PS-restraint males and similar trend in PS-random males. It should be noted that we have measured only total testosterone; there may be alterations in steroid hormone binding globulin levels that could influence testosterone's actions on the HPA axis. Sex steroids were not altered in females, consistent with no measurable change in basal ACTH with prenatal stress. Females in the current study were in diestrus, however, and perhaps changes in basal HPA hormones and gonadal steroids would be observed on a different day of the estrous cycle.

Prenatal stress: anxiety-related behaviors

The present study considered three aspects of anxiety-like behavior of prenatally stressed offspring: EPM (an index of passive anxiety-like behavior) (15); shock-elicited defensive burying test 1 (an index of active anxiety-like behavior) (17); and nonshock-elicited defensive burying test 2 (an index of conditioned aversion or avoidance retention) (16, 17). This strategy allowed us to identify distinct behavioral profiles of adult offspring that were gender and prenatal stress paradigm specific.

PS-restraint decreased open-arm exploration (increased passive anxiety-like behavior) on the EPM in females (consistent with Ref. 12). PS-restraint females also displayed an increase in shock-induced burying behavior on the first defensive burying test and were quicker to bury 24 h later when placed in the same environment. The shorter latency to bury in the second test suggests an enhanced conditioned aversion to adverse stimuli (*e.g.* the shock probe) in these animals (16, 17). Corticosterone is known to consolidate conditioned fear learning (53), and this behavioral characteristic fits well with the neuroendocrine data, in which shock-induced corticosterone was prolonged in these animals. Increased anxiety-like behavior on the EPM has been reported in prenatally restraint stressed males (11, 12), but the behavioral changes observed on the EPM in PS-restraint males presented here were not anxiety specific and instead reflected reduced lo-

comotion. Many variables, including the strain of animal and time of testing, could contribute to the disparate findings.

Exposure to randomized stress during gestation generated a behavioral profile in adulthood that differed from repeated restraint. Passive anxiety-like behavior on the EPM was not different from control animals in either sex, and if anything, there was a trend of an increase in open-arm percent and time (reflecting decreased anxiety-like behavior) in males. PS-random females, and to a lesser extent males, elicited higher levels of active anxiety-like behavior (increased burying) in response to shock, and this behavioral profile is suggestive of heightened anxiogenic-like coping (and possibly aggressive) responding to a noxious stimulus (shock probe) in these animals (17).

Prenatal stress effects: contributing factors

The two stressing paradigms differ in the type of stress (*e.g.* injection and shocks being painful stressors, whereas restraint is not) as well as the predictability and repetitive nature of the stress schedule; any or all of these factors could potentially contribute to the different neuroendocrine and behavioral characteristics of adult offspring. Although the repeated restraint groups received a higher frequency of stress sessions (three restraint sessions per day), this factor alone does not explain the differences between groups because stress frequency was not positively correlated with active anxiety-like behavior in defensive burying, passive anxiety-like behavior in the EPM, or prolonged HPA responses to stress (simple regression analyses in PS-random animals; data not shown). In other words, PS-random animals with more restraint stress sessions were not more similar to PS-restraint animals than were PS-random animals with fewer restraint sessions. There may, however, be an influence of the type of stress with respect to shock because the frequency of shock, but not restraint or injection, was positively correlated with more anxiety-like behavior in the EPM ($r = 0.23$, $P < 0.01$).

The predictability of the stress paradigm also may contribute to the findings. Predictable and unpredictable stressors have different physiological consequences (18). Chronic daily exposure to predictable repeated immobilization induces hippocampal atrophy, enhanced dendritic arborization in the basal lateral nucleus of the amygdala (a nucleus critical for fear conditioning), and increased anxiety-like behavior on the EPM in adult male rats (54). Conversely, chronic daily exposure to a randomized sequence of stressors has little effect on hippocampus morphology and elicits dendritic atrophy in basal lateral nucleus neurons. Unlike predictable stress, randomized stress fails to increase anxiety-like behavior on the EPM, similar to PS-random offspring in the present study.

Alterations in the hormonal milieu and behavior of the dam may also underlie some prenatal stress effects. Daily injections of the synthetic glucocorticoid dexamethasone during the last week of pregnancy increases anxiety-like behavior and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) and central amygdala and decreases corticosteroid receptor expression in the hippocampus in adult offspring,

and inhibition of 11β -hydroxysteroid dehydrogenase, which serves as a fetoplacental barrier to maternal glucocorticoids reverses some of these effects (8, 55). Corticosterone levels are elevated in maternal and fetal blood several hours after restraint stress (56). Accordingly, exposure to high levels of corticosterone during fetal brain development is a potential mechanism by which prenatal stress influences neuroendocrine and behavioral function in adulthood.

If maternal corticosterone played a role in the neuroendocrine and behavioral changes reported here, it is somewhat surprising that the restraint stress paradigm elicited more robust effects on the HPA axis of adult offspring than did the randomized stress paradigm, given that repeated exposure to the same stressor can lead to adaptations of the HPA axis. However, as stated above, predictable repeated *vs.* unpredictable variable stress differentially alter neural systems outside of the PVN, and these effects could have substantial impact on the developing fetus either directly or indirectly through effects on the mother [e.g. changes in maternal behavior (57, 58)]. In addition, adaptation to repeated stress does not occur equally at all levels of the HPA axis. Whereas repeated exposure to a homotypic stressor (restraint) reduced transcriptional responses in the PVN (20, 21) and blunted ACTH release (19, 22), it did not dampen corticosterone release (22). In fact, corticosterone responses can persist in the face of blunted ACTH release, indicating enhanced adrenal function after repeated exposure to the same stressor (19). This suggests that corticosterone responses to restraint in PS-restraint dams may not have diminished over time in pregnant dams in the present study, consistent with studies in mice (56).

Females and anxiety

The higher susceptibility in females to the anxiogenic-like effects of prenatal stress corresponds well with human studies indicating a higher prevalence of affective disorders in women (5, 59). A plausible explanation of sexual dichotomies in vulnerability to early stress is differential exposure to glucocorticoids *in utero*. Indeed, serum corticosterone concentrations are higher in female, compared with male, mouse fetuses exposed to prenatal stress (60), a gender difference that is likely attributable to differential transport of corticosterone across the placenta (60). Accordingly, greater placental transport of maternal corticosterone to female fetuses is one means by which prenatally stressed females in the current study may display more profound neuroendocrine and anxiety-like behavioral responses, compared with males. The two prenatal stressing paradigms presented here can be used to model gender differences in the lasting effects of early adversity on the neurocircuitry of anxiety and neuroendocrine function in adulthood.

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References

1. Wittchen HU 2002 Generalized anxiety disorder: prevalence, burden, and cost to society. *Depress Anxiety* 16:162–171
2. Nutt DJ, Ballenger JC, Sheehan D, Wittchen HU 2002 Generalized anxiety disorder: comorbidity, comparative biology and treatment. *Int J Neuropsychopharmacol* 5:315–325
3. Brown GW, Harris TO 1993 Aetiology of anxiety and depressive disorders in an inner-city population. 1. Early adversity. *Psychol Med* 23:143–154
4. Brown GW, Harris TO, Eales MJ 1996 Social factors and comorbidity of depressive and anxiety disorders. *Br J Psychiatry* 30:50–57
5. Simonds VM, Whiffen VE 2003 Are gender differences in depression explained by gender differences in co-morbid anxiety? *J Affect Disord* 77:197–202
6. Young MA, Scheffner WA, Fawcett J, Klerman GL 1990 Gender differences in the clinical features of unipolar major depressive disorder. *J Nerv Ment Dis* 178:200–203
7. Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C, Sharma S, Seckl JR, Plotsky PM 1996 Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Dev Neurosci* 18:49–72
8. Welberg LA, Seckl JR, Holmes MC 2001 Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behaviour. *Neuroscience* 104:71–79
9. Weinstock M 2001 Alterations induced by gestational stress in brain morphology and behaviour of the offspring. *Prog Neurobiol* 65:427–451
10. Morley-Fletcher S, Rea M, Maccari S, Laviola G 2003 Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *Eur J Neurosci* 18:3367–3374
11. Vallee M, Mayo W, Dellu F, Le Moal M, Simon H, Maccari S 1997 Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. *J Neurosci* 17:2626–2636
12. Zimmerberg B, Blaskey LG 1998 Prenatal stress effects are partially ameliorated by prenatal administration of the neurosteroid allopregnanolone. *Pharmacol Biochem Behav* 59:819–827
13. Rimondini R, Agren G, Borjesson S, Sommer W, Heilig M 2003 Persistent behavioral and autonomic hypersensitivity to stress following prenatal stress exposure in rats. *Behav Brain Res* 140:75–80
14. Endler NS, Kocovski NL 2001 State and trait anxiety revisited. *J Anxiety Disord* 15:231–245
15. Pellow S, Chopin P, File SE, Briley M 1985 Validation of open/closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 14:149–167
16. Treit D, Pinel JP, Fibiger HC 1981 Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. *Pharmacol Biochem Behav* 15:619–626
17. De Boer SF, Koolhaas JM 2003 Defensive burying in rodents: ethology, neurobiology and psychopharmacology. *Eur J Pharmacol* 463:145–161
18. Foa EB, Zinbarg R, Rothbaum BO 1992 Uncontrollability and unpredictability in post-traumatic stress disorder: an animal model. *Psychol Bull* 112:218–238
19. Hauger RL, Lorang M, Irwin M, Aguilera G 1990 CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress. *Brain Res* 532:34–40
20. Ma XM, Lightman SL 1998 The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. *J Physiol* 510(Pt 2):605–614
21. Ma XM, Lightman SL, Aguilera G 1999 Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology* 140:3623–3632
22. Simpkins JL, Devine DP 2003 Responses of the HPA axis after chronic variable stress: effects of novel and familiar stressors. *Neuro Endocrinol Lett* 24:97–103
23. Kiyokawa-Fougia N, Antoniou K, Bekris S, Liapi C, Christofidis I, Papadopoulou-Daifotis Z 2002 The effects of stress exposure on the hypothalamic-pituitary-adrenal axis, thymus, thyroid hormones and glucose levels. *Prog Neuropsychopharmacol Biol Psychiatry* 26:823–830
24. Willner P 1997 Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* 134:319–329
25. Grippo AJ, Sullivan NR, Damjanoska KJ, Crane JW, Carrasco GA, Shi J, Chen Z, Garcia F, Muma NA, Van de Kar LD 2004 Chronic mild stress induces behavioral and physiological changes, and may alter serotonin 1A receptor

- function, in male and cycling female rats. *Psychopharmacology (Berl)* 179:769–780
26. McCormick CM, Linkroum W, Sallinen BJ, Miller NW 2002 Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. *Stress* 5:235–247
 27. Seale JV, Wood SA, Atkinson HC, Bate E, Lightman SL, Ingram CD, Jessop DS, Harbuz MS 2004 Gonadectomy reverses the sexually dimorphic patterns of circadian and stress-induced hypothalamic-pituitary-adrenal axis activity in male and female rats. *J Neuroendocrinol* 16:516–524
 28. Sapolsky RM, Meaney MJ, McEwen BS 1985 The development of the glucocorticoid receptor system in the rat limbic brain. III. Negative-feedback regulation. *Brain Res* 350:169–173
 29. Ward IL, Weisz J 1980 Maternal stress alters plasma testosterone in fetal males. *Science* 207:328–329
 30. Rivier CL, Grigoriadis DE, Rivier JE 2003 Role of corticotropin-releasing factor receptors type 1 and 2 in modulating the rat adrenocorticotropin response to stressors. *Endocrinology* 144:2396–2403
 31. Zorrilla EP 1997 Multiparous species present problems (and possibilities) to developmentalists. *Dev Psychobiol* 30:141–150
 32. Rivier J, Amoss M, Rivier C, Vale W 1974 Synthetic luteinizing hormone releasing factor. Short chain analogs. *J Med Chem* 17:230–233
 33. Rivier C, Vale W 1990 Cytokines act within the brain to inhibit luteinizing hormone secretion and ovulation in the rat. *Endocrinology* 127:849–856
 34. Rivest S, Rivier C 1994 Stress and interleukin-1 β -induced activation of *c-fos*, NGFI-B and CRF gene expression in the hypothalamic PVN: comparison between Sprague-Dawley, Fisher-344 and Lewis rats. *J Neuroendocrinol* 6:101–117
 35. Rivier C, Shen GH 1994 In the rat, endogenous nitric oxide modulates the response of the hypothalamic-pituitary-adrenal axis to interleukin-1 β , vasopressin, and oxytocin. *J Neurosci* 14:1985–1993
 36. Mandyam CD, Norris RD, Eisch AJ 2004 Chronic morphine induces premature mitosis of proliferating cells in the adult mouse subgranular zone. *J Neurosci Res* 76:783–794
 37. Han F, Ozawa H, Matsuda K, Nishi M, Kawata M 2005 Colocalization of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus and hypothalamus. *Neurosci Res* 51:371–381
 38. Baldwin HA, Rassnick S, Rivier J, Koob GF, Britton KT 1991 CRF antagonist reverses the “anxiogenic” response to ethanol withdrawal in the rat. *Psychopharmacology (Berl)* 103:227–232
 39. Menzaghi F, Howard RL, Heinrichs SC, Vale W, Rivier J, Koob GF 1994 Characterization of a novel and potent corticotropin-releasing factor antagonist in rats. *J Pharmacol Exp Ther* 269:564–572
 40. Moreau JL, Kilpatrick G, Jenck F 1997 Urocortin, a novel neuropeptide with anxiogenic-like properties. *Neuroreport* 8:1697–1701
 41. File SE, Zangrossi Jr H 1993 “One-trial tolerance” to the anxiolytic actions of benzodiazepines in the elevated plus-maze, or the development of a phobic state? *Psychopharmacology (Berl)* 110:240–244
 42. Fernandes C, File SE 1996 The influence of open arm ledges and maze experience in the elevated plus-maze. *Pharmacol Biochem Behav* 54:31–40
 43. Cruz AP, Frei F, Graeff FG 1994 Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacol Biochem Behav* 49:171–176
 44. Korte SM, Korte-Bouws GA, Bohus B, Koob GF 1994 Effect of corticotropin-releasing factor antagonist on behavioral and neuroendocrine responses during exposure to defensive burying paradigm in rats. *Physiol Behav* 56:115–120
 45. Schafer JL, Graham JW 2002 Missing data: our view of the state of the art. *Psychol Methods* 7:147–177
 46. Schafer JL 1999 Multiple imputation: a primer. *Stat Methods Med Res* 8:3–15
 47. Rubin DB 1996 Multiple imputation after 18+ years (with discussion). *J Am Stat Assoc* 91:473–489
 48. Viau V 2002 Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. *J Neuroendocrinol* 14:506–513
 49. Ferguson SA, Gray EP 2005 Aging effects on elevated plus maze behavior in spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley male and female rats. *Physiol Behav* 85:621–628
 50. Jezova D, Jurankova E, Mosnarova A, Kriska M, Skultetyova I 1996 Neuroendocrine response during stress with relation to gender differences. *Acta Neurobiol Exp (Wars)* 56:779–785
 51. Le Mevel JC, Abitbol S, Beraud G, Maniey J 1979 Temporal changes in plasma adrenocorticotropin concentration after repeated neurotropic stress in male and female rats. *Endocrinology* 105:812–817
 52. McCormick CM, Smythe JW, Sharma S, Meaney MJ 1995 Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. *Brain Res Dev Brain Res* 84:55–61
 53. Hui GK, Figueroa IR, Poytress BS, Roozendaal B, McGaugh JL, Weinberger NM 2004 Memory enhancement of classical fear conditioning by post-training injections of corticosterone in rats. *Neurobiol Learn Mem* 81:67–74
 54. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S 2002 Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 22:6810–6818
 55. Welberg LA, Seckl JR, Holmes MC 2000 Inhibition of 11 β -hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. *Eur J Neurosci* 12:1047–1054
 56. Montano MM, Wang MH, Even MD, vom Saal FS 1991 Serum corticosterone in fetal mice: sex differences, circadian changes, and effect of maternal stress. *Physiol Behav* 50:323–329
 57. Moore CL, Power KL 1986 Prenatal stress affects mother-infant interaction in Norway rats. *Dev Psychobiol* 19:235–245
 58. Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, Le Moal M 1995 Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. *J Neurosci* 15:110–116
 59. Palanza P 2001 Animal models of anxiety and depression: how are females different? *Neurosci Biobehav Rev* 25:219–233
 60. Montano MM, Wang MH, vom Saal FS 1993 Sex differences in plasma corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress. *J Reprod Fertil* 99:283–290