

# Estrogen potentiates adrenocortical responses to stress in female rats

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**Figueiredo HF, Ulrich-Lai YM, Choi DC, Herman JP.** Estrogen potentiates adrenocortical responses to stress in female rats. *Am J Physiol Endocrinol Metab* 292: E1173–E1182, 2007. First published December 19, 2006; doi:10.1152/ajpendo.00102.2006.—It is well established that estrogens markedly enhance the glucocorticoid response to acute stress in females. However, the precise mechanism responsible for this regulation is poorly understood. Here, we tested whether estrogens enhance the activation of the paraventricular nucleus (PVN) of the hypothalamus by measuring stress-induced *c-fos* mRNA expression in the PVN of restraint-stressed ovariectomized (OVX) rats treated with physiologically relevant doses of estradiol (E<sub>2</sub>), the major female estrogen. As expected, E<sub>2</sub> enhanced plasma corticosterone responses to restraint in OVX females. However, E<sub>2</sub> markedly attenuated the stress-induced *c-fos* gene expression in the PVN and inhibited plasma ACTH responses in these animals. Furthermore, E<sub>2</sub>-inhibitory effects were mimicked by progesterone (P) alone or in combination with E<sub>2</sub>. Interestingly, the suppressive central effects of both E<sub>2</sub> and P were apparently independent of basal paraventricular corticotropin-releasing hormone (CRH) transcription, since these ovarian steroids did not significantly affect PVN CRH mRNA expression in unstressed rats. These unexpected findings suggested that E<sub>2</sub> promotes glucocorticoid hypersecretion in females by additional peripheral (i.e., adrenal) mechanisms. Indeed, E<sub>2</sub> markedly enhanced plasma corticosterone responses and adrenal corticosterone content in dexamethasone-blocked OVX rats challenged with varying doses of exogenous ACTH. These results suggest that enhanced adrenal sensitive to ACTH is an important physiological mechanism mediating E<sub>2</sub>-related glucocorticoid hypersecretion in stressed females.

corticosterone; progesterone; *c-fos*; adrenal; paraventricular nucleus

THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS represents an important output in the neuroendocrine response to stress. This axis involves the coordinated activity of parvicellular neurons in the hypothalamic paraventricular nucleus (PVN), adrenocorticotrophic hormone (ACTH)-secreting corticotropes in the anterior pituitary, and glucocorticoid-secreting cells in adrenal gland to bring together the neuroendocrine response to stress (1, 48, 55, 56). Activity of the HPA axis is markedly influenced by sex steroids, as illustrated by the pronounced elevations in glucocorticoid levels exhibited in female rodents compared with male counterparts (7, 45). In ovariectomized (OVX) female rats, estradiol (E<sub>2</sub>) potentiates the corticosterone response to numerous stressors, including restraint (60). Because E<sub>2</sub>-dependent glucocorticoid hypersecretion to a certain extent parallels elevations in ACTH, it is believed (3, 4) that estrogen acts centrally to modulate the neuroendocrine responses to stress.

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The central sites and mechanisms responsible for E<sub>2</sub> actions on the stress-induced glucocorticoid surge remain elusive. However, there is evidence that altered neuronal activity in the PVN might be involved in mediating E<sub>2</sub> effects on HPA axis responses to stress. In male rats, systemic injections of E<sub>2</sub> enhance the expression of the immediate early gene *c-fos* in response to novelty (63) and to restraint (38). However, the relevance of these findings to HPA axis activity in females is not clear, especially considering pronounced organizational and activational sex differences in the rodent brain (14, 43, 49, 51). In addition, conflicting reports of E<sub>2</sub> effects on corticotropin-releasing hormone (CRH) synthesis in the PVN (42, 44, 47) make it difficult to integrate E<sub>2</sub>-induced central changes in hypothalamic activation to stress neurosecretory events. Thus, despite evidence supporting a role of E<sub>2</sub> in modulating PVN responses to stressful stimuli, the precise coordinated involvement of central sites in stress-induced corticosterone hypersecretion in females remains unclear.

In the present study, we examined whether E<sub>2</sub> modulates stress-induced activation of the PVN in a manner consistent with its role in female glucocorticoid hypersecretion. Thus, we measured the “activational” expression of *c-fos* mRNA in the PVN of restraint-stressed OVX female rats treated with E<sub>2</sub> and compared it with the plasma ACTH and corticosterone responses exhibited by these animals. Because the effects of E<sub>2</sub> on neuroendocrine systems can be sensitive to the presence of progesterone (P), we also evaluated the participation of this progestin in the female response to stress. Finally, we tested the direct role of E<sub>2</sub> on adrenal gland responses to varying doses of exogenous ACTH. We show here an unexpected, potentially dichotomous mechanism employed by ovarian steroids, notably E<sub>2</sub>, in the control of HPA axis responses to stress in females.

## MATERIALS AND METHODS

### Experimental Animals

Young female Sprague-Dawley rats (~200 g; Harlan, Indianapolis, IN) were used in the following experiments. These animals were OVX bilaterally via ventral incision under anesthesia (ketamine, 87 mg/kg ip; xylazine, 13 mg/kg ip) 1 wk after arrival. OVX rats were then housed two or three per cage, depending on experimental design, on a 12:12-h light-dark cycle (lights on from 0600 to 1800) with food and water available ad libitum. All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

### Hormone Treatments

**E<sub>2</sub> and P injections.** One week after OVX, rats were subcutaneously injected with vehicle (sesame oil),  $\beta$ -E<sub>2</sub> 3-benzoate (E<sub>2</sub>, 10

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$\mu\text{g}/100 \mu\text{l}$  oil; Sigma, St. Louis, MO), or P (500  $\mu\text{g}/100 \mu\text{l}$  oil; Sigma), depending on the experimental group (Oil,  $\text{E}_2$ ,  $\text{E}_2+\text{P}$ , or P). Specifically, animals in the Oil group were injected with sesame oil on days 1, 2, and 3. Animals in the  $\text{E}_2$  group received an injection of  $\text{E}_2$  on days 1 and 2 and a single injection of oil on day 3. Animals in the P group received an injection of sesame oil on days 1 and 2, followed by a single injection of P on day 3. Finally, animals in the  $\text{E}_2$  plus P group received an injection of  $\text{E}_2$  on days 1 and 2 and a single injection of P on day 3. This steroid regimen was used in experiments 1 and 3 (see below). Additionally, a separate batch of OVX rats was injected with  $\text{E}_2$  or oil, as described above, and used in experiment 4.

**$\text{E}_2$  pellet implants.** We also decided to verify the effects of  $\text{E}_2$  on the stress response of OVX females by subcutaneously implanting 21-day continuous release pellets (Innovative Research of America, Sarasota, FL) containing  $17\beta\text{-E}_2$  (one 0.5-mg pellet/rat) or placebo (one 150.0-mg pellet/rat) at the time of OVX surgery. The 0.5-mg  $\text{E}_2$  pellet clamps produced circulating  $\text{E}_2$  levels at  $\sim 160 \text{ pg/ml}$  (47). These rats were used in experiment 2 (see below).

### Experimental Design

**Experiment 1: effects of short-term ovarian steroid treatments on HPA axis responses to acute restraint.** Restraint stress experiments were performed in the morning (between 0900 and 1200) 4 h after the last hormone injection on day 3. For this experiment, all rats ( $n = 6$  females/steroid treatment group) were placed in well-ventilated plastic cylindrical restrainers for 30 min and subsequently returned to their home cage. Blood samples were collected by tail nicks at 0 and 30 min, while animals were in restrainers, and again at 60 and 120 min from onset of restraint by briefly and gently placing the animals into clean restrainers (collection time:  $\sim 4 \text{ min/rat}$ ) and then back in their respective home cages. Blood samples were collected in ice-cold microcentrifuge tubes containing  $10 \mu\text{l}$  of 15% ( $\text{K}_3$ )EDTA and subsequently centrifuged at 1,500 g. All plasma samples were stored at  $-20^\circ\text{C}$ .

**Experiment 2: effects of  $\text{E}_2$  pellet implants on HPA axis responses to restraint and elevated-plus-maze exposures.** This experiment was designed to verify the influences of  $\text{E}_2$  on the HPA axis of rats treated with a different  $\text{E}_2$  regimen (pellet implant) and submitted to two different putatively psychological stressors, elevated-plus-maze (EPM) exposure and restraint. The EPM exposure was utilized in these studies as a type of "mild" psychological stressor, in contrast with restraint, a more intense stressor. EPM exposure was not utilized as a test for anxiety-related behavior since this was not the focus of the work. As such, the number of animals utilized were selected for assessments of HPA axis function; the number of animals utilized would be inappropriate for behavioral analysis, which generally requires more subjects. Moreover, due to logistical limitations, this experiment was conducted on a different date from the acute injection studies (experiment 1). Thus, 1 wk after  $\text{E}_2$  or placebo pellet implant, OVX rats were placed in EPM for 5 min under dim light and returned to their home cages. Tail nick blood samples were collected from these rats at 30, 60, and 120 min from the onset of EPM exposure, as described in experiment 1. One week after EPM exposure, these same rats were submitted to restraint stress for 30 min and subsequently returned to their home cage. Similarly to the EPM exposure, tail nick blood samples were collected at 30, 60, and 120 min from onset of restraint. All tests were performed between 0800 and 1200, and rats were tested only once on each paradigm.

**Experiment 3: effects of short-term ovarian steroid treatments on *c-fos* and CRH mRNA expression.** To investigate whether changes in plasma ACTH secretion in steroid-treated rats are accompanied by concomitant changes in PVN function, we measured stress-induced *c-fos* mRNA and basal CRH mRNA expressions in this nucleus. Although work by others utilizing combined immunohistochemistry and hybridization histochemistry (35) indicate that transcription of these genes at least are not temporally linked, we chose to analyze

them as two distinct measures of PVN function. For this experiment, female rats were injected with ovarian steroids 1 wk after OVX and randomly assigned into stressed ( $n = 24$ ; 6 females/steroid treatment) and unstressed ( $n = 24$ ; 6 females/steroid treatment) groups. Rats in the stressed group were placed in plastic restrainers for 30 min, similarly to experiment 1, and subsequently returned to their home cages for an additional 30 min prior to death. This 60-min time point was chosen on the basis of a previous study from our laboratory (14) and on the known inducibility of the *c-fos* gene (8). Unstressed animals remained in their home cages and were not restrained or disturbed. All animals were killed by rapid decapitation on the same day. Trunk blood samples were immediately collected into Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) containing 15% ( $\text{K}_3$ )EDTA and centrifuged at 1,500 g. Plasma samples were stored frozen at  $-20^\circ\text{C}$ . Brains were quickly removed from all animals, frozen in isopentane ( $-40^\circ$  to  $-50^\circ\text{C}$ ), and subsequently stored at  $-80^\circ\text{C}$ .

**Experiment 4: effect of  $\text{E}_2$  on adrenal sensitivity to ACTH.** One week after OVX, rats were injected with either  $\text{E}_2$  ( $n = 42$  rats) or oil ( $n = 41$  rats) as described above. On the morning of the third day, rats were subcutaneously injected with dexamethasone phosphate (400  $\mu\text{g}$ ; Sigma) to block endogenous ACTH release. Two hours later, randomized groups of rats were injected with exogenous rat ACTH (sc, pH 7.4; Bachem Bioscience, San Diego, CA) at doses of 0, 75, 150, 225, 300, or 3,000 ng. Fifteen minutes after ACTH injection, rats were killed by decapitation and trunk blood samples collected for measurement of plasma corticosterone. Adrenal glands were quickly collected, cleaned, weighed, and stored frozen for measurement of adrenal corticosterone content.

### In Situ Hybridization Procedures

Brains were sectioned at  $16 \mu\text{m}$  using a Microm cryostat (Kalamazoo, MI), mounted on Gold Seal slides (BD Biosciences, Portsmouth, NH), and stored at  $-20^\circ\text{C}$ . For *c-fos* in situ hybridization, brain sections from unstressed and stressed rat groups were used; for CRH in situ hybridizations, brain sections from unstressed rats were used. Briefly, sections were fixed in 4% phosphate-buffered paraformaldehyde for 10 min and rinsed twice in 5 mM potassium phosphate-buffered saline (KPBS) for 5 min each, twice in 5 mM KPBS with 0.2% glycine for 5 min each, and again in KPBS for 5 min each. Sections were then acetylated in 0.25% acetic anhydride (made in 0.1 M triethanolamine, pH 8.0) for 10 min, rinsed twice in 2X standard saline citrate (SSC) for 5 min each, and dehydrated through graded alcohols (for 2 min each) and chloroform (for 5 min).

The in situ hybridizations used antisense cRNA probes complementary to rat *c-fos* ( $\sim 700 \text{ bp}$ ) and CRH ( $\sim 300 \text{ bp}$ ) mRNAs. These probes were separately labeled by in vitro transcription using [ $^{35}\text{S}$ ]UTP. The *c-fos* fragment (original full-length cDNA from Dr. Tom Curran; St. Jude Children's Research Hospital, Memphis, TN) was cloned into pGem4Z vector, linearized with *Ava*II, and transcribed with SP6 RNA polymerase, resulting in a cRNA probe with a final length of 587 bp. The specificity of this probe has been validated in previous studies (8). The CRH fragment was cloned into a pGem4Z vector, linearized with *Ava*II, and transcribed with T7 RNA polymerase. The labeling reaction for *c-fos* or CRH consisted of  $5\times$  transcription buffer, 125  $\mu\text{Ci}$   $^{35}\text{S}$ , 200  $\mu\text{mol}$  of a mixture of nucleoside 5'-triphosphate (33% GTP, 33% CTP, 33% ATP, and 1% UTP), 100 mM dithiothreitol, 50 U ribonuclease inhibitor, 40 U SP6 or T7 RNA polymerase, and 2  $\mu\text{g}$  linearized DNA. The mixture was incubated for 90 min at  $37^\circ\text{C}$ , after which the template DNA was digested with ribonuclease-free deoxyribonuclease. The labeled probes were separated from free nucleotides by ammonium acetate precipitation and reconstituted in diethylpyrocarbonate-treated nanopure water.

Radiolabeled *c-fos* probes were diluted in hybridization buffer [50% formamide, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 335 mM NaCl,  $1\times$  Denhardt's solution, 200  $\mu\text{g/ml}$  salmon sperm DNA, 150

µg/ml yeast tRNA, 20 mM dithiothreitol, and 10% dextran sulfate] to yield 1,000,000 cpm/50 µl buffer. Diluted aliquots of 50 µl were applied to each slide, coverslipped, and hybridized overnight at 55°C in a polystyrene chamber over filter paper saturated with 50% formamide. The next day, the slides were briefly soaked in 2X SSC to remove coverslips, rinsed in 2X SSC for 20 min, and then incubated in 100 µg/ml ribonuclease A for 30 min at 37°C. Slides were briefly rinsed in 2X SSC, rinsed three times in 0.2X SSC for 15 min each, and then incubated in 0.2X SSC at 65°C for 1 h. Finally, the slides were dehydrated in graded alcohols, air dried, and exposed to Kodak Biomax MR-2 film (Packard Instruments, Meriden, CT) for 14 (c-fos) or 21 (CRH) days.

#### Image Analysis

Images of brain sections were captured from in situ hybridization autoradiographs by using a Cohu High Performance CCD Camera (Cohu, San Diego, CA) and Scion Image for Windows version Beta 4.0.2 (Scion, Frederick, MD). Semiquantitative analyses were conducted on every fifth brain section containing the anatomical region of interest and ranged from four to 10 sections depending on the size of the region. The number of sections analyzed in this study was kept consistent for each region. Mean gray level (signal) was quantified bilaterally in the region of interest and in an adjacent region with low hybridization signal (background). All measured signals were within linear range of detectability, as measured by calibration curves from ARC 146-<sup>14</sup>C standards (American Radiolabeled Chemicals, St. Louis, MO). Finally, signals from anatomical regions were corrected by subtracting the corresponding background signal and expressed as corrected gray level. In all cases, the mean corrected gray level values were calculated for each animal and used in the statistical analysis. All in situ quantifications were performed in a blinded fashion.

#### Plasma Hormone Assay

ACTH plasma concentration was measured by radioimmunoassay (RIA) using <sup>125</sup>I RIA kit from DiaSorin (Stillwater, MN). Corticosterone and P plasma concentrations were measured by <sup>125</sup>I RIA kits from ICN Biochemicals (Cleveland, OH). E<sub>2</sub> plasma concentration was measured by <sup>125</sup>I RIA DSL-4800 kit from Diagnostic Systems Laboratories (Webster, TX). For each assay performed, control samples with known concentrations of hormone (usually low, normal, and high; provided by the manufacturer) were included to assess performance and reliability.

#### Determination of Adrenal Corticosterone Content

To measure adrenal corticosterone, adrenal glands were homogenized in ethanol (20%, in saline) and centrifuged (4,000 g for 20 min), as previously described (54). Supernatants were diluted and measured by RIA as described above.

#### Statistical Analysis

Data are expressed as means ± SE. Hormone data from *experiments 1* and *2* were analyzed by repeated-measures ANOVA (main effect: hormone; repeated measure: time). Areas under the curve were calculated as the integrated areas under the plasma ACTH (pg/ml × 120 min) or corticosterone (ng/ml × 120 min) responses to restraint and analyzed by one-way factorial ANOVAs (main effect: hormone). Because c-fos expression was near background levels in the brains of unstressed animals as expected, c-fos expression data were analyzed only in the restraint-stressed animal group by a one-way ANOVA (main effect: hormone). CRH mRNA expression data were analyzed by a one-way factorial ANOVA in unstressed rats (main effect: hormone). Corticosterone data from *experiment 4* were analyzed by factorial ANOVA (main effects: ACTH dose, hormones). Significant treatment effects and/or interactions were further analyzed by Fisher's protected least significant difference test. For all comparisons, statis-

tical significance was set at  $P < 0.05$ . Statistical analyses were performed by using StatView (SAS Institute, Cary, NC) and GB Stat Version 9.0 (Dynamic Microsystems, Silver Spring, MD) software.

## RESULTS

### Ovarian Steroid Concentrations in Experimental Rats

Because of limitations in blood sampling volumes, E<sub>2</sub> and P plasma concentrations were measured only in rat blood samples obtained by decapitation (*experiment 1*). E<sub>2</sub> and P administration yielded appreciable levels of these hormones in the plasma of OVX rats (Table 1). As expected, E<sub>2</sub> injections resulted in higher plasma 17β-E<sub>2</sub> levels in the E<sub>2</sub> and E<sub>2</sub> plus P groups compared with Oil and P groups ( $F_{3,41} = 9.4$ ,  $P < 0.05$ ). Concurrently, P injections resulted in higher plasma P levels in the P and E<sub>2</sub> plus P groups compared with Oil and E<sub>2</sub> groups ( $F_{3,36} = 31.4$ ,  $P < 0.05$ ); however, the levels of P were higher when this hormone was injected alone (P) than when combined with E<sub>2</sub> ( $P < 0.05$ ). Neither E<sub>2</sub> nor P plasma levels were affected by stress ( $F_{1,37} = 3.1$ ,  $P > 0.5$ ; and  $F_{1,32} = 1.1$ ,  $P > 0.5$ , respectively). The steroid doses used in this study produced approximate E<sub>2</sub> and P blood levels observed during proestrus in regularly cycling female rats (40).

### Effects of Short-Term Ovarian Steroid Injections on HPA Axis Responses to Acute Restraint

Ovarian steroid replacement significantly altered restraint-induced HPA axis activity in OVX females (Fig. 1). Compared with oil-treated rats, plasma ACTH concentrations were significantly lower in steroid-treated animals at 30 min (E<sub>2</sub>, E<sub>2</sub> + P, and P;  $P < 0.05$ ) and 60 min (E<sub>2</sub> + P, P;  $P < 0.05$ ) from onset of stress ( $F_{3,95} = 4.3$ ,  $P < 0.05$ ). No differences between steroid treatment groups occurred at 0 or 120 min. Surprisingly, the corticosterone response profiles exhibited by these animals did not parallel that of ACTH (Fig. 1B). Specifically, plasma corticosterone concentrations were significantly higher in E<sub>2</sub>-treated rats at 0 and 60 min compared with oil ( $P < 0.05$ ). Additionally, corticosterone responses were significantly higher at 120 min in the E<sub>2</sub> plus P group compared with E<sub>2</sub>, P, and Oil groups ( $P < 0.05$ ). The apparent plasma corticosterone increase in P-treated rats at 0 and 120 min were not statistically significant compared with Oil groups.

The dissociation between the plasma ACTH and corticosterone time courses is further reflected by the integrated responses to restraint (Fig. 2). Thus, the net plasma ACTH response was significantly lower in steroid-treated rats compared with oil treatment ( $P < 0.05$ ). On the other hand, the net corticosterone responses exhibited by E<sub>2</sub> and E<sub>2</sub> plus P (but not P) groups were significantly higher than the oil group.

Table 1. Plasma ovarian steroid concentrations in experimental rats

Plasma Steroid Conc.	Oil	E <sub>2</sub>	E <sub>2</sub> + P	P
17β-E <sub>2</sub> , pg/ml	14.3 ± 1.6	166.7 ± 40.9*	191.4 ± 47.6*	34.5 ± 8.1
P, ng/ml	13.4 ± 3.1	20.5 ± 3.1	68.6 ± 14.1†	109.8 ± 13.1†‡

Values represent means ± SE of pooled unstressed and restraint-stressed ovariectomized (OVX) rats from *experiment 2*. Conc., concentration; E<sub>2</sub>, estradiol; P, progesterone. \* $P < 0.05$  vs. Oil and P; † $P < 0.05$  vs. Oil and E<sub>2</sub>; ‡ $P < 0.05$  vs. E<sub>2</sub> plus P.



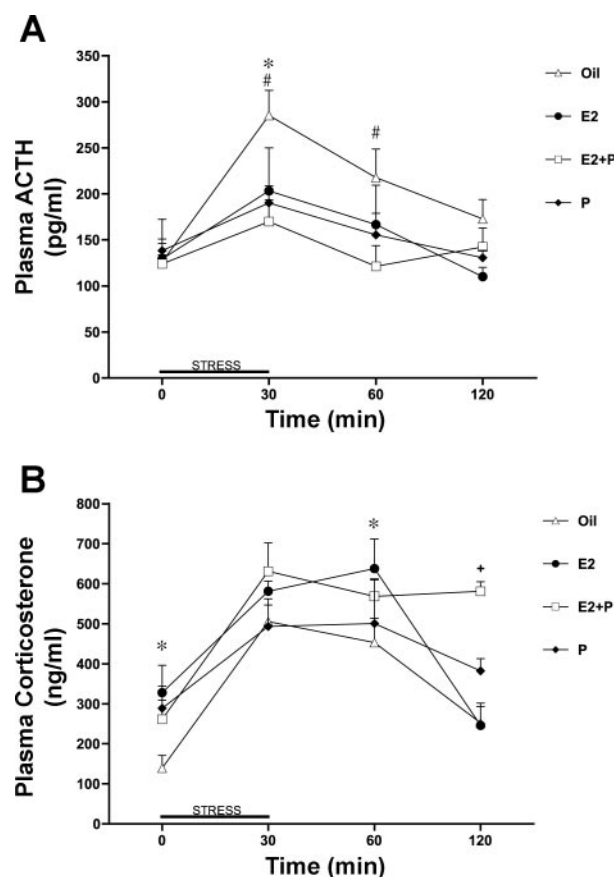


Fig. 1. Ovarian steroids inhibit ACTH but enhance corticosterone responses to restraint stress in ovariectomized (OVX) rats. Plasma ACTH (A) and plasma corticosterone (B) concentrations were measured at 0, 30, 60, and 120 min from onset of a 30-min restraint stress in OVX rats injected with vehicle oil or with 10  $\mu$ g of estradiol (E<sub>2</sub>) and/or 500  $\mu$ g of progesterone (P). Data represent means  $\pm$  SE ( $n = 5-6$  rats/group). \* $P < 0.05$ , Oil vs. E<sub>2</sub>; # $P < 0.05$ , Oil vs. E<sub>2</sub> plus P and P; + $P < 0.05$ , E<sub>2</sub> plus P vs. Oil, E<sub>2</sub>, and P.

#### Effects of E<sub>2</sub> Pellet Implants on HPA Axis Responses to Restraint and EPM Exposures

Because the previous experiment showed an unexpected dissociation between plasma ACTH and corticosterone levels, notably in E<sub>2</sub>-injected rats, we thought it would be important to verify the generality of this response in OVX rats with E<sub>2</sub> (0.5 mg) pellet implants, an alternative treatment shown to continuously produce E<sub>2</sub> plasma levels to  $\sim 160$  ng/ml (47). Moreover, we also decided to expose these pellet-implanted rats to restraint and EPM, another putative psychological stressor. In response to the 5-min EPM exposure, E<sub>2</sub> implants tended to inhibit plasma ACTH levels in OVX rats, although nonsignificantly ( $P = 0.0545$ ), and they effectively enhanced plasma corticosterone levels ( $F_{3,19} = 4.3$ ,  $P < 0.05$ ; Fig. 3). Compared with placebo, E<sub>2</sub> implants significantly attenuated plasma ACTH levels at 60 min ( $F_{1,2} = 6.7$ ,  $P < 0.05$ ) and significantly heightened corticosterone level at 120 min from commencement of 30-min restraint ( $F_{3,19} = 4.3$ ,  $P < 0.05$ ) (Fig. 3). Thus, under different experimental treatments, we were able to replicate the dissociated stress hormone responses in E<sub>2</sub>-treated rats.

#### Effects of Short-Term Ovarian Steroid Treatments on Stress-Induced *c-fos* mRNA Expression in the PVN

Our unexpected results (experiments 1 and 2) suggested that E<sub>2</sub> acts centrally to inhibit ACTH secretion. To gain insight on the mechanism mediating this response, we measured the expression of *c-fos* in the PVN of OVX rats treated with ovarian steroids. The immediate early gene *c-fos* is widely used as a marker of stimulus-dependent neuronal activity in the central nervous system (32). Here, we use a *c-fos* in situ hybridization strategy to assess the influence of ovarian hormones and stress in the PVN and other HPA-related brain regions of OVX animals replaced with E<sub>2</sub>, P, or E<sub>2</sub> plus P. In this study, levels of *c-fos* mRNA were near background values in brain regions of unstressed animals, regardless of hormone treatment (data not shown), and consistent with the inducible expression of this gene after stress (8, 35). In restraint-stressed rats, induction of *c-fos* signal was evident in the medial parvocellular PVN of all animals (Fig. 4). E<sub>2</sub>, E<sub>2</sub> plus P, and

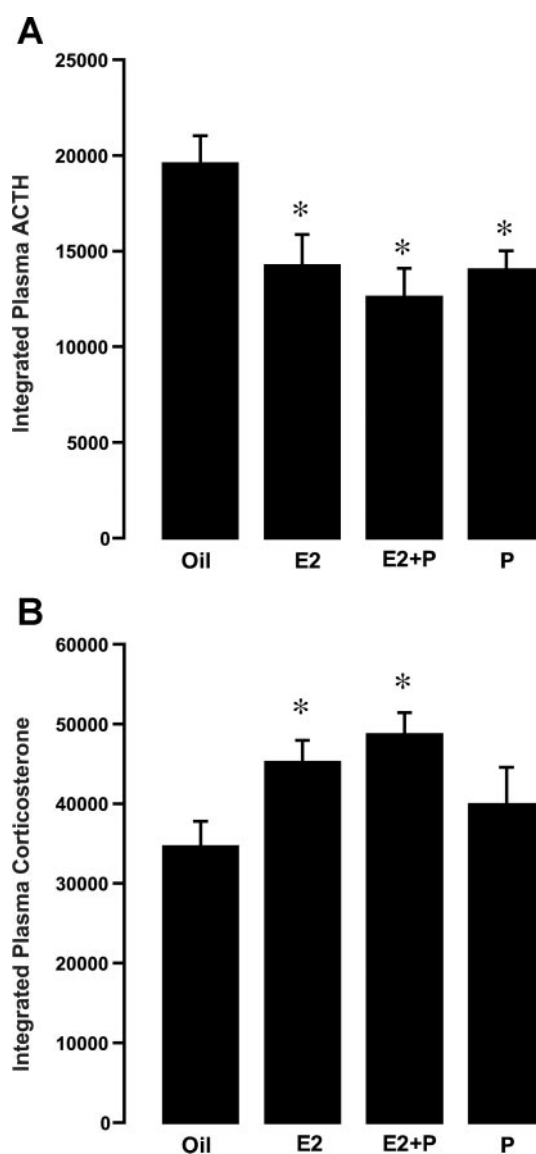


Fig. 2. Integrated plasma ACTH (A) and corticosterone (B) responses from Fig. 1. Data represent means  $\pm$  SE ( $n = 5-6$  rats/group). \* $P < 0.05$  vs. Oil.

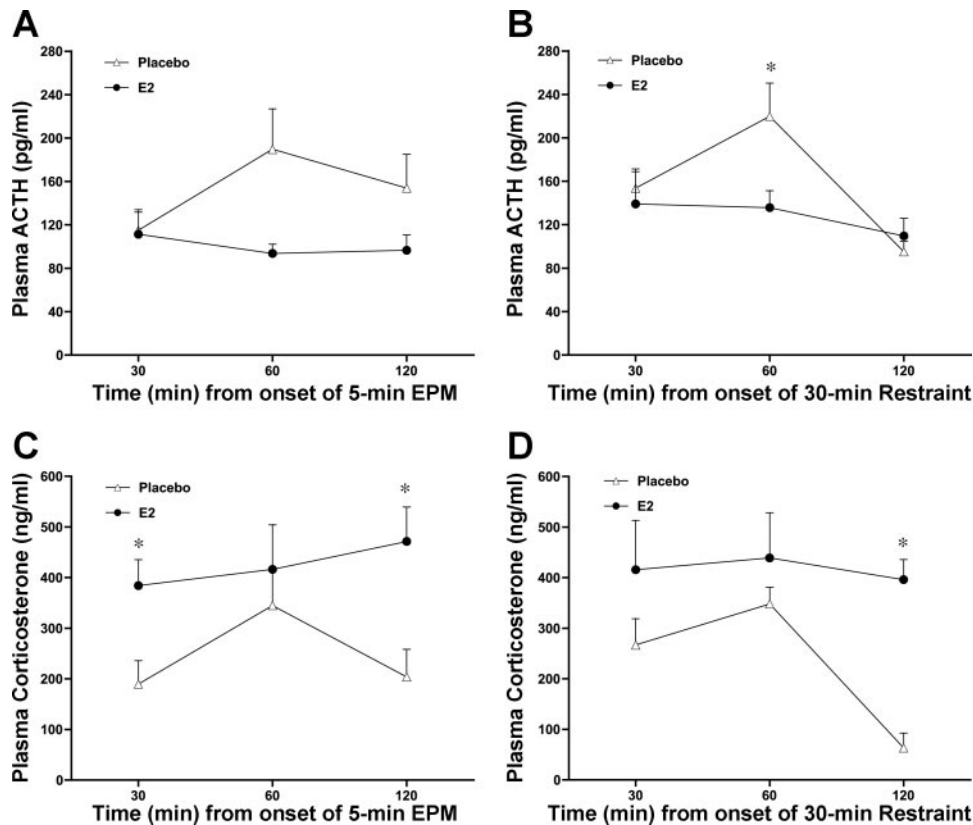


Fig. 3. E<sub>2</sub> (0.5-mg pellet) inhibits ACTH and enhances corticosterone responses to elevated-plus-maze (EPM) exposure and to restraint stress. Plasma ACTH (A and B) and plasma corticosterone (C and D) concentrations were measured in placebo-implanted or E<sub>2</sub> pellet-implanted OVX rats at 30, 60, and 120 min from onset of EPM (A and C) or restraint stress (B and D). Data represent means  $\pm$  SE ( $n = 5-6$  rats/group). \* $P < 0.05$ , E<sub>2</sub> vs. Oil.

P treatments markedly lowered *c-fos* mRNA expression in the PVN of restrained animals compared with the Oil group ( $F_{3,16} = 5.0$ ,  $P < 0.05$ ), indicating a general suppressive effect of ovarian steroids on the *c-fos* gene in the PVN. Furthermore, hormone treatments had no effect on *c-fos* mRNA expression in the cerebral cortex of these animals (Table 2), demonstrating the regional specificity of ovarian steroids on restraint-induced *c-fos* expression.

Because CRH in the PVN is a major regulator of pituitary ACTH secretion (48), we decided to determine whether basal synthesis of this secretagogue was altered in the PVN of OVX rats treated with ovarian hormones. Basal PVN CRH mRNA expression was not significantly increased in E<sub>2</sub>-treated rats ( $F_{3,14} = 1.5$ ,  $P > 0.05$ ; Fig. 5).

#### E<sub>2</sub> Effect on Adrenal Sensitivity and Adrenal Corticosterone Content

The central inhibitory actions of E<sub>2</sub> on PVN activation and CRH mRNA levels did not explain the observed E<sub>2</sub> enhancement of plasma glucocorticoid responses to stress. Thus, we reasoned that glucocorticoid hypersecretion in response to stress may result from E<sub>2</sub> actions on the adrenal gland. Our results show that E<sub>2</sub> increased the plasma corticosterone response to submaximal doses of exogenous ACTH (75–300 ng/rat) in dexamethasone-blocked rats in a dose-dependent manner ( $F_{1,59} = 8.7$ ,  $P < 0.05$ ), indicating that adrenal sensitivity was increased in these animals. Similarly, E<sub>2</sub> increased the adrenal corticosterone responses to exogenous ACTH ( $F_{1,58} = 11.1$ ,  $P < 0.05$ ; Fig. 6). E<sub>2</sub> did not affect the plasma corticosterone or adrenal corticosterone responses to maximal (3,000 ng/rat) doses of ACTH.

#### DISCUSSION

Using two different methods of E<sub>2</sub> administration, we demonstrate here that E<sub>2</sub> increases plasma corticosterone response to both acute restraint and EPM stress and concomitantly inhibits restraint-induced PVN activation (as measured by *c-fos* mRNA expression) and suppresses ACTH responses to stress in OVX female rats. These latter observations agree well with the emerging evidence that physiologically-relevant doses of E<sub>2</sub> inhibit ACTH responses to stress (10, 47, 62). We further extended these findings to show that P similarly inhibits paraventricular and pituitary responses to restraint. Interestingly, the suppressive effects of ovarian steroids are apparently not reflected by changes in CRH transcription. Furthermore, by measuring plasma and adrenal corticosterone responses to varying doses of exogenous ACTH in dexamethasone-blocked OVX females, we provide evidence that E<sub>2</sub> enhances adrenal sensitivity to ACTH. Together, our study demonstrates that the effects of sex hormones on glucocorticoid secretion are mediated, at least in part, through peripheral mechanisms involving changes in adrenal gland sensitivity to ACTH.

Our finding that ovarian steroids inhibit ACTH responses to stress is at odds with some reports indicating that E<sub>2</sub> enhances stress-inducible ACTH responses in OVX female rats (3, 4, 60) but agrees well with others (10, 47, 62) ascribing an inhibitory role to E<sub>2</sub>. Here, we used two distinct methods of E<sub>2</sub> replacement, 7-day pellet implant and short-term steroid replacement that produced physiologically relevant E<sub>2</sub> or P plasma concentrations comparable with earlier studies (plasma E<sub>2</sub> 160 pg/ml; 7-day treatment) (47, 62). We selected this short-term regimen because it delivers steroid concentrations at physiological lev-

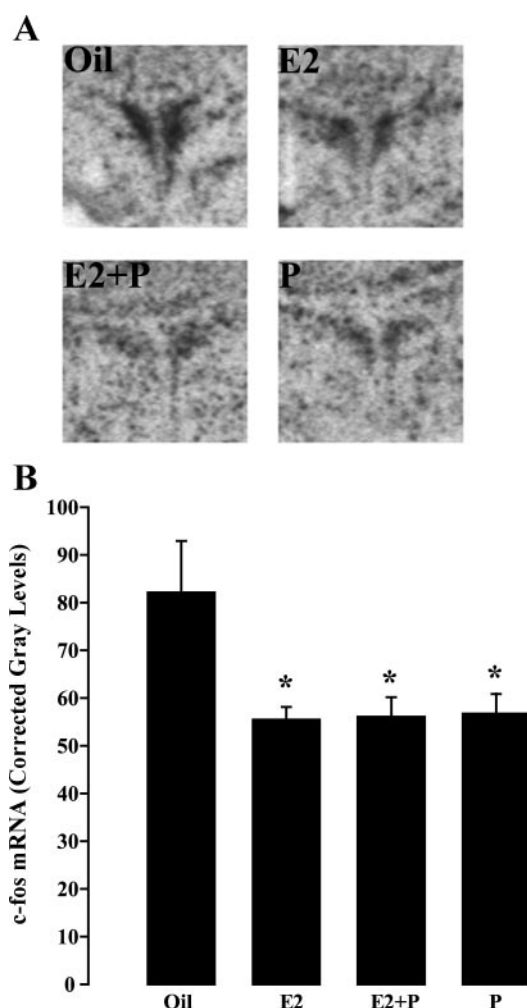


Fig. 4. Ovarian steroids attenuate paraventricular nucleus (PVN) activation in restraint-stressed OVX rats. Representative autoradiographic images (A) and semiquantitative measurements (B) of PVN *c-fos* mRNA expression in restraint-stressed OVX rats at 60 min from onset of 30-min restraint. OVX rats were injected with vehicle oil or with 10  $\mu$ g of E<sub>2</sub> and/or 500  $\mu$ g of P. Data represent means  $\pm$  SE ( $n$  = 5–6 rats/group). \* $P$  < 0.05 vs. Oil.

els and models changes in ovarian steroid levels such as those that occur in normally cycling female rats. On the other hand, earlier studies used either supraphysiological doses of E<sub>2</sub> (~500 pg/ml) (60) or longer E<sub>2</sub> exposure time (21 days) (3). It should be noted that the physiological effects of E<sub>2</sub> are particularly sensitive to specific experimental paradigms (2, 11, 23, 39, 53, 61), and it is very likely that the discrepancy among these studies arises from differences in steroid dosage and exposure time. Importantly, despite the fact that E<sub>2</sub> effects can be sensitive to the replacement protocol, the present work shows consistent HPA axis effects using two different E<sub>2</sub> replacement methods. Alternatively, it is also possible that our sampling time course may have missed an earlier peak in ACTH secretion. However, this is unlikely considering that E<sub>2</sub>, alone or in combination with P, also inhibits ACTH responses at early time points (5, 10, and 20 min) from commencement of a foot-shock stress (47).

At least two nonmutually exclusive mechanisms may explain the enhanced adrenal sensitivity to ACTH and glucocorticoid hypersecretion in our E<sub>2</sub>-treated rats. First, E<sub>2</sub> could have

initially acted centrally in these animals to stimulate hypothalamic and/or pituitary drive, with subsequent increase in adrenal sensitivity to ACTH and corticosterone secretion. In this scenario, the E<sub>2</sub>-treated rats may have had subsequently greater corticosterone-mediated inhibition of the HPA axis than oil-treated controls in such a way that, at the time of testing by restraint or EPM, the E<sub>2</sub>-treated rats displayed greater inhibition of hypothalamic activity (i.e., decreased *c-fos* expression in the PVN and decreased ACTH levels). Indeed, E<sub>2</sub> causes further decline in corticosterone-induced suppression of hippocampal GR expression (but does not alter CRH expression in PVN) in OVX-plus adrenalectomized (ADX) rats given supraphysiological doses of corticosterone (42), indicating that E<sub>2</sub> is capable of potentiating the negative feedback effects of corticosterone. Therefore, the enhancement of adrenal sensitivity to ACTH in our E<sub>2</sub>-treated rats could have been triggered by an earlier increase in central drive.

Second, E<sub>2</sub> may have acted via peripheral mechanisms by either decreasing corticosterone clearance or by increasing corticosterone synthesis at the levels of the adrenal gland. Because the glucocorticoid hypersecretion in our stressed females treated with E<sub>2</sub> cannot be easily explained by clearance mechanisms [E<sub>2</sub> increases plasma corticosterone clearance (60)], we reasoned that E<sub>2</sub> enhances glucocorticoid secretion by altering the synthetic response of the adrenal gland to ACTH. This possibility has been proposed by earlier works (29–31) to explain the well-known glucocorticoid hypersecretion in female rodents and is corroborated by recent *in vitro* experiments (37, 41) and *in vivo* studies assessing HPA response to a single dose of ACTH (57). Here, to our knowledge, we show for the first time that E<sub>2</sub> markedly increases the plasma corticosterone response to ACTH in dexamethasone-blocked OVX females injected with varying doses of exogenous ACTH. Importantly, our results also show that E<sub>2</sub> increases adrenal corticosterone content, indicating that E<sub>2</sub> acts, at least in part, via increasing glucocorticoid synthesis by the adrenal gland. These preliminary findings agree well with earlier studies (27, 29) and with recent work (37) and indicate that increases in adrenal gland sensitivity to ACTH are an important mechanism mediating E<sub>2</sub>-related potentiation of glucocorticoid response in females.

In the present study, we have used expression of the immediate early gene *c-fos* to evaluate PVN activation in response to stressful stimuli (5, 6, 8). Thus, the immediate early gene profile exhibited by our steroid-treated females further supports the previous evidence that E<sub>2</sub> inhibits hypothalamic activation in stressed female rats (9). This finding however, contrasts with the stimulatory effect of E<sub>2</sub> on PVN activation in male rats (38,

Table 2. Semiquantitative analysis of *c-fos* mRNA expression in brain cortex of restraint-stressed OVX rats treated with sex steroids

Brain Region (cortex)	Oil	E <sub>2</sub>	E <sub>2</sub> + P	P
Cingulate	89.5 $\pm$ 7.8	75.6 $\pm$ 8.4	93.9 $\pm$ 6.0	70.2 $\pm$ 9.2
Prelimbic	84.7 $\pm$ 9.7	74.7 $\pm$ 6.8	83.9 $\pm$ 6.9	61.0 $\pm$ 9.0
Infralimbic	80.6 $\pm$ 10.4	73.2 $\pm$ 6.4	80.1 $\pm$ 6.2	53.0 $\pm$ 10.0
Piriform	116.1 $\pm$ 3.6	97.9 $\pm$ 7.8	112.9 $\pm$ 3.6	90.1 $\pm$ 10.7

Data represent means  $\pm$  SE ( $n$  = 4–6 rats/group). No statistical differences were observed for these brain regions.

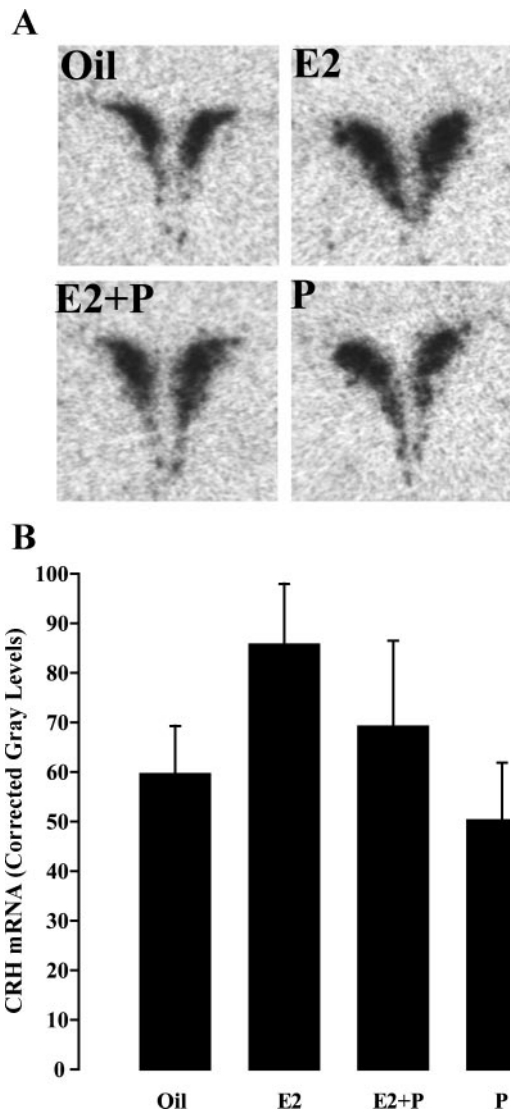


Fig. 5. Ovarian steroids did not alter basal corticotropin-releasing hormone (CRH) mRNA level in OVX females. Representative autoradiographic images (A) and semiquantitative analysis (B) of CRH mRNA expression (in situ hybridization) in the PVN of unstressed OVX rats treated with 10  $\mu$ g of E<sub>2</sub> and/or 500  $\mu$ g of P. Data represent means  $\pm$  SE ( $n = 4-6$  rats/group). There were no significant differences among treatment groups.

63) and highlights an important sex difference in the HPA axis reactivity to stress at the PVN level. Additionally and consistent with our study, a recent report demonstrates that cyclic E<sub>2</sub> administration attenuates *c-fos* protein expression in the PVN of OVX rats (17). In our study, the decrements in PVN *c-fos* expression after steroid treatment cannot be accounted for by a generalized downregulatory effect of ovarian steroids on *c-fos* gene transcription, since these hormones did not alter the stress-induced expression of *c-fos* mRNA in the limbic cortexes, ER-containing regions believed to relay inhibitory influences during restraint stress (12, 13). Moreover, it remains to be determined whether other "activational" markers (e.g., cAMP response-element binding protein and NGF-1) (6, 34) are similarly affected by sex steroids.

Although we have not closely examined the neuronal phenotypic expression of PVN *c-fos*, our radiographs suggest that

the sex steroid-related downregulation of the PVN was extensive enough to include the parvocellular neurosecretory cells that innervate the median eminence. However, because 1) the PVN lacks expression of estrogen receptors ER- $\alpha$  (50), 2) very few PVN CRH neurons, if any, express ER- $\beta$  (24), and 3) expression of progesterone receptor A (PRA) and progesterone receptor B (PRB) in this nucleus is uncertain, it remains to be determined whether steroid-mediated inhibition of this nucleus occurs directly via nongenomic mechanisms (25, 26, 36, 58), indirectly via transsynaptic influences, or indirectly via enhanced glucocorticoid negative feedback (33). Nevertheless, the overall suppressive effect of E<sub>2</sub> and P on PVN *c-fos* transcription and ACTH response speaks for a central inhibitory mechanism promoted by sex hormones in HPA axis regulation.

Moreover, despite our observations that ovarian steroids are capable of inhibiting hypothalamic-pituitary responses to emotional stressors, we found no evidence that they altered basal CRH transcription in the PVN. Thus, it remains that the inhibitory effects of sex steroids on ACTH secretion cannot be explained by a mechanism involving regulation of basal hypo-

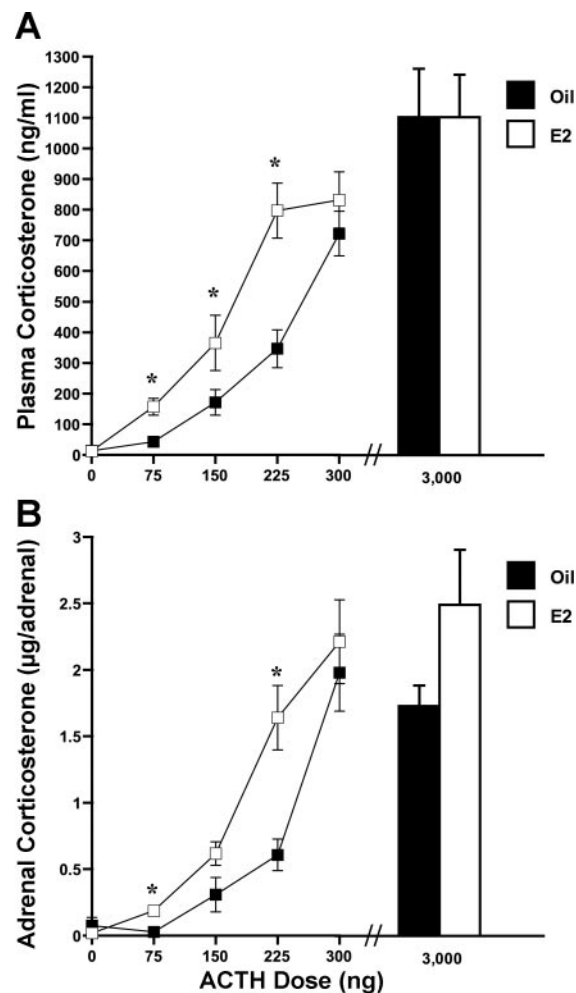


Fig. 6. E<sub>2</sub> increases adrenal gland sensitivity to submaximal (75–300 ng) doses of ACTH. Plasma corticosterone (A) and adrenal corticosterone content (B) after administration of exogenous ACTH to dexamethasone-blocked OVX rats treated with oil or 10  $\mu$ g E<sub>2</sub> (sc). Data represent means  $\pm$  SE ( $n = 5-7$  /group). \* $P < 0.05$  E<sub>2</sub> vs. Oil.



thalamic CRH gene regulation. In this context, it is important to note that the role of gonadal steroids on paraventricular CRH synthesis in nonstressed as well as in stressed female rats is at best controversial. Earlier studies indicated that  $E_2$  decreases hypothalamic CRH immunoreactivity (21, 22), has no effect on hypothalamic CRH mRNA expression (47), or decreases its basal expression (43, 44) in adrenal-intact female rats. Also,  $E_2$  caused no changes in OVX/ADX rats regardless of corticosterone supplementation (42). Whether sex steroids inhibit hypothalamic responses to stress through direct influences on CRH/ACTH secretion or via other HPA-regulating peptides (e.g., arginine vasopressin) is yet to be conclusively established.

Plasma corticosterone is affected by synthesis, corticosterone-binding globulin (CBG), clearance, and metabolism. Adrenal corticosterone content shows that  $E_2$  acts, at least in part, to increase adrenal corticosterone synthesis. It is possible that  $E_2$  also acts to alter CBG and clearance or metabolism. However, although we did not measure plasma CBG levels in this study, it is unlikely that  $E_2$  treatment reduced free corticosterone levels in our female rats. First, one study (44) shows that, although  $E_2$  treatment elevated total plasma corticosterone concentrations in OVX females, it did not change the binding capacity or the affinity in these animals, suggesting that  $E_2$  may actually elevate circulating free corticosterone in female rats.  $E_2$ , however, increases CBG activity in males (16), suggesting that estrogen's effects on CBG are sex dependent. Second, in contrast with earlier work utilizing longer  $E_2$  treatments (10 days) (16), shorter (3 days) subcutaneous administration of  $E_2$  to male rats did not significantly change serum CBG or hepatic CBG mRNA levels (52). Thus, it is likely that the shorter (2 days)  $E_2$  treatment used in our OVX females had little, if any, impact on free corticosterone levels in these animals.

Furthermore, corticosterone hypersecretion in females cannot be easily explained by sex differences in hepatic clearance of corticosterone. Female rats display far more rapid hepatic clearance of corticosteroids than males (19, 20), as indicated by the shorter biological half-life and increased hepatic reduction of corticosterone (29) in females. Thus, the increased rate of steroid clearance in females would decrease rather than increase circulating corticosterone levels in these animals. In addition,  $E_2$  stimulates hepatic clearance in OVX females (28), further suggesting that increased corticosterone levels in females result from mechanisms (e.g., increased corticosterone secretion) other than changes in hepatic clearance.

In our study, it is important to bear in mind that all females were OVX. We chose to use this experimental design on the basis of classical studies (see, for example, Refs. 3, 47, and 60), and this classical approach is an important first step toward investigating the effects of ovarian hormones in the female rat. The inclusion of gonadally intact animals in such studies has recently become appreciated (see Ref. 59), and future studies are planned to assess the role of  $E_2$  on the HPA axis function in intact female rats. This will determine whether  $E_2$  has similar effects in OVX and intact animals.

Moreover, the physiological relevance of enhanced glucocorticoid levels in OVX females treated with  $E_2$  is unclear. Importantly, it remains to be determined how the present findings relate to the HPA axis responses in ovarian-intact females, especially considering that OVX procedures can produce morphological and functional changes in the brain (15). However, because intact females show higher plasma cortico-

sterone concentration than intact males (7, 29), and because circulating glucocorticoid levels are notably enhanced during proestrus (the estrus phase of highest  $E_2$  and P secretion) (14, 46, 60), it is very likely that the enhancement of adrenal sensitivity to ACTH by  $E_2$  is a physiologically relevant mechanism mediating sex or estrus cycle differences in the HPA axis.

To date, it is unknown whether  $E_2$  potentiates corticosterone secretion in vivo via direct actions on the adrenal gland (by directly acting on adrenocortical cells or by indirectly acting via adrenomedullary cells, via adrenal splanchnic innervation, via adrenal vasculature, and/or via ACTH presentation rate) or via increases in central drive prior to increased glucocorticoid negative feedback. Regarding mechanisms at the level of the adrenal gland, we investigated changes in adrenal steroidogenic acute regulatory and peripheral-type benzodiazepine receptor proteins in groups of  $E_2$ -treated and oil-treated OVX rats and found no significant effects (data not shown). Thus, it remains to be determined whether  $E_2$  enhances adrenal sensitivity to ACTH by altering the expression of the ACTH receptor (melanocortin 2 receptor). Since the estrogen receptor  $ER\alpha$  is present in medullary but not cortical cells of female (but not male) rat adrenals (18), it is likely that  $E_2$  may as well act via the adrenal medulla.

In summary, the present study unexpectedly revealed potentially divergent central and peripheral mechanisms underlying sex steroid effects on the HPA axis. Our experiments demonstrate that physiologically relevant doses of  $E_2$  or P inhibit stress-induced activation of the PVN in OVX female rats. This effect, paralleled by decreases in ACTH secretion, emerged despite enhanced glucocorticoid levels in  $E_2$ -treated rats. Importantly,  $E_2$  dramatically enhanced adrenal sensitivity in OVX rats. These findings demonstrate the important involvement of peripheral steroid-sensitive mechanisms in mediating  $E_2$  effects on the stress axis. As such, future studies should reexamine the precise role of adrenal gland sensitivity to ACTH, particularly in  $E_2$ -dependent glucocorticoid hypersecretion in females.

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