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 (E2), the major female estrogen. As expected, E2 enhanced plasma corticosterone responses to restraint in OVX females. However, E2 markedly attenuated the stress-induced c-fos gene expression in the PVN and inhibited plasma ACTH responses in these animals. Furthermore, E2-inhibitory effects were mimicked by progesterone (P) alone or in combination with E2. Interestingly, the suppressive central effects of both E2 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 unstrained rats. These unexpected findings suggested that E2 promotes glucocorticoid hypersecretion in females by additional peripheral (i.e., adrenal) mechanisms. Indeed, E2 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 sensitivity to ACTH is an important physiological mechanism mediating E2-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), adrenocorticotropic 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 (E2) potentiates the corticosterone response to numerous stressors, including restraint (60). Because E2-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.

The central sites and mechanisms responsible for E2 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 E2 effects on HPA axis responses to stress. In male rats, systemic injections of E2 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 E2 effects on corticotropin-releasing hormone (CRH) synthesis in the PVN (42, 44, 47) make it difficult to integrate E2-induced central changes in hypothalamic activation to stress neurosecretory events. Thus, despite evidence supporting a role of E2 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 E2 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 E2 and compared it with the plasma ACTH and corticosterone responses exhibited by these animals. Because the effects of E2 on neuroendocrine systems can be sensitive to the presence of progesterone (P), we also evaluated the participation of this progesterin in the female response to stress. Finally, we tested the direct role of E2 on adrenal gland responses to varying doses of exogenous ACTH. We show here an unexpected, potentially dichotomous mechanism employed by ovarian steroids, notably E2, 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; xylazine, 13 mg/kg) 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

E2 and P injections. One week after OVX, rats were subcutaneously injected with vehicle (sesame oil), β-E2 3-benzoate (E2, 10

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E1173
were performed in the morning (between 0900 and 1200) 4 h after the
These rats were used in
females/steroid treatment group) were placed in well-ventilated plas-
with a different E2 regimen (pellet implant) and submitted to two
E2 pellet implants. We also decided to verify the effects of E2 on
the stress response of OVX females by subcutaneously implanting
21-day continuous release pellets (Innovative Research of America,
Sarasota, FL) containing 17β-E2 (one 0.5-mg pellet/rat) or placebo
(one 150.0-mg pellet/rat) at the time of OVX surgery. The 0.5-mg E2
pellet clamps produced circulating E2 levels at ~160 ng/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 plas-
tic 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: ~4 min/rat) and then back in their
respective home cages. Blood samples were collected in ice-cold
microcentrifuge tubes containing 10 μl of 15% (K3)EDTA and
subsequently centrifuged at 1,500 g. All plasma samples were stored
at ~20°C.

Experiment 2: effects of E2 pellet implants on HPA axis responses
to restraint and elevated-plus-maze exposures. This experiment was
designed to verify the influences of E2 on the HPA axis of rats treated
with a different E2 regimen (pellet implant) and submitted to two
different putatively psychologically stressful, 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 re-
quires 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 E2 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, simi-
larly 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% (K3)EDTA and centrifuged at 1,500 g. Plasma samples were stored
frozen at ~20°C. Brains were quickly removed from all animals,
frozen in isopentane (~40° to ~50°C), and subsequently stored at
~80°C.

Experiment 4: effect of E2 on adrenal sensitivity to ACTH. One week
after OVX, rats were injected with either E2 (n = 42 rats) or oil (n = 42
rats) as described above. On the morning of the third day, rats were
subcutaneously injected with dexamethasone phosphate (400 μg;
Sigma) to block endogenous ACTH release. Two hours later, random-
ized 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 measure-
ment of plasma cortisol. 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 μm using a Microm cryostat (Kalama-
zoo, MI), mounted on Gold Seal slides (BD Biosciences, Portsmouth,
NH), and stored at ~20°C. For c-fos in situ hybridization, brain
sections from unstressed and stressed rats groups were used; for CRH
in situ hybridization, brain sections from unstressed rats were used.
Briefly, sections were fixed in 4% phosphate-buffered paraformalde-
yde 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).

In situ hybridizations used antisense cRNA probes comple-
mentary to rat c-fos (~700 bp) and CRH (~300 bp) mRNAs. These
probes were separately labeled by in vitro transcription using
[35S]UTP. The c-fos fragment (original full-length cDNA from Dr.
Tom Curran; St. Jude Children’s Research Hospital, Memphis, TN)
was cloned in pGem4Z vector, linearized with AvaII, and trans-
scribed 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). TheCRH fragment was cloned into a pGem4Z
vector, linearized with AvaII, and transcribed with T7 RNA polyme-
rase. The labeling reaction for c-fos or CRH consisted of 5× transcrip-
tion buffer, 125 μCi [35S]UTP, 200 μmol 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 μg linearized DNA. The mixture was incubated for
90 min at 37°C, after which the template DNA was digested with
ribonuclease-free deoxyribonuclease. The labeled probes were sepa-
rated 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× Denhardt’s solution, 200 μg/ml salmon sperm DNA, 150
µg/100 μl oil; Sigma, St. Louis, MO), or P (500 µg/100 μl oil;
Sigma), depending on the experimental group (Oil, E2, E2+P, or P).
Specifically, animals in the Oil group were injected with sesame oil on
days 1, 2, and 3. Animals in the E2 group received an injection of E2
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 E2 plus P
group received an injection of E2 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 E2 or oil, as described above, and used in experiment 4.

E2 pellet implants. ...
and P plasma concentrations were measured by 125I RIA kits from ICN Biochemicals (Cleveland, OH). E2 plasma concentration was measured by radioimmunoassay (RIA) using 125I RIA kit fromDiaSorin (Stillwater, MN). Corticosterone and P plasma concentrations were measured by 125I RIA kits from ICN Biochemicals (Cleveland, OH). E2 plasma concentration was measured by 125I 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 ANOVA (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 groups 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, statistical significance was set at $P < 0.05$. Statistical analyses were performed by usingStatView (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, E2 and P plasma concentrations were measured only in rat blood samples obtained by decapitation (experiment 1). E2 and P administration yielded appreciable levels of these hormones in the plasma of OVX rats (Table 1). As expected, E2 injections resulted in higher plasma 17ß-E2 levels in the E2 and E2 plus P groups compared with Oil and P groups (F3,41 = 9.4, $P < 0.05$). Concurrently, P injections resulted in higher plasma P levels in the P and E2 plus P groups compared with Oil and E2 groups (F3,36 = 31.4, $P < 0.05$); however, the levels of P were higher when this hormone was injected alone (P) than when combined with E2 ($P < 0.05$). Neither E2 nor P plasma levels were affected by stress (F1,37 = 3.1, $P > 0.5$; and F1,32 = 1.1, $P > 0.5$, respectively). The steroid doses used in this study produced approximate E2 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 (E2, E2 + P, and P; $P < 0.05$) and 60 min (E2 + P; $P < 0.05$) from onset of stress (F3,95 = 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 E2-treated rats at 0 and 60 min compared with oil ($P < 0.05$). Additionally, corticosterone responses were significantly higher at 120 min in the E2 plus P group compared with E2, 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 E2 and E2 plus P (but not P) groups were significantly higher than the oil group.

Table 1. Plasma ovarian steroid concentrations in experimental rats

<table>
<thead>
<tr>
<th>Plasma Steroid Conc.</th>
<th>Oil</th>
<th>E2</th>
<th>E2 + P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>17ß-E2, pg/ml</td>
<td>14.3 ± 1.6</td>
<td>166.7 ± 40.9*</td>
<td>191.4 ± 47.6*</td>
<td>34.5 ± 8.1</td>
</tr>
<tr>
<td>P, ng/ml</td>
<td>13.4 ± 3.1</td>
<td>20.5 ± 3.1</td>
<td>68.6 ± 14.1</td>
<td>109.8 ± 13.1†‡</td>
</tr>
</tbody>
</table>

Values represent means ± SE of pooled unstressed and restraint-stressed ovariec-tomized (OVX) rats from experiment 2. Conc., concentration: E2, estradiol; P, progesterone. *$P < 0.05$ vs. Oil and P; †$P < 0.05$ vs. Oil and E2; ‡$P < 0.05$ vs. E2 plus P.
Effects of E2 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 E2-injected rats, we thought it would be important to verify the generality of this response in OVX rats with E2 (0.5 mg) pellet implants, an alternative treatment shown to continuously produce E2 plasma levels to ~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, E2 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, E2 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 E2-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 E2 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 E2, P, or E2 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). E2, E2 plus P, and
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 E2-treated rats ($F_{3,14} = 1.5, P > 0.05$; Fig. 5).

### E2 Effect on Adrenal Sensitivity and Adrenal Corticosterone Content

The central inhibitory actions of E2 on PVN activation and CRH mRNA levels did not explain the observed E2 enhancement of plasma glucocorticoid responses to stress. Thus, we reasoned that glucocorticoid hypersecretion in response to stress may result from E2 actions on the adrenal gland. Our results show that E2 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, E2 increased the adrenal corticosterone responses to exogenous ACTH ($F_{1.58} = 11.1, P < 0.05$; Fig. 6). E2 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 E2 administration, we demonstrate here that E2 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 E2 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 E2 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 E2 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 E2. Here, we used two distinct methods of E2 replacement, 7-day pellet implant and short-term steroid replacement that produced physiologically relevant E2 or P plasma concentrations comparable with earlier studies (plasma E2 160 pg/ml; 7-day treatment) (47, 62). We selected this short-term regimen because it delivers steroid concentrations at physiological lev-
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 E2-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 E2-treated rats displayed greater inhibition of hypothalamic activity (i.e., decreased c-fos expression in the PVN and decreased ACTH levels). Indeed, E2 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 E2 is capable of potentiating the negative feedback effects of corticosterone. Therefore, the enhancement of adrenal sensitivity to ACTH in our E2-treated rats could have been triggered by an earlier increase in central drive.

Second, E2 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 E2 cannot be easily explained by clearance mechanisms [E2 increases plasma corticosterone clearance (60)], we reasoned that E2 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 E2 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 E2 increases adrenal corticosterone content, indicating that E2 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 E2-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 E2 inhibits hypothalamic activation in stressed female rats (9). This finding however, contrasts with the stimulatory effect of E2 on PVN activation in male rats (38, 50).

Previously, we showed consistent HPA axis effects using two different E2 replacement methods. Alternatively, it is also possible that our sampling time course may have missed an earlier peak in hypothalamic activity (i.e., decreased c-fos expression in the PVN). Therefore, the enhanced adrenal sensitivity to ACTH and glucocorticoid hypersecretion in our E2-treated rats. First, E2 could have

 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 E2 (~500 pg/ml) (60) or longer E2 exposure time (21 days) (3). It should be noted that the physiological effects of E2 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 E2 effects can be sensitive to the replacement protocol, the present work shows consistent HPA axis effects using two different E2 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 E2, 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 E2-treated rats. First, E2 could have

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

<table>
<thead>
<tr>
<th>Brain Region (cortex)</th>
<th>Oil</th>
<th>E2</th>
<th>E2 + P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cingulate</td>
<td>89.5 ± 7.8</td>
<td>75.6 ± 8.4</td>
<td>93.9 ± 6.0</td>
<td>70.2 ± 9.2</td>
</tr>
<tr>
<td>Prefrontal</td>
<td>84.7 ± 9.7</td>
<td>74.7 ± 6.8</td>
<td>83.9 ± 6.9</td>
<td>61.0 ± 9.0</td>
</tr>
<tr>
<td>Infralimbic</td>
<td>80.6 ± 10.4</td>
<td>73.2 ± 6.4</td>
<td>80.1 ± 6.2</td>
<td>53.0 ± 10.0</td>
</tr>
<tr>
<td>Piriform</td>
<td>116.1 ± 3.6</td>
<td>97.9 ± 7.8</td>
<td>112.9 ± 5.6</td>
<td>90.1 ± 10.7</td>
</tr>
</tbody>
</table>

Data represent means ± SE (n = 4–6 rats/group). No statistical differences were observed for these brain regions.
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 E2 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 cortices, 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-α (50), 2) very few PVN CRH neurons, if any, express ER-β (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 E2 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. 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 μg of E2 and/or 500 μg of P. Data represent means ± SE (n = 4–6 rats/group). There were no significant differences among treatment groups.

Fig. 6. E2 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 μg E2 (sc). Data represent means ± SE (n = 5–7/group). *P < 0.05 E2 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 E2 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, E2 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 E2 acts, at least in part, to increase adrenal corticosterone synthesis. It is possible that E2 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 E2 treatment reduced free corticosterone levels in our female rats. First, one study (44) shows that, although E2 treatment elevated total plasma corticosterone concentrations in OVX females, it did not change the binding capacity or the affinity in these animals, suggesting that E2 may actually elevate circulating free corticosterone in female rats. E2, 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 E2 treatments (10 days) (16), shorter (3 days) subcutaneous administration of E2 to male rats did not significantly change serum CBG or hepatic CBG mRNA levels (52). Thus, it is likely that the shorter (2 days) E2 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 explained by sex differences in hepatic clearance of corticosterone. Female rats display significantly lower basal corticosterone levels 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, E2 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 E2 on the HPA axis function in intact female rats. This will determine whether E2 has similar effects in OVX and intact animals.

Moreover, the physiological relevance of enhanced glucocorticoid levels in OVX females treated with E2 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 corticosterone concentration than intact males (7, 29), and because circulating glucocorticoid levels are notably enhanced during estrus (the estrus phase of high E2 and P secretion) (14, 46, 60), it is very likely that the enhancement of adrenal sensitivity to ACTH by E2 is a physiologically relevant mechanism mediating sex or estrus cycle differences in the HPA axis.

To date, it is unknown whether E2 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 E2-treated and oil-treated OVX rats and found no significant effects (data not shown). Thus, it remains to be determined whether E2 enhances adrenal sensitivity to ACTH by altering the expression of the ACTH receptor (melanocortin 2 receptor). Since the estrogen receptor ERα is present in medullary but not cortical cells of female (but not male) rat adrenals (18), it is likely that E2 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 E2 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 E2-treated rats. Importantly, E2 dramatically enhanced adrenal sensitivity in OVX rats. These findings demonstrate the important involvement of peripheral steroid-sensitive mechanisms in mediating E2 effects on the stress axis. As such, future studies should reexamine the precise role of adrenal gland sensitivity to ACTH, particularly in E2-dependent glucocorticoid hypersecretion in females.

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