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Stress Activation of Cortex and Hippocampus Is Modulated by Sex and Stage of Estrus

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Sex plays a major role in stress integration and stress-related affective disease states. Notably, neurocircuits regulating organismic responses to stress are prime targets for central gonadal steroid action. To assess the roles of sex and estrous cycle in central stress integration, we analyzed c-*fos* mRNA expression in hypothalamic-pituitary-adrenocortical-related regions of stressed male and cycling female (proestrous, estrous, and diestrous) rats. At 60 min after the onset of acute restraint stress, all animal groups showed induction of c-*fos* mRNA in the frontal cortex, cingulate cortex, piriform cortex, hippocampus, hypothalamic paraventricular nucleus (PVN), medial amygdala, and lateral septum. However, the magnitude of c-*fos* induction in cortical and hippocampal regions was substantially lower in proestrous and estrous females compared with males and diestrous females. Sex- and estrus cycle-related changes are region specific, as no difference in c-*fos* induction occurred in the hypothalamic PVN, medial amygdala, or ventrolateral septum in any group. Furthermore, induction of c-*fos* mRNA in limbic cortexes (but not hippocampus) was positively correlated with progesterone and negatively correlated with ACTH levels. Taken together, this study indicates that cortical structures are differentially stress activated in females depending on the phase of the estrous cycle, perhaps in a progesterone-dependent fashion. (*Endocrinology* 143: 2534–2540, 2002)

THE IMMEDIATE-EARLY gene (IEG) c-*fos* has been validated as a powerful marker of neuronal activation and, consequently, as a valuable tool for functional mapping of stress neurocircuitry (1–4). In different acute stress paradigms, c-*fos* is induced (along with other IEGs) in numerous brain regions, including the cerebral cortex, hippocampus, and amygdala. Many of these are regions known to be involved in cognitive and emotional processing (5, 6) and may play roles in the initiation or cessation of the behavioral/neuroendocrine stress response. Furthermore, stress induction of c-*fos* expression is typically transient and dependent on stressor modality (7), indicating the utility of c-*fos* as a marker for stimulus specificity and intensity.

Despite the widespread uses of c-*fos* mRNA and protein as reliable indicators of neuronal excitation, few reports have investigated whether their stress inducibility is affected by sex and/or phase of the estrous cycle. There are reasons to expect that IEG expression following stress would be affected by changes in the gonadal hormone milieu. For example, induction of c-*fos* mRNA expression after restraint stress fluctuates during specific times of lactation and pregnancy (8) as well as after estrogen administration (8, 9). Affected regions included the cingulate cortex, hippocampus, and medial amygdala, all known to play important roles in the hypothalamic-pituitary-adrenocortical axis (HPA) response to stress. Moreover, these regions are known to express estrogen and progesterone receptors (10–14), further supporting a role for gonadal steroids in neural regulation of the HPA axis.

Abbreviations: GABA, γ-Aminobutyric acid; HPA, hypothalamic-pituitary-adrenocortical; IEG, immediate-early gene; KPBS, potassium PBS; MR, mineralocorticosteroid receptor; PVN, paraventricular nucleus; SSC, standard saline citrate.

In this report we investigated whether stress-induced c-*fos* expression in HPA-related brain regions is affected by sex and gonadal hormone fluctuations occurring during the estrous cycle. We used *in situ* hybridization to semiquantitatively analyze the expression of c-*fos* in HPA-related forebrain regions of male and cycling female (proestrous, estrous, and diestrous) rats submitted to acute restraint stress and to correlate differences in neural activation with changes in circulating hormone levels.

Materials and Methods

Experimental animals

Eighteen adult male and 54 adult female Sprague Dawley rats were included in this study. All rats were housed three per cage on a 12-h light, 12-h dark cycle (lights on from 0600–1800 h) with food and water available *ad libitum*. The stage of the estrous cycle was determined by vaginal smears sampled daily between 0800 and 1000 h following the cytological pattern described previously (15, 16). Only females demonstrating two consecutive 4-d estrous cycles were used in this study. All animal protocols were approved by the University of Kentucky institutional animal care and use committee according to NIH Guide on the Care and Use of Animals.

Acute stress protocol

Animals were randomly assigned to three stress groups. The unstressed group was not exposed to stress before death and thus represented the control group. The 60 min group was placed in plastic restraint tubes for 30 min, returned to their home cage, and subsequently killed 30 min later. The 120 min group was exposed to 30-min restraint stress, returned to their home cage, and killed 90 min later. All rats were killed by rapid decapitation between 0900 and 1100 h. On the day of experiment the phase of the estrous cycle was verified immediately after death of the animals. Trunk blood samples were collected in Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) containing 15% (K_3)EDTA and centrifuged at 1500 × *g*, and plasma samples were frozen at −20 C. All brains were removed and frozen in isopentane cooled on dry ice at −40 to −50 C and were stored at −80 C until processing. The procedures

were conducted on separate days over a period of time and included the following number of animals: unstressed group: males, n = 6; proestrus, n = 6; estrus, n = 6; diestrus, n = 5; 60-min group: males, n = 6; proestrus, n = 6; estrus, n = 6; diestrus, n = 6; and 120-min group: males, n = 6; proestrus, n = 5; estrus, n = 6; diestrus, n = 8.

Plasma hormone assay

Plasma from trunk blood samples were processed for RIA using ^{125}I RIA kits from ICN Biochemicals, Inc. (Cleveland, OH) to determine estrogen (17β-estradiol), progesterone, and corticosterone plasma levels and using a ^{125}I RIA kit from DiaSorin, Inc. (Stillwater, MN) to determine ACTH plasma levels according to the manufacturer's instructions. 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.

In situ hybridization

Brains were sectioned at 16 μm using a Microm cryostat (Kalamazoo, MI), mounted on Gold Seal slides (BD Biosciences, Portsmouth, NH), and stored at −20 C. For *in situ* hybridization, sections were fixed in 4% phosphate-buffered paraformaldehyde for 10 min and rinsed twice in 5 mM potassium PBS (KPBS) for 5 min, twice in 5 mM KPBS for 5 min with 0.2% glycine, and two more times in KPBS for 5 min. Sections were then acetylated in 0.25% acetic anhydride (suspended in 0.1 M triethanolamine, pH 8.0) for 10 min, rinsed twice in 2× standard saline citrate (SSC) for 5 min, and dehydrated through graded alcohols.

Antisense rat c-*fos* probe was generated by *in vitro* transcription using [^{35}S]UTP as label. The c-*fos* DNA construct was a fragment of an original full-length cDNA obtained from T. Curran, cloned into pGEM4Z. The specificity of this probe has been validated in previous studies (17). Briefly, plasmid was linearized with *Ava*II and transcribed with SP6 RNA polymerase, giving a cRNA probe with a final length of 587 bp. The transcription reaction consisted of 10× transcription buffer; 125 μCi [^{35}S]UTP; 200 μM ATP, CTP, and GTP; 10 μM cold UTP; 100 mM dithiothreitol; and 40 U/μl SP6 RNA polymerase. The mixture was incubated for 90 min at 37 C, after which the template DNA was digested with ribonuclease-free deoxyribonuclease, and probe was separated from free nucleotides by ammonium acetate precipitation.

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/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, whereupon the slides were coverslipped and incubated overnight at 50 C in humidifier chambers containing 50% formamide. The next day coverslips were removed in 2× SSC, and slides were incubated in 100 μg/ml ribonuclease A for 30 min at 37 C. Slides were briefly rinsed in 2× SSC, washed three times in 0.2× SSC (65 C), dehydrated, and exposed to Cyclone Storage Phosphor Screens (Packard Instrument Co., Inc., Meriden, CT) for 5 d.

Image analysis

Semiquantitative analyses of *in situ* hybridization images (obtained from Cyclone Storage Phosphor Screens) were performed using OptiQuant Image Analysis Software (Packard Instrument Co., Inc.). Briefly, anatomical regions of interest were determined based on Paxinos and Watson's rat brain atlas (18). Signals from these regions (measured as digital light units per square millimeter) were corrected by subtracting background signal as determined by sampling nonhybridized regions of each section. In all cases the mean value of two to four sections through a given region (four to eight individual measurements) was calculated for each animal and used in the statistical analysis. All *in situ* quantifications were performed in a blinded fashion, with the person performing the measurements unaware of the group assignments.

Statistical analysis

A 3 × 4 factorial ANOVA was conducted to analyze the effects of stress (unstressed *vs.* 60 *vs.* 120 min) and sex and estrous cycle (male *vs.* proestrus *vs.* estrus *vs.* diestrus) on the mean plasma concentration of each hormone (17β-estradiol, progesterone, ACTH, and corticosterone). Significant effects and/or interactions were further analyzed by separate one-way ANOVAs conducted for each stress group, followed by Fisher's least significant difference test. One-way ANOVAs were used to analyze the effects of sex and estrous cycle on the mean c-*fos* mRNA level in forebrain regions of interest, followed by Fisher's least significant difference test. The Pearson correlation coefficient was conducted to analyze relationships among hormone levels and mean c-*fos* induction. The Fisher's r to z transformation was performed to determine statistical significance of correlations. All animals in the 60 min group, including the males, were included in the correlation analysis. All data were analyzed by using StatView software (SAS Institute, Inc., Cary, NC).

Results

17β-Estradiol, progesterone, ACTH, and corticosterone levels

To verify the levels of circulating gonadal steroid in the experimental animals at the time of death, 17β-estradiol and progesterone blood levels were determined. The 17β-estradiol level was significantly higher in proestrous females compared with estrous females, diestrous females, and males in all stress groups ($F_{3,60} = 59.157$; $P < 0.05$), as expected (Fig. 1A). The 17β-estradiol plasma level was also higher in estrous and proestrous females compared with males across all stress groups ($P < 0.05$). In general, 17β-estradiol levels were higher than what has been previously reported (19, 20) despite the high reliability of our assay (tested with controls provided by manufacturer) and the low sample/group variability. Although the RIA kit used in this study is specifically designed for 17β-estradiol detection, 20.0% cross-reactivity occurs with estrone (manufacturer's data), potentially inflating our values. Furthermore, considerable differences in gonadal hormone levels have been recently observed among commercially available RIA kits (21).

The progesterone level was significantly higher in diestrous females compared with estrous females and males ($F_{3,60} = 21.142$; $P < 0.05$; Fig. 2B). The progesterone level was also significantly higher in diestrous females than proestrous females in the 60 and 120 min groups ($P < 0.05$). Earlier studies have shown that progesterone levels during the estrous cycle of rats peak on the morning of diestrus and on the afternoon of proestrus (19). Thus, the lack of elevated progesterone levels in the proestrous group (Fig. 2B) is most likely due to the time of sampling, which lagged behind the surge seen on the afternoon of proestrus.

The overall ANOVA indicated that ACTH levels were affected by stress ($F_{2,50} = 4.408$; $P < 0.05$), but not by sex and estrous cycle (Fig. 1C). However, *post hoc* analysis showed that this effect was significant only between stressed males (*i.e.* males in the 60 min group had significantly higher ACTH levels than males in the 120 min group; $F_{2,14} = 4.809$; $P < 0.05$). Nevertheless, basal levels in unstressed males were not different from those in either 60 or 120 min groups. No differences occurred among females either within or among stress groups. Corticosterone levels, on the other hand, significantly changed among stress groups ($F_{2,52} = 5.454$; $P < 0.05$) as well as according to sex and estrous cycle ($F_{3,52} = 2.717$; $P < 0.05$; Fig. 2D). The 60 min male group had higher levels of corticosterone than both unstressed and 120 min male groups ($F_{3,16} = 5.372$; $P < 0.05$), and unstressed estrous females had significantly lower levels than both 60 and 120 min stressed estrous females. No statistically significant dif-

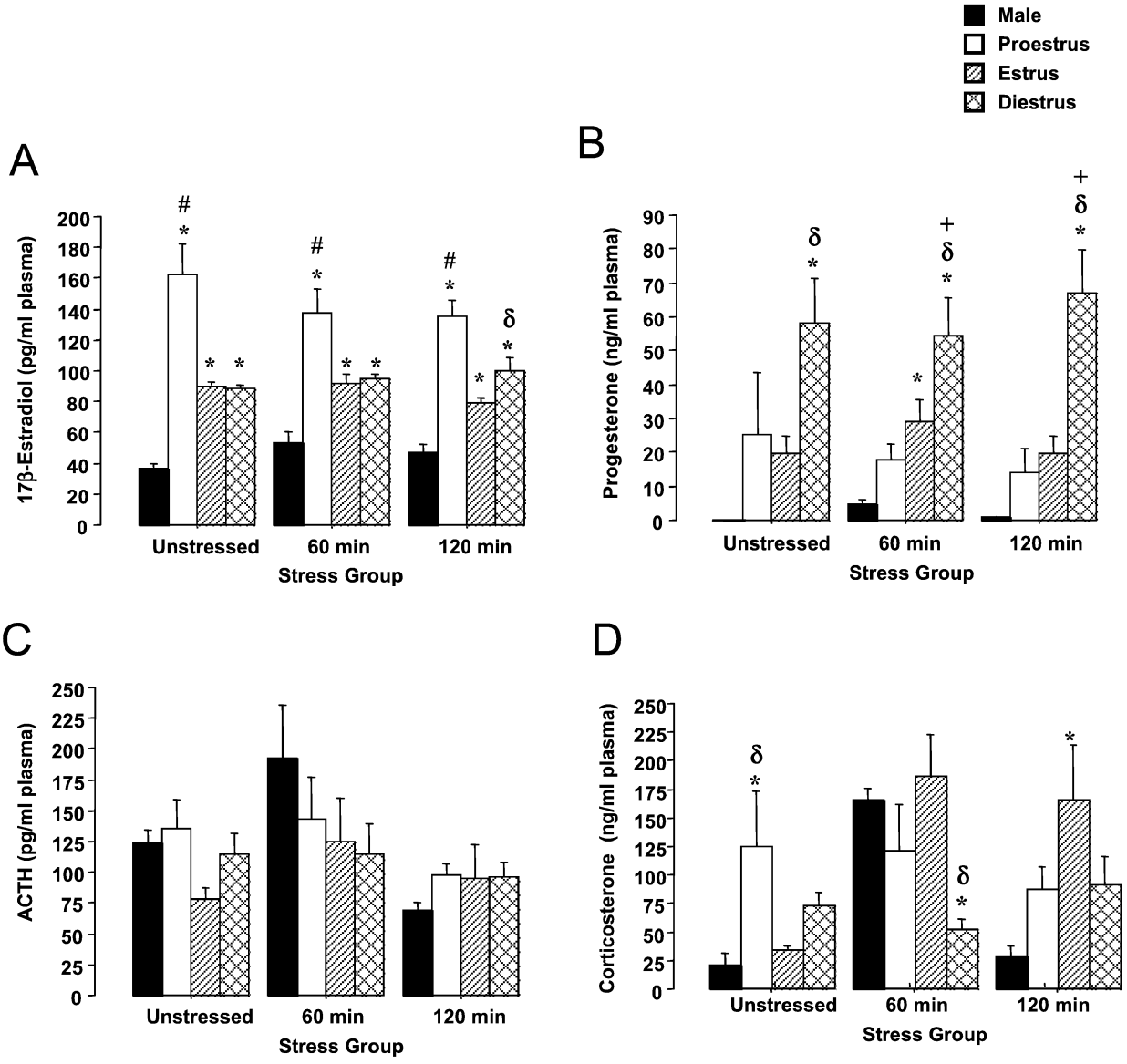

FIG. 1. Mean ± SEM concentrations of plasma 17β-estradiol (A), progesterone (B), ACTH (C), and corticosterone (D) in unstressed and stressed (60 and 120 min from the onset of 30-min restraint stress) male and female (proestrous, estrous, and diestrous) rats. *, $P < 0.05$ *vs.* male; #, $P < 0.05$ *vs.* estrus and diestrus; δ, $P < 0.05$ *vs.* estrus; +, $P < 0.05$ *vs.* proestrus. n = 3–8 animals/sex or phase of estrus/stress.

ferences were observed in either proestrous or diestrous females across the three stress groups studied. Within the unstressed group, corticosterone levels were higher in proestrous females compared with males and estrous animals ($F_{3,18} = 3.065$; $P < 0.05$). In the 60 min stress group, corticosterone was lower in the diestrous females compared with males and estrous females ($F_{3,16} = 5.372$; $P < 0.05$). Finally, in the 120 min group, corticosterone levels remained higher in cycling females than in males, with a significant increase in estrous females ($F_{3,17} = 2.997$; $P < 0.05$).

Effects of sex and estrous cycle on the expression of c-fos mRNA in forebrain regions

We analyzed the effects of animal group (males and cycling females) on neuronal activity in stressed animals using c-*fos in situ* hybridization. In this study, the c-*fos* signal was nearly absent in the forebrain of unstressed animals, was strongly expressed 30 min after restraint (*i.e.* 60 min group), and was markedly reduced 90 min after stress (*i.e.* 120 min group; data not shown; Figs. 2A and 3A). This pattern is consistent with early reports showing c-*fos* mRNA induction peaks between 30 and 60 min from the onset of stress (17). Semiquantitative analysis performed in sections from the 60 min stress group revealed that the c-*fos* mRNA level was markedly decreased in the cingulate cortex of proestrous and estrous females compared with males ($F_{3,20} = 3.645$; $P < 0.05$; Fig. 2). c-*fos* mRNA expression in the frontal cortex and piriform cortex was similarly affected, with proestrous females having significantly lower c-*fos* induction relative to males and diestrous females ($F_{3,20} = 3.41$; $P < 0.05$ and $F_{3,20} = 3.703$; $P < 0.05$, respectively; Fig. 2). Because c-*fos* mRNA levels

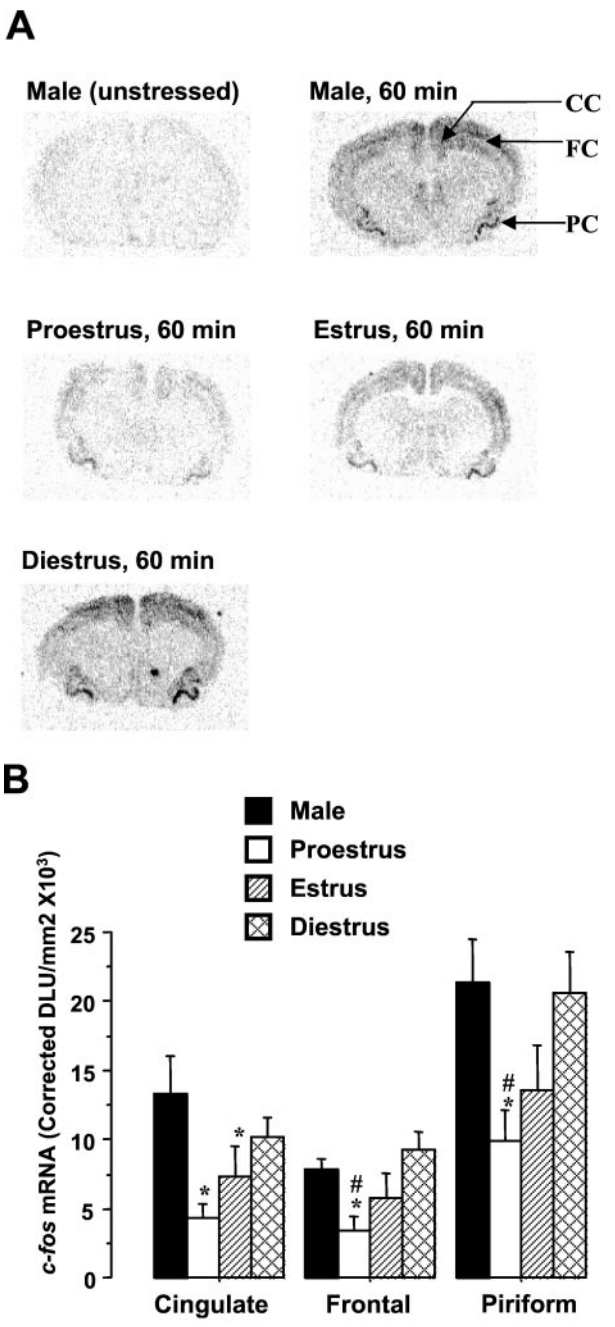

FIG. 2. A, Phosphor screen images showing c-*fos* mRNA hybridization signal in the cingulate (CC), frontal (FC), and piriform (PC) cortexes of male and cycling female rats at 60 min from the onset of stress. Note decreased c-*fos* mRNA signal in proestrous and estrous females compared with males and diestrous females. Also note the absence of c-*fos* mRNA signal in unstressed (control) male rat brain. B, Semiquantitative measurements of c-*fos* mRNA hybridization signal in the cingulate, frontal, and piriform cortexes of males and cycling female rats at 60 min from the onset of stress. Data are the mean ± SEM corrected digital light units (DLU) per square millimeter. *, $P < 0.05$ *vs.* male; #, $P < 0.01$ *vs.* diestrus. n = 6 animals/sex or phase of estrus/region.

in autoradiographs of unstressed and 120 min animals were at or near background levels for the areas under study, we did not assess sex/estrous cycle effects in those groups.

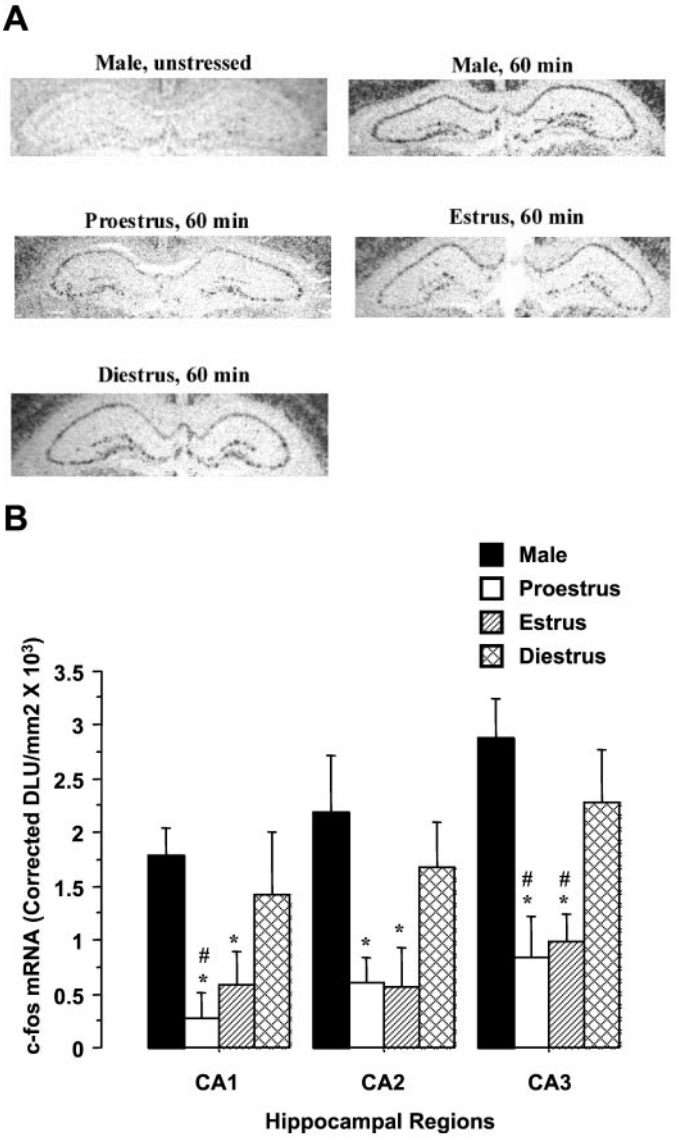

FIG. 3. A, Autoradiographs showing c-*fos* mRNA hybridization signal in the hippocampus (CA1, CA2, and CA3 regions) of male and cycling female rats at 60 min from the onset of stress. Note decreased c-*fos* mRNA signal in proestrous and estrous females compared with males and diestrous females. Also note the absence of c-*fos* mRNA signal in unstressed (control) male rat brain. B, Semiquantitative measurements of c-*fos* mRNA hybridization signal in the hippocampus (CA1, CA2, and CA3 regions) of male and cycling female rats at 60 min from the onset of stress. Data are the mean ± SEM corrected digital light units (DLU) per square millimeter. *, $P < 0.05$ *vs.* male; #, $P < 0.05$ *vs.* diestrus. n = 6 males animals/sex or phase of estrus/region.

Hippocampal c-*fos* induction in the 60 min group was differentially affected by region ($F_{2,60} = 3.829$; $P < 0.05$) and by sex and phase of estrous cycle ($F_{3,60} = 14.352$; $P < 0.05$; Fig. 3). *Post hoc* analyses performed on each region indicated that c-*fos* mRNA induction was significantly lower in proestrous and estrous animals than in males ($P < 0.05$). In addition, c-*fos* mRNA induction in the CA1 and CA3 hippocampal regions of proestrous females as well as that in the CA3 region of estrous females were significantly lower than that in their counterpart diestrous females ($P < 0.05$).

In contrast with cortex and hippocampus, there was no group effect on c-*fos* mRNA expression in the hypothalamic PVN, medial amygdala, and lateral septal nucleus of male and female rats at 60 min from the onset of restraint (Table 1).

Correlational analysis

Tables 2 and 3 show Pearson's correlation coefficients for the hormone and c-*fos* induction data from the 60 min group of animals. Fisher's r to z transformations revealed a significant positive correlation between progesterone and cortical (cingulate, frontal, and piriform) c-*fos* induction and a significant negative correlation between ACTH levels and c-*fos* induction in the cingulate and frontal cortexes (Table 2). There was no statistically significant correlation between the estradiol level and c-*fos* mRNA expression in these cortical regions. In contrast with cortex, hippocampal c-*fos* mRNA expression was not correlated with ACTH, progesterone, or estradiol levels (Table 3). In addition, there were no correlations between corticosterone levels and either c-*fos* expression or gonadal hormone levels (data not shown).

Discussion

The present study shows that stress-induced c-*fos* mRNA expression in cortex and hippocampus varies markedly according to sex and phase of the estrous cycle. Thus, 30 min after restraint stress, c-*fos* mRNA expression in the frontal cortex, cingulate cortex, piriform cortex, and hippocampus was markedly lower in proestrous and estrous females relative to males and diestrous females. These changes were region specific, as no changes in c-*fos* induction occurred in the hypothalamic PVN or subcortical limbic structures, including the lateral septum and medial amygdala. Considering that the proestrous and diestrous females used in this study exhibited high levels of estrogen and progesterone, respectively, it is likely that the sex and estrous cycle differences in cortical and hippocampal activation during stress result from complex interactions between gonadal steroids and ongoing neuronal signaling processes.

The activational changes observed in the cortex and hippocampus in our animals are consistent with the involvement of these structures in the regulation of the HPA axis (reviewed in Refs. 5 and 6). Importantly, both cingulate cortex and hippocampus are known to inhibit HPA activity (5, 22). Thus, it is possible that low activity of these regions during proestrus may result in decreased HPA inhibitory outflow, thereby contributing to enhancement of the HPA activity known to occur in proestrous females (23–26). Because no direct anatomical substrate between hippocampus/cortex and PVN parvocellular neurons have yet been successfully identified, it is likely that cortico-hippocampal influences on PVN hypophysiotropic neurons are indirectly achieved via subcortical relay neurons. For instance, hippocampal influences on PVN neurons may be indirectly relayed via projections to the bed nucleus of stria terminalis, preoptic area, anterior hypothalamus, subparaventricular area, and dorsal medial hypothalamic nucleus (27–30).

The widespread modulation of cortical excitability in accordance with phase of estrus indicates that the female gonadal steroid milieu may profoundly affect neuronal and, by extension, psychological responses to stress. Indeed, a recent report evaluating the antidepressant effects of exogenously administered estradiol also found this capable of influencing c-*fos* expression levels after forced swimming (9), a paradigm known to be stressful for rats. Estradiol administration to these animals promoted a significant reduction in the mean number of c-*fos*-immunoreactive cell nuclei in the anterior cingulate cortex and hippocampus (dorsal CA1 and dorsal CA3), but did not change those in the basomedial amygdala, central amygdala, cortical amygdala, and lateral septum (9), all consistent with our findings in rats with high estradiol levels (*i.e.* proestrous group).

The observed changes in c-*fos* expression could be due to several mechanisms, including direct influences of gonadal hormones on cellular excitability, thereby impinging on c-*fos* induction. In this scenario, c-*fos* expression could be affected at the level of transcription not only by genomic (*i.e.* via classical receptor ligand), but also by nongenomic, pathways (31), as estradiol has shown to rapidly potentiate kainate-induced currents in hippocampal neurons (32, 33). Alternatively, the differences in stress-induced c-*fos* expression could be secondary to plastic changes in synaptic transmission promoted by estrous cycle fluctuations. In the hippocampus, dendritic spine density increases on the day of proestrus (34), and this effect has been attributed to estrogen (35, 36), presumably by enhancing neuronal sensitivity to glutamatergic inputs (37) and, more importantly, by reducing γ-aminobutyric acid (GABA)-ergic influences (38, 39). Interestingly, these estrogen effects on hippocampal neurons are reversed by progesterone and its metabolites (40), which parallels the hippocampal and cortical c-*fos* expression patterns seen in our animals on different days of estrus. Furthermore, progesterone metabolites interact directly with the GABA-A receptor in an agonistic manner, suggesting that elevated progesterone levels may increase GABAergic tone and thereby reduce limbic excitation (41).

The relationship between sex/estrous cycle and stress-induced HPA activation appears complex. The effects of sex/cycle on corticosterone secretion are most pronounced 120 min after stress induction, suggesting a delayed shut-off of the HPA axis in proestrous and estrous females. Impor-

TABLE 1. Semiquantitative analyses of c-*fos* mRNA hybridization signal in the hypothalamic PVN, medial amygdala, and lateral septal nucleus of male and cycling female rats at 60 min from onset of stress

	Male	Proestrus	Estrus	Diestrus
Hypothalamic PVN	22803.2 ± 4414.7	24092.3 ± 4373.1	25711.7 ± 6884.8	16636.5 ± 3908.5
Medial amygdala	22339.9 ± 3911.0	27856.7 ± 2861.9	27297.8 ± 4512.9	20908.9 ± 1450.8
Lateral septal nucleus	7755.5 ± 873.7	5462.2 ± 2036.6	5078.1 ± 1193.5	6784.9 ± 1070.3

Data are the mean ± SEM corrected digital light units (DLU) per square millimeter. No statistically significant differences were observed. n = 6 animals/sex or phase of estrus/region.

TABLE 2. Statistical correlation among hormone levels and c-*fos* mRNA expression in cortexes after 60 min from onset of stress

	E2	Progesterone	ACTH	Cingulate cortex	Frontal cortex	Piriform cortex
E2	1.000					
Progesterone	−0.331	1.000				
ACTH	−0.298	−0.179	1.000			
Cingulate cortex	−0.300	0.490[a]	−0.502[a]	1.000		
Frontal cortex	−0.300	0.532[a]	−0.580[a]	0.863[a]	1.000	
Piriform cortex	−0.246	0.502[a]	−0.440	0.698[a]	0.836[a]	1.000

[a] $P < 0.05$.

TABLE 3. Statistical correlation among hormone levels and c-*fos* mRNA expression in CA1, CA2, and CA3 hippocampal regions 60 min after the onset of stress

	E2	Progesterone	ACTH	CA1	CA2	CA3
E2	1.000					
Progesterone	−0.331	1.000				
ACTH	−0.298	−0.179	1.000			
CA1	−0.205	0.120	0.013	1.000		
CA2	−0.208	0.336	−0.260	0.811[a]	1.000	
CA3	−0.353	0.404	−0.168	0.767[a]	0.800[a]	1.000

[a] $P < 0.05$.

tantly, diestrous females show minimal stress responsivity (60 min group), consistent with a blunting of the HPA stress response during this portion of the estrous cycle. When combined with the observed sex/estrous cycle patterns of c-*fos* expression, the data are consistent with the hypothesis that high levels of cortical and hippocampal activation occur within the context of enhanced stress axis inhibition. Moreover, a study by Carey *et al.* (42) suggested that gonadal steroids modulate HPA activity via the mineralocorticosteroid receptor (MR), but not by the glucocorticoid receptor. More specifically, estradiol decreases hippocampal MR mRNA expression and binding, whereas progesterone reverses this effect (42). Thus, because of the important role of adrenocorticosteroid receptors in corticosterone negative feedback, it is possible that sex/estrous cycle differences in MR capacity may contribute to the elevated corticosterone levels in proestrous females (unstressed group) and to the blunted response in stressed diestrous females.

In contrast with corticosterone, blood ACTH levels in stressed (60 and 120 min groups) animals did not significantly differ from those of unstressed animals regardless of sex/phase of estrus. This lack of stress effects on basal ACTH levels is most likely due to the time points selected for sampling. A time-course study of cannulated animals has shown that ACTH rapidly reaches its peak within minutes from the onset of stress and sharply declines to basal values between 30 and 60 min; corticosterone levels, on the other hand, rise gradually to reach a maximum at 30 min from initiation of stress, falling afterward to basal levels in 60 min (43). By the same token, robust estrous cycle differences and gonadal hormone effects on ACTH blood levels have been reported to occur only within the first the first 30 min poststress, but not thereafter (42, 44). Therefore, by sampling our animals at an optimal time point for c-*fos* mRNA detection (*i.e.* 60 min from onset of stress), we may have missed the peaks in ACTH levels as well as sex/gender differences.

In our study sex and estrous cycle differences in cortical activation after stress were not followed by activational changes in the PVN, a critical HPA regulatory region known to be influenced by estrous cyclicity and gonadal hormones (45–48). There are several possible explanations for the discrepancy between secretion data and c-*fos* induction. The PVN sits at the end of the forebrain cascade of the stress response and show minimal expression of estrogen and progesterone receptors. Thus, effects on PVN may be manifest as changes in the duration of the c-*fos* response, rather than its magnitude; analysis of a single poststress time point may have missed differences occurring earlier or later in time. Sex and estrous cycle may also affect HPA secretory activity without modifying the magnitude of c-*fos* gene transcription at the PVN. Such effects may be achieved by enhancing the release of CRH and/or AVP directly at median eminence terminals, increasing pituitary ACTH release, or enhancing corticosterone secretion directly at the adrenal. Finally, restraint may produce a maximal induction of c-*fos* mRNA in the PVN, whereby sex- or estrous cycle-related increases in cellular activation are not reflected by this measure.

In any case, the significant positive correlation between plasma progesterone levels and c-*fos* mRNA expression in the cingulate, frontal, and piriform cortexes found in our study further indicates the impact of changing hormonal milieu on suprahypothalamic stress-related neurocircuitry. In addition, a significant negative correlation was found between ACTH and the studied cortical regions, supporting a relationship between factors affecting cortical excitability and elaboration of the stress response. Although estrogen has been shown to decrease the activity of several HPA inhibitory structures (9), our data showed no correlation between the level of estrogen and c-*fos* expression in cortex. Thus, sex and estrous cycle variations in the stress response may result from combinatorial influences of fluctuating gonadal hormonal levels on hypophysiotropic neurons of the PVN.

In summary, the results of this study indicate estrous cycle dependence in cellular activation of brain regions controlling mood, reward, and memory. Decreases in restraint-induced cortical and hippocampal c-*fos* mRNA expression during estrus and proestrus predict estrous cycle-dependent differences in behavioral responses to stress. This hypothesis is supported by recent data showing altered development of learned helplessness in rats (enhanced in diestrus) (49) and social exploration in mice (reduced in estrus and diestrus) (49, 50). As such, estrous cycle-related changes in cortical and hippocampal activation may impart differential susceptibility to stress in female rats at the psychological as well as the physiological level.

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