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Research Report

Expression of c-*fos* and BDNF mRNA in subregions of the prefrontal cortex of male and female rats after acute uncontrollable stress

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Abstract

Women exhibit higher lifetime prevalences of stress-related disorders than men. These disorders have been associated with changes in prefrontal cortex structure and function. Here, we examine the effects of acute inescapable stress, an animal model of behavioral depression and post-traumatic stress disorder, on plasma corticosterone (CORT) and on c-*fos* mRNA and brain-derived neurotrophic factor (BDNF) mRNA in regions of the prefrontal and frontal cortex in male and cycling female rats. Inescapable stress consisted of 100 1 mA tailshocks, and no-stress controls remained in their home cages. Rats were sacrificed immediately (0 min) or 60 min after termination of the stressor. CORT levels were increased at both 0 and 60 min post-stress termination relative to controls, and the increase was greater in females at both time points. c-*fos* mRNA expression increased at 0 min in prefrontal cortical regions, but this increase was greater in males than estrus and proestrus females. At 60 min, c-*fos* mRNA levels were lower than at 0 min in males but not females. No correlations between CORT and c-*fos* mRNA levels in prefrontal regions were observed in females in the stress groups, but significant correlations were observed in males in several prefrontal regions. BDNF mRNA expression was greater in control females than control males. Inescapable stress increased BDNF mRNA expression at 0 but not 60 min in males, but there was no effect of inescapable stress on BDNF mRNA in females. These results reveal sex differences in inescapable stress-induced gene expression that may have implications for differences in vulnerability to stress-related disorders.
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Theme: Endocrine and autonomic regulation
Topic: Hypothalamic-pituitary-adrenal regulation

Keywords: c-*fos*; BDNF mRNA; Prefrontal cortex; Uncontrollable stress

1. Introduction

Women exhibit higher lifetime prevalences than men for stress-related disorders such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) [4–6]. The etiology of MDD and PTSD is often argued to involve the experience of stressful events [28,51], and preclinical studies have revealed sex differences in responses to acute [33] and chronic [2] stress, yet much remains unknown about the neurobiology of the acute response to stress in females relative to males. Because even a single very stressful event can precipitate the onset of these disorders in vulnerable individuals [51], it is of interest to examine sex differences in responding to an acute intense stressor.

The medial prefrontal cortex (mPFC) is known to be highly sensitive to stressors and modulates many behavioral and physiological responses to such events (see [44] for review). Stress can also cause lasting changes in the mPFC, such as decreased dendritic arborization [11]. These changes may affect the ability of the mPFC to modulate subsequent responses to stressors. In support of this idea, stress-related disorders have been associated with differences in both the

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structure and function of the mPFC [3,12,13,30]. Functional MRI studies show hypofunction of the mPFC in both MDD [12] and PTSD [3] patients, and cell loss in the mPFC has been observed in post-mortem studies of patients with these disorders [13,30]. It is possible that differential responding of the mPFC during a precipitating stressful event may contribute to the vulnerability of females to stress-related disorders. Ovarian hormones have been shown to impact mPFC function. For example, estrogen exacerbates stress-induced deficits on a PFC-dependent task in rats [31], while progesterone can enhance performance on a PFC-dependent task in humans [38].

In the present study, male and estrous cycling female rats were exposed to a single session of uncontrollable tailshock to examine stress-induced changes in the PFC. This stressor produces long-lasting changes in brain and behavior that closely resemble symptoms of depression [48] and PTSD [18,43]. Uncontrollable tailshock has been shown to produce sexually dimorphic behavioral deficits [39,40], and effects of the estrous cycle on vulnerability to the effects of this stressor have also been reported [22].

Females have long been known to exhibit higher stress-induced levels of the adrenal hormone corticosterone (CORT) [24], especially during proestrus [46], but the effect of uncontrollable tailshock on CORT in females is unknown. Thus, we measured plasma levels of CORT to assess hypothalamic–pituitary–adrenal (HPA) axis activation. To assess neuronal activation of the mPFC after uncontrollable stress, we measured mRNA expression of the immediate early gene c-*fos*. c-*fos* is a proto-oncogene that is induced in response to diverse stimuli [32] and has been widely used as a marker of neuronal activation resulting from stress exposure [7,10]. We also assessed mRNA expression of brain-derived neurotrophic factor (BDNF), which is known to be regulated by stress, although this has been best characterized in the hippocampus [37]. BDNF is involved in neuroprotection as well as plasticity and is of special interest because of its proposed role in depression [14,37]. Although it has been reported that acute stress does not affect BDNF mRNA expression in the mPFC of females [9], it has also been reported that acute stress increases BDNF mRNA expression in the mPFC of males [26]. To our knowledge, a direct comparison between sexes has not been previously reported, and the effects of an intense stressor on BDNF mRNA expression are unknown. The mPFC is a heterogeneous structure, comprising of distinct anatomical and functional subdivisions [41,42,45]. Because of this diversity, we examined the anterior cingulate (AC), infralimbic (IL), prelimbic (PL), and ventral orbital (VO) subdivisions of the PFC. We also examined primary motor cortex (M1) as a comparison region for general cortical activity. To measure the expression of c-*fos* and BDNF mRNA, we used in situ hybridization both immediately and 60 min after termination of the stressor as well as in unstressed (no stress, NS) controls.

2. Materials and methods

2.1. Subjects

Adult male (n = 18) and female (n = 43) Harlan Sprague–Dawley rats were used. They were purchased at 75 days old and thus were approximately 90 days old at the onset of vaginal lavage or handling. Rats were housed 2 per cage on a 12 h light–dark cycle (on 07:00 h:off 19:00 h) with food and water freely available. Males and females were housed in separate rooms. Rats were allowed to acclimate to the colony for 2 weeks. After this, daily vaginal lavage (females) or handling (males) occurred between 10:00 and 10:30 h for 2 weeks before experimentation began. Stage of estrous was determined by examining vaginal cytology with light microscopy. Diestrus I and II were characterized by the presence of leukocytes, proestrus by round nucleated cells, and estrus by cornified, irregularly shaped cells with degenerate nuclei. Rats in diestrus I and II were pooled into a single group. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee. All efforts were made to minimize discomfort as well as the number of animals used.

2.2. Inescapable stress

Inescapable stress occurred in Plexiglas restraint tubes which measured 17.5 cm in length and 7.0 cm in diameter. The front end of the tube was blocked by a Plexiglas plunger containing several air holes. Each rat's tail extended from the rear of the tube and was taped to a Plexiglas rod measuring 4.0 cm in length. Two copper strips were coated with a small amount of electrode paste, wrapped 4.0 cm apart around the midsection of the tail, and attached to two electrodes. Tailshocks (100 trials of 1 mA, 5 s each with a variable inter-trial interval averaging 60 s) were delivered using a Precision-Regulated Animal Shocker with Graphic State 3.0 software (Coulbourn Instruments, Allentown, PA). Each stress session lasted approximately 100 min. Males and females were run concurrently but in separate, adjacent shock rooms that were counterbalanced by sex across days. These rooms are identical in size, lighting, temperature, and background noise. Four separate cohorts of rats were tested, and each cohort was tested across 4 separate days such that females of each of the three estrous stages (diestrus, proestrus, and estrus) and males were represented each day. To avoid the effects of handling stress on the day of the experiment, female rats were pre-selected to be tested on a specific day based on the stage predicted from the previous 2 weeks of monitoring. Thus, the rats selected for that day's experiment were not subjected to lavage (female) or handling (male), before the experiment, but all remaining rats were. Lavage was performed post-mortem to verify stage of estrus.

2.3. Tissue collection

Rats were sacrificed either immediately following the stress session or 60 min later or at equivalent times for the no stress controls. Rats in the 60 min group were returned to their home cages before sacrifice. All animals were assigned to groups as cage-mate pairs such that, upon removal from or return to the home cage, no rat was left isolated. Rats were sacrificed by rapid decapitation. Trunk blood was collected in heparinized tubes and placed in wet ice. Brains were immediately removed, rapidly frozen in dry-ice-chilled isopentane, and placed in a −80 °C freezer for later analysis. Blood samples were centrifuged, and plasma was removed and stored at −20 °C until later analysis. Brain sections (10 μm) were taken using a −20 °C cryostat and thaw-mounted on poly-L-lysine-coated slides. Brain sections were returned to the −80 °C freezer after thaw-mounting.

2.4. In situ hybridization

Sections were fixed in a buffered 4% paraformaldehyde solution for 1 h at room temperature. Slides were washed in 2× saline sodium citrate (SSC) and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min to minimize nonspecific hybridization by reducing positive charge on tissue and polylysine-coated slides. Slides were then washed again in distilled water, dehydrated in a series of graded ethyl alcohol concentrations, and air-dried. ^{35}S-UTP/^{35}S-CTP labeled cRNA probes were generated for c-*fos* mRNA or BDNF mRNA from cDNA subclones in transcription vectors using standard in vitro transcription methodology. c-*fos* and BDNF cDNA were kindly provided by Dr. Tom Curran (St. Jude Children's Research Hospital, Memphis, TN, USA) and Dr. James Herman, (University of Cincinnati Medical Center, Cincinnati, OH, USA), respectively.

Riboprobes were diluted in hybridization buffer to a concentration of approximately 1 million counts per slide (65 μl/slide). Diluted probe was applied, and a coverslip was placed on each slide. Slides were placed in sealed plastic boxes lined with chromatography paper moistened with 50% formamide in ddH_2O and were incubated overnight at 55 °C. The next day, coverslips were floated off, and slides were rinsed several times in 2× SSC. The slides were then incubated in RNAseA (Sigma, St. Louis, MO, USA) at 37 °C for 1 h to remove unhybridized RNA fragments. Slides were then washed successively in decreasing concentrations of SSC, incubated in a stringent wash of 0.1× SSC at 70 °C for 1 h to denature weakly bound hybrids, and then dehydrated in graded concentrations of EtOH. Once the slides were dry, they were exposed to Kodak XAR X-ray film (Kodak, Rochester, NY, USA).

2.5. Image analysis

Semi-quantitative analyses were performed for c-*fos* and BDNF mRNA expression. Images from X-ray films were scanned and digitized using Image-J (NIH Image, http://www.rsb.info.nih.gov/nih-image/). See Fig. 1 for examples of autoradiographs of sections labeled for c-*fos* mRNA (Fig. 1A) and BDNF mRNA (Fig. 1B). A standard sized square region of interest (150 × 150 pixels) was centered over each subregion (AC, PL, IL, VO, and M1; see Fig. 1C). Within each region of interest, the average optical density was computed using Image-J. For each section, a value was also obtained from white matter; this was considered to be background and subtracted from each cortical value. Eight

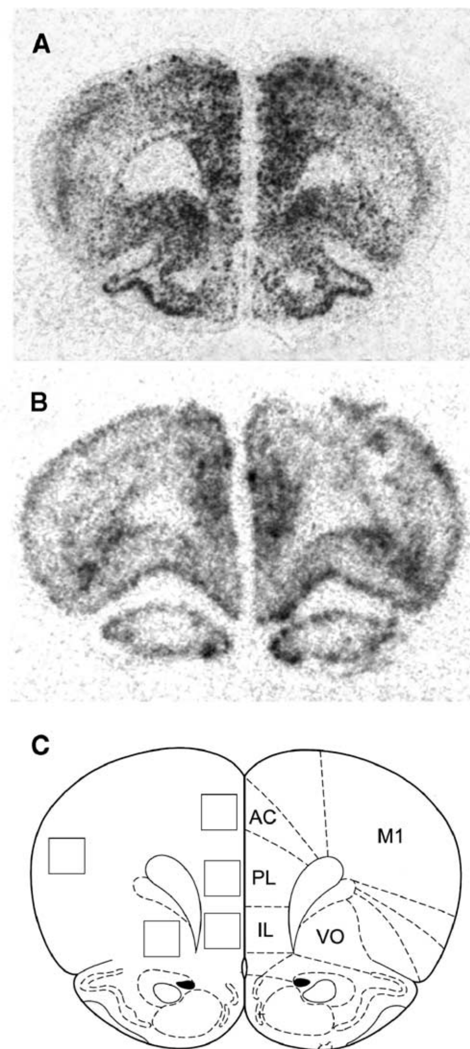

Fig. 1. Representative autoradiograms showing in situ hybridization signal for (A) c-*fos* mRNA and (B) BDNF mRNA. (C) Representation of the prefrontal and frontal cortical subregions sampled in the analysis, corresponding to 3.20 mm anterior to bregma and adapted from the atlas of Paxinos and Watson [29]. AC, anterior cingulate; PL, prelimbic; IL, infralimbic; VO, ventral orbital; and M1, primary motor cortex. The left side depicts a representation of the boxes drawn to sample each region.

measurements were taken per rat for each region (two measurements, right and left hemisphere, from each of 4 sections), and an average was computed for each region.

2.6. Plasma corticosterone

Total plasma CORT was measured by radioimmunoassay. 20 μl plasma samples were diluted in 0.01 M PBS (1 ml) and heat-inactivated at 75 °C for 1 h. Samples and standards (25–2000 pg/tube) were then incubated overnight with antiserum (rabbit antibody B3–163; Endocrine Sciences, Inc., Tarzana, CA) and [^{3}H] CORT (20,000 cpm/tube). Free CORT was separated from antibody-bound CORT with 500 μl dextran-coated activated charcoal. Antibody-bound CORT was then mixed with scintillation cocktail (3 ml) and counted with a liquid scintillation counter (Packard, 1600TR). The assay sensitivity was approximately 0.5 μg/ml for a 20 μl plasma sample.

2.7. Statistical analysis

c-*fos* and BDNF mRNA are presented as average optical density, and plasma CORT is presented as μg/100 ml. Stress groups consisted of NS controls, 0 min post-stress termination, and 60 min post-stress termination. To determine the effects of inescapable stress and sex independent of stage of estrus, data were first analyzed with females pooled across estrus stage, thus a 3 (NS, 0 min, 60 min) by 2 (male, female) between subjects ANOVA was performed on each measure. To determine effects of estrus stage, data were also analyzed with stage of estrus as a factor along with males, thus a 3 (NS, 0 min, 60 min) by 4 (diestrus, proestrus, estrus, male) between subjects ANOVA was performed on each measure. When a significant interaction was observed, post hoc tests (Fisher's LSD, with alpha set at .05) were performed. Correlation analyses were performed on c-*fos* mRNA, BDNF mRNA, and CORT, with only the stress groups (0 min and 60 min) included to avoid cluster effects caused by low levels in NS controls. Correlations analyses were also performed on these measures separately for males and females.

3. Results

3.1. CORT

Inescapable stress produced large increases in plasma CORT at both 0 min and 60 min post-stress termination; these increases were greater in females than in males (Fig. 2). ANOVA revealed a significant sex by stress interaction on plasma CORT [$F(2,56) = 3.52$, $P < .05$]. Post hoc Fisher's LSD tests (alpha set at .05) indicated that plasma CORT increased significantly in both male and female rats at 0 min post-stress relative to NS controls, and this increase was greater in females than in males. CORT levels remained elevated 60 min post-stress relative to NS; again, they were greater in females than in males. There was no significant difference in plasma CORT between NS males and females. There was no effect of estrus stage on plasma CORT across stress groups (see Table 1).

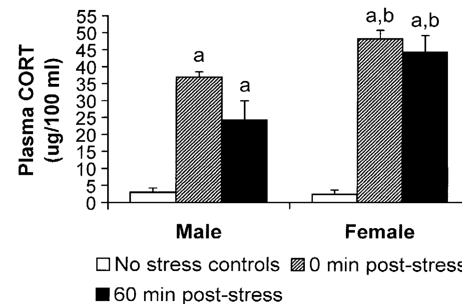

Fig. 2. Plasma CORT (μg/100 ml) in males and females after NS control treatment, 0 min post-stress termination, and 60 min post-stress termination. CORT was significantly increased at 0 min and at 60 min in males and females, and the increase was greater in females at both time points. Values represent means ± SEMs of 6 per group for males and 11–18 per group for females (estrous stages are pooled, see Table 1 for breakdown of estrous stage groups). a: different from own sex, no stress, $P < .05$. b: different from males at same time point, $P < .05$.

3.2. c-fos mRNA

Overall, inescapable stress produced large increases in c-*fos* mRNA expression in males at the 0 min post-stress termination relative to NS controls; this increase was smaller in females (Fig. 3). At 60 min post-stress termination, c-*fos* mRNA levels were decreased relative to 0 min post-stress in males, but not in females. ANOVA on each region revealed significant sex by stress interactions in AC [$F(2,56) = 4.19$, $P < .05$], IL [$F(2,56) = 4.96$, $P = .01$], PL [$F(2,56) = 9.80$, $P < .01$], and VO [$F(2,56) = 5.762$, $P < .01$], but not M1. Post hoc Fisher's LSD tests performed on AC, IL, PL, and VO indicated that in each region males had significantly greater c-*fos* mRNA expression at 0 min and 60 min relative to NS; moreover, males had significantly less c-*fos* mRNA at 60 min than at 0 min. Females had significantly greater c-*fos* mRNA expression at 0 and 60 min than NS; at 60 min, c-*fos* levels were not different than 0 min except in PL, where levels at 60 min were greater than 0 min. Males had significantly greater c-*fos* mRNA expression than females at 0 min but not at 60 min or in the NS condition.

When analyzed with estrous groups separated, there were significant group by stress interactions on c-*fos* mRNA expression in PL [$F(6,50) = 5.00$, $P < .01$], IL [$F(6,50) = 2.43$, $P < .05$], and VO [$F(6,50) = 2.45$, $P < .05$] and a trend in AC ($P = .07$), but not in M1. Post hoc Fisher's LSD tests indicated that these interactions reflected higher levels of c-*fos* mRNA in males compared to estrus females in AC,

Table 1
Levels of plasma CORT (μg/100 ml), c-*fos* mRNA (optical density), and BDNF mRNA (optical density) in female rats in each estrous stage

	NS controls			0 min post-stress			60 min post-stress		
	Diestrus (4)	Proestrus (4)	Estrus (3)	Diestrus (5)	Proestrus (7)	Estrus (6)	Diestrus (6)	Proestrus (4)	Estrus (4)
CORT	0.20 ± 0.16	7.02 ± 3.6	0.90 ± 0.4	50.60 ± 4.3	44.14 ± 2.3	57.75 ± 4.8	46.11 ± 6.8	39.50 ± 10.7	48.00 ± 16.7
c-*fos* mRNA									
AC	0.61 ± 0.11	1.16 ± 0.42	0.54 ± 0.24	3.65 ± 0.65	3.28 ± 0.49	2.70 ± 0.53	3.03 ± 0.48	3.79 ± 0.42	2.60 ± 0.59
PL	1.34 ± 0.12	1.87 ± 0.29	1.20 ± 0.25	2.73 ± 0.33	2.21 ± 0.23	2.39 ± 0.55	3.08 ± 0.23	3.99 ± 0.34	3.98 ± 0.96
IL	0.86 ± 0.12	1.00 ± 0.49	0.49 ± 0.15	3.50 ± 0.77	3.44 ± 0.31	2.48 ± 0.59	2.91 ± 0.38	3.78 ± 0.42	2.52 ± 0.43
VO	1.28 ± 0.07	1.84 ± 0.38	0.73 ± 0.19	4.23 ± 0.71	3.38 ± 0.47	3.08 ± 0.49	3.86 ± 0.31	3.89 ± 0.82	3.17 ± 0.70
M1	0.52 ± 0.11	0.86 ± 0.35	0.32 ± 0.15	1.97 ± 0.31	1.94 ± 0.24	1.57 ± 0.47	1.77 ± 0.23	2.05 ± 0.10	2.08 ± 0.47
BDNF mRNA									
AC	2.45 ± 0.44	2.59 ± 0.37	2.46 ± 0.47	2.31 ± 0.34	2.26 ± 0.38	2.38 ± 0.24	2.52 ± 0.32	3.05 ± 0.65	2.28 ± 0.68
PL	2.72 ± 0.29	2.88 ± 0.35	2.55 ± 0.43	2.72 ± 0.33	2.74 ± 0.43	2.63 ± 0.23	2.86 ± 0.22	3.69 ± 0.72	2.75 ± 0.56
IL	2.92 ± 0.28	2.74 ± 0.38	2.56 ± 0.09	2.68 ± 0.38	2.78 ± 0.56	2.77 ± 0.24	2.98 ± 0.19	3.56 ± 0.59	2.77 ± 0.14
VO	3.29 ± 0.27	2.73 ± 0.31	2.98 ± 0.16	2.87 ± 0.36	2.70 ± 0.21	2.93 ± 0.33	3.01 ± 0.27	3.85 ± 0.79	3.09 ± 0.58
M1	2.70 ± 0.36	2.58 ± 0.27	2.18 ± 0.28	2.23 ± 0.23	2.40 ± 0.44	2.30 ± 0.24	2.63 ± 0.26	2.91 ± 0.49	2.36 ± 0.59

Rats in the stress groups were sacrificed either 0 or 60 min after termination of the stress session. Values are means ± SEMs of (n) per group.

PL, IL, and VO at the 0 min time point (Fig. 4). In addition, males had greater c-*fos* mRNA expression than proestrus females in PL and VO at 0 min ($P < .05$). There were no significant differences in the NS or 60 min groups. Females in the estrous stage groups did not differ significantly from each other in any stress groups (see Table 1).

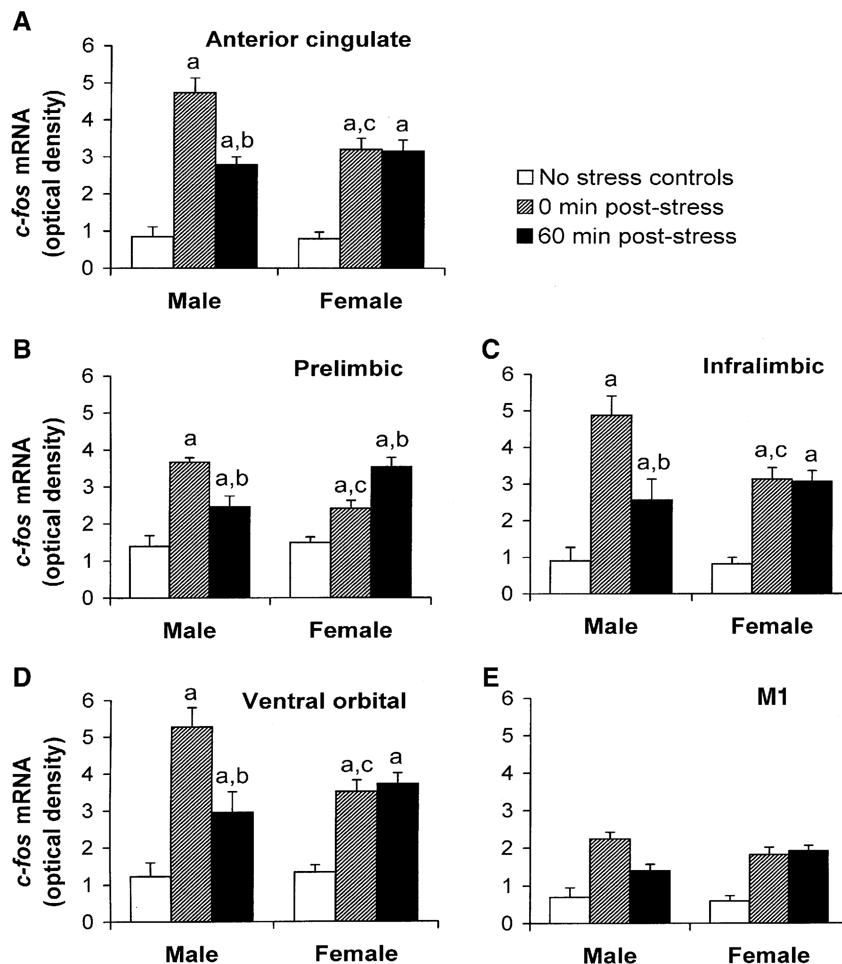

Fig. 3. c-*fos* mRNA expression (means ± SEMs) in male and female rats after no stress, 0 min post-stress termination, or 60 min post-stress termination, in subregions of the prefrontal (A–D) and frontal (E) cortex. In prefrontal subregions, c-*fos* mRNA levels were significantly increased at 0 min and 60 min. The increase was greater at 0 min in males than females. a: different from own sex, no stress, $P < .05$. b: different from own sex, 0 min, $P < .05$. c: different from male, 0 min, $P < .05$.

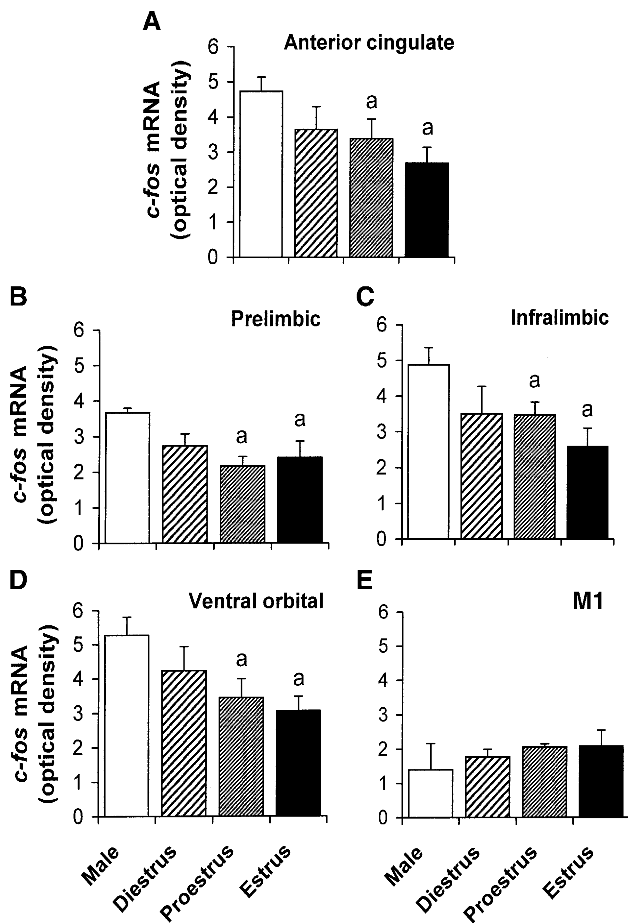

Fig. 4. c-*fos* mRNA expression (means ± SEMs of 5 to 8 per group) in subregions of the prefrontal (A–D) and frontal (E) cortex of male and female rats immediately after termination of inescapable stress (0 min post-stress). Female rats are grouped by stage of estrus as determined by vaginal lavage. Males had higher levels of c-*fos* mRNA than females in proestrus and estrus in prefrontal subregions. a: different from male, $P < .05$.

3.3. BDNF mRNA

Overall, inescapable stress produced increases in BDNF mRNA expression at the 0 min time point relative to NS in males, but not in females (Fig. 5). BDNF mRNA expression decreased to NS levels in 60 min post-stress in males. ANOVA on each region revealed significant sex by stress interactions in AC [$F(2,56) = 6.43$, $P < .01$], IL [$F(2,55) = 3.56$, $P < .05$], PL [$F(2,56) = 5.24$, $P < .01$], and VO [$F(2,56) = 4.90$, $P = .01$], and a strong trend for M1 ($P = .052$).

When analyzed with estrous groups separated, there were significant group by stress interactions on BDNF mRNA expression in AC [$F(6, 50) = 2.55$ $P < .05$], PL [$F(6,50) = 3.09$ $P = .01$], and VO [$F(6,50) = 2.51$, $P < .05$) and a trend in IL ($P = .07$), but not in M1. Post hoc Fisher's LSD tests indicated that these interactions reflected lower levels of BDNF mRNA in males than proestrus females at 60 min post-stress in PL, IL, and VO (all $P < .05$), but not in AC or M1. NS males were lower than all NS estrus stages in PL and IL ($P < .05$). There were no group differences at 0 min post-stress. Females in the estrous stage groups did not differ significantly from each other in any stress groups (see Table 1).

3.4. Correlations between plasma CORT, c-fos mRNA, and BDNF mRNA

There were no significant correlations between c-*fos* mRNA and plasma CORT in any regions with males and females pooled. When separate analyses were performed for males and females (Fig. 6), the correlation between CORT and c-*fos* was significant in males in AC and IL (both $P < .05$), and there was a strong trend in VO ($P = .07$). There were no significant correlations between c-*fos* and CORT in any region in females. Table 1 shows correlations of c-*fos* and CORT for each region separated by sex. *t* tests performed on the slopes [52] indicated that there were significant differences between males and females ($P < .01$) for AC, PL, IL, and VO, but not M1.

There were no significant correlations between c-*fos* and BDNF, or CORT and BDNF (Table 2).

4. Discussion

The present results reveal a pattern of sexually differentiated responses to intense inescapable stress in plasma CORT, as well as in expression of c-*fos* and BDNF mRNA in regions of the prefrontal cortex immediately or 60 min after termination of the stressor relative to unstressed controls. Primary motor cortex (M1) showed a similar pattern, but the responses were smaller in this region. Inescapable stress resulted in a greater increase in plasma CORT in females than in males. Conversely, levels of inescapable stress-induced c-*fos* mRNA expression were higher in males than in females immediately after the stressor but were reduced relative to this time point by 60 min post-stress in males, but not in females. Interestingly, c-*fos* mRNA expression in PFC regions correlated with plasma CORT in males, but not in females. Levels of BDNF mRNA in NS controls were higher in females than in males and were not affected by inescapable stress in females, while they were increased following the stressor in males. Reliable differences were not found between females in the different estrus stages on any measure, but post-stress sex differences in c-*fos* mRNA were primarily due to lower stress-induced levels in estrus and proestrus females.

Inescapable stress-induced plasma CORT levels were greater in female than in male rats immediately following termination of the stressor. This finding is consistent with several reports in which various stressors affect the CORT response differently in males and females. Females are known to exhibit a greater HPA response than males to restraint stress [23] and footshock [47], as well as to exogenous cytokines [19]. We did not observe statistically

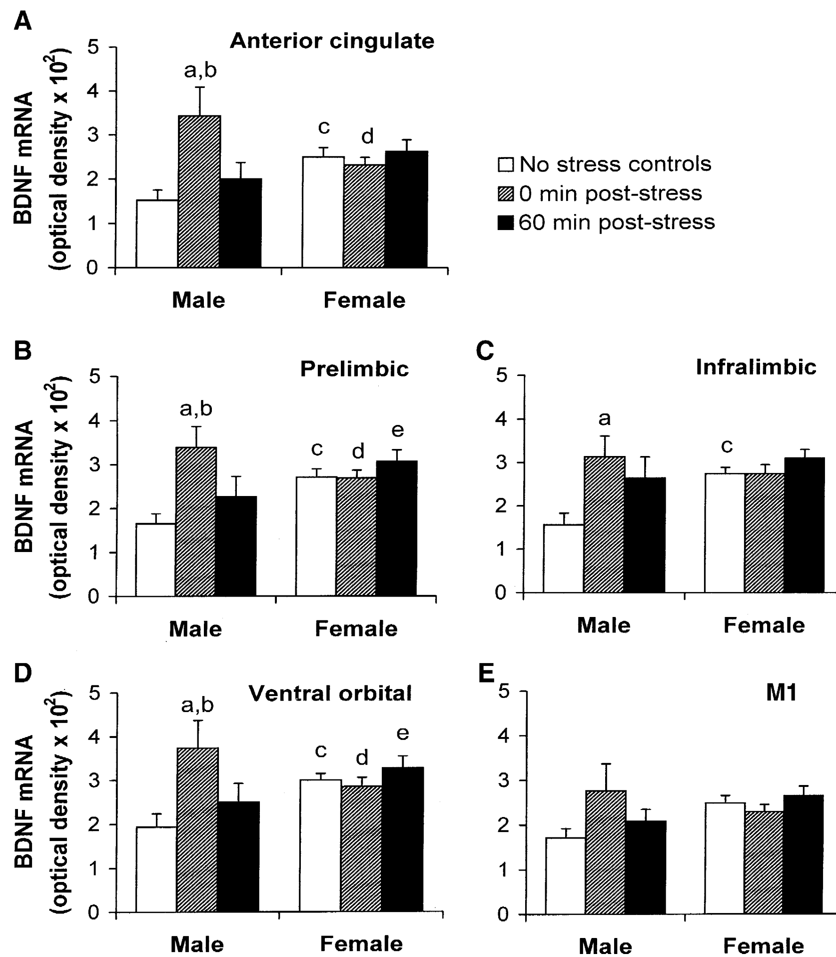

Fig. 5. BDNF mRNA expression in male and female rats after no stress, 0 min after inescapable stress, or 60 min after inescapable stress in subregions of the prefrontal (A–D) and frontal (E) cortex. BDNF mRNA levels were higher in no stress females than no stress males in prefrontal regions. Males, but not females, had increased levels of BDNF mRNA at 0 min relative to no stress in prefrontal subregions. In prelimbic (B) and ventral orbital (D) cortex, females had increased levels of BDNF mRNA at 60 min compared to males at the same time point. Values represent means ± SEMs of 6 per group for males and 11–18 per group for females (estrous stages are pooled, see Table 1 for breakdown of estrous stage groups). a: different from own sex, no stress, $P < .05$. b: different from own sex 60 min, $P < .05$. c: different from male, no stress, $P < .05$. d: different from male, 0 min, $P < .05$. e: different from male, 60 min, $P < .05$.

significant effects of estrous stage on CORT. Others have found that CORT levels were higher after brief (20 min) restraint in either early [46] or late proestrus [8]. It is possible that the very intense stressor used here resulted in ceiling levels of CORT that could not be further influenced by ovarian hormones.

The present results revealed similar patterns of c-*fos* induction within subregions of the mPFC, and these differed between males and females. Overall, in males, there was a large increase in c-*fos* immediately after the stressor; this effect was greatest in AC with a 558% increase and in IL with an increase of 540% relative to NS controls. By 60 min post-stress, these levels were somewhat reduced compared to the immediate time point, though not yet back to control levels. In contrast, c-*fos* mRNA induction in females immediately after the stressor was increased relative to control but significantly less than that of males at the same time point; the increase in AC was 400% in AC and 384% in IL relative to NS controls. This effect appears to be due to lower levels of stress-induced c-*fos* in proestrus and estrus females; diestrus females did not differ from males in any region. c-*fos* levels did not decrease at 60 min post-stress in females, in fact, in PL, c-*fos* levels were significantly greater at 60 min than at 0 min post-stress. These results are consistent with those found in the cingulate cortex in the study of Figueiredo et al. [16] In that study, animals were subjected to restraint for 30 min. In situ hybridization revealed that c-*fos* mRNA expression was significantly greater in males than in females in estrus and proestrus, but not diestrus. The present results extend that result by examining rostral PFC regions, which have been strongly linked to emotional regulation and stress-related disorders. In addition, these results indicate that, after an intense stressor, inescapable tailshock, c-*fos* mRNA expression is still elevated in both sexes 60 min after the stress session. This is in contrast to the finding of Figueiredo et al. [16] in which c-*fos* mRNA expression returned to baseline levels by 60 min after restraint stress.

Across all PFC subregions (but not M1), females in estrus showed less c-*fos* activation than males at the 0 min

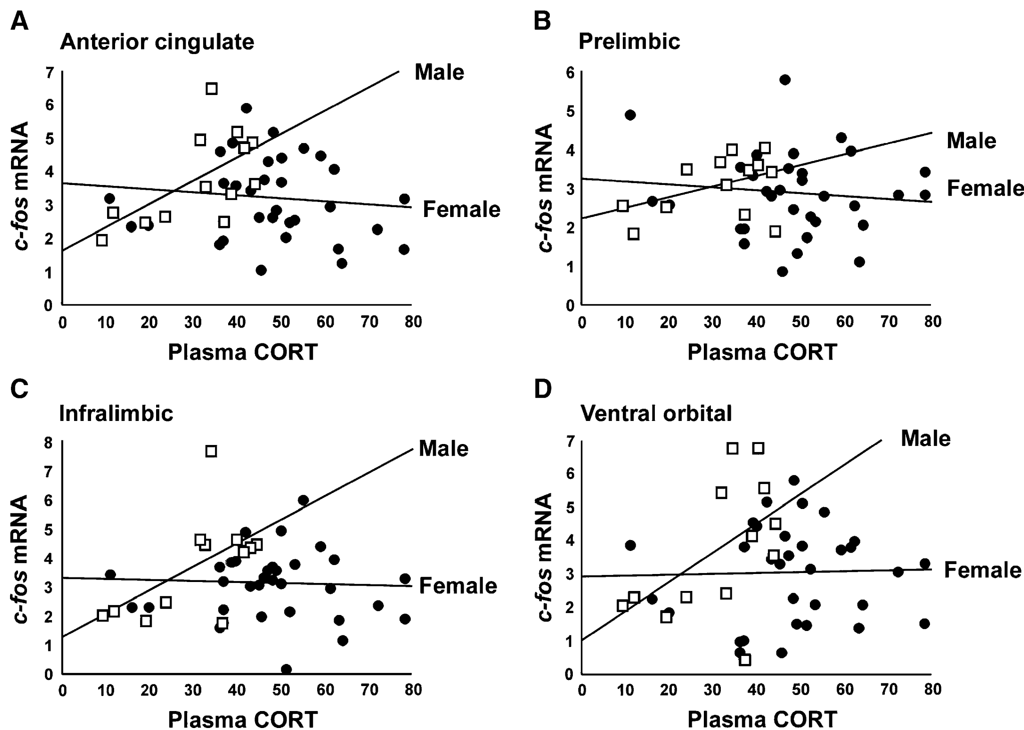

Fig. 6. Scatterplots of plasma CORT and c-*fos* mRNA in each PFC subregion (A–D), split by sex (females, ●; males, □). Significant correlations ($P < .05$) were observed in males in AC (A) and IL (C), and a strong trend ($P = .07$) was observed in VO (D). No correlations were observed in females. The slopes of males and females were significantly different in each region ($P < .01$).

post-stress time point. Jenkins et al. [22] reported that females in estrus were resistant to the development of learned helplessness in the shuttle-box escape test after a tailshock regimen similar to that used here. Females in estrus are also resistant to the effects of inescapable shock on immobility during the forced swim test [25]. It should be noted that, in studies of sex differences in stress-related behaviors, measures of performance on many of these tasks may be confounded by the greater activity levels in female rats relative to male rats and by changes in activity levels across the estrous cycle [17]. Nonetheless, these findings suggest the possibility that the PFC may be involved in the induction of learned helplessness, and further, that progesterone, which is high during estrus, may blunt this induction. However, an alternative explanation is that stress responsiveness may be more actively regulated by androgens than by ovarian hormones. In support of this idea, male rats exposed to a testosterone antagonist during development respond to stress exposure during adulthood similarly to females [35].

Table 2
Correlations between plasma CORT and c-*fos* mRNA expression in regions of the PFC and M1 in male and female rats after inescapable stress

Region	Male (n = 13)	Female (n = 32)
AC	.607*	−.105
PL	.412	−.109
IL	.562*	−.050
VO	.512	.036
M1	.273	−.129

0 min post-stress and 60 min post-stress groups have been pooled, and females in all the estrus stages have been pooled.
* $P < .05$.

Interestingly, the present results reveal a clear dissociation between males and females in the relationship between c-*fos* expression in the mPFC and plasma CORT. There was a significant correlation between CORT and c-*fos* in IL and AC in stressed males, as well as a strong trend in VO. No significant correlations were observed in any region of stressed females. Statistical analysis of the slopes revealed significant differences between males and females in all PFC regions. A similar dissociation has been seen between male and female rats on levels of plasma CORT and behavioral outcomes after stress exposure [50]. In that study, there was a close relationship between CORT and the percentage of conditioned responses in a trace conditioning task in males, but not in females. It is notable that this dissociation is also apparent in human populations. A study by Wolf et al. revealed a correlation between CORT and memory performance after the Trier Social Stress test in male, but not female, subjects [49].

Few studies have specifically examined stress effects on BDNF in the mPFC, although it has long been known that stress decreases BDNF mRNA expression in the hippocampus, and we have observed a decrease in BDNF mRNA in the hippocampus after inescapable stress (Maier et al., unpublished data). Cavus and Duman used 2 h restraint in female ovariectomized rats with and without β-estradiol benzoate treatment and observed no effect of stressor

exposure on BDNF mRNA expression, measured using in situ hybridization, in the mPFC in either of these groups [9]. There was no comparison with male rats. In contrast to this, the same 2 h restraint protocol produced a significant increase in BDNF mRNA, measured using RNase protection assay, 1 h after termination of the stressor (the only time point examined) by a different group in adult male rats [26]. The present results revealed that, after inescapable tailshock, BDNF mRNA levels were significantly increased only in males and only immediately after the stress session; levels were slightly but not significantly elevated 1 h later. Increases were observed immediately after stress in AC (226%), PL (205%), IL (194%), and VO (195%), relative to no stress controls. In contrast, BDNF mRNA levels in females were slightly decreased immediately after stress, with an average of 96% of control when averaged across regions; no increases were observed in any region, and there were no differences across estrus stage at this time point.

In the NS controls, males had significantly lower levels of BDNF expression than females of all estrous stages in the PL and IL and near significantly lower levels than all estrous stages in the AC and VO. There were no significant differences in BDNF mRNA in the mPFC across the stages of estrous in our NS treatment groups, in contrast to Cavus and Duman [9]. That group found that levels of BDNF mRNA are decreased in proestrus relative to estrus in the mPFC of unstressed females. This disparity could be due to differences in the time of day of the experiments. The rat estrous cycle is short (4–5 days), and thus small differences in the time of day that estrous is monitored can have a large impact on hormonal status. Other groups have also observed that BDNF mRNA in frontal cortical regions is not modulated by hormonal status. Gibbs [21] found no effect of ovarian hormones on BDNF mRNA expression in the frontal cortex, consistent with the present results, although effects were seen in the hippocampus. Similarly, Singh et al. [36] reported that, although ovariectomy reduced BDNF mRNA expression in the frontal cortex and hippocampus, estradiol replacement reversed this effect only in the hippocampus. Effects of estrous stage have also been reported in other forebrain regions [20].

Sexual dimorphism in adults can be the result either of organizational effects of gonadal hormones during development or of activational effects of circulating gonadal hormones. Both mechanisms can be responsible for sex differences in stress responsiveness. For example, estrogen has been shown to have an activational effect on learned helplessness behaviors [34]. Organizational effects of testosterone in utero on the effects of stress exposure on learning occur in adults [35]. Furthermore, estradiol can have both organizational and activational effects on stress-induced learning deficits [2]. In the present study, stress-induced c-*fos* mRNA expression was most different from males during stages when levels of estrogen and progesterone are high, yet estrous stage groups did not differ from each other, suggesting both organizational and activational effects of hormones on stress induction of this gene. BDNF levels in the PFC of females appear to be largely (though not completely) independent of estrus stage, and constitutive levels are different in males and females, suggesting an organizational effect of sex hormones.

In conclusion, inescapable stress produced greater effects on plasma CORT in females than males, and CORT is related to c-*fos* mRNA expression in males but not females. Conversely, inescapable stress had greater effects on PFC c-*fos* and BDNF mRNA expression in males than females. These genes have numerous downstream effects that can induce long-lasting changes in neuronal morphology, survival, and synaptic transmission [1,14,27], processes that have been implicated in mood disorders [15]. The use of females in future studies of stress reactivity is critical for developing sex-specific interventions for stress-related disorders.

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