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Chronic stress produces enduring sex- and region-specific alterations in novel stress-induced c-Fos expression

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ABSTRACT

Prolonged or repeated exposure to stress increases risk for a variety of psychological disorders, many of which are marked by dysfunction of corticolimbic brain regions. Notably, women are more likely than men to be diagnosed with these disorders, especially when onset of symptoms follows stressful life events. Using rodent models, investigators have recently begun to elucidate sex-specific changes in the brain and behavior that occur immediately following chronic stress. However, little is known regarding the lasting sequelae of chronic stress, as well as how potential changes may impact responsivity to future stressors. We recently demonstrated that male and female rats show different patterns of dendritic reorganization in medial prefrontal cortex in the days following chronic stress. Here, we examined the immediate and lasting effects of chronic restraint stress (CRS; 3 h/day, 10 days) on neuronal activation, across several corticolimbic brain regions, induced by novel acute stress exposure. Chronically stressed male and female rats were exposed to acute elevated platform stress (EPS) either 1 (CRS-EPS) or 7 (CRS-Rest-EPS) days after CRS. Compared to rats exposed to EPS only, significant reductions in acute stress-induced c-Fos expression were observed in the medial prefrontal cortex, hippocampus, and paraventricular nucleus of the hypothalamus (PVN) in CRS-EPS male rats, some of which persisted to 7 days post-stress. In contrast, we found little modulation of novel stress-induced c-Fos expression in CRS-EPS female rats. However, CRS-Rest-EPS female rats exhibited a significant enhancement of acute stress-induced neuronal activity in the PVN. Together, these data show that prior chronic stress produces sex- and region-specific alterations in novel stress-induced neuronal activation, which are dependent on the presence or absence of a rest period following chronic stress. These findings suggest that the post-stress rest period may give rise to sex-specific neuroadaptations to stress, which may underlie sex differences in stress susceptibility versus resilience.

1. Introduction

Prolonged exposure to stress increases risk for psychological disorders, including major depressive disorder (Kessler, 1997) and generalized anxiety disorder (Kessler et al., 2003). These disorders are associated with alterations in the structure and function of several corticolimbic brain regions, including prefrontal cortex (PFC), orbitofrontal cortex (OFC), hippocampus, and the amygdala (Michael et al., 2003a, b; Price and Drevets, 2010; Shin and Liberzon, 2010). Together, these regions mediate a variety of processes, including cognition, emotion regulation, and physiological responses to stress, many of which are disrupted in individuals with stress-related disorders (Yamasue et al., 2003; Vasic et al., 2008; Rock et al., 2014; Scott et al., 2015).

Women are twice as likely as men to be diagnosed with many stress-related psychological disorders (Cover et al., 2014), although the neurobiological basis for this female-biased vulnerability remains unclear. Using rodent models, investigators have begun to elucidate sex differences in chronic stress effects on corticolimbic circuitry. For example, immediately following chronic stress male rats have apical dendritic retraction in pyramidal neurons in medial prefrontal cortex (mPFC; Cook and Wellman, 2004; Radley et al., 2005; Garrett and Wellman, 2009; Moench and Wellman, 2017) and dorsal hippocampus (DHC; Galea et al., 1997). Concurrently, chronically stressed male rats have deficits in behavioral tasks mediated by these regions (Conrad et al., 1996; Bowman et al., 2003; Nikiforuk and Popik, 2011, 2014; Mika et al., 2012; Wei et al., 2014). In contrast, following chronic stress female rats show little dendritic remodeling (Galea et al., 1997; Moench and Wellman, 2017) or opposite patterns of remodeling (Garrett and Wellman, 2009) in these regions compared to male rats, and often do

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not have stress-induced behavioral deficits (Bowman et al., 2003; Kitraki et al., 2004; Wei et al., 2014; Snyder et al., 2015).

While many studies have examined the immediate effects of chronic stress on the brain and behavior, there is a dearth of information regarding how chronic stress may lead to lasting changes in the function of corticolimbic brain regions. Chronic stress-induced dendritic atrophy in DHC of male rats is ameliorated following a 10-day rest period (Conrad et al., 1999), and behavioral deficits are reversed by 30 days post-stress (Sousa et al., 2000). We have shown sex-specific dendritic remodeling in rat mPFC in the days following chronic stress. Following a 7-day rest period, chronically stressed male rats have dendritic outgrowth compared to unstressed male rats, which is absent after a 10-day rest period. In contrast, chronically stressed female rats showed little dendritic reorganization during this same post-stress time frame (Moench and Wellman, 2017). These data suggest that exposure to another stressor during this post-chronic stress rest period may differentially affect stress-sensitive brain regions in male and female rats. Indeed, in chronically stressed male rats, exposure to a novel acute stressor produces persistent changes in c-*fos* mRNA expression in several key regions in stress-regulation (Ostrander et al., 2006, 2009). However, it is unclear how these changes in activation may differ between males and females during the post-chronic stress period.

Here, we exposed chronically stressed male and female rats to a novel acute stressor either one or seven days following the cessation of chronic stress. Using c-Fos expression, we assessed changes in acute stress-induced activation of several corticolimbic regions that are important in cognitive and emotional processing, as well as regulation of stress physiology: the prelimbic (PL) and infralimbic (IL) subregions of PFC; OFC; basolateral amygdala (BLA); DHC subfields CA1, CA3 and dentate gyrus (DG); as well as the paraventricular nucleus of the hypothalamus (PVN). Our findings show region-, rest-, and sex-specific patterns of novel stress-induced cellular activation. These data indicate that chronic stress differentially alters acute stress-induced activation of corticolimbic circuitry in males and females in a manner that is dependent on the presence or absence of a rest period.

2. Methods

2.1. Animals and stress manipulations

Male and female Sprague Dawley rats (approximately 10 weeks of age at start; Envigo, Indianapolis, IN) were group-housed (3/cage) in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23–25 °C, free access to food and water, and a 12:12 light/dark cycle (lights on at 0800 h). All procedures were conducted between 8:00 a.m. and 6:00 p.m., were in accordance with NIH Guidelines, and were approved by the Bloomington Animal Care and Use Committee.

Rats were assigned to one of four stress conditions (see Fig. 1 for experimental design and timeline): No Stress (n = 6/sex); Elevated platform stress only (EPS Only; n = 9/sex); chronic stress followed by EPS 1 day later (CRS-EPS; n = 12 male; 9 female); or chronic stress followed by EPS 7 days later (CRS-Rest-EPS; n = 9 male; 8 female). Chronic stress consisted of daily restraint (3 h/day, 10 d). Rats were weighed daily throughout the stress procedure. Immediately after weighing, unstressed rats were returned to their home cages and left undisturbed in a separate room. Chronically stressed rats were placed in semi-cylindrical restrainers (male, 16 cm length × 6.5 cm width × 5 cm height; female, 15 cm length × 6 cm width × 4.5 cm height, modified so the tail piece locks into place; Braintree Scientific, Braintree, MA) in their home cages, with the time of restraint unpredictably varied over the light cycle. This manipulation produces significant increases in plasma corticosterone levels (Cook and Wellman, 2004). EPS consisted of placing each rat individually on a small platform (12 cm × 12 cm) elevated 90 cm off the floor for 30 min in a brightly lit room as previously described (Xu et al., 1997; Maroun and Richter-Levin, 2003; Maroun et al., 2013).

On the day of perfusion, vaginal lavages were performed and exfoliate cytology was examined immediately under light microscopy. Estrous phase was determined based on the morphology of cells present (Garrett and Wellman, 2009). Due to the small number of rats in proestrus and estrus (n = 1, No Stress; 2, EPS Only; 3, CRS-EPS; 3, CRS-Rest-EPS), we did not analyze our data relative to estrous phase.

Chronic stressors such as immobilization and restraint attenuate normal weight gain (Marti et al., 1994; Cook and Wellman, 2004). To verify our chronic stress manipulation, weight change (start weight – weight on the final day of restraint) was compared between stress conditions using a two-way ANOVA (sex × stress), followed by Fisher's protected LSD *post hoc* comparisons.

2.2. Immunohistochemistry

Approximately 60 min after the cessation of EPS, rats were deeply anesthetized with urethane and transcardially perfused with cold 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were removed and hemisected. One hemisphere per animal, counterbalanced across animals, was post-fixed in 4% PFA for 24 h. Hemispheres were cryoprotected in 30% sucrose and sectioned frozen at 44 µm on a sliding microtome and collected into 0.1 M PBS. For each hemisphere, 5 to 6 sections (approximately 264 µm apart) were collected through the rostral-caudal extent of mPFC, 3 to 4 sections (approximately 176 µm apart) were collected through the PVN, and 3 to 4 sections (approximately 396 µm apart) were collected through DHC and BLA.

Free-floating sections were stained for c-Fos using a modification of the protocol described by Lenz and Sengelaub (2010). Sections were incubated in blocking solution (0.1% Triton X-100, 5% NGS, and 5% bovine serum albumin in PBS) and 1% hydrogen peroxide for 30 min to block nonspecific binding and endogenous peroxidases. Sections were then incubated for 48 h at 4 °C in blocking solution and a rabbit polyclonal antibody for c-Fos (1:2000, Santa Cruz Biotech, sc-253). After rinsing, sections were incubated for 1 h in blocking solution and biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA). Sections were rinsed and incubated for 1 h in PBS with 0.1% Triton X-100 and ABC complex (Vector Laboratories). Staining was visualized using a nickel-intensified DAB reaction. After rinsing, sections were mounted, dehydrated, cleared, and coverslipped. Control sections incubated without the primary antibody demonstrated no staining.

To investigate the potential neuroendocrine ramifications of altered c-Fos expression in the PVN, we used a procedure similar to that of Smith et al. (2016) to perform double-label immunohistochemistry for c-Fos and corticotropin-releasing hormone (CRH) in a separate set of EPS Only, CRS-EPS, and CRS-Rest-EPS rats (n = 3/group/sex). Brains were prepared as described above. For immunohistochemistry, free-floating sections were incubated in blocking solution (0.1% Tween 20 and 5% NGS in 0.1 M PBS), followed by incubation 48 h at 4 °C in blocking solution with a polyclonal anti-CRH antibody raised in guinea pig (Peninsula Labs, 1:10,000). After rinsing, sections were incubated 1 h in blocking solution and biotinylated goat anti-guinea pig (1:200, Vector Laboratories). Sections were rinsed and incubated 1 h in PBS with 0.1% Triton X-100 and ABC complex (Vector Laboratories). CRH was visualized using a DAB reaction. Staining for c-Fos then proceeded as described above. Control sections incubated without primary antibodies demonstrated no staining.

2.3. Stereology

We examined the density of c-Fos + cells in PL, IL, and OFC; the pyramidal cell layer of CA1 and CA3 and the granule cell layer of DG; BLA; and PVN. In addition, we examined the density of c-Fos+, CRH+, and c-Fos+/CRH + cells in the PVN. Sampling occurred throughout

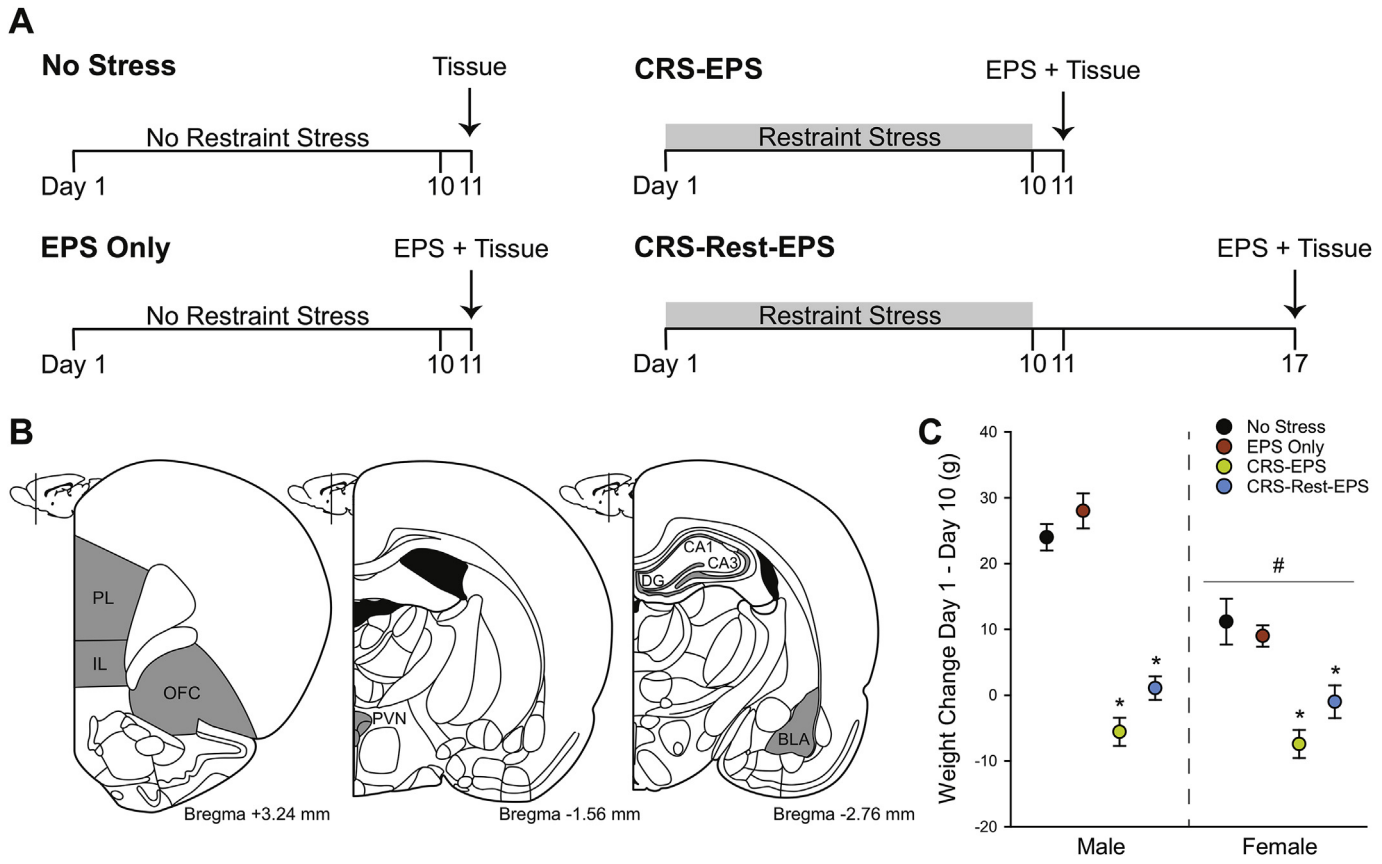

Fig. 1. (A) Experimental design and timeline of experimental procedures. Male and female rats were either left undisturbed or were exposed to CRS for 10 days. Rats either remained unstressed (No Stress) or were exposed to EPS (EPS Only). CRS rats were exposed to EPS either on the day following CRS (CRS-EPS) or 7 days following the cessation of CRS (CRS-Rest-EPS). (B) Schematic diagrams identifying regions examined. c-Fos expression was analyzed in PL, IL, OFC, PVN, CA1, CA3, DG, and BLA. Adapted from Paxinos and Watson (2007). (C) Chronic stress attenuates weight gain in both male and female rats. $^{*}p < 0.05$ compared to No Stress rats of same sex; $^{\#}p < 0.05$ compared to male rats.

the anterior-posterior extent of each region and was completed at a final magnification of 1800 × using the optical fractionator method and Stereo Investigator (MBF Biosciences Inc., Williston, VT). The counting frame for all regions was 50 μm × 50 μm. Sampling grid size for each region was as follows: PL, 150 μm × 300 μm; IL, 200 μm × 200 μm; OFC, 300 μm × 300 μm; CA1, 150 μm × 150 μm; CA3, 150 μm × 150 μm; DG, 150 μm × 150 μm; BLA, 150 μm × 250 μm; PVN, 100 μm × 100 μm. Guard zones were set with a centered-probe thickness of 15 μm for each region. Counts were performed with the experimenter blind to sex and stress condition.

For all regions, the estimated density of c-Fos + cells was calculated and analyzed using two-way ANOVAs (sex × stress). Significant effects were followed by Fisher's protected LSD *post hoc* comparisons. To assess the co-localization of c-Fos in CRH + cells in the PVN, we calculated the percentages of cells labeled only for c-Fos (c-Fos + cells), cells labeled only for CRH (CRH + cells) and cells double-labeled for c-Fos and CRH (c-Fos + /CRH + cells). These data were analyzed using three-way repeated measures ANOVAs (immunolabeling × stress × sex, immunolabeling as the repeated measure), followed by Fisher's protected LSD *post hoc* comparisons. Additionally, Spearman correlations were used to examine potential relationships of basal and acute stress-induced c-Fos expression between all brain regions examined.

3. Results

3.1. Chronic restraint stress attenuates weight gain in males and females

Weight comparisons included rats used for both single- and double-label immunohistochemistry. Male rats gained significantly more weight than female rats (Fig. 1C; main effect of sex, $F_{(1,\ 78)} = 23.97$, $p < 0.001$) and stress altered weight change in both males and females (main effect of stress, $F_{(3,\ 78)} = 53.19$, $p < 0.001$), although this effect was more pronounced in males (sex × stress interaction, $F_{(3,\ 78)} = 6.77$, $p < 0.001$). In males, weight gain did not differ between No Stress and EPS Only rats. In contrast, male rats exposed to chronic stress gained significantly less weight than No Stress male rats (CRS-EPS, $p < 0.001$; CRS-Rest-EPS, $p < 0.001$). Similarly, in female rats, weight change did not differ between No Stress and EPS Only rats, whereas in chronically stressed females, weight change was significantly reduced relative to No Stress female rats (CRS-EPS, $p < 0.001$; CRS-Rest-EPS, $p = 0.004$).

3.2. Chronic stress produces region- and sex-specific alterations in acute stress-induced c-Fos expression

3.2.1. Prefrontal cortex

For mPFC, animals were excluded from analyses when hemisection resulted in tissue damage to the region of interest. Thus, final n's for PL were as follows: male No Stress, n = 5; EPS Only, n = 7; CRS-EPS, n = 11; CRS-Rest-EPS, n = 9; and female No Stress, n = 5; EPS Only, n = 8; CRS-EPS, n = 8, CRS-Rest-EPS, n = 6. Final n's for IL were as follows: male No Stress, n = 5; EPS Only, n = 7; CRS-EPS, n = 11; CRS-Rest-EPS, n = 7; and female No Stress, n = 5; EPS Only, n = 8; CRS-EPS, n = 8, CRS-Rest-EPS, n = 6.

In PL (Fig. 2B), females had greater overall c-Fos expression than males (main effect of sex, $F_{(1,\ 51)} = 6.26$, $p = 0.02$). Stress significantly altered the density of c-Fos + cells (main effect of stress, $F_{(3,\ 51)} = 21.61$, $p < 0.001$), although the interaction between sex and stress was not significant ($F_{(3,\ 51)} = 0.83$, n.s.). Follow up comparisons

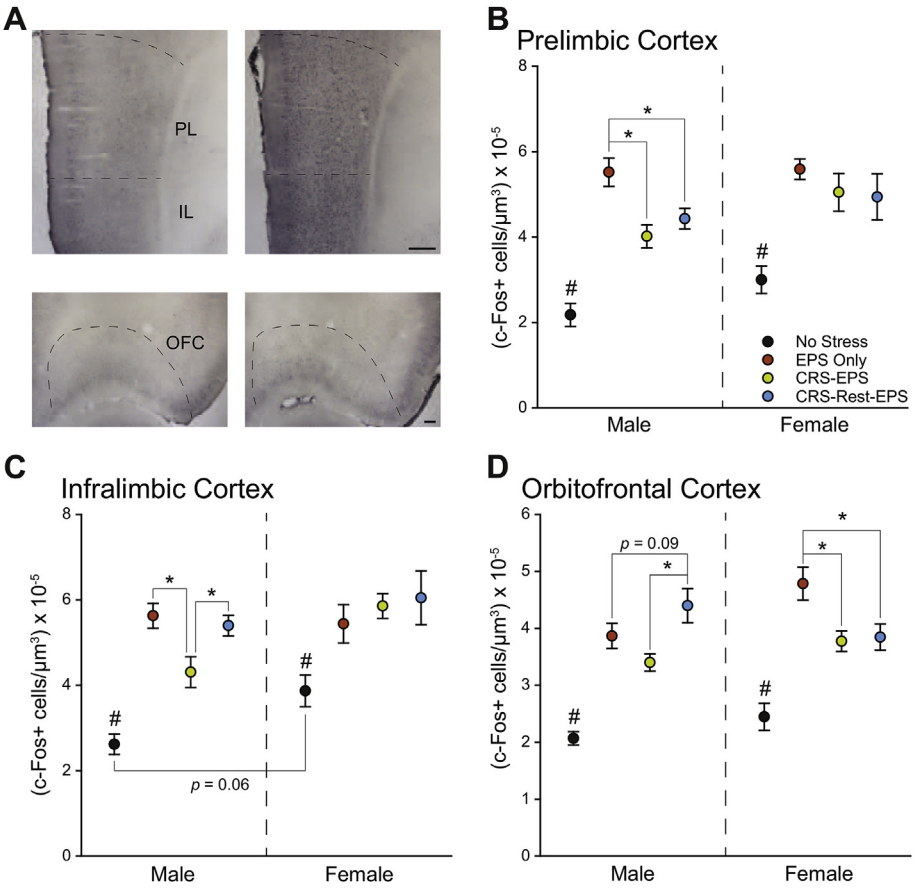

Fig. 2. (A) Representative photomicrographs of c-Fos immunohistochemistry in PL and IL (*Top*) and OFC (*Bottom*) of No Stress (*Left*) and EPS Only (*Right*) male rats. Scale bar = 250 μm. (B) In both male and female rats, EPS increases c-Fos expression in PL. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. (C) Female rats tended to have greater basal c-Fos expression in IL compared to male rats. In both male and female rats, EPS increases c-Fos expression in IL. This increase is reduced in CRS-EPS males, but is unaltered in CRS-Rest-EPS males. In female rats, EPS-induced c-Fos expression is unaltered in both CRS groups. (D) In both male and female rats, EPS increases c-Fos expression in OFC. In males, this increase is slightly enhanced in CRS males given a rest period. In female rats, EPS-induced c-Fos expression is reduced in both CRS groups. $^{*}p < 0.05$; $^{\#}p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

revealed that the density of c-Fos + cells did not differ between No Stress male and female rats. In males, EPS significantly increased c-Fos expression compared to No Stress males (p's < 0.001), although this increase was attenuated in male rats previously exposed to CRS (EPS Only v CRS-EPS, $p = 0.001$; EPS Only v CRS-Rest-EPS, $p = 0.02$). This effect was not dependent on a rest period, as the CRS groups did not differ from each other. EPS also resulted in increased c-Fos expression in female rats (p's ≤ 0.001), but this was not altered by previous chronic stress exposure regardless of rest period.

In IL (Fig. 2C), the density of c-Fos + cells was significantly higher in females than males (main effect of sex, $F_{(1, 49)} = 8.25$, $p = 0.01$), and was altered by stress (main effect of stress, $F_{(3, 49)} = 13.13$, $p < 0.001$), although the interaction of sex and stress was not significant ($F_{(3, 49)} = 2.13$, n.s.). Follow up comparisons indicated a non-significant trend towards increased c-Fos expression in No Stress females compared to males ($p = 0.06$). In males, EPS increased c-Fos expression in all stress groups (p's < 0.001). This effect was blunted in CRS males not given a rest period (EPS Only v CRS-EPS, $p = 0.01$), but not in those with a rest period. Further, CRS-Rest-EPS had greater c-Fos expression that CRS-EPS males ($p = 0.03$). EPS also increased the density of c-Fos + cells in all female stress conditions (p's ≤ 0.01), although this was not altered by previous chronic stress exposure regardless of rest period.

In OFC (Fig. 2D), females tended to have greater overall c-Fos expression than males (main effect of stress, $F_{(1, 60)} = 2.88$, $p = 0.09$). Further, stress altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 26.72$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 60)} = 3.68$, $p = 0.02$). Follow up comparisons indicated that c-Fos expression did not differ between No Stress males and females. In males, EPS significantly increased c-Fos expression across all stress conditions (p's < 0.001). Although CRS-EPS males did not differ from EPS Only males, CRS-Rest-EPS males tended to have enhanced c-Fos expression compared to EPS Only males ($p = 0.09$). Further, CRS-Rest-EPS males had a significantly greater density of c-Fos + cells compared to CRS-EPS ($p = 0.001$). In females, EPS significantly increased c-Fos expression in all stress conditions (p's < 0.001). This effect was blunted in all chronically stressed females regardless of rest period (EPS Only v CRS-EPS, $p = 0.002$; EPS Only v CRS-Rest-EPS, $p = 0.005$).

3.2.2. Hippocampus

In CA1 (Fig. 3B), female rats had greater c-Fos expression than males (main effect of sex, $F_{(1, 60)} = 5.70$, $p = 0.02$). Stress altered the density of c-Fos + cells (main effect of stress, $F_{(3, 60)} = 31.88$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3, 60)} = 3.35$, $p = 0.03$). In males, EPS increased c-Fos expression across all stress conditions (p's ≤ 0.004). Prior chronic stress, regardless of rest period, blunted this increase (EPS Only v CRS-EPS, $p = 0.02$; EPS Only v CRS-Rest-EPS, $p = 0.001$). EPS also increased c-Fos expression across all stress conditions in females (p's < 0.001), but this effect was not altered by chronic stress at either timepoint.

In CA3 (Fig. 3C), the density of c-Fos + cells did not differ between males and females (main effect of sex, $F_{(1, 60)} = 0.15$, n.s.). Stress altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 12.72$, $p < 0.001$), and this effect did not differ between males and females (sex × stress interaction, $F_{(3, 60)} = 0.21$, n.s.). In both males and females, EPS increase c-Fos expression across all stress conditions (p's < 0.01), although there were no differences between stress conditions.

In DG (Fig. 3D), the density of c-Fos + cells did not differ between males and females (main effect of sex, $F_{(1, 60)} = 0.14$, n.s.). Stress significantly altered c-Fos expression (main effect of stress, $F_{(3, 60)} = 27.26$, $p < 0.001$), and this effect differed between males and

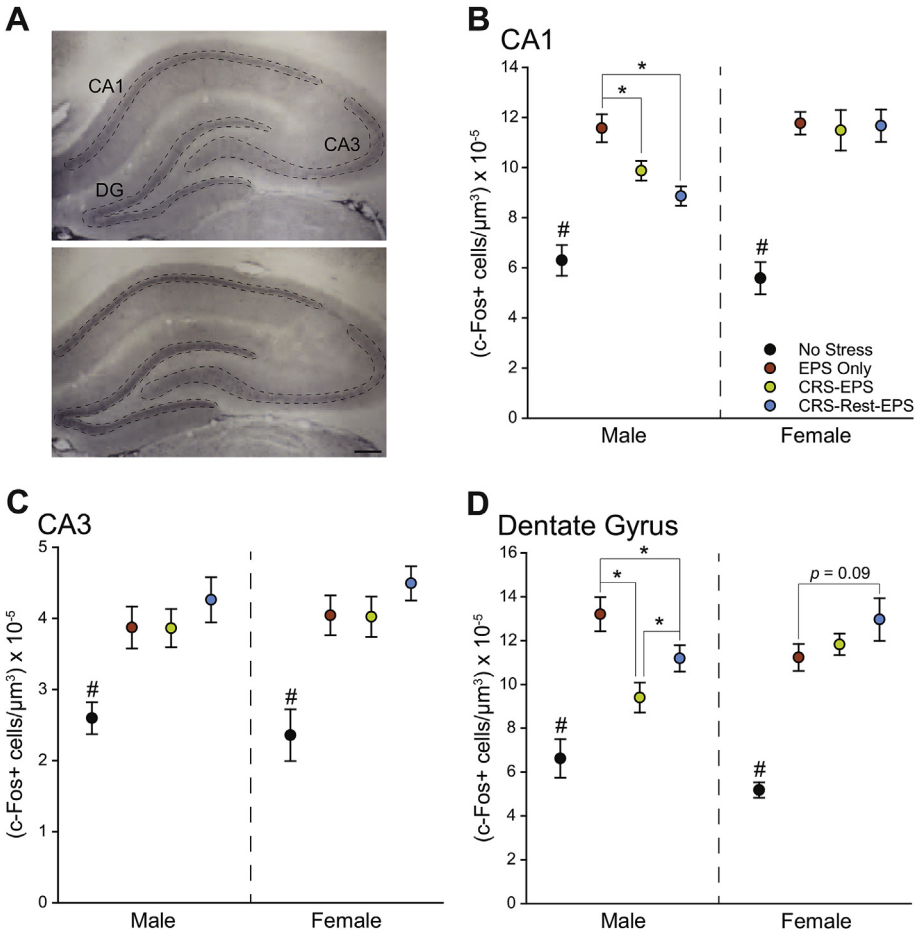

Fig. 3. (A) Representative photomicrographs of c-Fos immunohistochemistry in CA1, CA3, and DG of No Stress (*Top*) and EPS Only (*Bottom*) male rats. Scale bar = 250 μm. (B) In both males and females, EPS increases c-Fos expression in CA1. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. (C) In both male and female rats, EPS increases c-Fos expression in CA3. This increase in unaltered by CRS in both males and females. (D) In both males and females, EPS increases c-Fos expression in DG. This increase is reduced in both CRS male groups, but is unaltered in CRS female groups. $^{*}p < 0.05$. $^{\#}p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

females (sex × stress interaction, $F_{(3, 60)} = 5.04$, $p = 0.003$). Follow up comparisons indicated that in males, EPS increased c-Fos expression across all stress conditions (p's < 0.01). This increase was blunted in all males exposed to CRS (EPS Only v CRS-EPS, $p < 0.001$; EPS Only v CRS-Rest-EPS, $p = 0.04$), although this was more pronounced in CRS-EPS males (CRS-EPS v CRS-Rest-EPS, $p = 0.05$). In females, EPS also resulted in increased c-Fos expression across all stress conditions (p's < 0.001). This was largely unchanged by chronic stress, although a non-significant trend towards an increase in EPS-induced c-Fos expression was observed (EPS Only v CRS-Rest-EPS, $p = 0.09$). CRS-EPS and CRS-Rest-EPS females did not differ from each other.

3.2.3. Basolateral amygdala

In BLA (Fig. 4), female rats had greater c-Fos expression compared to males (main effect of sex, $F_{(1, 60)} = 5.49$, $p = 0.02$). Stress altered the density of c-Fos + cells (main effect of stress, $F_{(3, 60)} = 31.88$, $p < 0.001$), although this effect did not differ between males and females (sex × stress interaction, $F_{(3, 60)} = 1.32$, n.s.). In males, EPS increased c-Fos expression across all stress conditions (p's ≤ 0.004). Prior chronic stress without a rest period tended to blunt this response (EPS Only v CRS-EPS, $p = 0.08$). Chronically stressed males given a rest period did not differ from EPS Only males, although they tended to have greater c-Fos expression then those without a rest period ($p = 0.07$). EPS also increased c-Fos expression across all stress

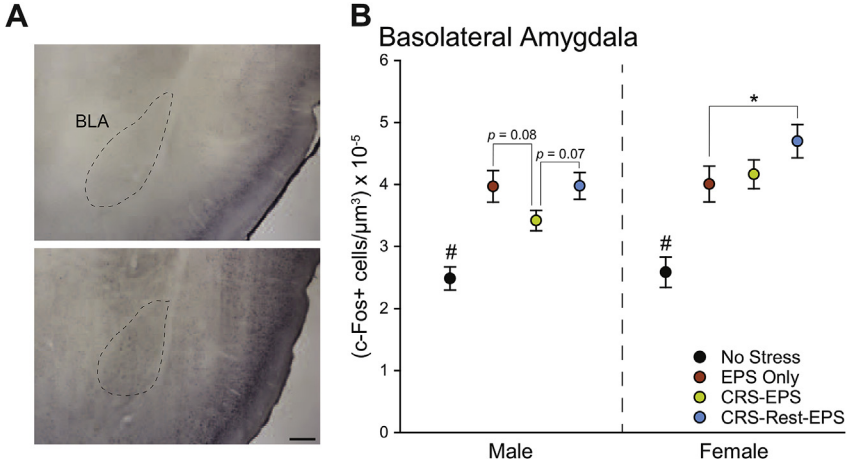

Fig. 4. (A) Representative photomicrographs of c-Fos immunohistochemistry in BLA of No Stress (*Top*) and EPS Only (*Bottom*) male rats. Scale bar = 250 μm. (B) In male rats, acute stress increases c-Fos expression in BLA. In CRS-EPS males this increase is slightly reduced. Likewise, EPS increases c-Fos expression in BLA. In CRS-Rest-EPS females this increase is enhanced. $^{*}p < 0.05$. $^{\#}p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM.

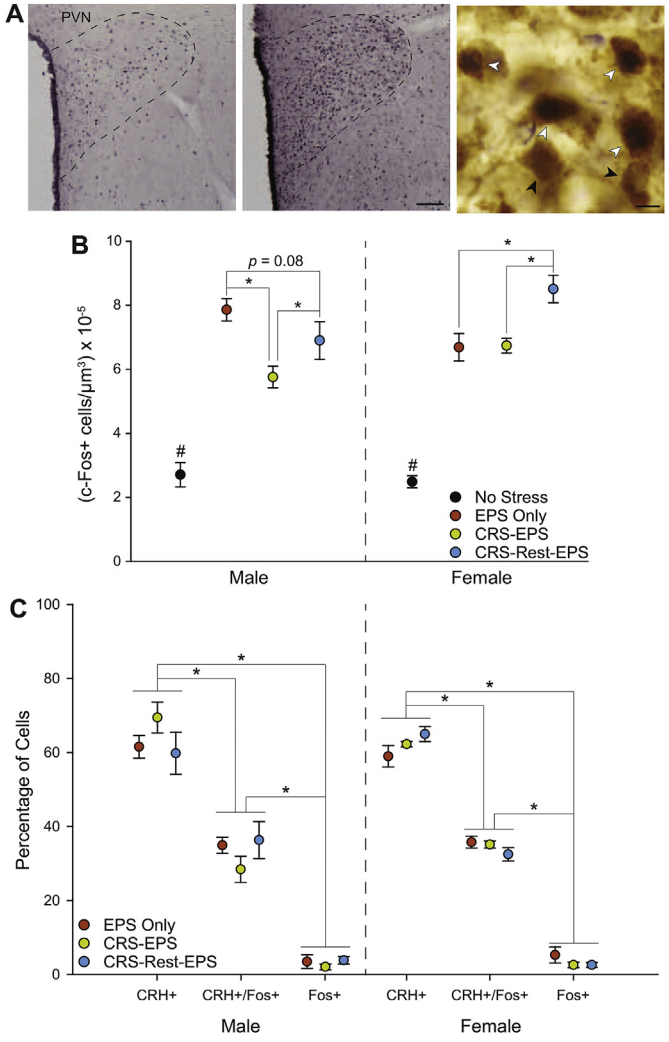

Fig. 5. (A) Representative photomicrographs of c-Fos immunohistochemistry in the PVN of No Stress (*left*) and EPS Only (*middle*) male rats. Scale bar = 100 μm. (*Right)* Photomicrograph of c-Fos (black) and CRH (brown) labeled cells within the PVN. Black arrowheads indicate single-label CRH immunopositive cells. White arrowheads indicate double-labeled cells. Scale bar = 10 μm. (B) Chronic stress effects on EPS-induced c-Fos expression in the PVN in male and female rats. In both male and female rats, EPS increases c-Fos expression in the PVN. This increase is significantly reduced in CRS-EPS male rats, and to a lesser extent in CRS-Rest-EPS males. In females, the acute stress-induced increase is enhanced in CRS-Rest-EPS rats. (C) In male and female rats, the majority of c-Fos immunopositive cells are co-localized with CRH across all stress conditions. $^{*}p < 0.05$. $^{\#}p < 0.05$ compared to all other stress conditions of same sex. Error bars represent SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conditions in females (*p*'s < 0.001). This effect was not altered in CRS-EPS females. In contrast, CRS-Rest-EPS females had enhanced EPS-induced c-Fos expression (EPS Only v CRS-Rest-EPS, $p = 0.05$), but this increase did not reach significance compared to CRS-EPS rats.

3.2.4. Paraventricular nucleus

As in mPFC, a small number of animals were excluded from analyses due to tissue damage resulting from hemisection. Thus, final n's for PVN were as follows: male No Stress, n = 5; EPS Only, n = 9; CRS-EPS, n = 9; CRS-Rest-EPS, n = 8; and female No Stress, n = 6; EPS Only, n = 9; CRS-EPS, n = 9, CRS-Rest-EPS, n = 7. The density of c-Fos + cells in the PVN (Fig. 5B) did not differ between males and females (main effect of sex, $F_{(1,\ 54)} = 1.10$, n.s.). Stress significantly altered c-Fos expression (main effect of stress, $F_{(3,\ 54)} = 53.79$, $p < 0.001$), and this effect differed between males and females (sex × stress interaction, $F_{(3,\ 54)} = 5.21$, $p = 0.003$). Follow up comparisons indicated that in males, EPS increased c-Fos expression across all stress conditions (*p*'s < 0.001). This increase was blunted in CRS-EPS males (EPS Only v CRS-EPS, $p < 0.001$), with a non-significant trend in the same direction in CRS-Rest-EPS males (EPS Only v CRS-Rest-EPS, $p = 0.08$), resulting in a significant difference between chronically stressed males without and with a rest period ($p = 0.04$). In females, EPS also resulted in increased c-Fos expression across all stress conditions (*p*'s < 0.001). This effect was not altered in CRS-EPS females. In contrast, CRS-Rest-EPS females had enhanced EPS-induced c-Fos expression compared to both EPS Only ($p = 0.002$) and CRS-EPS ($p = 0.002$) females.

3.3. In the PVN, c-Fos and CRH immunoreactivity are co-localized

In the PVN, single-versus double-labeled cells were readily discriminable by focusing through the tissue. Three-way repeated measures ANOVA revealed a main effect of immunolabeling in the PVN (Fig. 5C; $F_{(2,\ 24)} = 483.47$, $p < 0.0001$), but not sex ($F_{(1,\ 12)} = 0.50$, n.s.) or stress ($F_{(2,\ 12)} = 3.50$, n.s.). There were no significant interactions ($Fs \leq 3.50$, all n.s.). Follow-up pairwise comparisons revealed that there was a higher percentage of CRH+ (62.83 ± 1.41) cells compared to either CRH+/c-Fos+ (33.85 ± 1.17; $p < 0.001$) or c-Fos+ (3.32 ± 0.55; $p < 0.001$) cells. In addition, CRH+/c-Fos + cells were more significantly more numerous than c-Fos + cells ($p < 0.001$).

3.4. Stress alters inter-region correlations in a sex-dependent manner

The relationships among c-Fos expression across brain regions were examined using correlational analyses (Fig. 6). In No Stress male rats, strong and significant positive correlations between c-Fos expression in PL and IL ($r_{(5)} = 0.98$, $p < 0.01$), CA1 and CA3 ($r_{(6)} = 0.83$, $p = 0.04$), and CA1 and DG ($r_{(6)} = 0.82$, $p = 0.04$) were observed. A negative correlation between OFC and PVN ($r_{(5)} = -0.80$, $p = 0.10$) and a positive correlation between BLA and DG ($r_{(6)} = 0.78$, $p = 0.07$) approached significance. In No Stress females, c-Fos expression in PL and OFC ($r_{(5)} = 0.90$, $p = 0.04$), CA3 and DG ($r_{(5)} = 0.87$, $p = 0.02$), and CA3 and BLA ($r_{(5)} = 0.89$, $p = 0.02$) were strongly positively correlated, whereas expression in IL and DG ($r_{(5)} = -0.90$, $p = 0.04$) and IL and BLA ($r_{(5)} = -0.90$, $p = 0.04$) were strongly negatively correlated. Positive correlations between c-Fos expression in CA1 and DG ($r_{(6)} = 0.75$, $p = 0.08$) and CA1 and BLA ($r_{(6)} = 0.77$, $p = 0.07$) approached significance. Thus, in unstressed male and female rats, the pattern of correlated activity across the corticolimbic regions examined varies in strength and direction.

In EPS Only male rats, only one correlation reached significance: c-Fos expression in CA1 and PVN was negatively correlated ($r_{(9)} = -0.80$, $p = 0.01$). A positive correlation between PL and IL ($r_{(7)} = 0.67$, $p = 0.10$) approached significance, as did a negative correlation between IL and BLA ($r_{(7)} = -0.80$, $p = 0.07$). In EPS Only female rats, c-Fos expression in IL and DG ($r_{(8)} = 0.71$, $p = 0.05$) was positively correlated, whereas c-Fos expression in PL and PVN was negatively correlated ($r_{(8)} = -0.91$, $p < 0.01$); positive correlations between PL and CA1 ($r_{(8)} = 0.67$, $p = 0.07$) and CA1 and CA3 ($r_{(9)} = 0.59$, $p = 0.10$) approached significance. Thus, acute stress alone alters the pattern of activity across the corticolimbic brain regions examined, decreasing the number and strength of associations between regions in both male and female rats. Again, however, the pattern of correlated activity across regions varies between males and females.

In CRS-EPS male rats, c-Fos expression in PL and IL ($r_{(11)} = 0.70$, $p = 0.02$), CA1 and CA3 ($r_{(12)} = 0.67$, $p = 0.02$), CA1 and BLA ($r_{(12)} = 0.69$, $p = 0.01$), CA3 and BLA ($r_{(12)} = 0.66$, $p = 0.02$), and OFC and BLA ($r_{(12)} = 0.62$, $p = 0.03$) were significantly and positively correlated. In CRS-EPS female rats, c-Fos expression in PL and IL

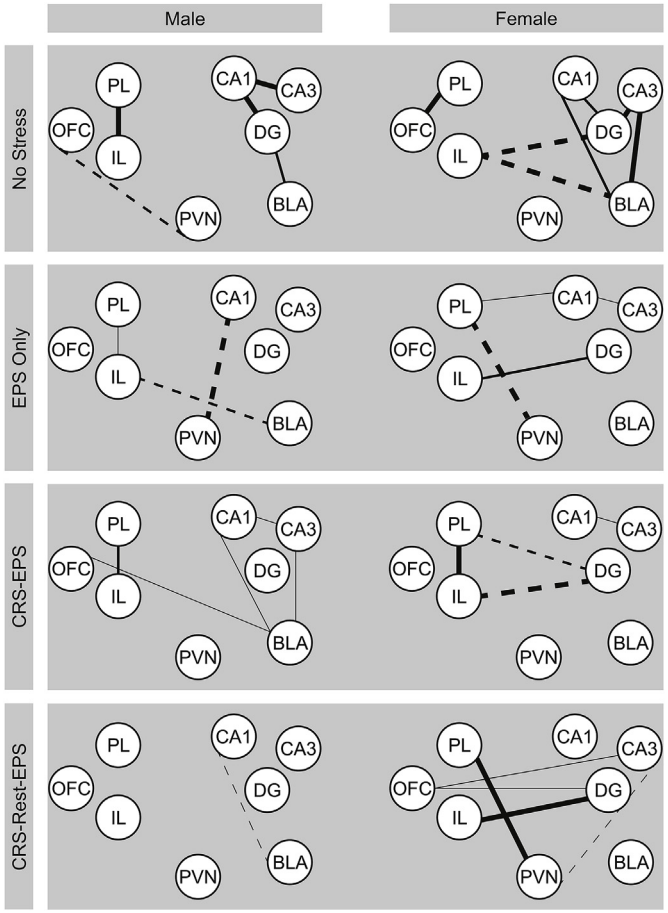

Fig. 6. Summary of correlational analyses of c-Fos expression across corticolimbic brain regions. Spearman correlations were used to assess potential associations in EPS-induced neuronal activation between OFC, PL, IL, PVN, CA1, CA3, DG, and BLA in males and females. Positive and negative correlations are represented by solid and dashed lines, respectively. The strength of each correlation is represented by line weight (——, — —, $r = \pm 0.81$–1.0; ——, — —, $r = \pm 0.71$–0.80; —, – –, $r = \pm 0.60$–0.70).

($r_{(8)} = 0.91$, $p < 0.01$) were significantly and positively correlated, while c-Fos expression in DG was significantly and negatively correlated with activation in both PL ($r_{(8)} = -0.76$, $p = 0.03$) and IL ($r_{(8)} = -0.91$, $p < 0.01$). A positive correlation between activation in CA1 and CA3 approached significance ($r_{(9)} = 0.58$, $p = 0.10$). Thus, in both males and females, chronic stress produced different patterns of correlated activity across the corticolimbic regions examined relative to acute stress. Furthermore, these patterns were markedly different in CRS-EPS males and females.

In CRS-Rest-EPS male rats, only one correlation approached significance (CA1-BLA, $r_{(9)} = -0.60$, $p = 0.09$). In contrast, in CRS-Rest-EPS female rats, c-Fos expression in PL and PVN ($r_{(5)} = 0.87$, $p = 0.05$) and in IL and DG ($r_{(6)} = 0.87$, $p = 0.02$) were significantly and positively correlated. Positive correlations between OFC and CA3 ($r_{(8)} = 0.65$, $p = 0.08$) and OFC-DG ($r_{(8)} = 0.62$, $p = 0.10$) approached significance, as did a negative correlation between PVN and CA3 ($r_{(7)} = -0.69$, $p = 0.09$). Thus, EPS-induced c-Fos expression in chronically stressed male rats given a rest period showed little association across the corticolimbic regions examined, whereas in chronically stressed female rats given a rest period, EPS produced an increase in correlated activation across these areas.

4. Discussion

Our results demonstrate sex differences in the modulation of acute stress-induced cellular activation by chronic stress with and without a rest period in a number of corticolimbic brain regions (see Table 1). In chronically stressed males not given a rest period, acute stress-induced activation was blunted in PL, IL, CA1, DG, the PVN, with a similar but nonsignificant tendency in the BLA. This blunted response was also present in PL, CA1, and DG after a 7-day rest period, whereas acute stress-induced activation in IL, BLA, and PVN was comparable to that seen in EPS-only males. In contrast, in female rats, prior chronic stress resulted in blunted acute stress-induced activation only in OFC. Notably, following a rest period, chronically stressed female rats had elevated c-Fos expression in BLA and the PVN, with a similar but nonsignificant tendency present in the DG following a novel acute stressor.

Table 1
Effects of chronic stress without and with a rest period on acute stress-induced c-Fos expression in corticolimbic brain regions.

Sex:	Male		Female	
Stress:	**No Rest**	**Rest**	**No Rest**	**Rest**
Prelimbic Cortex	↓	↓	-	-
Infralimbic Cortex	↓	-	-	-
Orbitofrontal Cortex	-	↑	↓	↓
CA1	↓	↓	-	-
CA3	-	-	-	-
Dentate Gyrus	↓	↓	-	↑
Basolateral Amygdala	↓	-	-	↑
Periventricular Nucleus	↓	↓	-	↑

Note: ↑ (green) indicates increased c-Fos expression compared to EPS Only rats of same sex; ↓ (red) indicates decreased c-Fos expression compared to EPS Only rats of same sex; darker shading, $p \leq 0.05$; lighter shading, $p \leq 0.09$.

4.1. Region-specific, persistent reduction of novel stress-induced c-fos expression in chronically stressed male rats with and without a rest period

Interest in the plasticity of adult rodent brain in the aftermath of chronic stress is growing. Conrad et al. (1999) provided the first evidence suggesting that chronic stress-induced changes were not permanent, demonstrating that in males, chronic stress-induced dendritic retraction in hippocampal CA3 pyramidal neurons was no longer present 10 days after the cessation of chronic stress. Further, chronic stress-induced deficits in spatial memory are also ameliorated following a post-stress rest period (Sousa et al., 2000). In contrast to the hippocampus, chronic stress leads to increased dendritic arborization in BLA (Vyas et al., 2002, 2003, 2004; Johnson et al., 2009), although this effect may depend on stressor intensity and/or duration, as others have found dendritic retraction (Grillo et al., 2015). In the case of dendritic outgrowth, a 21-day rest period is not sufficient to reverse chronic stress-induced changes (Vyas et al., 2004). In mPFC, chronic stress-induced retraction is ameliorated following a rest period in males (Radley et al., 2005; Moench and Wellman, 2017), suggesting that the reversibility of chronic stress-induced changes in dendritic architecture is region-specific. We recently showed that, in males, the process of post-stress dendritic remodeling in mPFC is dynamic – chronically stressed male rats given a 7-day rest period exhibit dendritic outgrowth beyond unstressed lengths (Moench and Wellman, 2017). This finding raises the possibility that a novel stress challenge during the post-stress rest period could have important functional implications for mPFC and other stress-sensitive brain regions. The present findings provide further support for this notion.

Previous studies have found reduced stress-evoked c-*fos* mRNA expression in a number of brain regions involved in stress regulation following exposure to repeated homotypic stress (e.g., Melia et al., 1994; Campeau et al., 2002; Girotti et al., 2006). However, few studies have examined the effects of prior chronic stress on heterotypic stress-induced neuronal activation. Those that have reveal conflicting findings. For example, male rats exposed to chronic cold stress have greater

c-Fos induction in the periventricular thalamus and amygdala following a restraint challenge (Bhatnagar and Dallman, 1998). Similar results have been found in male rats exposed to chronic restraint stress prior to an acute social defeat stressor (Chung et al., 2000). In contrast, chronic variable stress followed by exposure to a novel environment results in reduced c-*fos* mRNA expression in males in a number of cortical and non-cortical regions, including lateral septum, lateral hypothalamus, anterior cingulate cortex, PVN, and PL (Ostrander et al., 2009). A similar finding was reported in BLA of male rats exposed to a novel stress challenge one day following the cessation of CRS (Reznikov et al., 2008). The reason for discrepant findings among other studies is unclear, but may reflect differences in the intensity and/or salience of the novel stress challenge in relation to the chronic stress paradigm. On the other hand, differences in *c-fos* mRNA may not directly reflect changes in c-Fos protein expression. For instance, repeated, severe immobilization produced changes in *c-fos* mRNA but not c-Fos immunostaining in several brain regions (Ons et al., 2010). However, it is unknown if this is also the case following a heterotypic stress challenge such as the paradigm used here.

Indeed, our findings of reduced c-Fos expression in multiple corticolimbic brain regions in response to a novel stress challenge following chronic restraint stress are in agreement with Ostrander et al. (2009) and Reznikov et al. (2008), and suggest that changes in *c-fos* mRNA parallel changes in the protein following a heterotypic acute stressor. This regional specificity in chronic stress modulation of cellular excitability in response to an immediate novel stress challenge may indicate dynamic underlying neurobiological changes that differ across regions and may aid in stress adaptation. Together, these data indicated that in males, chronic stress produces a prolonged period of neuronal hyporesponsivity that varies by region and post-chronic stress time point. Further, they suggest, as recently argued by Ortiz and Conrad (2018), that post-stress changes in corticolimbic regions do not constitute a recovery—that is, a return to baseline—but rather reflect a new functional state for these structures, which is distinct from either the unstressed or stressed condition.

4.2. In females, chronic stress without a rest period does not modulate novel stress-induced activation, but novel stress-induced activation is potentiated following rest

In contrast to the effects of chronic stress in males, in females, chronic stress largely did not modulate neuronal activity in response to a heterotypic acute stressor. The notable exception here is the reduction found in c-Fos expression in OFC, which was one of just two regions we examined in which chronic stress only marginally altered c-Fos expression in male rats.

This is the first study to our knowledge to examine prior chronic stress effects on neuronal activity following a heterotypic stress challenge in adult females. Notwithstanding, our results align with a growing body of literature suggesting that chronic stress produces few neurobiological and behavioral changes in adult female rodents. Indeed, chronic stress does not disrupt behaviors mediated by hippocampus (Bowman et al., 2003) or mPFC (Wei et al., 2014; Snyder et al., 2015), and does not produce dendritic retraction in these regions (Galea et al., 1997; Moench and Wellman, 2017). These studies and others have led some to conclude that female rats are resilient to chronic stress paradigms, especially with regards to cognitive changes following stress (Luine et al., 2017). Our finding here that, across many corticolimbic brain regions that are involved in cognitive processing, chronic stress does not modulate novel acute stress-induced neuronal activity is consistent with this notion.

Surprisingly, however, chronically stressed female rats given a rest period showed *enhanced* c-Fos expression following a novel acute stressor, most notably in the PVN and BLA. Although it is possible that we overestimated the number of double-labeled cells due to nonspecific binding of the CRH antibody, the percentage of CRH-immunoreactive cells that were c-Fos-positive was in line with previous findings (e.g., Romeo et al., 2006). Thus, given that many CRH-immunoreactive cells in the PVN were c-Fos positive, and the possibility that projections from the BLA may act in a feed-forward manner to facilitate the PVN response to stress (Bhatnagar et al., 2004), this pattern of findings could reflect HPA axis hyperresponsivity to novel stress during the post-chronic stress rest period in female rats. Ostrander et al. (2009) demonstrated that reduced novel stress-induced c-*fos* expression in the PVN was associated with reduced plasma corticosterone in male rats. This suggests that changes in neuronal activation in the PVN in our model of two-hit stress likely result in altered neuroendocrine functioning. Although it is beyond the scope of the current study, future work should determine if these findings in females represent an exaggerated HPA response to a novel stressor in previously stressed female rats, and if so, what behavioral and neurobiological ramifications might result from such a response.

4.3. Implications of differential post-stress alterations in males and females

An excellent review of the effects of post-stress rest periods on hippocampal structure and function recently highlighted the notion that changes in the brain post-stress likely do not constitute a simple reversal of the effects of chronic stress and a return to basal conditions (Ortiz and Conrad, 2018). Indeed, Gray et al. (2014) recently showed that, compared to unstressed rats, over 700 genes within the hippocampus are altered in both chronically stressed male rats and chronically stressed male rats given a post-stress rest period. Interestingly, only 36 of the altered genes in these two groups overlapped. This suggests that, in males, the response to novel stressors during the post-stress rest period could involve the recruitment of a separate set of stress-adaptation mechanisms compared to stress-naïve and chronically stressed males. In contrast, the lack of modulation of novel stress-induced neuronal activation in chronically stressed female rats that we observed could represent an inability to recruit these same adaptive mechanisms. This hypothesis is consistent with the potentiated activation to novel stress seen in PVN in the chronically stressed females in the present study. Future research should aim to understand the ramifications of post-stress changes in the adult brain, and importantly, how these changes differ between males and females, leading to sex differences in risk and resilience in the face of multiple stressful experiences.

Our correlational analyses of c-Fos across brain regions revealed striking patterns of associations and dissociations between regions in chronically stressed male and female rats without and with a rest period compared to stress-naïve rats. These patterns suggest that there are sex-specific circuit-level changes in the neural response to an acute stress challenge following both chronic stress and a rest period, and highlight the unique pattern of activation across brain regions after EPS stress in CRS-Rest females. For instance, in females, chronic stress appears to produce a striking “uncoupling” of BLA from PFC and hippocampal areas. Interestingly, a similar pattern of uncoupling has been found in healthy adults following prolonged periods of occupational stress (Liston et al., 2009; Jovanovic et al., 2011), suggesting that chronic stress may reduce functional connectivity between corticolimbic brain regions. It is interesting to speculate that this might result in increased effects of a subsequent novel stressor on anxiety-like and/or fear behavior, perhaps reflecting less inhibitory control from PFC to BLA. Similarly, the altered relationship between BLA and hippocampus could have implications for contextual fear, discrimination, or generalization. Recent work from our lab has shown that there are also sex differences in microglia activation across corticolimbic brain regions, with the number, strength, and direction of correlations between brain regions differing following either acute or chronic stress (Bollinger et al., 2017). While the ramifications of these differing patterns of inter-regional associations are presently unclear, they nonetheless highlight the need to examine sex differences in the effects of stress not only in single regions of interest, but also at the level of potential circuit-wide changes that

might contribute to stress adaptation or maladaptation. Indeed, several recent reviews have highlighted the growing necessity for neuroscience research to move beyond single neuronal populations, and even individual brain regions (Yuste, 2015; Krakauer et al., 2017), to truly elucidate how neural circuits give rise to healthy and pathological behavioral states.

4.4. Limitations and future directions

This study has a few limitations that present opportunities for future research. First, as with all studies using c-Fos expression as a measure of neuronal activation, it is difficult to determine the functional consequences of altered c-Fos expression in our paradigm, as 1) c-Fos antibodies likely label not only c-Fos protein, but also Fos-family proteins, and therefore changes in expression could represent changes in other cellular processes aside from neuronal activation; 2) potential changes in constitutive c-Fos expression post-chronic stress could contribute to our group differences; and 3) we did not analyze the phenotype of c-Fos positive cells in HPC, PFC, and BLA. Given that there is evidence that chronic stress can result in sex-specific changes in proteins related to glutamatergic (Wei et al., 2014) and GABAergic (Shepard et al., 2016; Shepard and Coutellier, 2018) neurotransmission, it is possible that the changes in acute stress-induced c-Fos expression found here could represent the activation of different neuronal populations in males and females. Identifying sex-specific changes in particular neuronal subpopulations should shed light on the functional implications of the differences in the effects of two-hit stress that we demonstrate here.

In addition, because the majority of females in our study were in diestrus at the time of tissue collection, we did not examine potential differences in novel stress-induced patterns of neuronal activation across the estrous cycle. Ovarian hormones can influence both basal and stress-induced HPA axis activity. Under basal conditions, gonadally intact females have higher plasma ACTH and corticosterone levels compared to ovariectomized females, an effect that is prevented with estradiol replacement (Lesniewska et al., 1990; Seale et al., 2004). Likewise, intact females also have greater stress-induced increases in plasma corticosterone compared to ovariectomized females, an effect that is estradiol-dependent (Seale et al., 2004). Further, estradiol treatment in ovariectomized females enhances c-*fos* mRNA in PVN following acute restraint stress (Weiser and Handa, 2009). In contrast, progesterone can inhibit HPA axis activity (Viau and Meaney, 1991). Given that levels of both of these hormones were relatively low in most of the females in this study, future studies should examine how patterns of acute stress-induced c-Fos expression in stress-sensitive regions might differ across the estrous cycle.

5. Conclusions

In this study, we demonstrated that chronic stress modulates novel acute stress-induced c-Fos expression in a sex-, region-, and rest-dependent manner. Notably, while chronic stress tended to produce immediate reductions in acute stress-induced neuronal activity in a number of corticolimbic brain regions in male rats, we found little modulation of neuronal activity in several corticolimbic regions in female rats. An important exception to this pattern: following a post-stress rest period, chronically stressed female rats had increased activation in the BLA and PVN, which could contribute to an exaggerated HPA axis response to a novel stress challenge. These results suggest that the post-stress rest period may give rise to sex-specific mechanisms underlying stress adaptation and underscore the necessity of understanding not only the immediate consequences of chronic stress, but also the lasting sequelae of stress, and how these changes may modulate the brain's response to future perturbations.

Author contributions

KMM and CLW designed the experiment. KMM and MRB collected data. KMM analyzed data. KMM and CLW wrote and revised the manuscript. All authors approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

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