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Prior stress followed by a novel stress challenge results in sex-specific deficits in behavioral flexibility and changes in gene expression in rat medial prefrontal cortex.

Kelly M. Moench^{1,2,3}, Michaela R. Breach¹, Cara L. Wellman^{1,2,3}

¹Department of Psychological & Brain Sciences, Indiana University, Bloomington, IN, USA

²Program in Neuroscience, Indiana University, Bloomington, IN, USA

³Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN, USA

Abstract

Chronic stress leads to sex-specific changes in the structure and function of rat medial prefrontal cortex (mPFC). Little is known about whether these effects persist following the cessation of chronic stress, or how these initial effects may impact responses to future stressors. Here we examined attentional set-shifting in male and female rats following chronic restraint stress, a post-chronic stress rest period, and an acute novel stress challenge. Chronic stress resulted in a reversible impairment in extradimensional set-shifting in males, but had no effect on attentional set-shifting in females. Surprisingly, chronically stressed female, but not male, rats had impaired extradimensional set-shifting following a novel stress challenge. Alterations in the balance of excitation and inhibition of mPFC have been implicated in behavioral deficits following chronic stress. Thus, in a separate group of rats, we examined changes in the expression of genes related to glutamatergic (NR1, NR2A, NR2B, GluR1) and GABAergic (Gad67, parvalbumin, somatostatin) neurotransmission in mPFC after acute and chronic stress, rest, and their combination. Stress significantly altered the expression of NR1, GluR1, Gad67, and parvalbumin. Notably, the pattern of stress effects on NR1, Gad67, and parvalbumin expression differed between males and females. In males, these genes were upregulated following the post-chronic stress rest period, while minimal changes were found in females. In contrast, both males and females had greater GluR1 expression following a rest period. These findings suggest that chronic stress leads to sex-specific stress adaptation mechanisms that may contribute to sex differences in response to subsequent stress exposure.

Keywords

glutamate; GABA; attentional set-shifting; acute stress; prefrontal cortex

Corresponding Author: Cara L. Wellman, Author Contact: wellmanc@indiana.edu, 812-855-4922, Author Affiliation: Indiana University, 1101 E. 10th St. Bloomington, IN, 47405.

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1. Introduction

Stress is one of many well-known risk factors for the development of a number psychological disorders (Lian et al., 2014; Risch et al., 2009). There are notable sex differences in the prevalence, severity, persistence, and symptomatology of these disorders. For example, women are more likely to be diagnosed with mood and anxiety disorders, whereas men are more likely to suffer from substance use disorders (Cover et al., 2014). These disorders are associated with dysfunction of a number of corticolimbic brain regions, including prefrontal cortex, leading to deficits in emotion regulation and cognition (Butters et al., 2004; Joormann and Gotlib, 2010).

In rats, chronic or repeated stress leads to sex-specific changes in the structure and function of medial prefrontal cortex (mPFC). For example, daily restraint stress—with chronicity ranging from 7 to 21 days—induces apical dendritic retraction in males (Cook and Wellman, 2004; Garrett and Wellman, 2009; Radley et al., 2005), but either no change (Moench and Wellman, 2017) or dendritic outgrowth in females (Garrett and Wellman, 2009). Further, chronically stressed male rats have deficits in behaviors mediated by mPFC including extinction learning (e.g., Miracle et al., 2006), behavioral flexibility (Jett et al., 2017; Liston et al., 2006; McKlveen et al., 2016; Nikiforuk and Popik, 2011, 2013), object recognition (Bowman et al., 2009), and delayed alternation (Hains et al., 2009). These deficits are remarkably consistent across a range of stressors and chronicities. In contrast, chronically stressed female rats often do not show these same behavioral impairments (Baran et al., 2009; Bowman et al., 2009; Snyder et al., 2015).

Altered glutamatergic neurotransmission in mPFC has been linked to chronic stress-induced dendritic and behavioral changes (reviewed in Popoli et al., 2011). In males, chronic stress results in a downregulation of the NMDA receptor subunits NR1, NR2A, and NR2B (Lee and Goto, 2011; Shepard and Coutellier, 2018). These changes are associated with deficits in temporal order working memory in adolescent rats (Wei et al., 2014). Downregulation of the AMPA receptor subunit GluR1 also has also been reported following chronic stress and is associated with depressive-like behaviors in male rats (Li et al., 2011). Thus, reduced glutamate neurotransmission likely plays an important role in stress-induced deficits in behaviors that are mPFC-mediated. Notably, the effect of stress on glutamatergic neurotransmission appears to be more pronounced in males than females (Wei et al., 2014).

In contrast to the effects of chronic stress on glutamatergic neurotransmission, recent studies have suggested that chronic stress-induced changes in GABAergic neurotransmission may be more pronounced in mPFC of females. For instance, female mice exposed to 2 weeks of chronic unpredictable mild stress have an increase in the expression of parvalbumin mRNA in mPFC, which corresponded to a decrease in overall neuronal activation as measured by c-Fos expression (Shepard et al., 2016). Further, chronically stressed female mice have greater glutamatergic transmission onto parvalbumin-expressing neurons in mPFC (Shepard and Coutellier, 2018). Thus, the inhibitory tone in mPFC may be enhanced in chronically stressed female mice, which may contribute to deficits on an object-context mismatch test (Shepard and Coutellier, 2018; Shepard et al., 2016). Together, these studies suggest that

chronic stress-induced changes in the prefrontal GABAergic system may be pronounced in females, while changes in glutamatergic neurotransmission may be pronounced in males. This pattern likely contributes to sex differences in performance on tasks mediated by mPFC following chronic stress.

Far less is known about sex differences in the lasting effects of chronic stress, or how initial chronic stress-induced changes may contribute to sex-specific responsivity to a novel stress challenge. We and others have shown that chronically stressed male rats have persistent reductions in novel stress-induced c-Fos expression in mPFC (Moench et al., 2019; Ostrander et al., 2009), an effect not observed in females (Moench et al., 2019). Further, while chronically stressed male rats have a blunted neuroendocrine response to a novel stress challenge (Ostrander et al., 2006), chronically stressed females may instead have an enhanced response to the same challenge (Moench et al., 2019).

Here, we examined if these sex-specific responses to a novel stress challenge result in alterations in attentional set-shifting in chronically stressed males and females. The extradimensional shift in this task is mediated by the prelimbic subregion of mPFC (Birrell and Brown, 2000; Hamilton and Brigman, 2015), and is preferentially disrupted by chronic stress in male rats (Jett et al., 2017; Liston et al., 2006). We then assessed the effects of chronic stress exposure followed by a novel stress challenge on the expression of a number of genes involved in glutamatergic (NR1, NR2A, NR2B, GluR1) and GABAergic (Gad67, parvalbumin, and somatostatin) neurotransmission in prelimbic cortex. Given that 1) there are sex differences in the expression of these genes following chronic stress, and 2) the modulation of these genes and the proteins they code for plays an important role in learning and memory, it is possible that changes in the expression of these genes following a novel stress challenge in chronically stressed males and females could contribute to sex-specific behavioral changes during this time.

2. Materials and Methods

2.1. Animals and Stressors

Adult male and female Sprague Dawley rats (approximately 10 weeks of age at start; Envigo, Indianapolis, IN) were housed in standard laboratory cages (48 cm × 20 cm × 26 cm), with ambient temperature 23–25 °C, with free access to water, and a 12:12 light/dark cycles (lights on at 0800 h). In Experiment 1, rats were housed individually to allow for food restriction (85% of free-feeding weight), while rats in Experiment 2 were group-housed (3/ cage) and had free access to food. All procedures were conducted between 8:00 am and 6:00 pm, were in accordance with NIH Guidelines, and were approved by the Bloomington Animal Care and Use Committee.

Chronic stress consisted of daily restraint (CRS; 3 h/day, 10 d). In males, this manipulation results in dendritic remodeling and deficits in prefrontally-mediated behaviors that are comparable to those resulting from longer-term (e.g., 21 days) daily restraint (Baran et al., 2009; Cook and Wellman, 2004; Miracle et al., 2006; Moench and Wellman, 2017; Radley et al., 2005). 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 $\times$ 6.5 cm width $\times$ 5 cm height; female, 15 cm length $\times$ 6 cm width $\times$ 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 (Cook and Wellman, 2004). Elevated platform stress (EPS) was used as an acute stress manipulation and consisted of placing each rat individually on a small platform (12 cm $\times$ 12 cm) elevated 90 cm from the floor for 30 min in a brightly lit room as previously described (Maroun et al., 2013; Maroun and Richter-Levin, 2003; Xu et al., 1997).

2.2. Experiment 1: The effects of prior chronic stress on behavioral flexibility following a novel stress challenge.

For this experiment, a mixed between- and within-subjects design was used. Rats were assigned to one of three stress conditions, and all groups underwent behavioral testing at two timepoints (See Fig. 1A for experimental design and timeline). The first group was not exposed to CRS prior to the first test session, and thus, on test session 1, served as unstressed controls. Rats in this group were then exposed to EPS six days later, with the second test session on the day after EPS (No CRS-EPS; $n = 9$ male, 9 female). The second group underwent chronic stress followed by EPS on the day after chronic stress ended. The first test session for this group occurred the day after EPS and the second test occurred after a six-day rest period (CRS-EPS-Rest; $n = 8$ male, 10 female). The third group underwent chronic stress and was tested for the first time on the day after chronic stress. Rats in this group were then exposed to EPS six days later and the second test session occurred on the day after EPS (CRS-Rest-EPS; $n = 8$ male, 13 female). Thus, we assessed the effects of chronic versus acute stress, changes in performance after a rest period, and the effect of chronic stress with and without a rest period on subsequent effects of acute stress exposure.

Rats were trained on an attentional set-shifting task as previously described (Birrell and Brown, 2000; Lapiz-Bluhm et al., 2008). All training and testing took place under dimly lit conditions prior to stress on experimental Days 9 and 10, and before rats were given their daily food ration. This task consisted of training rats to dig in small terracotta pots (2.5 inches height $\times$ 3 inches width) for a food reward (BioServ pellets; Holton Industries, Frenchtown, NJ). The pot rims could be scented and/or covered with textures. Thus, pots could differ along two stimulus dimensions: odor and texture (see Table 1 for stimuli used). The testing apparatus was a rectangular black Plexiglas arena (24 in length $\times$ 16 in width $\times$ 8 in height) with a panel to divide one-third of the arena into two sections, in which pots were placed. A removable divider separated the start area from these two sections.

2.2.1 Habituation—On Day 9 of restraint stress (Day 9 of food restriction in No CRS rats) rats were habituated to the apparatus and trained to dig in unaltered pots. During habituation, unscented pots filled with digging medium (bedding) were placed in both testing sections with sucrose pellets placed on top. Rats were allowed to move about the arena freely. When both sucrose pellets were retrieved, rats were placed back into the start area, pellets were replenished, and rats were again allowed to move about the arena freely. Sucrose pellets were gradually buried further into the digging medium in order to shape

digging behavior. This continued until rats reliably retrieved both sugar pellets. Rats that did not develop digging behavior did not go on to simple discrimination training. This was the case for 4 rats (male, $n = 2$, CRS-EPS-Rest; female, $n = 2$, CRS-Rest-EPS).

2.2.2. Training—On Day 10 of restraint (Day 10 of food restriction in No CRS rats) rats were trained on both an odor and texture simple discrimination in which two pots were presented (e.g., cinnamon versus oregano or the texture of bubble wrap versus that of pipe cleaner) but only one was baited. A trial was initiated when the removable start area panel was lifted, and rats were given access to both pots. During the first four trials a dig first in the unbaited pot was recorded as an error but the rat was then permitted to dig in the baited pot to retrieve the reward. Beginning on the fifth trial, if the rat dug in the unbaited pot first, an error was recorded, and the trial was ended by placing the rat back in the start area. This continued until six consecutive correct trials were recorded. Digging was defined as the displacement of digging medium by either the nose or the nose and front paws. The stimuli used during training were not used again during testing.

2.2.3. Testing—On test days (see Fig. 1A) rats were tested on a series of increasingly difficult discriminations (see Table 2 for an example protocol). Each test session began with a simple discrimination where pots differed along only one dimension. For the compound discrimination, the second dimension was added, but the correct and incorrect stimuli from the simple discrimination phase remained constant. For the first reversal, the stimuli and correct dimension were unchanged from the compound discrimination stage, but the previously incorrect stimulus was rewarded. For the intradimensional shift, all the stimuli were replaced, but the correct dimension from the prior three phases remained constant. A second reversal followed the intradimensional shift in an identical manner to reversal 1. Finally, for the extradimensional shift, all the stimuli were replaced, and the correct dimension was shifted to the previously incorrect dimension. For rats that began testing with odor as the correct dimension, texture became the correct dimension in the extradimensional shift; for rats that began testing with texture as the correct dimension, odor became the correct dimension. The order of the extradimensional shift was counterbalanced between groups and within animals between testing sessions. Previous studies have demonstrated that this task can be used in a within-subjects design without significant facilitation of performance in the second testing session (Wallace et al., 2014).

During each stage of training and testing, trials continued until the rat made six consecutive correct choices, and trials to criterion were recorded. Three rats (male, $n = 1$, No CRS-EPS; female, $n = 2$, CRS-Rest-EPS) stopped making choices during the first test session, and therefore were not included in data analysis for this session. Data were collected from these rats during the second test session. One rat did not perform the task during the second test session (male, No CRS-EPS) but data from the first test session were included in analyses. Therefore, the final n 's per group for Test 1 are: No CRS-EPS, 9 male, 9 female; CRS-EPS-Rest, 8 male, 9 female; CRS-Rest-EPS, 8 male, 10 female. The final n 's per group for Test 2 are: No CRS-EPS, 8 male, 9 female; CRS-EPS-Rest, 8 male, 9 female; CRS-Rest-EPS, 8 male, 10 female.

2.2.4. Data Analysis—Data were first analyzed via a four-way repeated measures ANOVA (sex x stress condition x testing stage x testing session; stage and session as the repeating measures). To minimize the number of pairwise comparisons, the following strategy was used. Following the initial omnibus test, planned comparisons were used to determine the source of significant interactions. First, to determine if there was a basic sex difference in performance on any stage of the task, male and female No CRS-EPS (Test 1) were compared using a one-way ANOVA. Following this initial comparison, data from males and females were analyzed separately. One-way ANOVAs were used to compare groups within the same test session. Significant effects were followed by Fisher's protected LSD post hoc comparisons. Paired t-tests were used to compare data within each stress group across test sessions. For all analyses, data from Reversal 1 and Reversal 2 were averaged for each animal. To estimate effect sizes, η^2 (for between-subjects effects in ANOVAs), η^2_p (for within-subjects effects in ANOVAs), and Cohen's d (for two-group comparisons) were calculated.

2.3. Experiment 2: The effects of prior chronic stress on gene expression in prelimbic cortex following a novel stress challenge.

We also assessed the effects of chronic stress exposure followed by a novel stress challenge on the expression of several genes involved in glutamatergic and GABAergic neurotransmission. To avoid potential confounds due to behavioral training and testing, gene expression was assessed in a separate set of behaviorally naïve rats. Rats were assigned to one of six stress conditions (see Fig. 1B for experimental design): No Stress (n = 9/sex); EPS Only (n = 9/sex); chronic stress only (CRS Only; n = 8 male, 9 female); chronic stress followed by EPS 1 day later (CRS-EPS; n = 8 male; 9 female); chronic stress followed by a 7 day rest period (CRS-Rest; n = 9 male, 7 female); or chronic stress followed by EPS 7 days later (CRS-Rest-EPS; n = 9/sex). Body weight change (Day 10 – Day 1) was analyzed using a two-way ANOVA (stress x sex) followed by Fisher's protected LSD post-hoc comparisons to verify the chronic stress manipulation.

2.3.1. Tissue Collection and RNA Isolation—Animals were overdosed with urethane and rapidly decapitated. Brains were extracted, snap-frozen on dry ice, and stored at -80°C . Slices through prefrontal cortex (1 mm) were obtained using a precision brain slicer (Braintree Scientific Inc., Braintree, MA) that was equilibrated to -20°C . Prelimbic cortex was identified using gross anatomical landmarks (Paxinos and Watson, 1998) and micropunch samples (1.5 mm diameter) were obtained from both hemispheres (Fig. 1C). Total RNA was isolated using the Maxwell RSC simplyRNA Tissue Kit (Promega, Madison, WI) per the manufacturer's recommended protocol. RNA concentration was determined using the Take3 Micro-Volume Plate and BioTek spectrophotometer (BioTek, Winooksi, VT).

2.3.2. cDNA synthesis.—Aliquots of RNA samples (11 μL) were DNase treated for 30 min at 37°C followed by 65°C for 10 min. Samples were then chilled to 4°C prior to being incubated with oligo-dT primers (1.5 μL , 5 μM , Invitrogen) and dNTPs (0.5 μL , 25 mM, Invitrogen) for 10 min at 65°C . Reverse transcription into first strand cDNA then proceeded via the addition of a mixture of Superscript III Reverse Transcriptase (1 μL , Invitrogen), an

RNAse inhibitor (1 μ L, RNAseOUT, Invitrogen), First Strand Buffer (4 μ L, 5X, Invitrogen), and DTT buffer (1 μ L, 0.1M, Invitrogen). Samples were then incubated at 42 °C for 50 min followed by enzyme denaturation and reaction termination at 70 °C for 15 min. cDNA samples were stored at –20 °C.

2.3.3. Quantitative PCR.—Primers to measure the expression of genes of interest, as well as the reference gene glyceraldehyde-6-phosphate dehydrogenase (GAPDH), were selected from previous studies and optimized for the current study (Table 3). Primers were obtained from Eurofins Genomics (Eurofins MWG Operon LLC, Huntsville, AL). For each sample, triplicate reactions were performed in 384-well plates at a volume of 10 μ L. Reactions consisted of 3 μ L cDNA, 5 μ L PerfeCta SYBR Green SuperMix (Quanta BioSciences, Gaithersburg, MD), 1.2 μ L primer, and 0.8 μ L UltraPure water. Formation of PCR product was measured in real time using the Roche LightCycler 480 System (Roche Diagnostics, Indianapolis, IN) with cycling conditions as previously described (Bollinger et al., 2016). PCR cycling began with a pre-incubation phase (10 min, 95 °C) followed by 45 cycles of denaturation (15 s, 95 °C), annealing (40 s, 60 °C), and product extension (10 s, 72 °C). SYBR green I fluorescence was captured at 72 °C. Primer specificity and primer dimer formation were assessed post-cycling using melt curves.

2.3.4. Data Analysis—The relative abundance of mRNA was quantified using the 2^{-CT} method (Schmittgen and Livak, 2008). Mean C_T values were calculated across reaction triplicates for each sample. The mean C_T of the internal reference gene was subtracted from this value, which was then normalized via subtraction of the mean C_T of the internal reference gene from the respective gene of interest of the sample measured across all plates. To compare basal sex difference in the expression of genes of interest, data from No Stress female rats were expressed relative to No Stress male rats. These data were compared using independent samples t-tests. Following this initial comparison, data for all stress conditions were expressed relative to same-sex No Stress controls. These data were compared using one-way ANOVAs. To limit the number of irrelevant comparisons, significant ANOVAs were followed by planned comparisons that 1) compared all groups to the No Stress condition, 2) compared groups that were exposed to only one stressor, 3) compared all groups exposed to EPS, and 4) compared all groups exposed to CRS. To estimate effect sizes, η^2 (for ANOVAs) and Cohen's d (for two-group comparisons) were calculated.

3. Results

3.1. Experiment 1: The effects of prior chronic stress exposure on behavioral flexibility following a novel stress challenge.

3.1.1. Basal Sex Differences in Set-Shifting Performance—The four-way repeated measures ANOVA revealed main effects of test stage ($F_{(4, 172)} = 17.26, p < 0.001; \eta^2_p = 0.29$), and stress ($F_{(2, 43)} = 8.92, p = 0.001; \eta^2 = 0.27$), but not test session ($F_{(1, 43)} = 0.01, p = 0.92; \eta^2_p = 0.00$) or sex ($F_{(1, 43)} = 0.01, p = 0.92; \eta^2 = 0.00$). Several two-way interactions were significant, including test stage by sex ($F_{(4, 172)} = 5.22, p = 0.001; \eta^2_p = 0.11$), test stage by stress ($F_{(8, 172)} = 9.54, p < 0.001; \eta^2_p = 0.31$), test session by sex ($F_{(1, 43)}$

= 51.97, $p < 0.001$; $\eta^2_p = 0.55$), and sex by stress ($F_{(2, 43)} = 3.21$, $p = 0.05$; $\eta^2 = 0.10$), but not test session by stress ($F_{(2, 43)} = 2.41$, $p = 0.10$; $\eta^2_p = 0.10$) or test stage by test session ($F_{(4, 172)} = 1.16$, $p = 0.33$; $\eta^2_p = 0.03$). All three-way interactions were significant, including test stage by sex by stress ($F_{(8, 172)} = 3.49$, $p = 0.001$; $\eta^2_p = 0.14$), test session by sex by stress ($F_{(2, 43)} = 19.73$, $p < 0.001$; $\eta^2_p = 0.14$), test stage by test session by sex ($F_{(4, 172)} = 28.38$, $p < 0.001$; $\eta^2_p = 0.40$), and test stage by test session by stress ($F_{(8, 172)} = 2.61$, $p = 0.01$; $\eta^2_p = 0.11$). Finally, the four-way interaction (test stage by test session by sex by stress) was also significant ($F_{(8, 172)} = 10.85$, $p < 0.001$; $\eta^2_p = 0.34$). Trials to criterion on the first test session differed between No CRS-EPS males and females on the extradimensional shift (Fig. 2; $F_{(1, 16)} = 4.98$, $p = 0.04$; $\eta^2 = 0.24$; $d = 1.05$), such that females required fewer trials to complete this stage. Performance on all other stages was comparable ($F_s = 0.13$, all p 's > 0.80 ; all d 's < 0.17).

3.2.2. Stress Effects on Set-Shifting in Males—The effects of chronic stress on set-shifting in males were examined using a one-way ANOVA comparing trials to criterion on the first test session between the three groups. Trials to criterion did not differ on any stage of testing except for the extradimensional shift (Fig. 3A; extradimensional shift, $F_{(2, 21)} = 27.14$, $p < 0.001$; $\eta^2 = 0.72$; all other stages, $F_s = 2.22$, all p 's > 0.13 ; all η^2 's < 0.17). *Post hoc* analyses revealed that both CRS-EPS-Rest ($p < 0.001$) and CRS-Rest-EPS ($p < 0.001$) males required more trials to complete the extradimensional shift compared to No CRS-EPS, indicating that chronic stress preferentially disrupts performance on this stage.

Paired t-tests were then used to compare performance on each test stage within each group. Trials to criterion did not differ from test session one to test session two in No CRS-EPS male rats (Fig. 3B; $-0.33 \leq 1.04$, all p 's > 0.33 ; $0.04 \leq d$'s < 0.64), indicating that acute stress did not alter performance on any stage of the task. For CRS-EPS-Rest males, trials to criterion differed only on the extradimensional shift (Fig. 3C; $t_{(7)} = 8.37$, $p < 0.001$; $d = 4.44$) such that fewer trials to criterion were required on the second test session. Performance did not differ on any other test stage ($-0.57 \leq 1.00$, all p 's > 0.35 ; $0.19 \leq d$'s < 0.56). Thus, although chronic stress followed one day later by acute stress disrupted initial extradimensional set-shifting, this deficit was no longer present after a rest period. For CRS-Rest-EPS males, trials to criterion differed on both the intradimensional (Fig. 3D; $t_{(6)} = 2.57$, $p = 0.04$; $d = 1.43$) and extradimensional stages ($t_{(6)} = 8.37$, $p = 0.001$; $d = 3.11$), such that fewer trials to criterion were needed during the second test session. Performance did not differ on any other test stage ($-0.84 \leq 0.20$, all p 's > 0.43 ; $0.08 \leq d$'s < 0.35). Thus, although chronic stress initially disrupts extradimensional set-shifting, this deficit was no longer present after exposure to a novel acute stressor, whereas intradimensional set-shifting performance was facilitated in these males.

3.2.3. Stress Effects on Set-Shifting in Females—The effects of chronic stress on set-shifting in females were examined using a one-way ANOVA comparing trials to criterion on the first test session across the three groups. Trials to criterion did not differ on any stage of testing (Fig. 4A; $F_s = 1.51$, all p 's > 0.24 ; all η^2 's < 0.12). Thus, in females, chronic stress does not alter set-shifting performance.

Paired t-tests were then used to compare performance on each test stage within each group. Trials to criterion did not differ from test session one to test session two in No CRS-EPS female rats (Fig. 4B; $-1.90 \leq 0.73$, all p 's > 0.10 ; $0.00 \leq d$'s < 0.93), indicating that acute stress did not alter performance on any stage of the task. For CRS-EPS-Rest females, trials to criterion did not differ on any stage of the task between test session one and two (Fig. 4C; $-0.89 \leq 0.37$, all p 's > 0.40 ; $0.04 \leq d$'s < 0.36), indicating that the presence of a post-chronic stress rest period does alter set-shifting performance. For CRS-Rest-EPS females, trials to criterion differed between test session one and two on both the simple discrimination (Fig. 4D; $t_{(9)} = -2.53$, $p = 0.03$; $d = 0.80$) and extradimensional shift ($t_{(7)} = -6.60$, $p < 0.001$; $d = 2.94$) stages such that more trials to criterion were needed on both of these stages during the second test session. Performance did not differ on any other test stage ($-1.80 \leq -0.75$, all p 's > 0.12 ; $0.35 \leq d$'s < 1.02). Thus, although chronic stress does not alter performance on the set-shifting task, exposure to a novel acute stressor following a rest period results in deficits in both simple discrimination and extradimensional set-shifting.

3.3. Experiment 2: The effects of prior chronic stress exposure on gene expression in prelimbic cortex following a novel stress challenge.

3.3.1. Body Weight Analysis—Male rats gained significantly more weight than female rats (Fig. 5; main effect of sex, $F_{(1, 92)} = 6.64$, $p = 0.01$; $\eta^2 = 0.03$) and stress altered weight change in both males and females (main effect of stress, $F_{(5, 92)} = 38.45$, $p < 0.001$; $\eta^2 = 0.87$), although this effect was more pronounced in males (sex $\times$ stress interaction, $F_{(5, 92)} = 4.36$, $p = 0.001$; $\eta^2 = 0.10$). In males, weight gain did not differ between No Stress and EPS Only rats ($p = 0.64$; $d = 0.39$). In contrast, male rats exposed to chronic stress gained significantly less weight from Day 1 through Day 10 than No Stress male rats (CRS Only, $p < 0.001$, $d = 6.36$; CRS-EPS, $p < 0.001$, $d = 6.67$; CRS-Rest, $p < 0.001$, $d = 2.53$; CRS-Rest-EPS, $p < 0.001$, $d = 4.07$). Similarly, in female rats, weight change did not differ between No Stress and EPS Only rats ($p = 0.67$, $d = 0.39$), whereas in chronically stressed females, weight change was significantly reduced relative to No Stress female rats (CRS Only, $p < 0.001$, $d = 1.82$; CRS-EPS, $p < 0.001$, $d = 3.00$; CRS-Rest, $p < 0.001$, $d = 1.88$; CRS-Rest-EPS, $p = 0.004$, $d = 1.94$).

3.3.2. Basal Sex Differences in Gene Expression—The relative expression of NR1 was significantly greater in No Stress female rats compared to No Stress male rats (Fig. 6; $t_{(15)} = -2.81$, $p = 0.01$; $d = 1.37$). No other basal sex differences in relative gene expression were significant ($0.30 \leq t$'s ≤ 1.32 , all p 's > 0.77 ; $0.48 \leq d$'s < 1.03).

3.3.3. Stress Effects on Glutamatergic Gene Expression in Males—The relative expression of NR1 differed significantly as a result of stress in male rats (Fig. 7A; $F_{(5, 43)} = 7.18$, $p < 0.001$; $\eta^2 = 0.58$). Planned comparisons revealed that the expression of NR1 was greater in EPS Only ($F_{(1, 15)} = 18.69$, $p = 0.001$; $d = 2.10$), CRS Only ($F_{(1, 14)} = 5.94$, $p = 0.03$; $d = 1.23$), CRS-Rest ($F_{(1, 14)} = 18.69$, $p < 0.001$; $d = 6.83$), and CRS-Rest-EPS ($F_{(1, 14)} = 10.79$, $p = 0.005$; $d = 1.64$) males compared to No Stress males, while expression in CRS-EPS males did not differ from controls ($F_{(1, 14)} = 1.82$, $p = 0.20$; $d = 0.67$). The EPS-induced increase in the expression of NR1 was prevented by CRS, but only if a rest period was not present (EPS Only v CRS-EPS, $F_{(1, 15)} = 4.40$, $p = 0.05$, $d = 1.01$; EPS Only v CRS-Rest-

EPS, $F_{(1, 15)} = 0.63$, $p = 0.44$, $d = 0.39$). The CRS-induced increase in NR1 expression was enhanced following a rest period (CRS Only v CRS-Rest, $F_{(1, 14)} = 14.44$, $p = 0.002$, $d = 3.60$), but this enhancement was blunted following exposure to EPS (CRS-Rest v CRS-Rest-EPS, $F_{(1, 14)} = 8.15$, $p = 0.01$, $d = 2.38$). Despite this, NR1 expression was greater in CRS-Rest-EPS males compared to CRS-EPS males ($F_{(1, 14)} = 4.51$, $p = 0.05$, $d = 1.06$). No other planned comparisons reached significance (F 's ≤ 1.25 , all p 's ≥ 0.28 , all d 's ≤ 0.67). The relative expression of NR2A and NR2B (Table 4) did not differ significantly as a result of stress in male rats (NR2A, $F_{(5, 45)} = 1.78$, $p = 0.14$, $\eta^2 = 0.17$; NR2B, $F_{(5, 45)} = 1.42$, $p = 0.24$, $\eta^2 = 0.14$).

Stress significantly altered the relative expression of GluR1 in males (Fig. 7C; $F_{(5, 43)} = 2.87$, $p = 0.03$, $\eta^2 = 0.24$). Planned comparisons revealed that EPS increased expression of GluR1 (EPS Only v No Stress, $F_{(1, 16)} = 5.12$, $p = 0.04$, $d = 1.06$). GluR1 expression was not altered by CRS alone but was increased in CRS males following a rest period (No Stress v CRS Only, $F_{(1, 15)} = 0.52$, $p = 0.48$, $d = 0.35$; No Stress v CRS-Rest, $F_{(1, 16)} = 4.46$, $p = 0.05$, $d = 1.00$). The EPS-induced increase in the expression of GluR1 was prevented by CRS, but only if a rest period was not present (EPS Only v CRS-EPS, $F_{(1, 15)} = 13.93$, $p = 0.002$, $d = 1.81$; EPS Only v CRS-Rest-EPS, $F_{(1, 15)} = 0.13$, $p = 0.72$, $d = 0.18$), resulting in a significant difference between CRS-EPS and CRS-Rest-EPS males ($F_{(1, 14)} = 7.05$, $p = 0.02$, $d = 1.38$). No other planned comparisons reached significance (F 's ≤ 2.63 , all p 's ≥ 0.13 ; d 's ≤ 1.19).

3.3.4. Stress Effects on Glutamatergic Gene Expression in Females—In females, stress significantly altered the relative expression of NR1 (Fig. 7B; $F_{(5, 43)} = 3.80$, $p = 0.006$, $\eta^2 = 0.31$). Planned comparisons revealed that whereas EPS and CRS alone did not alter NR1 expression (No Stress v EPS Only, $F_{(1, 15)} = 1.45$, $p = 0.25$, $d = 0.58$; No Stress v CRS Only, $F_{(1, 15)} = 0.03$, $p = 0.87$, $d = 0.09$), the combination of the two did, but only when a rest period was present (No Stress v CRS-EPS, $F_{(1, 16)} = 0.002$, $p = 0.96$, $d = 0.03$; No Stress v CRS-Rest-EPS, $F_{(1, 15)} = 7.56$, $p = 0.02$, $d = 1.19$; CRS-EPS v CRS-Rest-EPS, $F_{(1, 15)} = 8.05$, $p = 0.01$, $d = 1.15$). Further, NR1 expression was greater in CRS-Rest-EPS females compared to CRS Only ($F_{(1, 14)} = 7.76$, $p = 0.02$, $d = 1.07$) and CRS-Rest ($F_{(1, 13)} = 14.38$, $p = 0.002$, $d = 2.30$) females. No other planned comparisons reached significance (F 's ≤ 2.28 , all p 's ≥ 0.15 , d 's ≤ 1.29). The relative expression of NR2A and NR2B (Table 4) did not differ significantly as a result of stress in female rats (NR2A, $F_{(5, 43)} = 1.36$, $p = 0.26$, $\eta^2 = 0.14$; NR2B, $F_{(5, 44)} = 2.01$, $p = 0.10$, $\eta^2 = 0.19$).

The relative expression of GluR1 was significantly altered by stress in females (Fig. 7D; $F_{(5, 45)} = 2.36$, $p = 0.05$, $\eta^2 = 0.21$). Planned comparisons revealed that GluR1 expression was increased in CRS females either after a rest period (No Stress v CRS-Rest, $F_{(1, 14)} = 4.67$, $p = 0.05$, $d = 0.89$) or after exposure to EPS without a rest period (No Stress v CRS-EPS, $F_{(1, 16)} = 5.13$, $p = 0.04$, $d = 1.06$). GluR1 expression was also greater in CRS-EPS females compared to EPS only females ($F_{(1, 15)} = 8.79$, $p = 0.01$, $d = 1.44$). No other planned comparisons reached significance (F 's ≤ 3.34 , all p 's ≥ 0.09 , d 's ≤ 1.03).

3.3.5. Stress Effects on GABAergic Gene Expression in Males—The relative expression of Gad67 differed significantly as a result of stress in males (Fig. 8A; $F_{(5, 44)} =$

2.09, $p = 0.005$, $\eta^2 = 0.31$). Expression of Gad67 was not altered by CRS alone, but was increased in CRS males following a rest period (No Stress v CRS Only, $F_{(1, 15)} = 0.31$, $p = 0.59$, $d = 0.27$; No Stress v CRS-Rest, $F_{(1, 15)} = 6.90$, $p = 0.02$, $d = 1.27$; CRS Only v CRS-Rest, $F_{(1, 14)} = 8.14$, $p = 0.01$; $d = 1.42$). Further, Gad67 expression was greater in CRS-Rest-EPS males compared to CRS Only males ($F_{(1, 15)} = 5.59$, $p = 0.03$, $d = 1.14$). No other planned comparisons reached significance (F 's = 3.63, all p 's = 0.08, 0.03 d 's = 1.03).

The relative expression of parvalbumin in males also differed significantly as a result of stress (Fig. 8C; $F_{(5, 45)} = 5.73$, $p < 0.001$, $\eta^2 = 0.39$). Planned comparisons revealed that while EPS (No Stress v EPS Only, $F_{(1, 16)} = 3.55$, $p = 0.08$, $d = 0.86$) and CRS (No Stress v CRS Only, $F_{(1, 14)} = 0.01$, $p = 0.93$, $d = 0.22$) alone did not alter parvalbumin expression, both CRS-Rest and CRS-Rest-EPS males had greater parvalbumin expression than No Stress males (CRS-Rest, $F_{(1, 16)} = 15.23$, $p = 0.001$, $d = 1.75$; CRS-Rest-EPS, $F_{(1, 16)} = 5.73$, $p = 0.03$; $d = 1.12$), and CRS Only males (CRS-Rest, $F_{(1, 14)} = 12.76$, $p = 0.003$, $d = 1.85$; CRS-Rest-EPS, $F_{(1, 14)} = 4.81$, $p = 0.05$, $d = 1.22$). Further, parvalbumin expression was significantly reduced in CRS-EPS males compared to EPS Only males ($F_{(1, 15)} = 6.01$, $p = 0.03$, $d = 1.14$) and CRS-Rest-EPS males ($F_{(1, 15)} = 7.79$, $p = 0.01$, $d = 1.35$). No other planned comparisons reached significance (F 's = 3.14, all p 's = 0.10, 0.17 d 's = 0.99). The relative expression of somatostatin in males did not differ across stress conditions (Table 4; $F_{(5, 44)} = 1.25$, $p = 0.30$, $\eta^2 = 0.12$).

3.3.6. Stress Effects on GABAergic Gene Expression in Females—In female rats, the relative expression of Gad67 (Fig. 8B) and somatostatin (Table 4) did not differ significantly as a result of stress (Gad67, $F_{(5, 44)} = 2.00$, $p = 0.10$, $\eta^2 = .18$; SST, $F_{(5, 46)} = 1.19$, $p = 0.33$, $\eta^2 = 0.11$). In contrast, the relative expression of parvalbumin was significantly altered by stress in females (Fig. 8D; $F_{(5, 46)} = 2.38$, $p = 0.05$, $\eta^2 = 0.21$). Planned comparisons revealed that chronic stress significantly increased the expression of parvalbumin in females ($F_{(1, 16)} = 7.13$, $p = 0.02$, $d = 1.17$), and this increase was still present following a rest period ($F_{(1, 14)} = 6.95$, $p = 0.02$, $d = 1.32$). However, this chronic stress-induced increase in parvalbumin expression was no longer present following EPS in females given a post-CRS rest period ($F_{(1, 16)} = 4.41$, $p = 0.05$, $d = 0.91$). No other planned comparisons reached significance (F 's = 3.09, all p 's = 0.10, 0.18 d 's = 0.82).

4. Discussion

In Experiment 1 we showed that chronic stress impaired extradimensional set-shifting performance in male, but not female, rats. This deficit in chronically stressed males was ameliorated following a 7 day rest period. In chronically stressed female rats, exposure to a novel stress challenge following a post-chronic stress rest period resulted in impaired extradimensional set-shifting. This effect was absent in male rats. In Experiment 2, we demonstrated that acute and chronic stress, as well as their combination, alter the expression of NR1, GluR1, Gad67, and parvalbumin genes in prelimbic cortex a sex-specific manner.

4.1. In males, chronic stress-induced deficits in extradimensional set-shifting are reversible.

Previous studies have shown that chronic stress impairs performance on a number of tasks that are mediated by mPFC in male rats. For example, repeated restraint stress results in deficits in object recognition (Bowman et al., 2003; Bowman et al., 2009), response withholding (Mika et al., 2012), working memory (Mika et al., 2012), and extradimensional set-shifting (Liston et al., 2006). Findings from lesion and inactivation studies assessing the neural substrates of each rule change in the attentional set-shifting task show that extradimensional set-shifting is mediated by the prelimbic and infralimbic subregions of mPFC (Birrell and Brown, 2000), intradimensional shifts are mediated by anterior cingulate cortex (Ng et al., 2007), and reversal learning is mediated by orbitofrontal cortex (McAlonan and Brown, 2003). Chronic stressors typically disrupt extradimensional set-shifting (Jett et al., 2017; Liston et al., 2006; Nikiforuk and Popik, 2011, 2013, 2014), although reversal learning can also be affected when more severe physical stressors are used (e.g., chronic intermittent cold stress; Danet et al., 2010; Lapiz-Bluhm et al., 2009). Our data agree with findings from the former studies, as we demonstrated that male rats exposed only to chronic stress or to chronic stress followed one day later by acute stress have deficits in extradimensional set-shifting. Together with previous research (Holmes and Wellman, 2009), these data suggest that mPFC of male rats is sensitive to the immediate effects of chronic stress.

The first studies to examine the reversibility of chronic stress effects on behavior assessed hippocampus-dependent behaviors. Performance on spatial working memory tasks, including the Morris water maze and the radial arm maze, is disrupted following chronic stress in males (Bowman et al., 2003; Conrad et al., 1996; Hoffman et al., 2011; Luine et al., 1994; McFadden et al., 2011; Sousa et al., 2000). Luine and colleagues (1994) demonstrated that chronic stress-induced deficits in the radial arm maze were not found in rats that were tested 18 days after chronic stress ended. Since this initial finding, many studies have shown that chronic stress-induced deficits in hippocampus-dependent tasks are reversible in males (Hoffman et al., 2011; McFadden et al., 2011; Ortiz et al., 2018; Ortiz et al., 2014; Ortiz et al., 2015; Sousa et al., 2000). Here, we demonstrated that deficits in extradimensional set-shifting induced by chronic stress followed one day later by acute stress are also reversible following a 7-day no-stress rest period, which is consistent with our previous study demonstrating that chronic stress-induced dendritic retraction is also ameliorated at this time point (Moench and Wellman, 2017). However, we cannot rule out the possibility that exposure to the elevated platform one day after the chronic stress altered brain circuits to promote recovery or to facilitate cognitive flexibility. Regardless, these results add to a growing literature suggesting that at least some of the deleterious effects of chronic stress in adulthood are reversible.

In contrast to males, chronic stress did not alter performance on any phase of the attentional set-shifting task in females. This is consistent with prior work showing no effect of chronic stress on mPFC-mediated behaviors in female rats. For example, 21 days of restraint stress does not impair or object recognition memory (Bowman et al., 2003; Bowman et al., 2009), although one recent study reported deficits in delayed alternation following 14 days of

chronic variable stress (Anderson et al., 2019). Additionally, unlike in male rats, chronic stress does not disrupt hippocampus-dependent tasks in female rats, and instead often results in *enhanced* performance. For example, Morris water maze performance is facilitated following either 10 days of chronic unpredictable stress (McFadden et al., 2011) or 21 days of restraint stress (Bowman et al., 2001). These and other studies showing no change in hippocampus-dependent behaviors following chronic stress in females (Kitraki et al., 2004; McLaughlin et al., 2010), combined with mounting evidence that chronic stress does not alter performance on mPFC-mediated tasks, suggests that female rats may be somewhat resistant to the immediate effects of chronic stress on hippocampus- and mPFC-mediated behaviors.

Note that rats were individually housed and food restricted to increase motivation on the attentional set shifting task (e.g., Lapiz-Bluhm et al., 2008). It is possible that weight loss due to stress may have contributed to deficits on set-shifting performance. However, this is unlikely, as others have shown that neither isolation housing nor food restriction interfere with the development of a stress phenotype in male rats (Flak et al., 2011). Further, stressed rats did not show deficits in acquisition of the simple discrimination, suggesting that motivation was comparable across groups. In contrast, isolation housing can augment the effects of chronic stress in females (Westenbroek et al., 2005). Thus, we cannot rule out the possibility that the females in Experiment 1 experienced increased stress relative to males. However, this possibility does not undermine our major finding that, unlike in males, females do not show immediate deficits in extradimensional set-shifting after chronic stress, but instead these deficits emerge after exposure to a novel acute stressor after a rest period.

4.2 Chronic stress results in both immediate and lasting sex-specific changes in gene expression in prelimbic cortex.

We found that chronic stress increased NR1 mRNA expression in males, but not females. Chronic stress has been shown to downregulate the expression of NR1 protein in males (Wei et al., 2014; Yuen et al., 2012), while others have reported no change in NR1 mRNA expression 48 hours after the cessation of stress (Shepard and Coutellier, 2018). In the present study, tissue was taken on the final day of stress, and acute stress has been shown to increase NR1 protein expression (Yuen et al., 2009). Thus, one possible explanation for the discrepant results is that the increase in NR1 mRNA found here may reflect a transient, acute stress-induced increase in expression that would not have been present 24-48 hours post-stress. Alternatively, NR1 mRNA was even higher after CRS-Rest, and one might expect that an effect of acute stress alone might have moderated to control levels after a rest period. Perhaps a more likely explanation is that post-translational modifications of mRNA can result in poor correlations between gene expression changes and protein levels following a number of experimental manipulations (Vogel and Marcotte, 2012). Thus, the discrepancy in mRNA expression found here and the reduction in NR1 protein found in previous studies (Wei et al., 2014; Yuen et al., 2012), and suggests that there may be post-translational modifications that result in an overall downregulation of protein. In contrast to males, chronic stress did not alter NR1 expression in prelimbic cortex in females. This lack of change following chronic stress is consistent with a lack of change in surface protein in mPFC that has been previously reported following chronic restraint stress in adolescents

(Wei et al., 2014) and mRNA expression following chronic unpredictable mild stress in adults (Shepard and Coutellier, 2018).

In contrast to the pattern of change we observed in NR1 mRNA expression, we found that chronic stress increased parvalbumin expression in females, but not males. Parvalbumin-expressing interneurons account for ~50% of cortical interneurons and play a critical role in maintaining the balance of excitation and inhibition in cortical brain regions by modulating the spike timing of excitatory neurons (reviewed in Ferguson and Gao, 2018b). Previous studies examining the expression of parvalbumin in prelimbic cortex in response to stress have yielded mixed findings. No changes in parvalbumin mRNA (Shepard et al., 2016), and no change (Czéh et al., 2018; Shepard and Coutellier, 2018), increases (Shepard et al., 2016), and decreases (Todorovi et al., 2019) in the number of parvalbumin-positive cells in mPFC of males have been found, while others have shown a decrease in the total amount of parvalbumin protein (Banasr et al., 2017). In contrast, in females, an increase in both parvalbumin-positive cells and parvalbumin mRNA occurs following chronic mild stress (Shepard and Coutellier, 2018; Shepard et al., 2016). Together, our data, along with these previous findings suggest that chronic stress may lead to increased inhibitory tone in mPFC of females but not males. However, others have suggested increased inhibitory tone in the infralimbic subregion of mPFC in males following chronic stress (McKlveen et al., 2016), suggesting there may be regional specificity to the effects of chronic stress on mPFC neurophysiology. In turn, the different patterns of gene expression seen in males and females immediately after chronic stress may contribute to the different patterns of attentional set-shifting performance seen in males and females at this time point post-stress. While this hypothesis is intriguing, note the experimental methods used in Experiments 1 and 2 were not identical. For instance, rats in Experiment 1 were individually housed and food restricted, whereas rats in Experiment 2 were not. Therefore, caution must be used in linking results from Experiments 1 and 2.

In males, parvalbumin-expressing neurons have been implicated in cognitive flexibility, with decreases in parvalbumin-dependent inhibitory transmission predicting impaired cognitive flexibility deficits (Ferguson and Gao, 2018a; Murray et al., 2015; Sparta et al., 2014). Here, we found impaired cognitive flexibility in males after CRS, which coincided with higher levels of parvalbumin expression. Furthermore, females also demonstrated increased parvalbumin expression, but did not demonstrate deficits in cognitive flexibility immediately after CRS. These seemingly contradictory findings may be reconciled by a consideration of the networks that underlie cognitive flexibility. That is, as described recently (Page and Coutellier, 2019), the *balance* of inhibition versus excitation may be critical in regulating cognitive flexibility—and thus perhaps the decreased inhibitory tone in females was offset by alterations—or lack of alterations—in other mPFC circuits that were not assessed in the present study. Finally, it is possible that different neural mechanisms underlie cognitive flexibility despite similar behavioral expression in males and females, thus resulting in different effects of stress despite some similarities in stress-induced changes in gene expression (Bangasser and Wicks, 2017; Becker and Koob, 2016).

Perhaps the most surprising findings from the current study are the sex differences that persist and/or emerge in gene expression following the cessation of chronic stress. In

chronically stressed males, the expression of NR1 was even greater following a rest period than that found immediately after chronic stress. Further, although we found no changes in GluR1, Gad67, or PV in males immediately after chronic stress, the expression of all of these genes was increased following a post-chronic stress rest period. We previously showed that dendritic remodeling in prelimbic cortex of chronically stressed male rats is highly dynamic during the post-stress rest period. Following a 7-day post-chronic stress rest period, chronically stressed male rats have apical dendritic outgrowth relative to unstressed males, despite initial chronic stress-induced dendritic retraction (Moench and Wellman, 2017). Upregulation of NR1 and GluR1 receptor subunits may reflect this increase in dendritic material, while the upregulation of Gad67 and parvalbumin may be indicative of compensatory changes involved in maintaining an optimal excitation-inhibition balance in prelimbic cortex. Given that the chronic stress-induced deficit in extradimensional set-shifting was no longer present at this time, these delayed changes in gene expression may also play a role in the functional recovery of prelimbic cortex following the cessation of chronic stress.

In females, we found that the chronic stress-induced increase in parvalbumin expression persisted through the post-chronic stress rest period, suggesting that the initial increase in inhibitory tone in prelimbic cortex following chronic stress may persist following the cessation of chronic stress. The expression of GluR1 was also increased following a post-chronic stress rest period, although this was the only other gene of interest that was altered in females at this time. This is consistent with our previous finding that, unlike in males, chronically stressed female rats have minimal dendritic remodeling in prelimbic cortex following the cessation of chronic stress (Moench and Wellman, 2017). These findings are consistent with the notion that in females, prelimbic cortex demonstrates less plasticity in response to chronic stress, and suggests different mechanisms of stress adaptation in males versus females.

4.3. A novel stress challenge following a post-chronic stress rest period disrupts extradimensional set-shifting in female rats and results in sex-specific changes in gene expression.

Although we found that chronic stress did not alter behavioral flexibility in females, exposure to a novel stress challenge after a rest period led to a deficit in extradimensional set-shifting. In contrast, while chronic stress reversibly disrupted extradimensional set-shifting in males, exposure to a novel stress challenge following a rest period did not affect set-shifting performance. This pattern suggests that mPFC-mediated behaviors in male rats may be more susceptible to the effects of chronic stress, while these behaviors in females may be more affected by the compounding effects of multiple stressors.

We have previously shown that there are sex differences in neuronal activation across a number of corticolimbic brain regions in chronically stressed rats that are exposed to a novel stress challenge (Moench et al., 2019). Notably, males have a persistent reduction in novel stress-induced neuronal activation in prelimbic cortex. In contrast, prior chronic stress does not modulate neuronal activation in prelimbic cortex in response to a novel stress challenge in females (Moench et al., 2019). In line with this, here we found that the post-chronic stress

increase in parvalbumin mRNA expression was sustained through a novel stress challenge in males, but not in females. Together, these data suggest that males may have enhanced inhibitory signaling in prefrontal cortex following a novel stress challenge that was not present immediately following chronic stress. In contrast, while females appear to have greater inhibitory tone after chronic stress, this inhibition may be reduced following a novel stress challenge, resulting in behavioral deficits that are not found in males.

We also found that chronic stress resulted in different patterns of change in NR1 and GluR1 expression in response to a novel stress challenge in males and females, and that these patterns differed with the presence or absence of a rest period. For example, in males, chronic stress increased NR1 mRNA expression. This increase was prevented when an acute stress challenge was presented on the day following chronic stress. Interestingly, NR1 expression was further increased one week after chronic stress; and the novel acute stress challenge at this time point produced a marked decrease from this new baseline—resulting in expression levels that were comparable in EPS-only and CRS-Rest-EPS males. In contrast, NR1 mRNA expression was only altered in chronically stressed females following a novel stress challenge, but this was only the case when a rest period was present—at which time, unlike in males, EPS induced an increase in NR1 expression. Thus, in chronically stressed males, the presence of a rest period results in an acute stress-induced decrease in NR1 mRNA expression, whereas in chronically stressed females, the presence of a rest period results in an acute stress-induced increase in NR1 mRNA expression that was not present following acute stress alone.

In chronically stressed males, we found a similar pattern of change in GluR1 mRNA expression, whereby the acute stress-induced increase was prevented when chronic stress immediately preceded the acute stress challenge, but not if a rest period was present. In females, although acute stress alone did not alter GluR1 mRNA expression, we found an increase in expression in chronically stressed females exposed to a novel stress challenge only when no rest period was present. Altogether, these data indicate that chronic stress results in sex-specific changes in gene expression in prefrontal cortex following a novel stress challenge, and that these changes are further modulated by the presence or absence of a post-chronic stress rest period.

4.5. Conclusions

Our results contribute to a growing interest in the lasting effects of stress on brain regions critical for executive functioning and emotion regulation (Ortiz and Conrad, 2018), and support the notion that the process of “recovery” from chronic stress does not simply involve a return to an unstressed state. Instead, it is likely that exposure to chronic stress results in the recruitment of stress adaptation mechanisms that influence responsivity to future stressors. Further, the findings from this study suggest that that this recruitment of stress adaptation mechanisms may be sex-specific. This may represent a point of divergence in risk and resilience between males and females, whereby males may be buffered to some degree against the deleterious effects of multiple stressors. In contrast, females may be more susceptible to the compounding effects of stress. This, in combination with other known risk factors, may increase susceptibility for the development of stress-related disorders.

Understanding sex differences in the lasting effects of chronic stress, and how other known risk factors interact with post-stress changes will contribute to a better understanding of the etiologies of stress-related psychological disorders, especially those in which prevalence differs between men and women.

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Highlights

- Chronic restraint stress (CRS) leads to a reversible deficit in extradimensional set-shifting in males.
- Acute stress following a post-CRS rest period leads to behavioral deficits in females.
- Sex-specific changes in gene expression in prelimbic cortex (PL) emerge after a post-CRS rest period.
- Post-CRS changes in PL may result in sex-specific responsivity to a novel stressor.
- Males and females likely recruit different stress adaptation mechanisms.

Figure 1.

(A) Experimental design and timeline for Experiment 1. Male and female rats were either left undisturbed (No CRS-EPS) or exposed to chronic restraint stress for 10 days (CRS-EPS-Rest/CRS-Rest-EPS). The first test session for No CRS-EPS and CRS-Rest-EPS rats occurred on the day following chronic stress, or the corresponding day in unstressed rats. Rats in the CRS-EPS-Rest group were exposed to elevated platform stress (EPS) on the day following chronic stress and the day prior to the first test session. No CRS-EPS and CRS-Rest-EPS rats were exposed to EPS following a rest period. The second test session occurred on day 18 for all groups. (B) Experimental design and timeline for Experiment 2. Male and female rats were either left undisturbed or exposed to CRS for 10 days. Rats either remained unstressed (No Stress) or were exposed to EPS (EPS Only). CRS rats were either 1) euthanized on the final day of restraint (CRS Only); 2) exposed to EPS on the day following CRS prior to euthanasia (CRS-EPS); 3) given a 7 day rest period following CRS (CRS-Rest); or 4) exposed to EPS 7 days following the cessation of CRS (CRS-Rest-EPS). (C) Schematic diagram depicting location of micropunch samples from prelimbic (PL) cortex. Samples were obtained using gross morphological criteria (Paxinos and Watson, 1998). For simplicity, only one hemisphere is shown.

Figure 2.

Basal sex differences in the attentional set-shifting test. For the extradimensional shift, unstressed male rats required more trials to reach criterion than unstressed females. SD, simple discrimination; CD, compound discrimination; Rev, reversal; IDS, intradimensional shift; EDS, extradimensional shift. * $p < 0.05$. Error bars represent SEM.

Figure 3.

The effects of stress on attentional set-shifting in male rats. (A) Chronic restraint stress (CRS) and CRS followed one day later by elevated platform stress (EPS) impairs performance on the extradimensional shift compared to unstressed males. (B) EPS alone does not alter performance on any phase of the test in previously unstressed rats. (C) Extradimensional shifting is no longer impaired following a rest period in male rats that were first tested immediately following CRS and EPS. (D) Extradimensional shifting is not impaired in male rats who underwent CRS, were given a rest period, and then exposed to EPS. Intradimensional shifting is also facilitated in the second test session in these males. SD, simple discrimination; CD, compound discrimination; Rev, reversal; IDS, intradimensional shift; EDS, extradimensional shift. * $p < 0.05$. Error bars represent SEM.

Figure 4.

The effects of stress on attentional set-shifting in female rats. (A) Chronic restraint stress (CRS) and CRS followed one day later by elevated platform stress (EPS) do not impair performance on any phase of the test compared to unstressed females. (B) EPS alone does not alter performance on any phase of the test in previously unstressed rats. (C) Task performance before and after a rest period is unchanged in females exposed to chronic stress followed one day later by EPS. (D) Extradimensional shifting is impaired in female rats who underwent CRS, were given a rest period, and then exposed to EPS. Trials to criterion are also increased in the simple discrimination phase in these females. SD, simple discrimination; CD, compound discrimination; Rev, reversal; IDS, intradimensional shift; EDS, extradimensional shift. * $p < 0.05$. Error bars represent SEM.

Figure 5.

Chronic stress attenuates weight gain in both male and female rats. Error bars represent SEM. CRS, chronic restraint stress; EPS, elevated platform stress. * $p < 0.05$ compared to No Stress rats of same sex. Error bars represent SEM.

Figure 6.

Basal sex differences in gene expression. Relative NR1 expression is higher in No Stress females than males. PV, parvalbumin; SST, somatostatin. * $p < 0.05$. Error bars represent SEM.

Figure 7.

Expression of NR1 and GluR1 in males and females. (A) In males, both elevated platform stress (EPS) and chronic restraint stress (CRS) alone increased NR1 expression. This increase following CRS was enhanced following a rest period. The combination of EPS and CRS also resulted in increased NR1, but only when a rest period was present. (B) In females, the combination of CRS and EPS increased the expression of NR1 only when a rest period was present. (C) EPS alone increased GluR1 expression in males, but this increase was prevented when EPS was immediately preceded by CRS. GluR1 expression was not

altered by CRS alone, but was greater in CRS-Rest males. (D) GluR1 expression was increased in CRS females after a rest period or after exposure to EPS without a rest period. Error bars represent SEM. * $p < 0.05$ compared to No Stress; + $p < 0.05$ compared to all other groups; ^ $p < 0.05$.

Figure 8.

Expression of Gad67 and parvalbumin (PV) in males and females. (A) Gad67 expression was not altered by elevated platform stress (EPS) or chronic restraint stress (CRS) alone, but was increased in CRS males following a rest period. This increase following CRS and rest period was also present following EPS. (B) Gad67 expression was not altered by stress in females. (C) PV expression was not altered by EPS or CRS, but was increased in CRS-Rest males. PV expression was also increased in males that were exposed to both EPS and CRS, but this was only the case if a rest period separated the two. (D) CRS significantly increased

the expression of PV in females, and this increase was still present following a rest period. In contrast, PV expression was reduced following EPS in CRS females after a post-CRS rest period. Error bars represent SEM. * $p < 0.05$ compared to No Stress; ^ $p < 0.05$.

Table 1.

Stimuli used in the AST.

	Training Pair	Test Pair 1	Test Pair 2	Test Pair 3
Odor	Cinnamon/Oregano	Blueberry/Lavender	Ginger/Nutmeg	Cherry/Lemon
Texture	Bubble wrap/Towel	Twine/Pebbles	Wood Balls/Pom poms	Glass tiles/Beads

Table 2.

Representative example of stimulus pairing on the AST.

Discrimination Stage	Dimensions		Example Combinations	
	Relevant	Irrelevant	(+)	(–)
Simple (SD)	Odor		Blueberry	Lavender
Compound (CD)	Odor	Texture	Blueberry /Twine Blueberry /Pebbles	Lavender/Pebbles Lavender/Twine
Reversal 1 (Rev1)	Odor	Texture	Lavender /Pebbles Lavender /Twine	Blueberry/Twine Blueberry/Pebbles
Intradimensional Shift (IDS)	Odor	Texture	Ginger /Wood Balls Ginger /Pom Poms	Nutmeg/Pom Poms Nutmeg/Wood Balls
Reversal 2 (Rev2)	Odor	Texture	Nutmeg /Pom Poms Nutmeg /Wood Balls	Ginger/Wood Balls Ginger/Pom Poms
Extradimensional Shift (EDS)	Texture	Odor	Lemon/ Beads Cherry/ Beads	Cherry/Tiles Lemon/Tiles

Table 3.

Primer specifications for qPCR.

Gene	Function	Primer Sequence (5' – 3')	Reference
NR1	NMDA receptor subunit	F – TGGTAGAGCAGAGCCCGACCC R – CCCCGGTGCTCGTGTCTTTGG	Lopes et al. (2013)
NR2A	NMDA receptor subunit	F – AGCCCCCTTCGTCATCGT R – GACAGGGCACCGTGTTCCT	Pershing et al. (2016)
NR2B	NMDA receptor subunit	F – CCCAACATGCTCTCTCCCTTAA R – CAGCTAGTCGGCTCTCTTGGTT	Lindenbach et al. (2015)
GluR1	AMPA receptor subunit	F – ATGCTGACCTCCTTCTGTGG R – TCCTGTAGTTCCGGGCGTAG	Sadri-Vakili et al. (2010)
Gad67	GABA synthesis enzyme	F – GCTGGAAGGCATGGAAGGTTTTA R – ACGGGTGCAATTCATATGTGAACATA	Jaenisch et al. (2014)
PV	Calcium-binding protein	F – AGCCTTTACTGCTGCAGACTCCTT R – AGTCATCCTCCTCAATGAAGCCA	Bastian et al. (2014)
SST	Neuropeptide	F – GCCACCGGAAACAGGAAGTGG R – GGGTGCCATGGCTGGGTTCG	Hou and Yu (2013)
GAPDH	Glycolysis	F – ACCACAGTCCATGCCATCACTG R – GATGACCTTGCCCACAGCCTT	Bollinger et al. (2016)

Table 4.

Stress effects on NR2A, NR2B, and SST gene expression of males and females.

Sex	Stress	NR2A ($2^{-CT} \pm \text{SEM}$)	NR2B ($2^{-CT} \pm \text{SEM}$)	SST ($2^{-CT} \pm \text{SEM}$)
Male	No Stress	1.00 ± 0.15	1.00 ± 0.15	1.00 ± 0.22
	EPS Only	1.97 ± 0.37	1.14 ± 0.18	1.32 ± 0.06
	CRS Only	1.14 ± 0.27	1.36 ± 0.29	1.31 ± 0.23
	CRS-Rest	1.56 ± 0.23	0.97 ± 0.15	1.00 ± 0.17
	CRS-EPS	1.22 ± 0.39	0.68 ± 0.12	0.78 ± 0.15
	CRS-Rest-EPS	1.83 ± 0.33	1.07 ± 0.17	1.17 ± 0.21
Female	No Stress	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.25
	EPS Only	1.42 ± 0.24	1.36 ± 0.23	1.17 ± 0.20
	CRS Only	1.08 ± 0.17	0.92 ± 0.21	1.13 ± 0.19
	CRS-Rest	1.78 ± 0.35	1.62 ± 0.31	1.40 ± 0.24
	CRS-EPS	1.27 ± 0.17	1.20 ± 0.26	1.09 ± 0.18
	CRS-Rest-EPS	1.17 ± 0.27	0.69 ± 0.16	0.70 ± 0.17