

Sex-Specific Effects of Stress on Mood-Related Gene Expression

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Abstract

Women are twice as likely as men to be diagnosed with major depressive disorder (MDD). Recent studies report distinct molecular changes in depressed men and women across mesocorticolimbic brain regions. However, it is unclear which sex-related factors drive distinct MDD-associated pathology. The goal of this study was to use mouse experimental systems to investigate sex-specific mechanisms underlying the distinct molecular profiles of MDD in men and women. We used unpredictable chronic mild stress to induce an elevated anxiety-/depressive-like state and “four core genotypes” (FCG) mice to probe for sex-specific mechanisms. As predicted, based on previous implications in mood, stress impacted the expression of several dopamine-, GABA-, and glutamate-related genes. Some of these effects, specifically in the prefrontal cortex, were genetic sex-specific, with effects in XX mice but not in XY mice. Stress also impacted gene expression differently across the mesocorticolimbic circuit, with increased expression of mood-related genes in the prefrontal cortex and nucleus accumbens, but decreased

expression in basolateral amygdala. Our results suggest that females are sensitive to the effects of chronic stress, partly due to their genetic sex, independent of gonadal hormones. Furthermore, these results point to the prefrontal cortex as the node in the mesocorticolimbic circuitry with the strongest female-specific effects.

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Introduction

Every year, 350 million people suffer from major depressive disorder (MDD) globally [1]. MDD is a leading cause of disease burden. In 2010, it had an estimated economic burden of \$210.5 billion, significantly higher than the \$173.2 billion estimate in 2005 [2]. MDD symptoms are characterized by overall emotion dysregulation, low mood, anhedonia, and poor affect, among others [3]. Current treatments are only effective at inducing remission in a portion of the population [4], and men

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and women respond differently to pharmacological treatment [5]. Cross-cultural epidemiological studies that control for reporting biases have found that depression affects women at twice the rate that it affects men. When diagnosed, women report a higher number of symptoms, and rate their symptoms more severely than men [6–10].

Traditional studies looking at the molecular mechanisms of depression have often investigated only a single brain region. However, this provides an incomplete picture, and looking at how pathways are affected across regions may be more informative in the understanding of the disease at the network and circuit level [11, 12]. By investigating stress-induced transcriptional changes across major nodes of a circuit, it may be possible to gain a greater insight into how these molecular pathways are affected in disease.

We therefore selected three regions from the mesocorticolimbic circuit implicated in depression neuropathology, i.e., the prefrontal cortex (PFC), basolateral amygdala (BLA), and nucleus accumbens (NAc) [13], with the intention of comparing gene pathway changes across regions. Prior studies suggest that these regions are affected in depression, with possible region-specific effects. For example, blood oxygenation level-dependent (BOLD) imaging studies report activity differences in these regions, with an increase in BLA activity at baseline, and a decrease in NAc and PFC activity during emotion tasks (e.g., [14–22]). At the molecular level, our lab and others have reported large transcriptional changes in MDD across the circuit. Furthermore, we recently reported vastly different molecular signatures between men and women in the PFC and BLA of postmortem brains from MDD subjects [23].

Within these regions, we looked at three candidate gene pathways that have been previously implicated in mood regulation: (1) γ aminobutyric acid (GABA), (2) glutamate, and (3) dopamine. We selected GABA, the major inhibitory neurotransmitter, as GABA dysfunction in MDD has been heavily implicated in both animal and human studies across the circuit [24–27]. We previously reported a sex-specific reduction in SST, a GABA interneuron marker, in the BLA of women with MDD, but not in men [28]. We also selected glutamate, the major excitatory neurotransmitter, as dysfunction in this pathway has also been implicated in MDD [27]. There appear to be sex differences within this pathway at the molecular level in the dorsolateral (DL)PFC. For instance, depressed women exhibit increased expression of glutamate-related genes compared to healthy women, but these genes are

not affected in depressed men [29]. Furthermore, surmounting evidence suggests sex-dependent effects of ketamine, an antidepressant believed to modulate glutamate signaling in the brain, thus potentially indicating a different underlying pathology in men and women [30, 31]. Lastly, we looked at dopamine-related genes due to the clear ties to reward and motivation circuitry [32, 33]. Anhedonia and amotivation are classical depressive symptoms in humans that may be linked specifically to dopamine pathway dysfunction within the NAc [34] but can also involve other regions such as the PFC [35]. Previous labs have linked dopamine neurons specifically to depressive behaviors in animals [36], further warranting an in-depth investigation.

The overall goal of this study was to use mouse experimental systems to investigate the potential sex-specific mechanisms underlying the distinct molecular profiles of depression that we revealed in men and women. We used unpredictable chronic mild stress (UCMS) to induce an elevated anxiety-/depressive-like state. Furthermore, to probe for sex-specific mechanisms, we used “four core genotypes” (FCG) mice, in which we could independently examine the effects of adult circulating hormones, the developmental organizational effects of hormones, and X/Y genetic sex [37]. We examined if males and females exhibited similar or distinct molecular changes in response to stress exposure, with a focus on whether the same molecular pathways were influenced similarly across the relevant brain regions.

Materials and Methods

Mice

FCG mice were used in all experiments. In humans and animals, the *Sry* gene on the Y-chromosome encodes for testes. Thus, genetic sex and gonadal sex are linked. However, in FCG mice, the *Sry* gene is on an autosome, making it possible to independently investigate developmental organizational effects of hormones (testes vs. ovaries) and genetic sex (XY vs. XX). In addition, it is possible to gonadectomize adult FCG mice to separate the effects of circulating hormones from organizational hormonal effects. The three sex differences can thus be examined independently of one another. The four possible genotypes are: XX females (XXF), XX-*Sry* males (XXM), XY*Sry* males (XYM), and XY females (XYF). Throughout our experiments, mice were group-housed (3–5 mice/cage) and maintained under standard conditions (12 h:12 h light-dark cycle; 22 ± 1 °C, food and water ad libitum).

Hormone Manipulation

At approximately 12 weeks of age, all mice underwent GDX surgeries to remove the gonads, ensuring the elimination of circulating gonadal hormones. At the time of surgery, mice were given

a silastic capsule (Dow Corning Corp., Midland, MI, USA) that contained either 5 mm of crystalline testosterone, or a size-matched blank capsule. Within each genotype, half of the mice were randomly assigned to be implanted with testosterone-filled capsules and half were implanted with blank capsules. A testosterone capsule of this size produces circulating testosterone levels that mimic those of a normal adult male [38]. After surgery, the mice were allotted 3 weeks of recovery before starting the experimentation process.

Unpredictable Chronic Mild Stress

We used the extensively validated model known as UCMS to induce an elevated depressive-/anxiety-like state in the mice [28, 38, 39]. UCMS elicits a state similar to that of MDD in humans. We exposed mice to a random schedule of psychosocial stressors over 8 weeks. Example stressors were altered light cycles, mild restraint, social stress, wet or no bedding, predator odor, reduced space, a forced bath, and a tilted cage. Weight and the state of the fur were monitored and recorded weekly to track the progression of UCMS syndrome. Nonstressed mice were not exposed to stressors, but their weight and the state of their fur were monitored weekly with mild handling.

Gene Selection and Primer Design

Genes were selected from three major mood-related pathways: GABA, glutamate, and dopamine. For GABA, we examined genes coding for three receptor subunits (GABA-A receptor subunit $\alpha 2$ [*Gabra2*], GABA-A receptor subunit $\alpha 5$ [*Gabra5*], and GABA-B receptor subunit 2 [*Gabbr2*]), GABA receptor-anchoring protein gephyrin (*Gphn*), GABA transporter (*Gat1*), GABA type A receptor-associated protein (*Gabarap*), and GABA type A receptor-associated protein-like 1 (*Gabarapl1*). For glutamate, we examined genes coding for two glutamate ionotropic α amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (glutamate receptors 1 [*Gria1*] and 3 [*Gria3*]), metabotropic glutamate receptor 1 (*Grm1*), ionotropic kainate 3 glutamate receptor (*Grik3*), N-methyl-D-aspartate (NMDA) receptor subunit 3A (*Grin3a*), mitochondrial glutamate carrier 1 (*Slc25a22*), and glutamate receptor-interacting protein 1 (*Grip1*). For dopamine, we examined genes coding for three dopamine receptors (*Drd1*, *Drd2*, and *Drd5*), catechol-O-methyltransferase (*Comt*), monoamine oxidases A (*Maoa*) and B (*Maob*), cAMP-responsive element-binding proteins 1 (*Creb1*) and 3 (*Creb3*), CREB-binding protein (*Crebbp*), and DOPA decarboxylase (*Ddc*).

Quantitative PCR

Upon sacrifice, brains were flash-frozen on dry ice and stored at -80°C . Rostrocaudal sections (160- μm -thick) were obtained using a cryostat, and tissue punches were used to isolate the PFC (bregma 2.34–0.50 mm; includes prelimbic and cingulate cortices), NAc (bregma 0.74–0.38 mm), and BLA (bregma -0.94 to -1.82 mm) [40]. The locations of tissue punches are shown in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000499105). RNA was isolated and extracted using RNeasy Plus Micro kits (Qiagen, Valencia, CA, USA) with Qiashtredder columns (Qiagen). RNA concentration was determined using a Qubit fluorometer (Invitrogen), and RNA integrity was measured with a bioanalyzer (Agilent). RNA from mice ($n = 3$) was pooled into a single sample to reduce variability across replicates. We used 3 replicates per group, giving a total of

144 samples (3 replicates \times 4 genotypes \times 2 hormone treatments \times 3 brain regions \times 2 stress conditions). RNA (15 μL /sample) was converted into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA), and quantities of cDNA were normalized across samples. Quantitative (q)PCR was run on a CFX 96 real-time PCR (BioRad) using SsoAdvanced Universal SYBR Green Supermix (BioRad). We ran each gene and sample combination in triplicate and derived the mean of the replicates. Results were then calculated as the geometric mean of the relative intensities compared to 2 housekeeping genes, cyclophilin and GAPDH. Importantly, these housekeeping genes do not differ by sex or stress. We then transformed this value to the arbitrary signal using the formula: $2^{-\Delta\Delta\text{CT}} \times 10,000$. Primer sequences are included in online supplementary Table 1.

Data Analysis

Data were analyzed in SPSS. We first examined whether expression values for each gene were normally distributed, using the Kolmogorov-Smirnov test for normality. If a gene's expression was not normally distributed, we transformed the data using a rank-based inverse normal transformation [41]. We then performed a 4-way ANOVA (stress \times genetic sex \times gonadal sex \times hormone). If an interaction of stress and a sex-related factor was identified, we performed a 2-way ANOVA (stress \times sex-related factor), followed by Tukey's post hoc test. We report interaction statistics (e.g., interaction of stress and genetic sex) from the 4-way ANOVA and post hoc statistics from the 2-way ANOVA. $p < 0.05$ was considered statistically significant and data are expressed as mean \pm standard error of the mean (SEM). To test for potential type I errors for main effects, we controlled the false discovery rate (set at 5%) using the Benjamini-Hochberg method [42]; the number of genes in each category was used as the total number of tests (7 for GABA-related genes, 7 for glutamate-related genes, and 10 for dopamine-related genes).

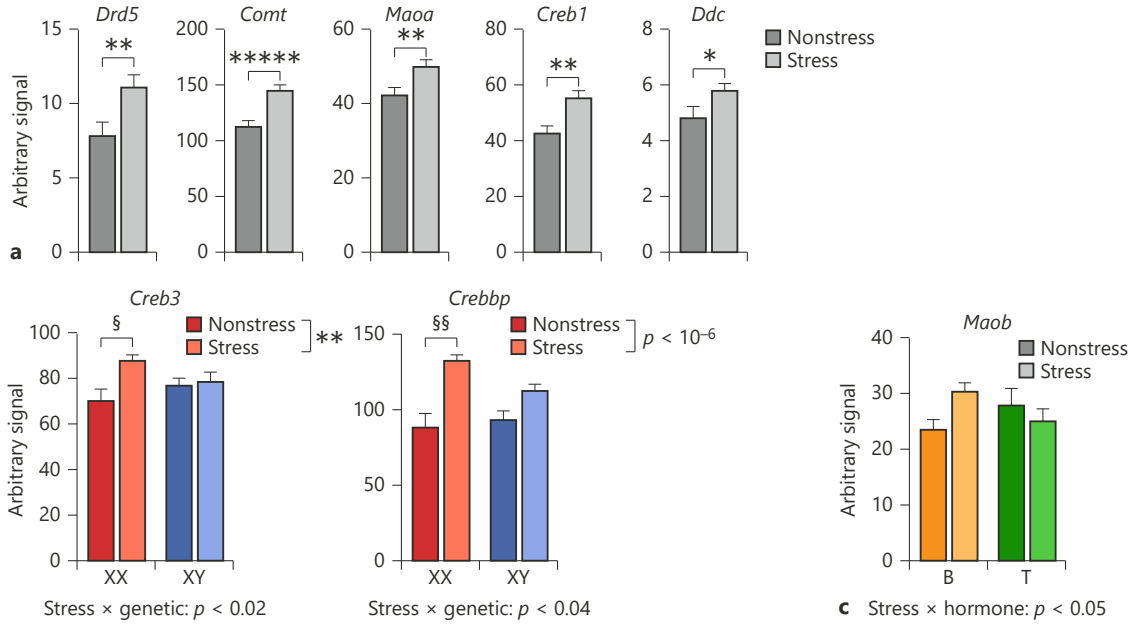
Heatmaps

We also report total patterns of gene expression changes using heatmap visualizations (of the effects of stress for each sex-related

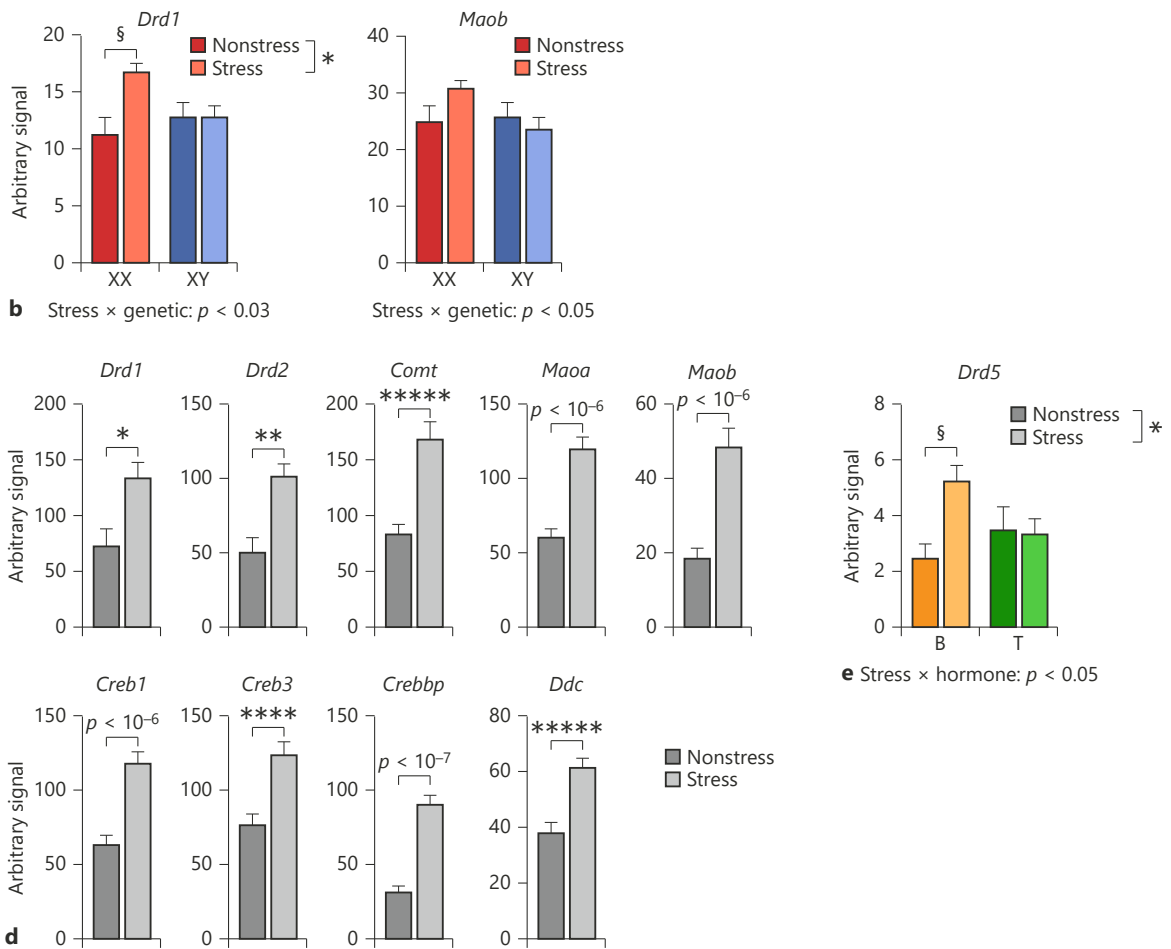
Fig. 1. Effects of chronic stress exposure on expression of dopamine-related genes. **a** In the PFC, stress induced an increase in expression of *Drd5* ($p < 0.01$), *Comt* ($p < 10^{-5}$), *Maoa* ($p < 0.006$), *Creb1* ($p < 0.002$), and *Ddc* ($p < 0.04$) ($n = 12/\text{group}$). **b** In the PFC, there were significant interactions between stress and genetic sex on expression of *Creb3* ($p < 0.02$), *Crebbp* ($p < 0.04$), *Drd1* ($p < 0.03$), and *Maob* ($p < 0.05$); stress increased expression of these genes in XX mice but not XY mice ($n = 6/\text{group}$). **c** In the PFC, there was a significant interaction of stress and testosterone exposure on expression of *Maob* ($p < 0.025$) ($n = 6/\text{group}$). **d** In the NAc, stress increased expression of *Drd1* ($p < 0.02$), *Drd2* ($p < 0.0025$), *Comt* ($p < 10^{-5}$), *Maoa* ($p < 10^{-6}$), *Maob* ($p < 10^{-6}$), *Creb1* ($p < 10^{-6}$), *Creb3* ($p < 10^{-4}$), *Crebbp* ($p < 10^{-7}$), and *Ddc* ($p < 10^{-5}$) ($n = 12/\text{group}$). **e** In the NAc, there was a significant interaction of stress and hormone exposure on expression of *Drd5* ($p < 0.03$). Main effects of stress: * $p < 0.05$; ** $p < 0.01$; **** $p < 10^{-4}$; ***** $p < 10^{-5}$. Post hoc 2-group tests performed after significant interaction: § $p < 0.01$; §§ $p < 0.0001$.

(For figure see next page.)

PFC



NAC



group). Heatmaps representing our expression data were created using the free browser program Matrix2png [43]. Data tables for each brain region were constructed by using the ratio of stress-to-nonstress of the means for all experimental groups (ovaries, testes, XX, XY, blank capsule, and testosterone capsule). Data are represented as positive and negative values based on the relative expression of the nonstress and stress groups.

Gene Networks

Gene networks were generated for XX nonstress, XX stress, XY nonstress, and XY stress conditions using the PFC gene expression data ($n = 12/\text{group}$). Two genes are considered coexpressed if their expression patterns are correlated across samples, which may reflect a shared function or shared upstream regulatory pathway [44, 45]. The correlation strength was measured using the Pearson correlation coefficient raised by power 3 (i.e., $|r|^3$). Networks were generated using coexpression values in which $|r|^3 > 0.3$; some genes are excluded from networks if none of the coexpression values hits this threshold. The node size is proportional to the degree for that gene, which is calculated as the number of edges linked to that gene. Edges between nodes are proportional to the correlation ($|r|^3$) between the two nodes. Within Cytoscape (3.5.0), edge-weighted spring embedded layout function using $|r|^3$ determined the network layout. Within each network, we calculated the density and global-clustering coefficient as global network parameters. The density of a network indicates how many edges are present in a network compared to the maximum possible number of edges, with a maximum value of 1. The global-clustering coefficient measures the extent to which nodes in a network tend to cluster together, with a maximum value of 1. To test whether global network parameters (density and clustering coefficient) differed between the nonstress and stress conditions, we generated a null distribution of clustering coefficients and density separately, through permutation, and then created confidence intervals (CIs). To compare 2 groups, we then determined if the CIs overlapped. If they did not overlap, we rejected the null hypothesis that the two groups would have the same clustering coefficient or density.

Results

The Effects of Stress on Dopamine-Related Genes

In the PFC, we found that stress affected expression of several dopamine-related genes. There was a main effect of stress on expression of several genes (Fig. 1a). Specifically, stress exposure caused an increase in expression of *Drd5* ($p < 0.01$), *Comt* ($p < 10^{-5}$), *Maoa* ($p < 0.006$), *Creb1* ($p < 0.002$), and *Ddc* ($p < 0.04$). There were also several genes with interactions of stress and a sex-related factor. There was a significant interaction of genetic sex and stress on expression of *Creb3* ($p < 0.02$), *Crebbp* ($p < 0.04$), *Drd1* ($p < 0.03$), and *Maob* ($p < 0.05$); stress increased expression in XX mice but not in XY mice (Fig. 1b). There was also a significant interaction of hormone exposure and stress on expression of *Maob* ($p < 0.025$; Fig. 1c), with stress increasing expression of *Maob*

in blank-treated mice but not in testosterone-treated mice (online suppl. Table 2: summary of statistical results).

In the BLA, we found that stress had less of an effect on dopamine-related genes than in the PFC. There were no main effects of stress and no effect of an interaction of stress and any sex-related factor on expression of dopamine-related genes in the BLA (online suppl. Table 2: summary of statistical results).

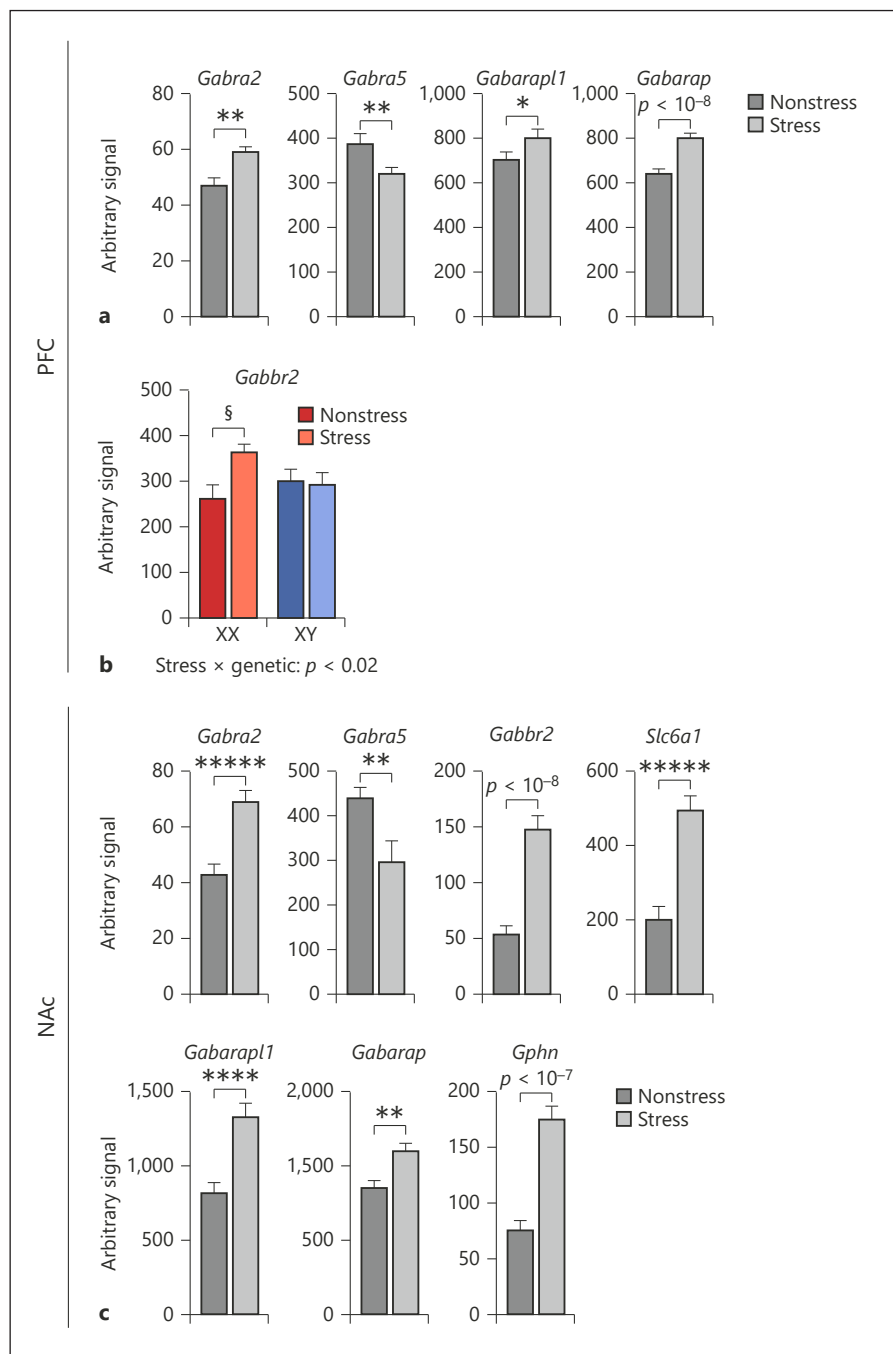
We found robust main effects of stress on expression of dopamine-related genes in the NAc (Fig. 1d). Specifically, we found that stress caused an increase in expression of *Drd1* ($p < 0.02$), *Drd2* ($p < 0.0025$), *Comt* ($p < 10^{-5}$), *Maoa* ($p < 10^{-6}$), *Maob* ($p < 10^{-6}$), *Creb1* ($p < 10^{-6}$), *Creb3* ($p < 10^{-4}$), *Crebbp* ($p < 10^{-7}$), and *Ddc* ($p < 10^{-5}$). There was a significant interaction of stress and hormone exposure on expression of *Drd5* ($p < 0.03$; Fig. 1e); stress increased expression in blank-treated mice but not in testosterone-treated mice (online suppl. Table 2: summary of statistical results).

The Effects of Stress on GABA-Related Genes

In the PFC, we found that stress affected expression of many GABA-related genes. First, we found main effects of stress on GABA-related genes (Fig. 2a). Specifically, stress exposure increased expression of *Gabra2* ($p < 0.003$), *Gabarapl1* ($p < 0.02$), and *Gabarap* ($p < 10^{-8}$); the only gene to be significantly decreased by stress was *Gabra5* ($p < 0.002$). We also observed a significant interaction of stress and genetic sex on expression of *Gabbr2* ($p < 0.02$; Fig. 2b), where stress increased expression in XX mice but not in XY mice (online suppl. Table 2: summary of statistical results).

We then looked at the BLA in the same mice to see how stress impacted expression of GABA-related genes. However, there were no main effects of stress or interactions of stress and any sex-related factor on these GABA-related genes in the BLA (online supplementary Table 2: summary of statistical results).

Within the NAc, we found that stress generally increased expression of GABA-related genes, with similar effects across sex-specific groups (Fig. 2c). There were main effects of stress increasing expression of *Gabra2* ($p < 10^{-5}$), *Gphn* ($p < 10^{-7}$), *Gabarap* ($p < 0.004$), *Gabarapl1* ($p < 10^{-4}$), *Gabbr2* ($p < 10^{-8}$), and *Gat1* ($p < 10^{-6}$). *Gabra5* was the only gene in the NAc with reduced expression upon stress exposure ($p < 0.01$). There was no significant interaction of a sex-related factor and stress on expression of GABA-related genes in the NAc (online suppl. Table 2: summary of statistical results).



The Effects of Stress on Glutamate-Related Genes

In the PFC, glutamate-related genes were generally upregulated by stress, with several of these effects being sex-specific. There were main effects of stress on expression of *Gria1* ($p < 10^{-5}$), *Slc25a22* ($p < 0.02$), and *Grip1* ($p < 0.003$) (Fig. 3a); stress increased expression of *Gria1* and *Grip1* and decreased expression of *Slc25a22*. We observed

a significant interaction of stress and genetic sex on expression of *Gria3* ($p < 0.03$) and *Grin3a* ($p < 0.005$); specifically, stress increased expression in XX mice but not in XY mice (Fig. 3b). We also observed a significant interaction of stress and hormone treatment on expression of *Gria3* ($p < 0.04$), where stress increased expression in blank-treated mice but with no effects in testosterone-

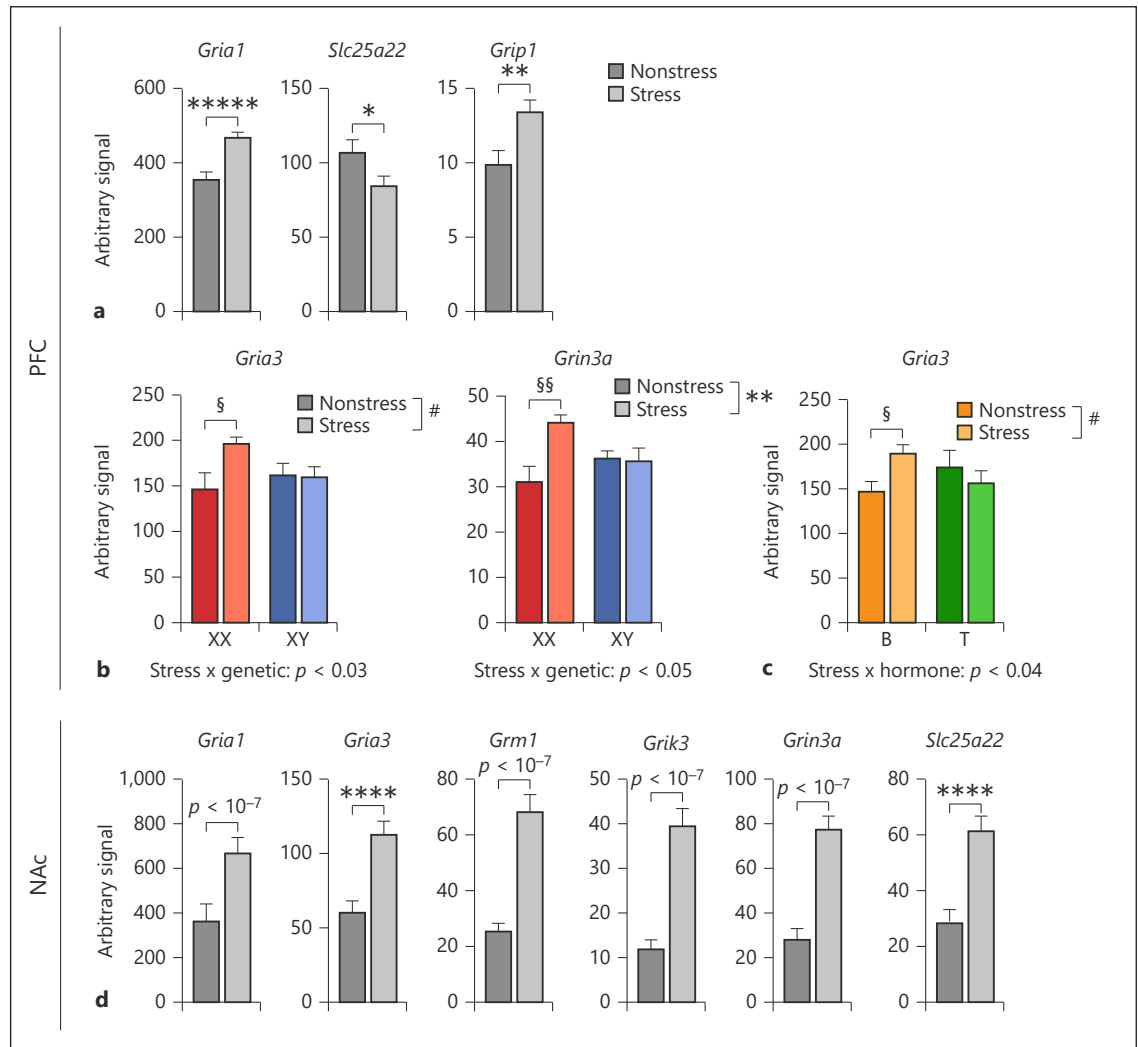


Fig. 3. Effects of chronic stress exposure on expression of glutamate-related genes. **a** In the PFC, stress increased expression of *Gria1* ($p < 10^{-5}$) and *Grip1* ($p < 0.003$) and decreased expression of *Slc25a22* ($p < 0.02$) ($n = 12/\text{group}$). **b** In the PFC, there was a significant interaction of stress and genetic sex on expression of *Gria3* ($p < 0.03$) and *Grin3a* ($p < 0.005$), with stress increasing expression only in XX mice ($n = 6/\text{group}$). **c** In the PFC, there was a significant interaction of stress and testosterone (T) exposure on expression

of *Gria3* ($p < 0.04$), with stress increasing expression in blank (B)-treated mice but not in T-treated mice ($n = 6/\text{group}$). **d** In the NAc, stress increased expression of *Gria1* ($p < 10^{-7}$), *Gria3* ($p < 10^{-4}$), *Grm1* ($p < 10^{-7}$), *Grik3* ($p < 10^{-7}$), *Grin3a* ($p < 10^{-7}$), and *Slc25a22* ($p < 10^{-4}$) ($n = 12/\text{group}$). Main effects of stress: # $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; **** $p < 10^{-4}$; ***** $p < 10^{-5}$. Post hoc 2-group tests performed after significant interaction: § $p < 0.05$; §§ $p < 0.01$.

treated mice (Fig. 3c) (online supplementary Table 2: summary of statistical results).

In the BLA, we did not identify any main effects of stress or interaction of stress and any sex-related factor on expression of glutamate-related genes in the BLA (online suppl. Table 2: summary of statistical results).

In the NAc, stress increased expression of glutamate-related genes regardless of sex (Fig. 3d). Specifically, there were main effects of stress increasing expression of *Gria1*

($p < 10^{-7}$), *Gria3* ($p < 10^{-4}$), *Grm1* ($p < 10^{-7}$), *Grik3* ($p < 10^{-7}$), *Grin3a* ($p < 10^{-7}$), and *Slc25a22* ($p < 10^{-4}$). There were no interactions of stress and any sex-related factor on expression of glutamate-related genes in the NAc (online suppl. Table 2: summary of statistical results).

Heatmap Representation of Gene Expression Changes

To summarize the gene expression data and extract the patterns of stress effects, we generated a heatmap in-

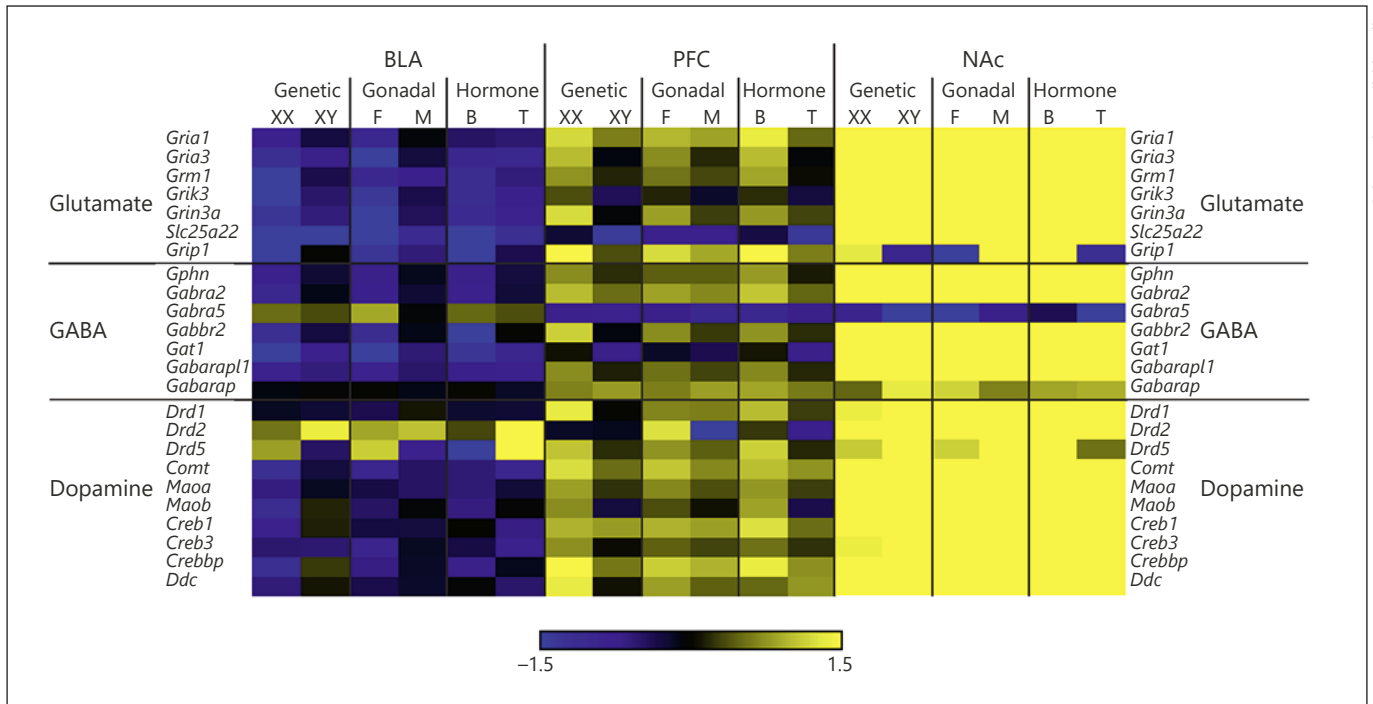


Fig. 4. Heatmap representation of gene expression results. The effect of chronic stress on gene expression in the BLA, PFC, and NAc. Yellow indicates that chronic stress increased gene expres-

sion, while blue indicates that chronic stress decreased gene expression. B, blank-treated; F, gonadal female; M, gonadal male; T, testosterone-treated.

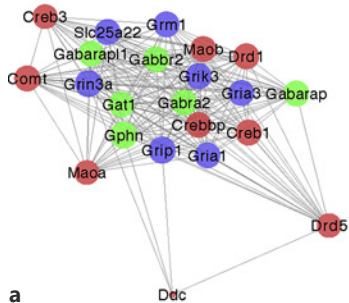
dicating these effects within each sex-related factor (Fig. 4). We also plotted results for each brain region, in order to extract general patterns that are consistent across brain regions or may be region-specific. The most striking feature of the heatmap is that stress influenced gene expression differentially across brain regions. Overall, stress increased expression of the investigated glutamate-/GABA-/dopamine-related genes in the PFC and NAc but decreased their expression in the BLA. There are, however, some exceptions to this pattern. For instance, *Gabra5* exhibited the opposite stress effect in each brain region, with stress decreasing expression in the PFC and NAc but increasing expression in the BLA. The other notable feature of the heatmap is that the effects of stress were often more pronounced in the female (XX, gonadal female, and/or blank-treated) than in the male (XY, gonadal male, and/or testosterone-treated) phenotypes. The strongest female-specific effects were in the PFC, followed by the BLA. Interestingly, however, stress impacted female and male phenotypes similarly in the NAc. There were also some genes for which stress affected male and female phenotypes in opposite directions within the same brain region; this pattern was pres-

ent for *Drd2* in the PFC, *Drd5* in the BLA, and *Grip1* in the NAc.

Stress Disrupted the Gene Coexpression Networks

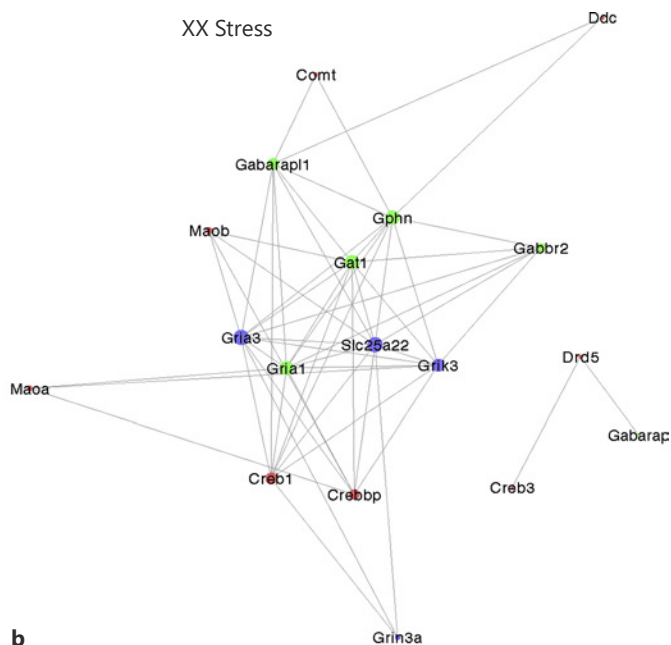
We next generated weighted gene coexpression networks to investigate the effect of stress on coordinated gene expression patterns. Gene coexpression networks can be useful when considering a disease state in which multiple genes are affected; in other words, several small changes in gene expression might converge to produce the disease state [12]. Given that there were several interactions of genetic sex and stress in the PFC (stress affected gene expression in XX mice but not in XY mice), we examined how stress influenced gene coexpression in the PFC for XX and XY mice. Nonstressed XX mice exhibited a highly coordinated gene expression network (Fig. 5a), indicated by the high clustering coefficient (0.978) and high global density (0.921). Interestingly, these network measures were significantly reduced in stressed XX mice (clustering coefficient = 0.677; density = 0.380; $p < 0.05$ for both measures; Fig. 5b), together indicating a much less coordinated gene network in stressed XX mice. In XY mice, the effect of stress on network statistics was not sig-

XX Nonstress



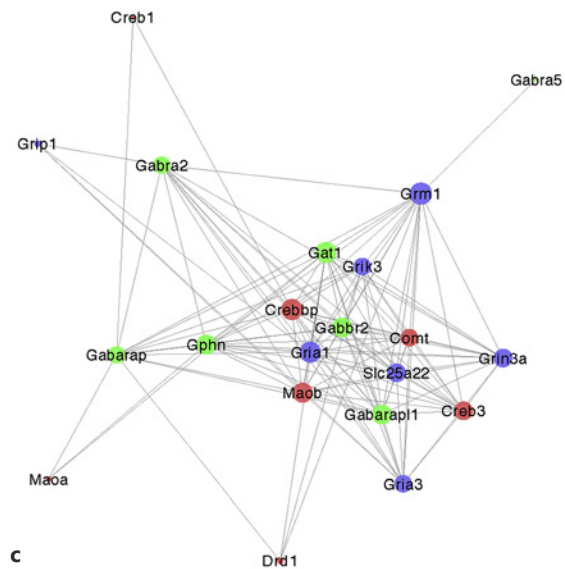
a

XX Stress



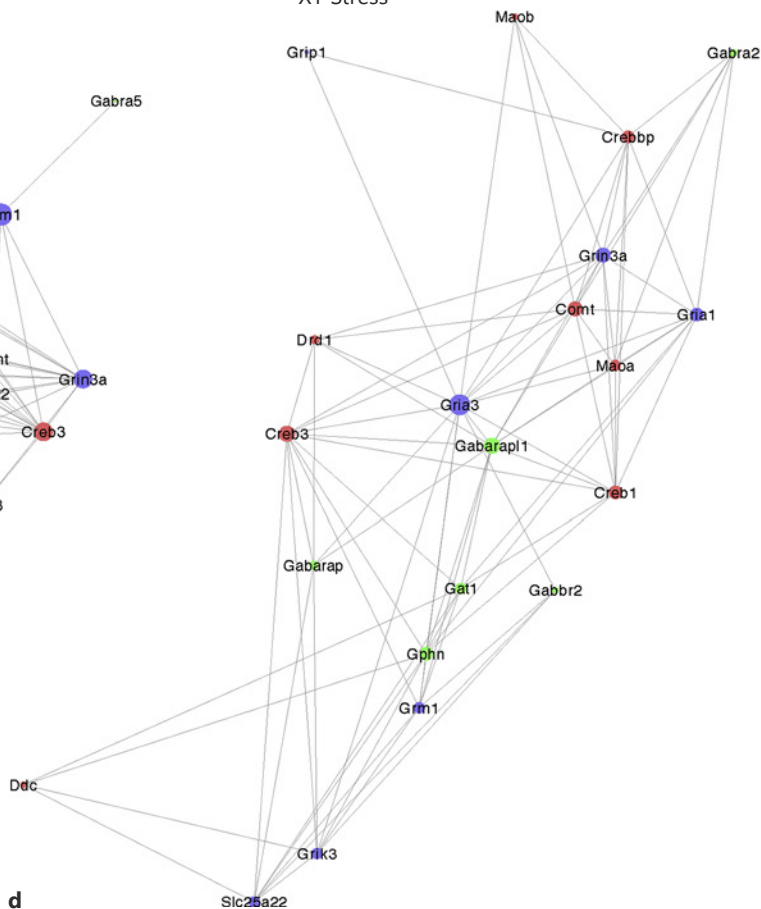
b

XY Nonstress



c

XY Stress



d

5

(For legend see next page.)

nificant (Fig. 5c, d). Specifically, stress elicited a nonsignificant reduction in the clustering coefficient for XY mice, from 0.833 to 0.726 ($p > 0.05$), and in density, from 0.593 to 0.424 ($p > 0.05$).

Discussion

Recent studies which examined the molecular pathology of MDD report distinct molecular changes in men and women across several mesocorticolimbic brain regions [23, 46]. However, it is unclear which sex-related factors might drive this distinct MDD-associated pathology in the brain. Here, we examined the effects of chronic stress on expression of sets of mood-related genes across the mesocorticolimbic circuit. Furthermore, we used the FCG mouse strain to determine whether the effects of stress were influenced by any sex-related factors. The overall goal was to identify sex-related factors that might drive the sex-specific MDD molecular pathology observed in humans. As predicted, based on previous evidence for these genes being implicated in mood, we found that stress impacted expression of several dopamine-, GABA-, and glutamate-related genes. Furthermore, we found that some of these effects, mostly in the PFC, were genetic sex-specific, with stress affecting gene expression in XX mice but not in XY mice. We also found that stress impacted gene expression differently across the mesocorticolimbic circuit; stress generally increased expression of mood-related genes in the PFC and NAc but decreased expression in the BLA.

Dopamine-Related Dysfunction in Mood Disorders

The brain's mesolimbic dopamine system plays an integral role in stress-induced physiological changes and depressive-like behaviors in animals and in human MDD

Fig. 5. Genetic sex-specific disruptions of gene networks in the PFC. Weighted gene coexpression networks for all examined dopamine-, GABA-, and glutamate-related genes. Networks were generated for XX nonstress (a), XX stress (b), XY nonstress (c), and XY stress (d) groups. Networks are split based on genetic sex and stress exposure. Lines connecting genes indicate coexpression of the 2 genes, with the weight of the line proportional to the level of coexpression. Only coexpression edges with $|r^3| > 0.3$ are shown; some examined genes are absent from certain networks if none of their coexpression values hit this threshold. The size of each node indicates the degree (sum of the edge weights of all connections) for that gene. Dopamine-related genes are shown in red, GABA-related genes in green, and glutamate-related genes in blue.

[47–53]. Dopamine neurons of the midbrain send projections to the amygdala, PFC, and NAc, and stress-induced changes in dopamine neurons in the ventral tegmental area (VTA) are linked to anhedonia and anxiety-like behavior in rodents [36, 49, 50, 54, 55]. In addition, anhedonia, a hallmark of MDD, is attributed, in part, to dysfunctional dopamine neurotransmission [56–61]. An overall reduction of dopamine neurotransmission within the mesocorticolimbic circuit has been described in patients with MDD and in multiple chronic stress paradigms in rodents. For example, rats undergoing 8–12 weeks of chronic mild stress, a paradigm similar to the one we used, led to depressive-like behaviors which could be reversed via phasic optogenetic activation of VTA dopamine neurons and specific to dopamine neurotransmission in the NAc [36]. In mice, chronic social defeat stress has been shown to lead to hyperexcitability of VTA dopamine neurons, and to depressive-like behaviors which could also be normalized by optogenetic activation of these neurons and the restoration of ionic currents [50]. Moreover, sex-dependent effects of stress on these circuits may be linked to sex differences in depressive-like behaviors [51]. In an effort to investigate whether there were sex-dependent effects on dopamine signaling pathways, we examined expression of dopamine-related genes in the PFC, BLA, and NAc. In the PFC and NAc, chronic stress led to an increase of *Ddc*, *Maoa*, and *Comt*, each of which are involved in dopamine metabolism. DDC catalyzes the conversion of DOPA to dopamine presynaptically, while MAOA and COMT metabolize dopamine postsynaptically, which, in the context of our findings, suggests an enhanced dopamine release into these brain regions. This would be consistent with an upregulation of *Drd1* and *Drd2* in the NAc. Sex-dependent effects were found for these dopamine-related genes in the PFC, with increased *Creb3*, *Crebbp*, *Drd1*, and *Maob* expression in XX mice but not in XY mice. Activation of CREB-dependent transcriptional pathways is important for the neural plasticity associated with stress, drug abuse, and learning [62–82]. Intriguingly, CREB3 is an endoplasmic reticulum-bound transcription factor activated via cAMP-dependent signaling and was recently found to modulate expression and activity of the glucocorticoid receptor (GR) [83]. CREB3 can act as a coactivator of GR and a direct transcriptional activator of the gene [83]. Given the known role of GR in stress-related pathologies and the female specificity of the effect of stress on *Creb3* expression in the PFC, investigating whether CREB3 is a key driver of stress sensitivity at the cellular level may reveal novel roles for this protein related to sex differences in anxiety and depression.

GABA-Related Dysfunction in Mood Disorders

Our results for the impact of chronic stress on GABA-related gene expression are largely consistent with what has previously been reported in humans. We found that *Gabarapl1* expression was increased by chronic stress in the PFC and NAc, consistent with results in human MDD in which expression is increased in Brodmann area 9 (BA9), BA10, BA20, and BA46 [84, 85]. Consistent with what we observed for the effects of stress on *Gabra2* expression in the PFC and NAc, subjects with MDD have increased *Gabra2* expression in the cerebellum [86]. While single-nucleotide polymorphisms (SNPs) in the *Gabbr2* gene are associated with MDD, human postmortem studies have not found altered expression of this gene in the brain (e.g., [85, 87]). Since we found increased expression of *Gabbr2* in the PFC of XX mice but not XY mice, it is tempting to speculate that human postmortem studies might find an effect on *Gabbr2* expression if analyses were stratified by sex. The *Gabra5* gene has SNPs with an association for suicide, but human postmortem brain studies report mixed results for changes in expression in subjects committing suicide and/or with MDD (increased expression in BA20 and BA46 [85], but no change in the amygdala [88]). Interestingly, we previously found that treating mice with a positive allosteric modulator for the $\alpha 5$ subunit of the GABA receptor (the subunit coded for by the *Gabra5* gene) decreased measures of anxiety-/depressive-like behaviors in female mice but not in male mice [89]. Here, we find that chronic stress decreased *Gabra5* expression in the PFC and NAc of both males and females. Together, this suggests that chronic stress impacts *Gabra5* in both sexes, but that boosting GABA signaling at receptors containing the $\alpha 5$ subunit is only effective in females.

Glutamate-Related Dysfunction in Mood Disorders

Here, we found effects of chronic stress on expression of many glutamate-related genes, with notable female-specific effects in the PFC. This female specificity for the effect of chronic stress is especially interesting, given the recent finding of female-specific increases in expression of several glutamate-related genes in the DLPFC of subjects with MDD. For instance, Gray et al. [29] reported a female-specific increase in DLPFC *GRIA3* expression, and we found that chronic stress increased PFC *Gria3* expression in XX and blank-treated mice, but not in XY or testosterone-treated mice. This suggests that the sex-specific human findings are driven by genetic and circulating-hormone differences. Alternatively, XY genetic sex and/or circulating testosterone might be protective

factors in males. There are also SNPs in the *GRIA3* gene which are associated with suicidal ideation [90, 91]. Gray et al. [29] also reported a female-specific increase in *GRM1* in the DLPFC of subjects with MDD. However, we found that chronic stress increased expression of *Grm1* in the PFC of both males and females, suggesting potential species differences. Our results for *Gria1* are consistent with reports in the literature. For instance, *GRIA1* expression is increased in BA9 [84, 87] and BA21 [85] in MDD subjects. We also saw increased *Gria1* expression in the PFC and NAc of chronically stressed mice.

Brain Region Specificity

A striking observation in our study is that chronic stress produces patterns of gene expression changes in the BLA opposite to those in the PFC and NAc; this is especially apparent when examining the heatmap in Figure 4. In the BLA, chronic stress almost universally led to decreased gene expression across these pathways, while in the PFC and NAc, it mostly caused increased gene expression. Interestingly, we previously reported a similar phenomenon in human MDD, where subsets of genes changed in opposite directions in the PFC and BLA [23]. BOLD imaging studies also suggest opposing activation patterns in these regions in MDD, with an increase in BLA activity at baseline, and a decrease in NAc and PFC activity during emotion-related tasks (e.g., [14–22]). These opposing changes between brain regions likely reflect stress-induced alterations of neurotransmission.

Conclusion

We report the effects of chronic stress on expression of several dopamine-, GABA-, and glutamate-related genes. Importantly, many of the effects of stress on gene expression are consistent with what has been reported in human MDD, suggesting that the UCMS paradigm recreates the relevant features of human MDD. Notably, we found that chronic stress affected gene expression similarly in males and females within the BLA and NAc, but that this was female-specific in the PFC; this suggests that future studies on sex-specificity of MDD could focus on the PFC. Finally, one of the most striking results of this study is that chronic stress produces differential effects across mesocorticolimbic brain regions, with the changes in the BLA being opposite in direction to those in the PFC and NAc. Together, our findings suggest that stress-induced altera-

tions in molecular signaling pathways of neurotransmission within these mood-related circuits are sex-dependent and region-specific.

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Statement of Ethics

Mouse experiments conform to internationally accepted standards and have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.L.S. and R.W.L. designed the experiments; K.B. and W.P. conducted the experiments; K.B., W.P., K.C., and M.L.S. analyzed the data; M.L.S., R.W.L., K.B., and W.P. wrote the paper.

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