The effect of sex-specific factors and stress exposure on expression of GABA-related genes in the basolateral amygdala of mice

by

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THE EFFECT OF SEX-SPECIFIC FACTORS AND STRESS EXPOSURE ON EXPRESSION OF GABA-RELATED GENES IN THE BASOLATERAL AMYGDALA OF MICE

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University of Pittsburgh, 2018

Major depressive disorder (MDD) is a common, often severe illness of mood regulation that affects approximately 350 million people worldwide. Women are twice as likely to be diagnosed with depression compared to men, but the molecular mechanisms underlying this sex difference are unclear. Previous human postmortem studies suggest that there is a dysfunction of inhibitory gamma-aminobutyric acid (GABA) function and brain-derived neurotropic factor (BDNF) function across corticolimbic brain regions, including the basolateral amygdala (BLA). Interestingly, post-mortem brain tissue from depressed patients suggests that there might be a sex difference in the way GABA is affected in depression, with more robust deficits in women with depression. Proper GABA function is also crucial during adolescence, as disruption could lead to susceptibility to mood disorders in adulthood. In our translational approach, we used the Four Core Genotypes (FCG) mouse model, in which genetic and gonadal sex are decoupled, to examine how sex-related factors influence GABA- and BDNF-related gene expression in the BLA in both adulthood and development. In a cohort of chronically-stressed adult mice (N=12-20/group) as well as in a cohort of gonadally-intact mice at three developmental time-points (N=19-26/group), we quantified the gene expression of five genes: *Sst,* a marker a marker of a GABA-interneuron subtype that preferentially targets distal dendrites of pyramidal cells; *Gad67* and *Gad65,* genes that code for rate-limiting enzymes of GABA synthesis; *TrkB*, a gene that codes for the BDNF receptor, *and Bdnf*, a gene that codes for BDNF protein. We found an

opposing effect of testosterone (similar to a typical male level) and male genetic sex (XY) on expression of *Sst*, with testosterone increasing *Sst* expression ($p<0.04$), but lower Sst expression in XY mice ($p<0.04$). This result also mirrored the behavioral results, with lower anxiety-like behavior in testosterone-treated mice, but higher anxiety-like behavior in XY mice. We also found genetic sex effects suggesting a differing developmental trajectory of the GABA and BDNF systems, where XY mice develop later than XX mice. Together, my findings highlight the complex relationship of sex-related factors on GABA- and BDNF-related gene expression through development that, if altered, could lead to adult psychopathology.

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PREFACE

Dedicated to my parents, Dr. Alexander and Marion Vasilakis, and my sister, Kristen Vasilakis. I am thankful for their unwavering support and guidance, providing me with countless opportunities to learn and grow, and their unconditional love throughout my whole life.

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research experience. I will take the skills and knowledge I have gained through this program with me as I continue on to medical school and beyond.

1.0 INTRODUCTION

1.1 MDD

Major Depressive Disorder (MDD) is a common, chronic, and often severe illness of altered mood regulation that affects approximately 350 million people worldwide [1]. Depression is characterized by sadness, irritability, low mood, and altered psychophysiology, which together disrupt normal daily functioning and familial relationships. Currently, there is no isolated cause of depression, no established mechanism, and an inconsistent response to present-day treatments [2].

1.2 BASOLATERAL AMYGDALA IN MDD

Human postmortem studies of depressed subjects suggest that the corticolimbic neural network of mood regulation is dysregulated in depression [3, 4]. The corticolimic brain regions include the dorsolateral prefrontal cortex (DLPFC; important for executive function, decision making, and mood regulation), the hippocampus (memory center), subgenual anterior cingulate cortex (sgACC; important for regulating emotional responses), and the basolateral amygdala (BLA; the “fear” center). The sgACC facilitates communication between the DLPFC and the

BLA [5, 6]. After processing emotionally salient stimuli from the environment, the BLA, together with cortical feedback, initiates behavioral responses [7]. fMRI neuroimaging studies suggest that patients with MDD abnormally process emotional stimuli and exhibit prolonged hyperactivity in the BLA in response to various adverse stimuli [8, 9]. However other studies report no significant difference in BLA activation between MDD patients and control subjects [10, 11]. Patients with other anxiety disorders, such as post-traumatic stress, social anxiety, and generalized anxiety have similar hyperactivity of the BLA [12].

1.3 SEX DIFFERENCES IN MDD

Women are twice as likely as men to be diagnosed with MDD in their lifetime. Symptom prevalence and severity differ between men and women, as women experience both more symptoms and higher symptom severity [13]. Additionally, women are more likely to have comorbid hyperphagia, hypersomnia, seasonal affective disorder, and an anxiety disorder along with MDD, but men are more likely to have comorbid substance abuse with MDD [14]. Interestingly, cross-cultural and epidemiological studies suggest that although women are more likely to report and seek medical assistance for their symptoms, this is not a contributing factor to the sex difference in MDD prevalence [15]. Thus, by controlling for potentially confounding variables, such as healthcare access, these studies suggest that the underlying cause for the sex difference in MDD incidence is biological. The sex differences in symptom prevalence and severity suggest that MDD progresses differently in men and women, with possibly different underlying biological mechanisms. Understanding depression in both sexes will not only

improve our understanding of MDD overall, but might also ultimately contribute to the development of sex-specific treatments for depression [16].

1.4 GABA AND BDNF DYSFUNCTION IN MDD

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and is present at each node of the corticolimbic circuit. Both magnetic resonance spectroscopy [17-19] and molecular studies [20, 21] demonstrate a GABA-deficit in MDD, which possibly leads to reduced GABA-mediated inhibition [22]. The decrease in GABA-inhibition within the circuit can lead to impaired excitation/inhibition balance in mood disorders [17, 20]. Previous studies conducted in our lab suggest a reduction in dendritic-targeting GABA interneurons in the sgACC [23, 24], DLPFC [25], and BLA [21] of MDD patients. Somatostatin (*SST*), a marker of a GABA-interneuron subtype that preferentially targets distal dendrites of pyramidal cells, was more robustly decreased in female subjects with MDD than men with MDD [23, 24, 26]. In the BLA, females MDD subjects have reduced *SST*, but depressed males do not [26]. *GAD67* and *GAD65* are genes that code for rate-limiting enzymes in GABA synthesis. There is a high level of *SST, GAD67,* and *GAD65* found in human postmortem brains of MDD patients, suggesting a common function of these three genes or shared common regulatory pathways e.g. hormone signaling or transcription factors [26, 27]. Our human studies are supported by causal mouse studies in which slight reduction in GABA-signaling induces depressive-like behaviors [28]. Brain-derived neurotropic factor (BDNF), known for growth and neuronal survival in the brain, also exhibits altered expression in MDD [21]. A robust decrease of BDNF expression is found in

sgACC of both sexes and in the BLA of females specifically [21, 24]. There is also reduced expression of *TrkB,* the gene that codes for the BDNF receptor, in subjects with MDD [24]. This suggests there may also be an sex difference in BDNF-related signaling in the BLA of MDD subjects [16].

1.5 STUDYING SEX DIFFERENCES

Our lab is interested in understanding the underlying biological mechanisms of these sex differences observed in MDD subjects. We investigate three types of sex differences: effects of genetic sex, effects of developmental gonadal hormone (organizational effects of hormones; leads to permanent, and thus irreversible, sex differences), and effects of adult circulating hormones (activational effects of hormones; leads to transient sex differences in adulthood) [29, 30]. We are limited in our ability to clearly identify the underlying sex dependent mechanisms that could explain the observed human sex differences in MDD. For example, genetic sex always determines gonadal sex in humans; consequently, it is impossible to distinguish if the observed sex differences are driven solely by genetic sex or gonadal sex. We also do not know how circulating hormone levels of our postmortem subjects varied over their lifetime or at the time of death. Combined, these limitations interfere with our ability to discern specifically which sex-related variables drive any of the observed sex differences in MDD. Moving our studies to mouse models allows us to tightly control different sex-related factors and easily manipulate our system of interest [31]. We can study analogous brain regions in a mouse model to expand our

understanding of the overall circuit, as the corticolimbic circuit is comparable to that of humans [32, 33].

1.6 FCG MICE

Genetic sex drives gonadal sex in typical wildtype mice, which makes it difficult to identify the three major types of sex differences explained earlier. Genetic engineering, however, allows for us to take advantage of genetically modified mouse strains, such as the Four Core Genotypes (FCG) mice. Genetic sex and gonadal sex are decoupled in this model. Crossing a C57BL/6J female mouse with a XY^{-}*Sry* (XYM) male (testes-determining gene, *Sry*, is located on an autosomal transgene instead of its endogenous location on the Y chromosome) allows for genetic sex and gonadal sex decoupling in this model. We can generate four different genotypes in which genetic sex does not determine gonadal sex: XXF (genetic and gonadal female), XYF (genetic male, gonadal female), XXM (genetic female, gonadal male), and XYM (genetic and gonadal male). Utilizing this model lets us independently analyze both genetic sex and gonadal sex effects, as well as better understand which component is responsible for observed sex differences. In addition to these genome manipulations, we also can control adult circulating hormone levels to examine activational effects of hormone.

Our lab previously studied *Sst*, *Gad67*, and *Gad65* expression in the frontal cortex of Four Core Genotypes (FCG) adult mice that were exposed to unpredictable chronic mild stress (see 'Methods'). Interestingly, we found that chronically stressed XY mice, regardless of gonadal sex or circulating hormone, had lower expression of *Sst, Gad67, and Gad65* compared

to XX mice. This was surprising to us given the increased female prevalence to MDD. However, XY mice also exhibited increased anxiety-like behavior, which is consistent with them having decreased *Sst*, *Gad67*, and *Gad65* expression. As we predicted given previous findings, testosterone treatment strongly decreased anxiety-like behavior [34], but testosterone did not affect gene expression in the frontal cortex [26, 35]. This led us to hypothesize that in a healthy male, circulating testosterone may override the underlying vulnerability to increased anxiety-like behaviors caused by male genetic sex.

It is interesting that while *SST* exhibits lower levels in the sgACC of both men and women, the *SST* reduction in the BLA is specific to females with MDD. This finding points to a potential sex difference in the BLA of MDD patients [26]. Human and mouse studies together support the hypothesis of an overall GABA dysfunction in MDD, but specifically a dysfunction of GABA in the BLA of females with MDD[16]. Additionally, there is a strong BDNF dysfunction in the BLA of female MDD patients, but not males [27]. Together, we hypothesized that the BLA is the brain region involved in the sex difference in MDD. In cohort 1, I aimed to test two alternative hypotheses: 1) there a brain-wide effect of genetic sex on GABA- and BDNF related genes; or 2) the genetic sex effect on GABA- and BDNF related genes is brain region-specific.

1.7 UNPREDICTABLE CHRONIC MILD STRESS

Our lab uses UCMS, a behavioral paradigm designed to strongly increase anxiety- and depressive-like behavior and produce homologous features that are associated with human major

depressive disorder (MDD)[26, 36, 37]. UCMS induces a UCMS syndrome in mice with construct validity, face validity, and predictive validity. Construct validity refers to the mice exposed to and coping with small stressors over a long period of time as is often true in depression, rather than a singular traumatizing event, like post-traumatic stress disorder. Face validity refers to the observable increased anxiety- and depressive-like behaviors of the mice. Predicative validity refers to the use of antidepressant treatments to reverse the UCMS syndrome, just like in depression: one dose of antidepressant treatment will not reverse the UCMS syndrome, but chronic exposure to antidepressants does reverse the UCMS syndrome [16, 26].

1.8 ADOLESCENCE AND GABA IN MOOD

Adolescence is a transitional time in development that includes various physiological and behavioral changes between pre-puberty and adulthood that are not fully understood. Child abuse and neglect leads to stress and trauma during early development, which can affect mood disorder susceptibility in adulthood. The development of the GABA system specifically affects both the structure and the communication of the cortex. GABA originates as an excitatory neurotransmitter at birth, but becomes inhibitory during pre-pubertal stages of development [38]. The developing GABA system also contributes to cortical activity synchronization in adolescence [39]. Dysregulated cortical synchronization is believed to perhaps contribute to the development of MDD, as subjects with the disorder have increased synchronization of neuron firing [40]. This suspected developmental window of vulnerability overlaps with the well-known

developmental window for sex differences. Differences between male and female GABA function during adolescence could influence sex-specific synaptic development and/or cortical synchronization later in life. Ultimately, this could negatively impact cortical development and foreshadow vulnerability in adulthood for psychiatric disease, like MDD [16]. In cohort 2, I aimed to determine if genetic sex and/or organizational hormone influence GABA-related genes expression in the BLA of pre- and peri-pubertal mice.

2.0 METHODS

2.1 MICE

This study utilized a unique strain of mice called the Four Core Genotypes (FCG) mice. In a wildtype mouse, genetic sex and gonadal sex are entirely linked due to the testes-determining gene, *Sry*, on the Y chromosome. This means that if a wildtype organism has a Y chromosome, they have *Sry,* and therefore, will develop gonadally male (both genetically and gonadally male). If a wildtype organism does not have a Y chromosome, they do not have *Sry*, and therefore, will develop gonadally female (both genetically and gonadally female). Using genetic engineering, the *Sry* gene was removed from the Y chromosome and placed on an autosome instead, thereby decoupling genetic and gonadal sex.

The FCG mice used in these studies (originating from Jackson Laboratories, Bar Harbor, ME, USA; B6.Cg- Tg(Sry)2Ei Srydl1Rlb/Arnoj) were generated by crossing a C57BL/6J female with an XY^{-}*Sry* male. Y^{-} indicates the absence of Sry on the Y chromosome, with Sry present instead as an autosomal transgene. Here, we use the male (M) and female (F) designation to indicate gonadal sex. This cross yielded four groups of mice: XXM (XX*Sry*), XXF (XX), XYM (XY^{-}*Sry*) and XYF (XY^{-}). These four genotypes allow us to investigate the effects of genetic sex and developmental gonadal sex (i.e., organizational effects of hormones) independent of the other, as genetic sex and gonadal sex are inherited independently in this model. The addition of a

testosterone or blank capsule (discussed below) after gonadectomy in adulthood also allows us to investigate the effects of circulating hormones (i.e., activational effects of testosterone). The mice were maintained under standard conditions (12-hour light and dark cycles; 22 ± 1°C, food and water ad libitum), in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2 EXPERIMENT 1: ADULTS (COHORT 1)

Adult FCG mice were bilaterally gonadectomized at 15 weeks under isoflurane anesthesia to remove the endogenous source of circulating gonadal hormone. Half of each genotype was implanted with a subcutaneous testosterone-filled capsule containing 5-mm crystalline testosterone (1.57-mm ID x 2.41-mm OD) or with a similarly sized blank capsule [16]. [XXF + B (n=12), XXF + T (*n* = 18), XYF + B (*n*=13), XYF + T (*n*=13), XXM + B (*n*=18), XXM + T (*n* = 13), XYM + B (*n*=12), XYM + T (*n* = 13)]. It is important to note that the concentration the testosterone capsules were at or slightly above physiologically normal male levels. After allowing 4 weeks for mice to recover from surgery and for hormone levels to equilibrate, the mice were exposed to 8 weeks of unpredictable chronic mild stress (UCMS; see details below). After 7 weeks of UCMS exposure, mice were assessed for anxiety-like behaviors in the elevated plus maze and open field. (Figure 1). Mice were exposed to UCMS during behavior testing, thus the mice were exposed to UCMS for a total of 8 weeks.

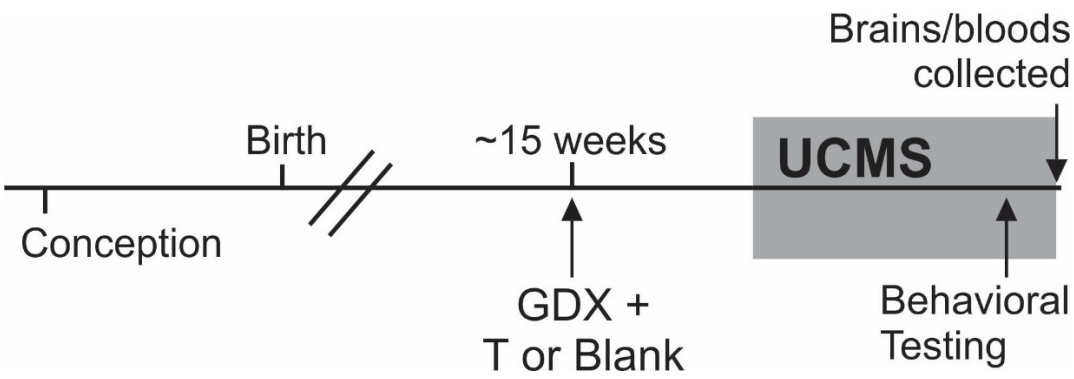

Figure 1: Experimental design for cohort 1.

Mice were gonadectomized (GDX) at ~15 weeks of age and implanted subcutaneously with either a testosterone (T)-filled or blank capsule. After GDX, mice in cohort 1 were exposed to 8 weeks of unpredictable chronic mild stress (UCMS) followed by behavioral testing and were then sacrificed. At the time of sacrifice, the brains were harvested for gene expression analyses and bloods were collected for hormone assays [16].

2.2.1 Unpredictable Chronic Mild Stress

Group-housed adult mice (gonadal sex and hormone treatment matched) were exposed to a randomized schedule of environmental stressors 7 days a week for 8 weeks, gradually increasing in intensity, starting with 1–2 separate stressors a day and ending with 4–5 stressors a day (separately and in tandem with one another) during the final week, as performed previously in our lab [16, 26]. Disturbances included light cycle disruption, tilted cage (45° tilt), social stress (rotate mice into previously occupied cages), reduced space (limiting mice to 1/3 of typical space in cage), aversive smell (20 min of exposure to bobcat or fox urine), no bedding or wet bedding overnight, mild restraint (50-mL conical tube with air hole for 15 min), and forced bath (approximately 2 cm of 21°C water for 15–45 min). We assessed body weight and fur weekly to track progression of the UCMS syndrome. Behavior testing occurred while mice were still being

exposed to UCMS. We previously reported frontal cortex gene expression results from this cohort [16, 26, 35].

2.2.2 Behavioral Testing

2.2.2.1 Elevated Plus Maze

The elevated plus maze is a behavioral test used to measure anxiety-like behavior [41]. This cross maze was constructed with 2 open and 2 closed 30 cm x 5 cm arms. Performed during the light phase, the mouse was initially placed at the intersection of the four arms. Over 10 minutes, the amount of time spent in the open arms and percent entries (entries into open arms divided by entries into open or closed arm x 100) in the open arms were recorded to measure anxiety-like behavior. An index of locomotor behavior was indicated by the total number of entries into any arm [26, 42].

2.2.2.2 Open Field Test

The open field test is a behavioral test used to measure anxiety-like behavior[43]. During the light phase, the open field test was performed in a 50.8 cm x 50.8 cm square arena. The centermost portion was identified at 25.4 cm X 25.4 cm of the arena. Behavior was tracked using ANY-Maze software (Stoelting; Wood Dale, IL, USA) and a ceiling-mounted camera. Over 10 minutes, the amount of time spent in the center of the arena and the percent distance (distance in the center divided by the total distance x 100) was recorded to measure anxiety-like behavior. An index of locomotor activity was indicated by the total distance traveled [26].

2.3 EXPERIMENT 2: DEVELOPMENTAL TRAJECTORY (COHORT 2)

We decided to study time-points clearly before puberty (late postnatal) through early adolescence. We used FCG mice aged postnatal day 16 (P16), P21, and P26 [P16 (pre-weaning), P21 (at time of weaning), and P26 (post-weaning)]. P16 had 79 gonadally intact FCG mice [XXF (n=20), XYF (n=19), XXM (n=20), XYM (n=20)]. P21 had 88 gonadally intact FCG mice [XXF (n=22), XYF (n=26), XXM (n=21), XYM (n=19)]. P26 had of 80 gonadally intact mice [XXF (n=20), XYF (n=20), XXM (n=20), XYM (n=20)]. These groups were not exposed to stress and did not have circulating hormone manipulations. At the time of sacrifice, mice were rapidly decapitated. Brains were collected for gene expression studies (qPCR) and bloods were collected for the ELISA testosterone assay (Figure 2).

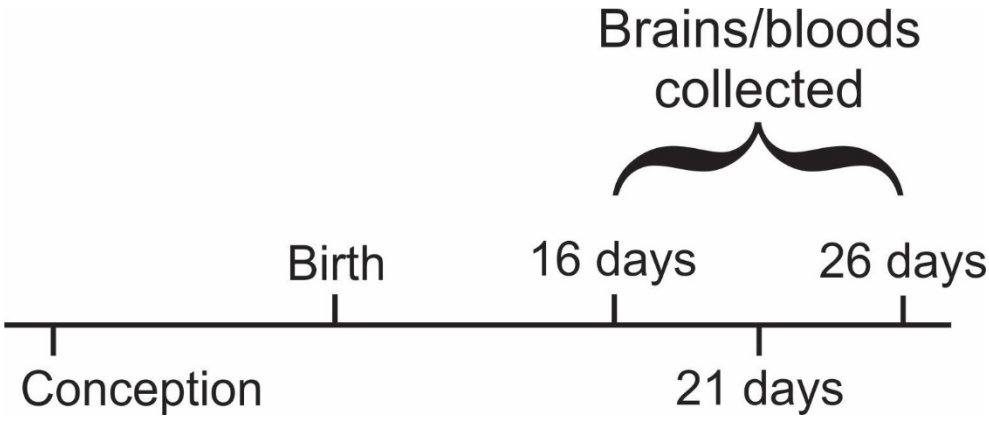

Figure 2: Experimental design for cohort 2.

Mice in cohort 2 were sacrificed at one of three developmental time-points: postnatal day 16, 21 or 26. P16 and P21 are both prior to the onset of puberty while P26 is closer to the onset of puberty. At the time of sacrifice, the brains were harvested for gene expression analyses and bloods were collected for testosterone hormone assays.

2.4 SACRIFICE AND TISSUE PROCESSING

Mice were sacrificed at postnatal day 16, postnatal day 21, postnatal day 26, and week 24 (while adults were still being exposed to stressors), and the brains and bloods were collected. Adult mice were anesthetized by isoflurane and both adult and developmental mice were rapidly decapitated. The brains were dissected out and immediately flash frozen on dry ice. Trunk blood was collected, allowed to clot at room temperature for 90 min, and the serum separated out to measure testosterone levels [16].

2.5 TESTOSTERONE ASSAY

Circulating testosterone levels of this cohort were measured from collected serum samples. In adults, the serum samples were sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (supported by the Eunice Kennedy Shriver NICHD/HIH (SCCPIR) Grant U54-HD28934), and an ELISA kit was used to determine testosterone concentration. Using the same ELISA kit and protocol as the ones used at University of Virginia, we measured circulating testosterone levels of P16, P21 and P26 mice by using a testosterone (mouse/rat) ELISA assay (IBL America; Minneapolis, MN) in accordance with kit instructions. Subjects found to be statistical outliers by using the Graphpad Grubb's Outlier Test were excluded from qPCR analysis [16].

2.6 PROCESSING OF BRAIN TISSUE: BLA DISSECTION AND GENE EXPRESSION ANALYSIS

Bilateral micropunches (1mm bore punch) of the BLA (between Bregma −0.94 and −1.82 mm; [31]) were obtained from approximately six 160-µm thick coronal tissue sections cut on a cryostat within the boundaries created by the internal and external capsule. All tools were treated with RNase Zap to prevent RNases contamination. Punches were stored in RNase free 1.5mL tubes at −80°C prior to RNA extraction [16].

2.7 RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was extracted from BLA tissue punches using RNeasy Plus Micro Kits (Qiagen; Valencia, CA, USA). RNA was reverse-transcribed into complementary DNA (cDNA) using QScript cDNA Supermix (olido(dT) and random primers (Quanta Biosciences, Gaithersburg, MD, USA)) [16].

2.8 QUANTITATIVE POLYMERASE CHAIN REACTION

In the adult cohort, small PCR products were amplified on a MJ Research (Waltham, MA, USA) DNA Engine Opticon System for qPCR using universal PCR conditions (65 to 59 °C touch-down and 40 cycles (10 s at 95°C, 10s at 59°C, and 10s at 72°C)). cDNA was amplified in 20-µL reactions (0.1 × SYBR Green, 3 mM MgCl2, 200 nM dNTPs, 200 nM primers, 0.25 unit

Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA)) [16]. In the developmental trajectory cohort, gene expression was measured using CFX96 Touch™ Real-Time PCR Detection System using the following PCR conditions (5s at 95°C, 30s at 60°C) for 39 cycles. cDNA was amplified in 20 μL reactions (10 μL supermix (add official name), 9 μL primer associated with gene of interest, and 1 μL cDNA in each well) [16].

Samples were run in triplicate, and results were calculated as the geometric mean of relative intensities compared to three internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cyclophilin). Actin, GAPDH, and cyclophilin are considered good housekeeping genes because sex-related factors do not influence their expression or abundance, thus we consider them to be internal controls. The results are expressed as arbitrary signal ($2^{-dCT} \times 10{,}000$). We eliminated actin from the P26 experiments because it was not strongly correlated with the other two housekeeping genes. The arbitrary signals in the P26 study, then, were calculated from the geometric mean of cyclophilin and GAPDH. Genes of interest (*Sst, Gad67, Gad65, TrkB, and Bdnf*) were selected based on our human postmortem brain findings in depression. Grubbs' outlier test determined statistical outliers in gene expression values. These outliers were excluded from qPCR analysis [16]. I performed qPCR for P16 and P26 groups, while another lab member ran qPCR for the P21 group.

2.9 CORRELATING GENE EXPRESSION AND ANXIETY-LIKE BEHAVIOR

A Spearman correlation was used to compare BLA expression of the five genes of interest to anxiety-like behavior tested in the adult cohort (behavior separately reported [26]). Results were

combined into anxiety-like *Z*-scores to reduce the complexity of the correlation analysis. The first step is to calculate the Z-score for each behavioral test measure (e.g., Z-EPM-PercentCrossesOpenArms or Z-OF-PercentTimeCenter); this is performed by normalizing an individual mouse's test measure to the mean and standard deviation of the comparison group. For all Z-scores, we used XX blank mice as the comparison group. The directionality of the Z-score was adjusted such that increased values represented increased anxiety-like behavior (e.g., decreased time in the open arms of the elevated plus maze was considered increased anxiety-like behavior). We next calculated the Z-score for each mouse per behavioral test by averaging the two individual Z-measures for each test (e.g., averaging Z-EPM-TimeOpenArms and Z-EPM-PercentCrossesOpenArms to get Z-EPM-Anxiety). Finally, the overall anxiety-like Z-score was calculated by averaging the Z-EPM-Anxiety and Z-OF-Anxiety scores. Correlations were performed between the Z-Anxiety measure for each mouse and expression of each gene (for the entire stressed cohort, as well as performed in groups based on main effects (XX vs. XY, ovaries vs. testes, and blank vs. testosterone treatment)) [16].

2.10 STATISTICAL ANALYSIS

In adult mice, we used a three-way analysis of variance (ANOVA), (genetic sex x developmental gonadal sex x hormone treatment) to analyze the gene expression data from this cohort. In the developmental trajectory cohort, we used a two-way ANOVA, (genetic sex x developmental gonadal) to analyze dependent measures. We did not examine the main effects of hormone in the P16, P21 and P26 groups, as these mice did not receive hormone manipulations. However, in the

developmental cohort, we correlated testosterone levels with gene expression using Pearson correlation to determine whether endogenous testosterone levels affected gene expression. If the ANOVA was significant for any main effect or interaction, we performed planned comparisons using Tukey's post hoc test. We did not correct for multiple testing in these studies, as we had a priori hypotheses for these genes to be altered based on our findings in the frontal cortex of FCG mice and in human postmortem brain tissue of patients with MDD [23, 24, 26]. Data are expressed as mean ± SEM, and significance was set at $p < 0.05$ [16].

3.0 RESULTS

3.1 EXPERIMENT 1: ADULTS (COHORT 1)

The adult FCG mice in cohort 1 were gonadectomized and given testosterone or blank capsules at 15-weeks old. Mice were subjected to 8 weeks of UCMS to induce elevated anxiety-/depressive-like behaviors. Importantly, adult mice treated with testosterone did not differ in testosterone levels and had significantly higher testosterone levels when compared to blank-treated mice [16]. Compared to XX mice, XY mice had significantly lower *Sst* expression in the BLA ($p < 0.04$; Fig. 3A). There was no effect of genetic sex on expression of *Gad67*, *Gad65*, *TrkB*, or *Bdnf* in the BLA ($p > 0.1$ for all comparisons; Fig. 3B-E). There were no gonadal sex differences on expression of *Sst*, *Gad67*, *Gad65*, *TrkB*, or *Bdnf* in the BLA ($p > 0.1$ for all comparisons; Fig. 3A-E). We found a main activational effect of testosterone exposure on *Sst*, with testosterone increasing *Sst* expression ($p < 0.04$; Fig. 3A). There was also a main effect of circulating testosterone on BLA *Bdnf* expression ($p < 0.03$; Fig. 3E) with testosterone decreasing BLA *Bdnf* expression. There was no effect of testosterone on expression of *Gad67*, *Gad65*, or *TrkB* expression the BLA ($p > 0.2$ for all comparisons; Fig. 3B-D). In graphs representing results in this cohort, we show graphs summarized by main effects [XX ($n = 61$), XY ($n = 51$); F (n=56), M ($n = 56$); B ($n = 55$), T ($n = 57$)].

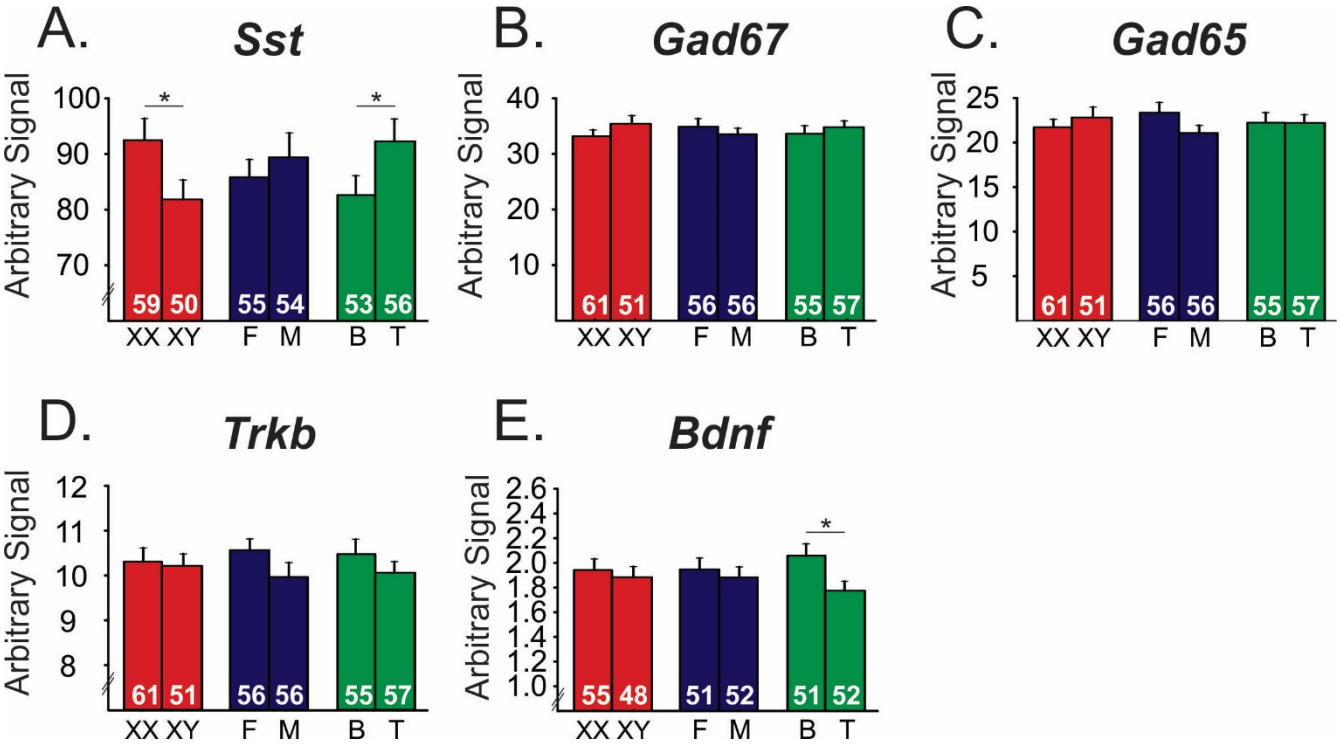

Figure 3: Main effects of sex-related factors on GABA- and Bdnf-related gene expression in the BLA of adult stressed mice.

(A) XY mice had lower expression of Sst. Additionally, mice treated with testosterone had higher Sst expression. There was no genetic sex effect on expression of Gad67 (B), Gad65 (C), TrkB (D), or Bdnf (E). Gonadal sex did not influence expression of any gene (A–E). Testosterone significantly increased expression of Sst (A) and decreased expression of Bdnf (E), but did not affect expression of Gad67 (B), Gad65 (C), or TrkB (D). Numbers at the base of the bars indicate N. *p < 0.05; #, p < 0.1. T, testosterone, B, blank, F, gonadal female, M, gonadal male[16]

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3.1.1 Correlation between gene expression and anxiety-like behavior

Given the potential links between GABA- and BDNF-related gene expression and mood (see Literature Review), we next probed for potential correlations between anxiety-like behavior and BLA gene expression. We previously studied these same genes in the frontal cortex of FCG

mice, but the gene expression results did not match the behavioral results. In the BLA, we found that *Sst* expression was negatively correlated with anxiety-like behavior ($p = 0.041$; (i.e., higher Sst expression levels were correlated with decreased anxiety-like behavior). There was also a trend ($p = 0.077$) for a positive correlation between *Bdnf* expression and anxiety-like behavior (i.e., higher *Bdnf* expression levels were correlated with increased anxiety-like behavior). There were no other significant correlations between anxiety-like behavior and expression of the remaining genes (*Gad67*, *Gad65*, *TrkB*; $p > 0.25$ for all comparisons). Anxiety-like behaviors (Fig. 4E) are measured in z-scores and separated by main effects of genetic, gonadal, and circulating hormone treatment. XY mice had significantly higher anxiety-like behavior than XX mice. There was no significant difference between gonadal females and gonadal males. Testosterone treated-mice had significantly lower anxiety-like behavior than blank-treated mice. *Sst* expression (Fig. 4F) shows that XY mice have lower expression than XX mice and mice that received a testosterone capsule had significantly increased *Sst* expression compared to blank-treated mice.

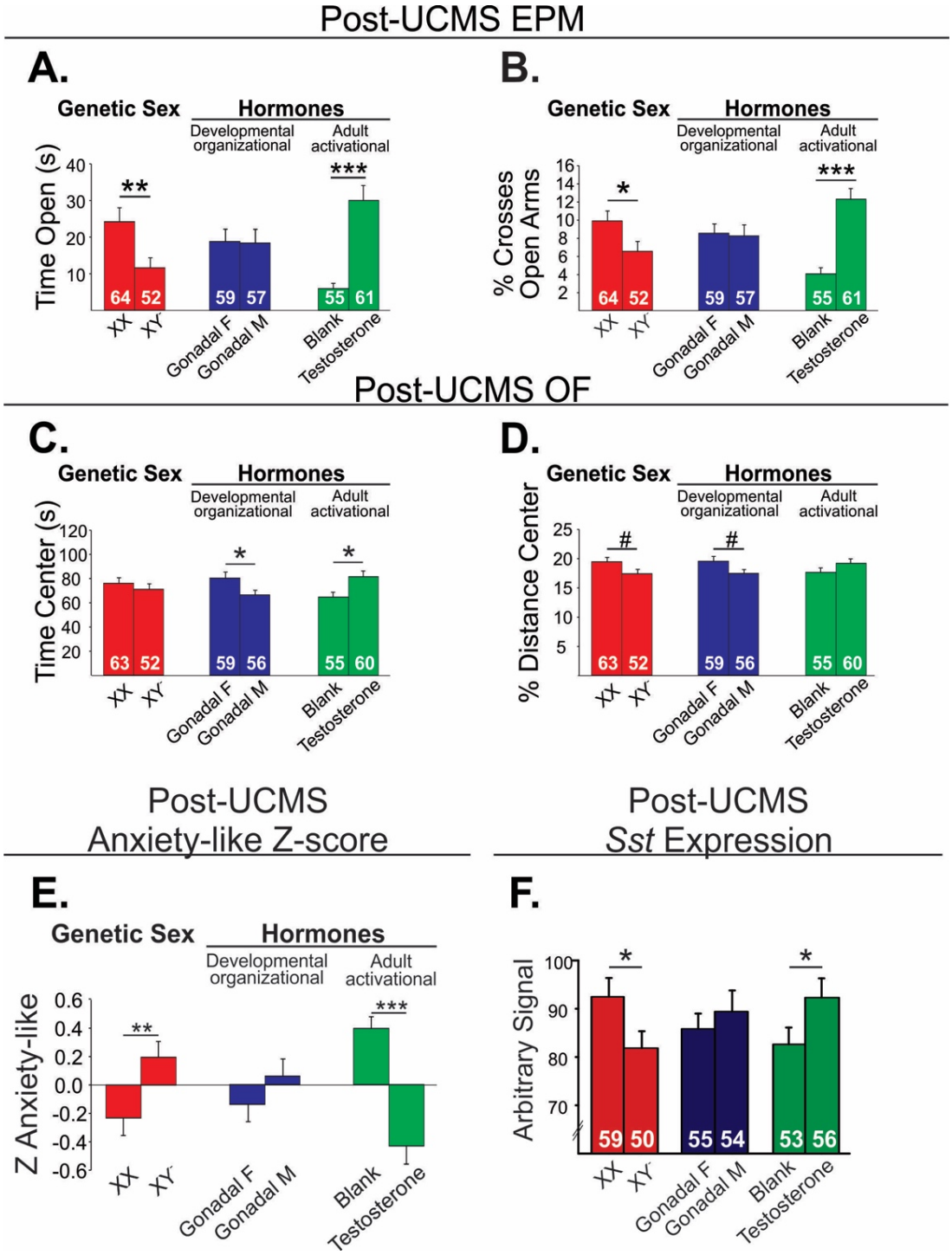

Figure 4: Behavioral assays after UCMS reveal an inverse relationship between anxiety-like behavior and expression of *Sst* in the BLA adult stressed mice.

(A) Post-UCMS elevated plus maze (EPM) results for time and (B) Percent crosses into open arms. (C) Post-UCMS Open Filed (OF) results for time and (D) Percent distance in the center[26]. (E) Data from A-D were combined to

calculate anxiety-like behavior *Z*-scores (see *Correlating gene expression and anxiety-like behavior* above). Genetic males exhibited more anxiety-like behaviors than genetic female mice. Testosterone (T)-treated mice exhibited lower anxiety-like behaviors than blank (B)-treated mice. (F) *Sst* expression is lower in XY mice compared to XX mice, but testosterone-treated mice had increased *Sst* expression in the BLA compared to blank-treated mice. Numbers at the base of the bars in the graph on the right indicate *N*. Error bars indicate mean ± SEM. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; #$p < 0.1$. F, gonadal female, M, gonadal male, B, blank, T, testosterone.

3.2 EXPERIMENT 2: DEVELOPMENTAL COHORT (COHORT 2)

Gonadally intact FCG mice (XXF, XXM, XYF, XYM) were sacrificed at three developmental time-points from late postnatal through early adolescence: P16 (pre-weaning), P21 (at time of weaning), and P26 (post-weaning). We then examined the effects genetic and gonadal sex (i.e., organizational effects of hormones) on expression of the same GABA- and BDNF-related genes in the BLA.

3.2.1 P16

We found an effect of genetic sex on expression of *Sst* ($p < 0.01$) and *TrkB* ($p < 0.001$) in the BLA, with XY mice having lower expression of both genes compared to XX mice (Fig. 5A, D). With respect to *Sst*, this is the same genetic sex effect we observed in the BLA of adult stressed mice. There was no effect of genetic sex on *Gad67* (Fig. 5B), *Gad65* (Fig. 5C), or *Bdnf* (Fig. 5E) expression in the BLA. Compared to gonadal females, there was a trend for gonadal males to have higher expression of *Bdnf* ($p = 0.088$; Fig. 5E). There was no significant main effect of gonadal sex, however, on expression of *Sst*, *Gad67*, *Gad65*, or *TrkB* in the BLA ($p > 0.3$ for all

comparisons; Fig. 5A-D). There were no significant interactions between genetic and gonadal sex ($p > 0.3$ for all comparisons). In graphs representing results for P16, P21, and P26, we show graphs summarized by main effects.

As expected, gonadal males had higher testosterone levels than gonadal females (M 0.233 ng/mL ± 0.02; F 0.170 ng/mL ± 0.07; $p < 0.01$; Fig. 6A). Four subjects were eliminated due to testosterone levels outside of the expected normal circulating hormone levels and were excluded from gene expression analysis. Importantly, there were no significant differences in circulating testosterone between XX and XY mice (XX 0.211 ng/mL ± 0.02; XY 0.192 ng/mL ± 0.01; $p > 0.3$) Further, testosterone levels at P16 did not differ between XXM and XYM mice (XXM 0.137 ng/mL ± 0.01; XYM 0.139 ng/mL ± 0.011; $p > 0.45$; Fig. 6A), suggesting that the genetic sex difference does not affect testosterone levels in gonadal males.

Additionally, circulating testosterone did not correlate with expression levels of any gene of interest ($p > 0.5$ for all correlation analyses). Together, this suggests that any difference in gene expression we might observe based on gonadal sex at P16 will be due to organizational effects of hormones rather than activational effects of testosterone.

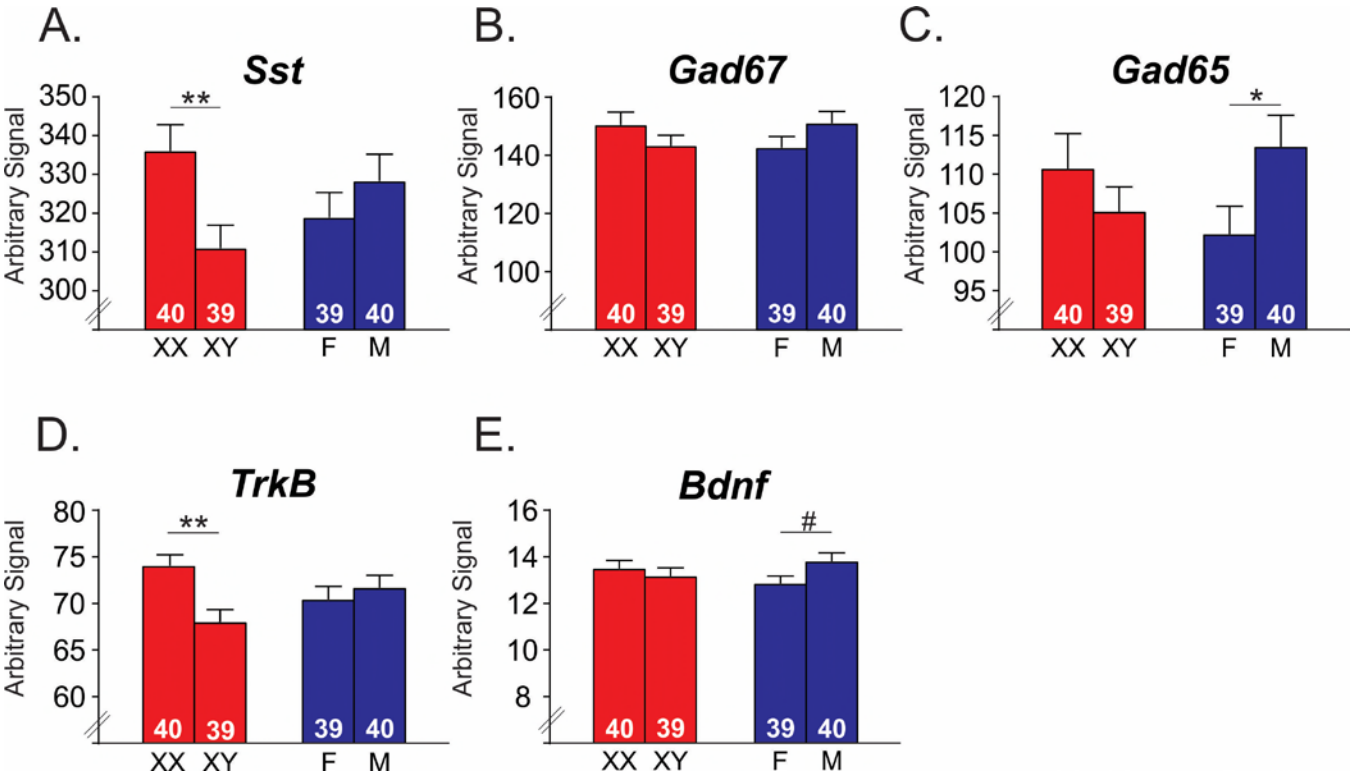

Figure 5: Main effects of GABA-related and BDNF-related gene expressin in the BLA of P16 Mice

XY mice had lower expression of *Sst* (A) and *TrkB* (D) than XX mice; there was no effect of genetic sex on *Gad67* (B), *Gad65* (C), or *Bdnf* expression (E). Gonadal males (M) had significantly higher expression of *Gad65* (C) and *Bdnf* (E); there were no gonadal sex effects on *Sst* (A), *Gad67* (B), and *TrkB* (D). Numbers at the base of the bars indicate *N*. **p < 0.01; *p < 0.05; #p < 0.1. F, gonadal female, M, gonadal male.

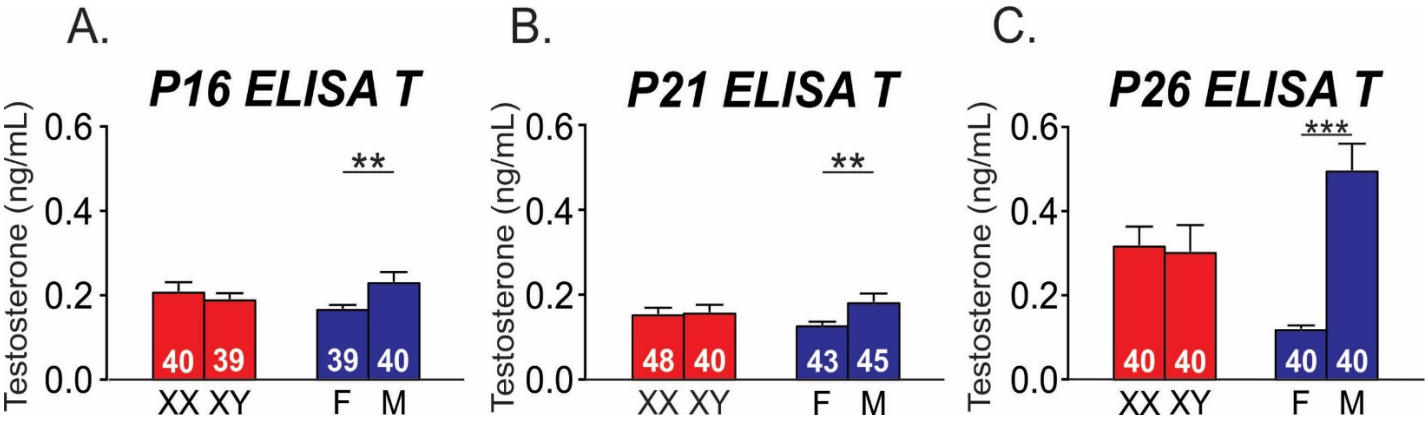

Figure 6: P16, P21, and P26 Testosterone Concentrations Compared Genetically and Gonadally

Gonadal males had higher testosterone levels at P16 (A), P21 (B), and P26 (C). There was no effect of genetic sex on testosterone levels at any age (A, B, C). Numbers at the base of the bars in the graph on the right indicate *N*. ***p < 0.001; **p < 0.01. F, gonadal female, M, gonadal male.

3.2.2 P21

We found an effect of genetic sex, with XY mice having lower expression of *Gad67* ($p = 0.01$; Fig. 7B), and *Gad65* ($p < 0.015$; Fig. 7C), in the BLA compared to XX mice. We also found a trend for an effect of genetic sex on Sst ($p < 0.07$; Fig. 7A) and *TrkB* ($p < 0.08$; Fig. 7D) where XX mice have higher levels compared to XY mice. There was no effect of genetic sex on *Bdnf* expression in the BLA (Fig. 7E, $p > 0.1$). Compared to gonadal females, gonadal males showed no significant difference in expression of *Sst*, *Gad67, Gad65, TrkB*, or *Bdnf* ($p > 0.3$ for all comparisons; Fig. 7A-E).

There was not a genetic sex effect on circulating testosterone (XX 0.156 ng/mL ± 0.01; XY 0.160 ng/mL ± 0.02; $p > 0.3$; Fig. 6B) but there was a main effect of gonadal sex (M 0.130 ng/mL ± 0.01; F 0.185 ng/mL ± 0.02; $p < 0.01$; Fig. 6B). Importantly, testosterone levels at P21 did not differ between XXM and XYM mice (XXM 0.181 ng/mL ± 0.02; XYM

0.19 ng/mL ± 0.03; $p > 0.45$), suggesting that genetic sex did not influence testosterone levels in gonadal males. Additionally, circulating testosterone did not correlate with expression levels of any gene of interest ($p > 0.5$ for all correlation analyses). Together, this suggests that any difference in gene expression we might observe based on gonadal sex in weanlings will be due to organizational effects of hormones rather than activational effects of testosterone [16].

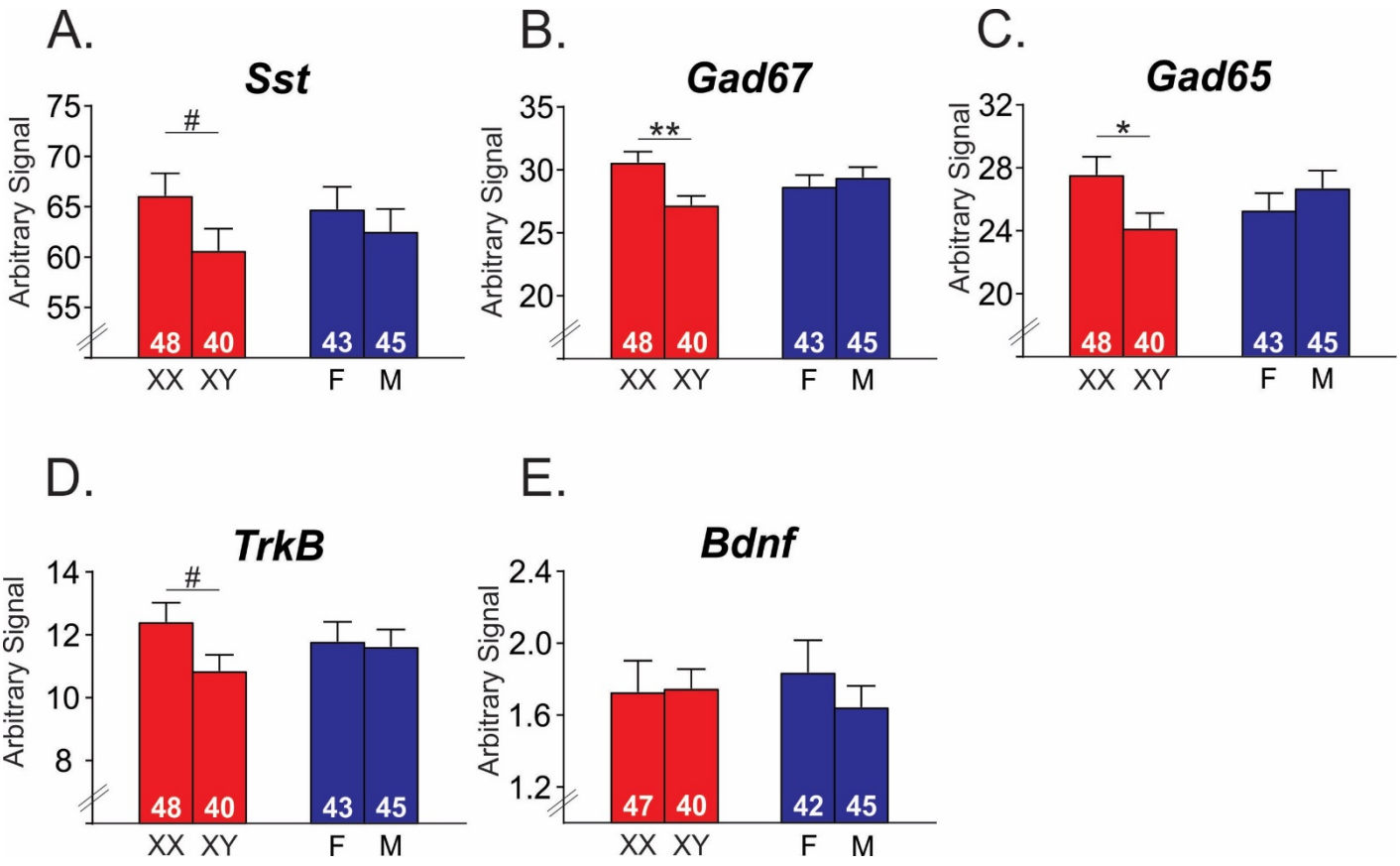

Figure 7: Sex differences in GABA-related and BDNF-related gene expression in the BLA of P21 mice

XY mice had lower expression of *Sst* (A), *Gad67* (B), and *Gad65* (C), and *TrkB* (D) compared to XX mice; there was no effect genetic sex on *Bdnf* expression (E). There were no organizational effects of hormones on expression of any gene investigated (A–E). Numbers at the base of the bars indicate *N*. **p < 0.01; *p < 0.05; #p < 0.1. F, gonadal female, M, gonadal male.

3.2.3 P26

There was no effect of genetic sex on expression of *Sst*, *Gad67*, *TrkB* or *Bdnf* ($p > 0.05$ for all comparisons; Fig. 8A, B, D, E). We did find, however, a trend for an effect of genetic sex on *Gad65*, with XY mice having higher expression than XX mice ($p = 0.068$; Fig. 8C). There was no effect of gonadal sex on expression of *Sst*, *Gad67*, *Gad65*, *TrkB*, or *Bdnf* in the BLA ($p > 0.1$; Fig. 8A-E). There were no significant interactions between genetic and gonadal sex ($p > 0.2$ for all comparisons).

At P26, gonadal males had significantly higher testosterone levels than gonadal females (M 0.499 ng/mL ± 0.06; F 0.122 ng/mL ± 0.007; $p < 10^{-8}$; Fig. 6C). Four subjects were eliminated using the Graphpad Grubb's Outlier Test due to testosterone levels outside the expected normal circulating hormone levels and were excluded from gene expression analysis. There were no genetic sex effects on circulating testosterone (XX 0.321 ng/mL ± 0.04; XY 0.301 ng/mL ± 0.06; $p > 0.3$). Additionally, there was no significant difference between XXM and XYM mice (XXM 0.513 ng/mL ± 0.06; XYM 0.485 ng/mL ± 0.11; $p > 0.3$), suggesting that the genetic sex difference does not affect testosterone levels in gonadal males. Additionally, circulating testosterone did not correlate with expression levels of any gene of interest ($p > 0.2$ for all correlation analyses).

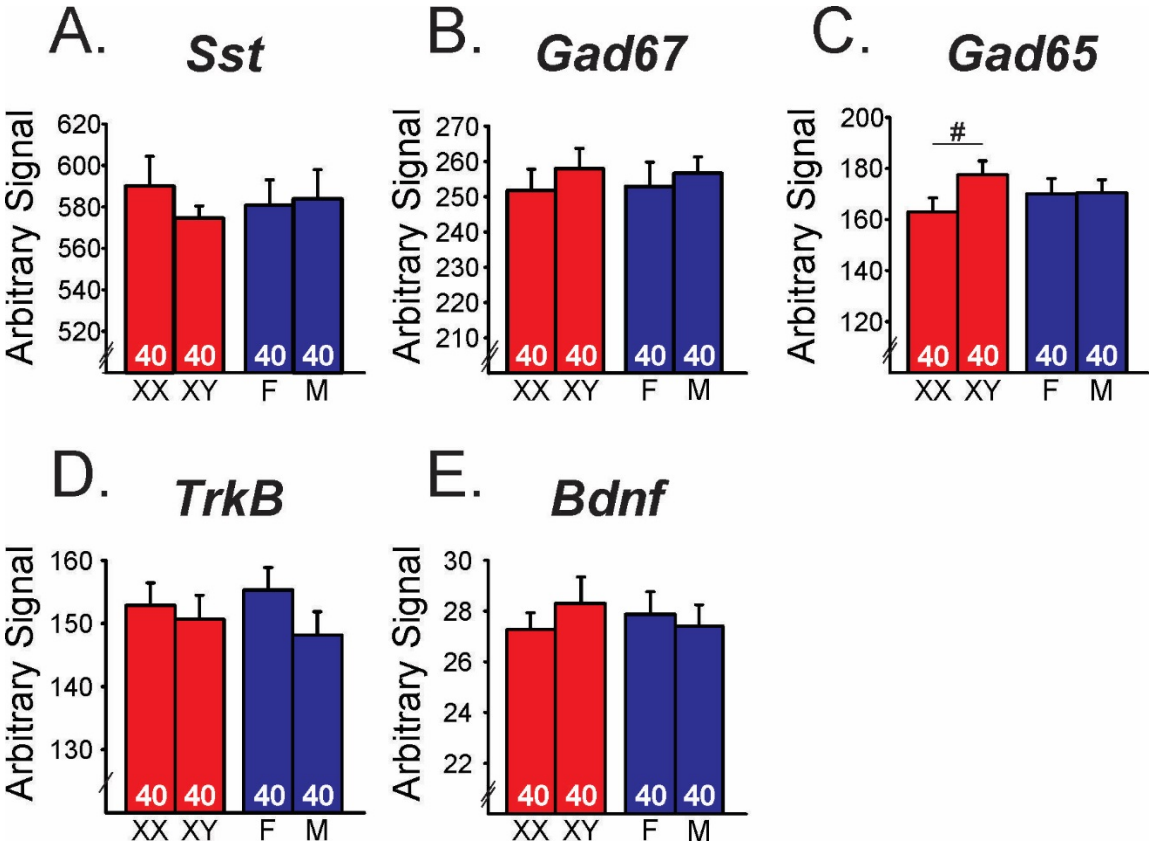

Figure 8: Sex differences in GABA-related and BDNF-related gene expression in the BLA of P26 mice

There was no effect of genetic sex on *Sst* (A), *Gad67* (B), *TrkB* (D), or *Bdnf* expression (E). XY mice had a trend for higher expression of *Gad65* expression (C) compared to XX mice. There were no organizational effects of hormone on expression of any gene investigated (A-E). Numbers at the base indicate *N*. #p < 0.1. F, gonadal female, M, gonadal male.

3.3 SUMMARY OF EXPERIMENTAL FINDINGS

Table 1: Summary of experimental results.

	Sst	*Gad67*	*Gad65*	*TrkB*	*Bdnf*	Testosterone
P16	** XX>XY	—	* F<M	** XX>XY	# F<M	** F<M
P21	# XX>XY	** XX>XY	* XX>XY	# XX>XY	—	** F<M
P26	—	—	# XX<XY	—	—	*** F<M
Non-Stressed Adults	—	—	—	* XX>XY	* B<T	—
Stressed Adults	* XX>XY B<T	—	—	—	* B>T	—

This table displays a summary of the experimental results of this study. Additionally, the results from a non-stressed adult cohort studied by another lab member are included to more completely visualize the proposed development trajectory [16]. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; #, $p < 0.1$; F, gonadal female, M, gonadal male, T, testosterone, B, blank.

4.0 DISCUSSION

We discovered several sex-related differences in the GABA and BDNF systems in the BLA (Table 1). To isolate the specific effects of genetic sex, development gonadal sex, and activational effects of adult hormones, we used the FCG mice. In cohort 1, we investigated GABA- and BDNF-related genes in gonadectomized adult mice exposed to unpredictable chronic mild stress. In cohort 2, we studied GABA- and BDNF-related genes in a developmental cohort across three time-points pre- and peri-pubertally: P16, P21, and P26.

In cohort 1 (Fig. 3), we found a main effect of genetic sex on *Sst* expression the BLA, with genetic females having higher expression of *Sst* than genetic males (XX > XY) and an opposing main effect of male-like testosterone levels, where testosterone-treated mice had higher *Sst* expression compared to blank-treated mice. Behavioral testing in these mice revealed that genetic sex significantly influenced behavior as well, with XY mice exhibiting higher anxiety-like behavior than XX mice (Fig. 4E). Also, mice with male-like testosterone exposure had significantly lower anxiety-like behavior compared to blank-treated mice (previously reported in [26]). Higher testosterone levels correlated with lower anxiety-like behavior in the mice. In cohort 2, we studied three developmental time-points. In P16 mice (Fig. 5), genetic sex significantly influenced expression of *Sst* and *TrkB* (XX > XY) and gonadal sex influenced *Gad65* and *Bdnf* expression (F < M). In P21 mice (Fig. 7), XY mice had significantly lower expression of *Gad67* and *Gad65,* and a trend for *Sst and TrkB* compared to XX mice. In P26

mice (Fig. 8), we found a trending main effect of genetic sex where genetic male mice had higher expression of *Gad65* than genetic female mice. Gonadal sex did not influence any of the five genes investigated. At all three developmental time-points, gonadal males had higher T levels compared to gonadal females (Fig. 6). There were no genetic sex differences in T levels at the three developmental time-points.

Our lab investigated GABA- and BDNF-related gene expression in the BLA of adult FCG mice under stressed conditions (cohort 1) to further investigate the relationship between gene expression and anxiety-like behavior. A previous study in our lab aimed to understand the same gene expression in the frontal cortex, and this is where we initially found the main effect of genetic sex on the expression of *Sst* where XX > XY [26]. However, we found that testosterone did not influence *Sst* expression in the frontal cortex despite testosterone strongly decreasing anxiety-like behavior. These results encouraged us to investigate gene expression in another brain region of the corticolimibic circuit, such as the BLA, in the same mice that could potentially correlate with these behavioral results. Our human postmortem work suggested that *SST* was reduced in the BLA of depressed women, but not depressed men. Thus, we turned our focus in mice on the BLA as the node in the corticolimbic network that might mediate sex differences in mood. The pattern of *Sst* expression that we found in the BLA of mice (lower *Sst* levels in XY mice, with testosterone increasing *Sst* expression) is consistent with the behavioral results. Lower *Sst* expression in XY mice is accompanied by higher anxiety-like behavior, while an increase in *Sst* expression with testosterone treatment is accompanied by a decrease in anxiety-like behavior. Our correlation analysis of these results revealed a significant negative correlation between *Sst* expression and anxiety-like behavior; moreover, higher *Sst* expression is correlated with lower anxiety-like behavior. Our *Sst* results suggest that male genetic sex and

male-like testosterone levels have opposing effects on BLA expression to modulate behavior. It seems that high circulating testosterone is compensating for the genetic vulnerability in XY mice. The compensation hypothesis suggests that gonadal hormones may act to abolish innate sex differences determined by genetic sex [44]. We believe testosterone acts to increase gene expression in the BLA to prevent the undesirable effects of lower expression in genetic males. Although XY mice may have a genetic deficit of *Sst* expression, testosterone serves to increase *Sst* expression and mask this underlying male vulnerability [16]. This pattern has been observed in other studies, which ultimately serves to decrease male-female differences. [44, 45]. For instance, mouse experiments reveal testosterone treatment increases male sex behavior, but having a Y chromosome decreases male sex behavior. It is thought that the genes on the X chromosome act to compensate for the unequal androgen experiences between males and females [45]. We previously found reduced *SST* expression in both the sgACC and DLPFC of both males and females with MDD, but reduced *SST* expression in the BLA was only found in females with MDD. The action of *Sst* that we revealed in cohort 1 may be in accordance with female vulnerability to MDD, as females do not typically have high levels of circulating testosterone [16].

Our testosterone findings in cohort 1 that show decreased anxiety-like behavior and increased expression of GABA-related genes are consistent with findings in both humans and rodents. For example, androgen insensitive males show higher levels of mood disorders ([46-50]). Low testosterone in human males is also associated with higher anxiety levels, but testosterone treatments reduce their anxiety [51, 52]. Rodent studies also report similar anti-anxiety effects of testosterone. Rats with testicular feminization mutation (Tfm) of the androgen receptor, both spontaneous and induced, have elevated anxiety-like behaviors compared to

wildtype males and females. Our results for the effect of testosterone on GABA and BDNF-related gene expression suggest the anti-anxiety effects of testosterone may be mediated by expression of these mood-related genes. Together, we hypothesize that men might be protected from anxiety and MDD via effects of testosterone on SST in the BLA.

When comparing non-stressed to stressed cohorts, we discovered interesting patterns of gene expression that allow us to construct an interpretation of the GABA and BDNF systems under these conditions. For example, the opposing effects of male genetic sex and testosterone on *Sst* expression discussed earlier only emerged under stressed conditions (Fig. 3). *Bdnf* expression was also affected based on stress conditions where testosterone increased expression under non-stressed conditions, but testosterone decreased expression in mice under chronically stressed conditions. Additionally, some sex-related factors are masked by chronic stress. Both the effect of genetic sex on expression of *TrkB* and the organizational effect on *Bdnf* expression disappear under chronic stress conditions [16]. These results signify the complex relationship between GABA- and BDNF-related gene expression and stress conditions.

Given the adult mice results, we asked whether there would be GABA- and BDNF-related sex differences in the BLA during development. We chose time-points considered to be pre- and peripubertal: P16, P21, and P26. Studying gonadally-intact developing mice allowed us to understand the GABA- and BDNF-related gene expression that exists pre- and peripubertally. We believe that events like abuse and neglect during critical periods of development could lead to pathologies in gene expression that would make one more vulnerable to mood disorders upon stressful situations in adulthood.

We found a main effect of genetic sex on expression of *Sst* and *TrkB* at P16. We also found a trend for a genetic sex effect of *Sst* and *TrkB* expression at P21. The main effect of

genetic sex in *Sst* and *TrkB* expression is present at P16, suggesting that this genetic sex difference originates at an earlier time point in the BLA (earlier than P16). The main effect of gonadal sex on circulating testosterone levels at P26 was more exaggerated than at P21, suggesting more pronounced gonadal hormone synthesis at this older age. It is interesting to note that even at these time-points before puberty, gonadal males still have higher testosterone levels than gonadal females. Our lab is conducting follow-up experiments to investigate gene expression of these same five genes in the BLA and circulating testosterone levels of P0 mice (the day of birth) to see if these sex effects are evident at birth.

The P16, P21, and P26 time-points are all within the organizational window in which sex differences in hormone exposure cause permanent sex differences. These time-points also, overlap with the well-known developmental trajectory of mood. The genetic sex differences in gene expression were present at P16 through P21. By P26, however, these differences almost completely disappear, with the exception of *Gad65* where there is a trending genetic sex difference (XX < XY). This pattern suggests a naturally differing developmental trajectory of the GABA system between XX and XY mice where the GABA system in the XY mice develops later than XX mice. We believe that disrupting this development could lead to adult psychopathology.

The developmental cohort results are quite interesting when considered in the context of previous studies conducted in our lab. We previously studied a cohort of gonadectomized mice under non-stressed conditions [16]. Interestingly, *TrkB* expression was influenced by genetic sex (XX > XY) and *Bdnf* expression was influenced by both gonadal sex and hormone treatment (F < M; blank > testosterone) in the BLA of non-stressed mice (Table 1). Additionally, we found that the *Sst* expression observed at P16 and P21 is similar to the *Sst* expression in adulthood

under chronic stress conditions (XX > XY). *Sst, Gad67, and Gad65* expression of the non-stressed cohort revealed no main effects, just like at P26. Interestingly, this main effect of genetic sex in *Sst* expression before puberty seems to disappear in adulthood, and only reemerges under stress conditions in adulthood. It appears that by P26, the gene expression in XY mice have caught up to XX mice. We predict that if we were to study a time-point between P26 and adulthood, we would find similar gene expression results to P26.

The peak of the effect of genetic sex on GABA- and BDNF-related gene expression appears to be at P21. Four out of the five genes of interest are significantly affected (XX > XY). As a general pattern, *Sst* expression and *TrkB* expression seem to move together over the course of development. In adulthood, the genetic sex effect on *TrkB* persists in non-stressed adults and the effect of stress eliminates this difference. The opposite happens for *Sst* expression; the genetic sex effect reappears under stressed conditions. Perhaps these results suggest an inverse relationship between the GABA and BDNF systems in adulthood under different stress conditions. *Gad65* and *Bdnf* expression also seem to move in concert with each other over the course of development. *Gad65* expression is significantly different in either genetic or gonadal sex before and during puberty, but this difference disappears in adulthood under both non-stressed and stressed conditions. While *Bdnf* is trending for an effect of gonadal sex at P16, expression is not significantly affected until adulthood. The effect of testosterone on *Bdnf* expression has opposite responses to stress conditions in adulthood: testosterone increases *Bdnf* in non-stressed mice, but decreases *Bdnf* expression in stressed mice. Our gene expression results highlight the intricate relationships between the GABA and BDNF systems over the course of development and under stress conditions.

There are a few limitations of our studies that I want to address. First, the FCG mice model is an artificial system that is not equivalent to male and female mice or humans. This model is necessary for our studies, however, to differentiate the sex-related factors that underlie the sex differences that are observed in wild-type mice and in humans. Testosterone levels also may vary by genetic sex during development. Although we did not test for testosterone levels at every point of development, we did not find a genetic sex difference in testosterone concentrations at P16, P21, or P26. Thus, our reported effects based on genetic sex are not confounded by potential differences in gonadal hormones. We used testosterone as our hormone treatment because it can be converted into estradiol in the brain. Thus, testosterone can act at both estrogen and androgen receptors. We will need to study receptor specificity in future studies, however, as our current study does not specify which receptors are in fact driving the effect of testosterone. We also did not compare our stressed and non-stressed cohorts at the same time. Using tissue homogenate of the BLA masks any cell-specific changes that may be occurring. We would have to change our methods moving forward, such as using laser microdissection of certain cell types, to identify cell-specific changes.

In conclusion, I found differential expression of GABA- and BDNF-related genes in the BLA that illustrates a different developmental trajectory of these systems between the genetic sexes. Together, my results suggest that gene expression pre- and peripubertally that make up the developmental trajectory overlap well with the developmental trajectory of mood. My studies support the hypothesis that testosterone acts on SST in the BLA to affect mood. The genetic sex differences in the developmental trajectory of the GABA system and the influence of circulating testosterone uncovered in my studies could affect adult susceptibility to mood disorders.

APPENDIX

THE EFFECT OF SEX-SPECIFIC FACTORS ON SEROTONIN- AND DOPAMINE-RELATED GENE EXPRESSION IN THE BASOLATERAL AMYGDALA OF ADULT STRESSED MICE

A.1 INTRODUCTION

A.1.1 Serotonin in Major Depressive Disorder

Serotonin (5-Hydroxytryptamine) is one of the monoamine neurotransmitters. Synthesis of serotonin depends on the level of the essential amino acid tryptophan and the activity of its rate limiting enzyme, tryptophan hydroxylase [53] (Figure 9).

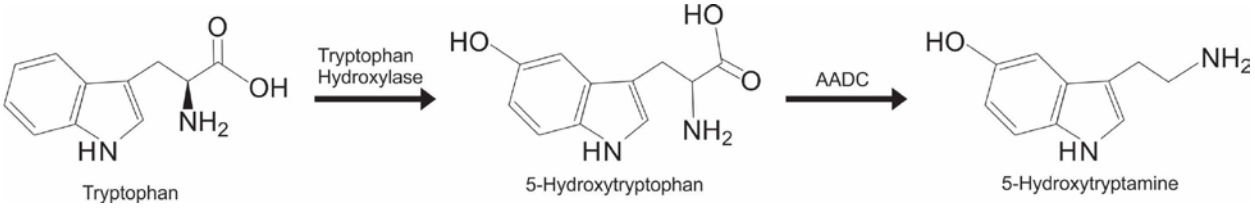

Figure 9: Serotonin synthesis pathway.

Serotonin is created by converting tryptophan into 5-hydroxytryptophan via the rate limiting enzyme tryptophan hydroxylase. This intermediate is then converted into 5-hydroxytrypamine (serotonin) via the enzyme aromatic amino acid decarboxylase (AADC).

Serotonin is largely found in the gastrointestinal tract and other tissues in the periphery[54]. Serotonin neurons in the brain are primarily located in the raphe nucleus and have widespread projections to have coordinated actions affecting mood, anxiety, and circadian rhythms [55, 56]. To measure serotonin metabolism from the central nervous system, a spinal tap is performed to measure 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin [57, 58]. Only 1-2% of total serotonin concentration is found in the brain, yet serotonin has been found to have an active role in mood regulation, behavior, and psychopathology etiology and treatment [59].

Evidence for serotonin involvement in major depressive disorder (MDD) includes decreased tryptophan, the amino acid from which serotonin is made [60, 61]. MDD also exhibits decreased serotonin metabolites, such as 5-Hydroxyindoleacetic acid (5-HIAA), in the cerebrospinal fluid [58]. Altered markers of transmission in the frontal cortex and of midbrain factors involved in serotonin production suggests ineffective subcortical-cortical serotonergic transmission [62]. Extensive rodent studies have explored the link between the serotonin system and anxiety-/depressive-like behaviors [63, 64]. Other studies revealed sex differences in the serotonin system in both human and rodent systems [65, 66]. Interestingly, studies have shown that depressed women respond better to selective serotonin reuptake inhibitors (SSRIs) than men [67].

A.1.2 Dopamine in Major Depressive Disorder

Dopamine is a catecholamine derived from the amino acid tyrosine, which is present at saturated concentrations in the body. The rate-limiting enzyme in this monoamine's synthesis pathway is tyrosine hydroxylase (Figure 10).

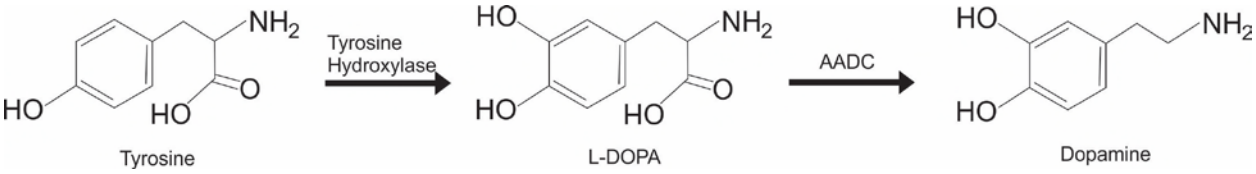

Figure 10: Dopamine synthesis pathway.

Dopamine is created by first converting tyrosine into L-DOPA via the rate limiting enzyme tyrosine hydroxylase. L-DOPA is then converted to Dopamine via the enzyme aromatic amino acid decarboxylase (AADC).

There is strong evidence that dopamine is involved in the anticipation of reward and the motivation to gain rewards [68-72]. Dopamine is commonly known to be involved in modulating these emotional responses, but it also has had implications in the pathophysiology of depression as well as antidepressant medication mechanisms [73, 74]. In response to antidepressant treatment, dopaminergic neurotransmission is altered [75-78]. To measure dopamine metabolism from the central nervous system, a spinal tap is performed to measure homovanillic acid (HVA) [79].

It has been hypothesized that altered dopamine function underlies anhedonia and amotivational behaviors in depression [73, 80]. Evidence for dopamine involvement in MDD includes both postmortem and imaging studies suggesting changes in receptor levels, yet these results remain uncertain [81-83]. Optogenetic studies demonstrate a causal link between ventral tegmental area (VTA) dopaminergic neuron firing and anhedonia-like behavior in mice [84].

Human imaging studies reveal dopamine-related sex differences in the frontal cortex of control subjects, where women have increased dopamine D2-like receptors compared to men [85]. There is conflicting evidence that indicates depressed patients have lower metabolites of dopamine in the cerebrospinal fluid, suggesting dopamine hypofunction [86]. However, subsequent rodent studies reveal sex differences in the dopamine system and report lower dopamine levels and higher turnover in the frontal cortex of female rats when compared to male rats (i.e., higher HVA/DA ratio in female rats) [35, 87].

A.1.3 Serotonin and Dopamine in the frontal cortex of FCG Mice

Our lab previously studied expression of serotonin- and dopamine-related genes in the frontal cortex of FCG mice exposed to unpredictable chronic mild stress (UCMS). Results were previously reported in [35]. Overall, we found main effects of genetic sex, gonadal sex, and circulating testosterone on expression of genes coding for serotonin and dopamine receptors, components of both the cAMP/PKA and AKT signal transduction pathways, and other serotonin related genes in the frontal cortex of these mice. Specifically, genetic sex was the main factor influencing serotonin- and dopamine-related gene expression in the frontal cortex under chronic stress conditions [35]. These results were quite interesting to us and led us to formulate two alternative hypotheses when studying expression of these same pathways in the basolateral amygdala (BLA). We aimed to determine if effects of these sex-specific factors are 1) brain-wide in serotonin- and dopamine systems or if 2) the genetic sex differences observed in the previous study were specific to the frontal cortex. The frontal cortex and the BLA are both regions involved in the corticolimbic neural network of mood regulation [3], with the frontal cortex

providing top-down processing and the amygdala providing bottom-up processing [88]. Top-down processing and bottom-up processing are two different responses to sensory stimuli in the environment. Top-down processing refers to the perception of information via cognition while bottom-up processing refers to the perception of information via the external stimulus itself [89]. There is a dynamic interplay between these two processes when one is tasked with processing sensory information. An emotionally salient stimulus that evokes a fear response, such as seeing a snake in the woods, is an example of bottom-up processing. Conversely, realizing that the "snake" in the woods was actually a stick is an example of top-down processing. Since our lab has studied gene expression in the frontal cortex extensively, we decided to study BLA, which is at the opposite end of this circuit, with the goal of understanding the relationship between these two regions.

A.2 METHODS

A.2.1 Mice

This study utilized a unique strain of mice called the Four Core Genotypes (FCG) mice. In a wildtype mouse, genetic sex and gonadal sex are entirely linked due to the testes-determining gene, *Sry*, on the Y chromosome. This means that if a wildtype organism has a Y chromosome, they have *Sry,* and therefore, will develop gonadally male (both genetically and gonadally male). If a wildtype organism does not have a Y chromosome, they do not have *Sry*, and therefore, will develop gonadally female (both genetically and gonadally female). Using genetic engineering,

the *Sry* gene was removed from the Y chromosome and placed on an autosome instead, thereby decoupling genetic and gonadal sex.

The FCG mice used in these studies (originating from Jackson Laboratories, Bar Harbor, ME, USA; B6.Cg- Tg(Sry)2Ei Srydl1Rlb/Arnoj) were generated by crossing a C57BL/6J female with an XY^{-}*Sry* male. Y^{-} indicates the absence of Sry on the Y chromosome, with Sry present instead as an autosomal transgene. Here, we use the male (M) and female (F) designation to indicate gonadal sex. This cross yielded four groups of mice: XXM (XX*Sry*), XXF (XX), XYM (XY^{-}*Sry*) and XYF (XY^{-}). These four genotypes allow us to investigate the effects of genetic sex and developmental gonadal sex (i.e., organizational effects of hormones) independent of the other, as genetic sex and gonadal sex are inherited independently in this model. The addition of a testosterone or blank capsule (discussed below) after gonadectomy in adulthood also allows us to investigate the effects of circulating hormones (i.e., activational effects of testosterone). The mice were maintained under standard conditions (12-hour light and dark cycles; 22 ± 1°C, food and water ad libitum), in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee [16]. We studied the GABA-related gene expression in this same cohort of mice previously (Cohort 1).

A.2.2 Gonadectomies and testosterone treatment

Adult FCG mice were bilaterally gonadectomized at 15 weeks under isoflurane anesthesia to remove the endogenous source of circulating gonadal hormone. Half of each genotype was implanted with a subcutaneous testosterone-filled capsule containing 5-mm crystalline testosterone (1.57-mm ID x 2.41-mm OD) or with a similarly sized blank capsule [16]. [XXF +

B (n=10), XXF + T (*n* = 16), XYF + B (*n*=12), XYF + T (*n*=12), XXM + B (*n*=15), XXM + T (*n* = 14), XYM + B (*n*=10), XYM + T (*n* = 12)]. It is important to note that the concentration the testosterone capsules were at or slightly above physiologically normal male levels. After allowing 4 weeks for mice to recover from surgery and for hormone levels to equilibrate, the mice were exposed to 8 weeks of unpredictable chronic mild stress (UCMS; see details below). After 7 weeks of UCMS exposure, mice were assessed for anxiety-like behaviors in the elevated plus maze and open field (Figure 11). Mice were exposed to UCMS during behavior testing, thus the mice were exposed to UCMS for a total of 8 weeks.

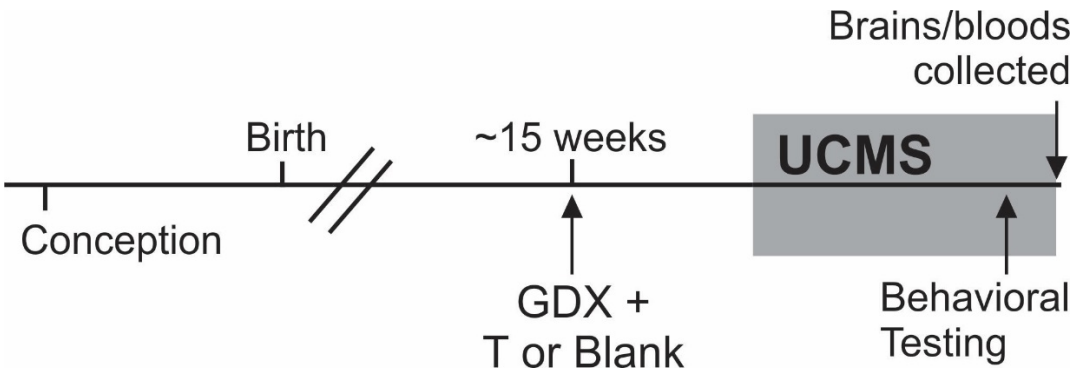

Figure 11: Experimental design for adult stressed mice.

Mice were gonadectomized (GDX) at ~15 weeks of age and implanted subcutaneously with either a testosterone (T)-filled or blank capsule. After GDX, mice in cohort 1 were exposed to 8 weeks of unpredictable chronic mild stress (UCMS) followed by behavioral testing and were then sacrificed. At the time of sacrifice, the brains were harvested for gene expression analyses and bloods were collected for hormone assay [16].

A.2.3 Unpredictable Chronic Mild Stress

Group-housed adult mice (gonadal sex and hormone treatment matched) were exposed to a randomized schedule of environmental stressors 7 days a week for 8 weeks, gradually increasing in intensity, starting with 1–2 separate stressors a day and ending with 4–5 stressors a day

(separately and in tandem with one another) during the final week, as performed previously in our lab [16, 26]. Disturbances included light cycle disruption, tilted cage (45° tilt), social stress (rotate mice into previously occupied cages), reduced space (limiting mice to 1/3 of typical space in cage), aversive smell (20 min of exposure to bobcat or fox urine), no bedding or wet bedding overnight, mild restraint (50-mL conical tube with air hole for 15 min), and forced bath (approximately 2 cm of 21°C water for 15–45 min). We assessed body weight and fur weekly to track progression of the UCMS syndrome. Behavior testing occurred while mice were still being exposed to UCMS. We previously reported frontal cortex gene expression results from this cohort [26, 35].

A.2.4 Processing of brain tissue: BLA dissection and gene expression analyses

Bilateral micropunches (1mm bore punch) of the BLA (between Bregma −0.94 and −1.82 mm; [31]) were obtained from approximately six 160-µm thick coronal tissue sections cut on a cryostat within the boundaries created by the internal and external capsule. All tools were treated with RNase Zap to prevent RNases contamination. Punches were stored in RNase free 1.5mL tubes at −80°C prior to RNA extraction [16].

A.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from BLA tissue punches using RNeasy Plus Micro Kits (Qiagen; Valencia, CA, USA). RNA was reverse-transcribed into complementary DNA (cDNA) using QScript cDNA Supermix (olido(dT) and random primers (Quanta Biosciences, Gaithersburg, MD, USA)) [16].

A.2.6 Gene Selection in the BLA

Similar to our frontal cortex study, we utilized the Allen Brain Atlas to determine which serotonin- and dopamine-related genes have high expression in the basolateral amygdala (BLA) expression of mice. These genes with high BLA expression were selected for quantitative polymerase chain reaction (qPCR) analysis. We examined 13 genes related to serotonin and dopamine in the BLA of FCG mice: *Adcy2, Adcy5, Adrbk2, Akt3, App, Cacna1a, Cdk5, Comt, Gsk3a, Gsk3b, Mapk1, Ppp1r1b,* and *Syn2* (See Table 2).

Table 2: Serotonin and Dopamine Gene Selection in the BLA of Adult Mice.

Gene	Name	Function	Studied in Frontal Cortex	Sex-Difference in Frontal Cortex
Adcy2	Adenylyl cyclase 2	Involved in cAMP/PKA signal transduction pathway	Yes	Yes
Adcy5	Adenylyl cyclase 5	Involved in cAMP/PKA signal transduction pathway	Yes	Yes
Adrkb2	G-protein coupled receptor 3	Involved in signal transduction pathways	—	—
Akt3	Serine/ threonine kinase 3	Regulator of P13K-AKT-mTOR pathway	Yes	Yes
App	Amyloid precursor protein	Involved in synapse formation	Yes	Yes
Cacna1a	Calcium voltage-gated channel subunit α 1A	P/Q Ca^{2+} channel equivalent	—	—
Cdk5	Cyclin dependent kinase 5	Phosphorylates Ppp1r1b in NMDA receptor	Yes	No
Comt	Catecol-O-methyltransferase	Metabolizes dopamine	—	—
Gsk3a	Glycogen synthase kinase 3 A	Inhibits glycogen synthase via phosphorylation	—	—
Gsk3b	Glycogen synthase kinase 3 B	Inhibits glycogen synthase via phosphorylation	—	—
Mapk1	Mitogen-activated protein kinase 1	Involved in MAP/ERK pathway	—	—
Ppp1r1b	Protein Phosphatase 1 Regulatory Inhibitory Subunit 1B	Involved in dopamine signaling	—	—
Syn2	Synapsin 2	Modulation of neurotransmitter release on the surface of synaptic vesicles	—	—

We selected serotonin- and dopamine-related genes that had high expression in the BLA according to the Allen Brain atlas. We previously analyzed expression of some of these genes in the frontal cortex of FCG mice [35].

A.2.7 Quantitative Polymerase Chain Reaction (qPCR)

Small PCR products were amplified on a MJ Research (Waltham, MA, USA) DNA Engine Opticon System for qPCR using universal PCR conditions (65 to 59 °C touch-down and 40 cycles (10 s at 95°C, 10s at 59°C, and 10s at 72°C)). cDNA was amplified in 20-μL reactions (0.1 × SYBR Green, 3 mM MgCl2, 200 nM dNTPs, 200 nM primers, 0.25 unit Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA)) [16].

Samples were run in triplicate, and results were calculated as the geometric mean of relative intensities compared to three internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cyclophilin). Actin, GAPDH, and cyclophilin are considered good housekeeping genes because sex-related factors do not influence their expression or abundance, thus we consider them to be internal controls. The results are expressed as arbitrary signal ($2^{-dCT} \times 10{,}000$). Grubbs' outlier test determined statistical outliers in gene expression values. These outliers were excluded from qPCR analysis [16].

A.2.8 Statistical analysis

We used a three-way analysis of variance (ANOVA), (genetic sex x developmental gonadal sex x hormone treatment) to analyze gene expression data. This is the same approach we used for Cohort 1 in the GABA study (see *Methods)*. If the ANOVA was significant for any main effect or interaction, we performed planned comparisons using the Least Significant Difference (LSD) post hoc test. We did not correct for multiple testing in these studies, as we had a priori hypotheses for these genes to be altered based on our findings in the frontal cortex of FCG mice

and in human postmortem brain tissue of patients with MDD[23, 24, 26]. Data are expressed as mean ± SEM, and significance was set at $p < 0.05$ [16].

A.3 RESULTS

Compared to XX mice, XY mice had significantly lower expression of *App* in the BLA ($p < 0.04$; Figure 4A). There was no effect of genetic sex on expression of *Adcy2, Adcy5, Adrbk2, Akt3, Cacna1a, Cdk5, Comt, Gsk3a, Gsk3b, Mapk1, Ppp1r1b,* or *Syn2,* ($p > 0.2$ for all comparisons). We found a trend for gonadal males to have higher expression of *Adrbk2* compared to gonadal females ($p < 0.09$; Figure 12B). There was no effect of gonadal sex on expression of *Adcy2, Adcy5, Akt3, App, Cacna1a, Cdk5, Comt, Gsk3a, Gsk3b, Mapk1, Ppp1r1b,* or *Syn2* ($p > 0.1$ for all comparisons). There was also a trend for testosterone increasing expression of both *Comt* ($p < 0.1$; Figure 12C) and *Mapk1* ($p < 0.1$; Figure 4D) in the BLA. There was no effect of testosterone on expression of *Adcy2, Adcy5, Adrbk2, Akt3, App, Cacna1a, Cdk5, Gsk3a, Gsk3b, Ppp1r1b,* or *Syn2* ($p > 0.1$ for all comparisons).

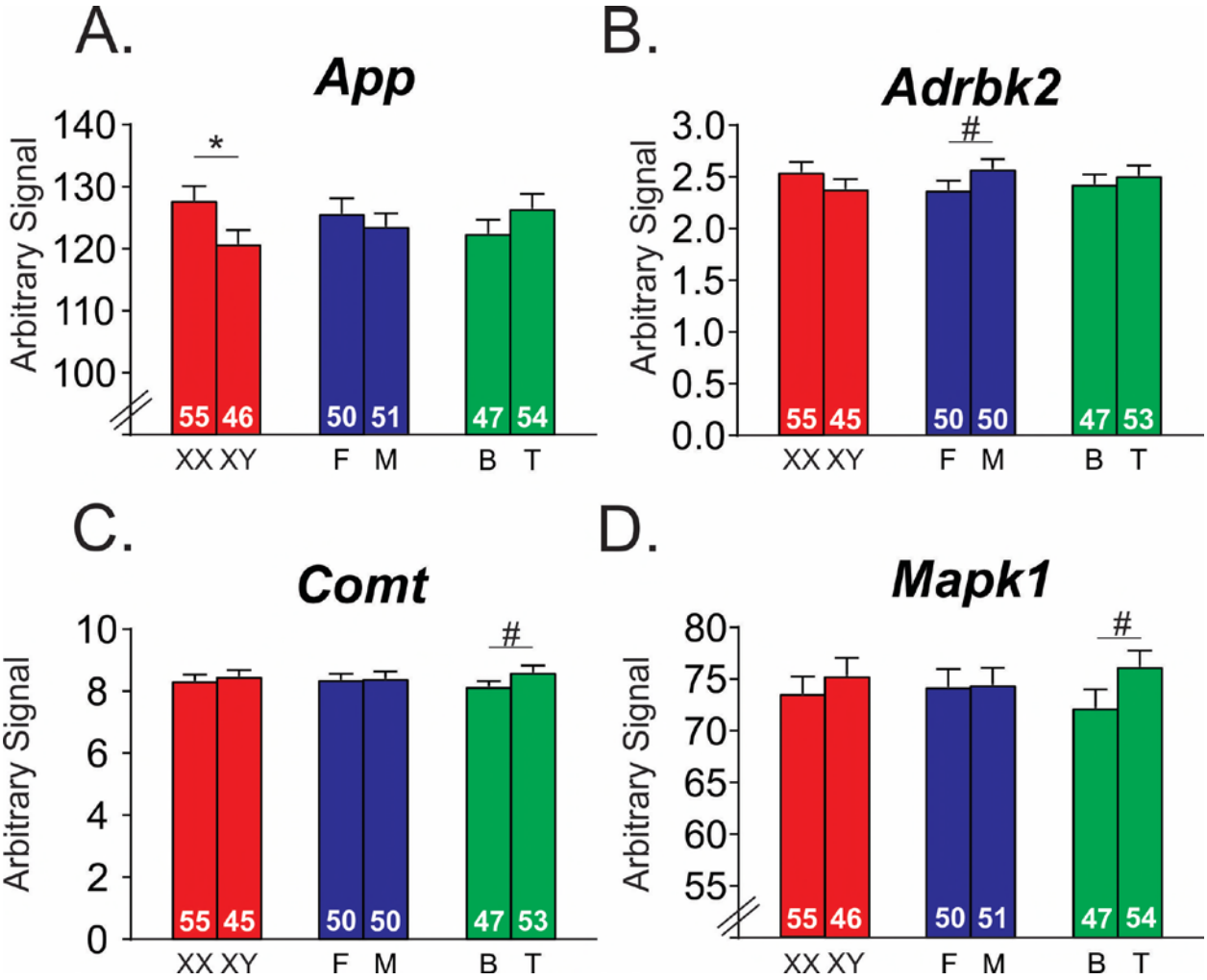

Figure 12: Main effects of sex-specific factors on the serotonin- and dopamine-related genes in the BLA of adult stressed mice.

(A) XY mice had lower expression of *App* than XX mice. There was no effect of genetic sex on *Adrbk2* (B), *Comt* (C), or *Mapk1* (D). (B) There was a trend for an effect of gonadal sex on *Adrbk2* expression. Gonadal sex did not influence expression of *App* (A), *Comt* (C), or *Mapk1* (D). There was a trend for an effect of testosterone exposure on expression of *Comt* (C) and *Mapk1* (D), but no effect of testosterone on expression of *App* (A) or *Adrbk2* (B). Numbers at the base of the bars indicate *N*. *$p < 0.05$; #, $p < 0.1$. T, testosterone, B, blank, F, gonadal female, M, gonadal male.

We also found some interesting interactions between the main effects on expression of the serotonin- and dopamine-related genes in the BLA. There was a trend for an interaction between genetic sex and testosterone on *Adcy2* expression ($p = 0.053$; Figure 13A); post hoc analysis revealed a trend for a difference between genetic females with and without testosterone treatment (XX+B vs. XX+T; $p = 0.053$) and a significant difference between genetic males treated with testosterone and genetic females treated with testosterone (XX+T vs. XY+T; $p = 0.041$). There was a significant interaction of gonadal sex and testosterone on *App* expression ($p = 0.031$; Figure 13B); post hoc analysis revealed a difference between gonadal females with and without testosterone treatment (F+B vs. F+T; $p = 0.012$) and a difference between gonadal males treated with testosterone and gonadal females treated with testosterone (F+T vs. M+T). There was a trend for an interaction between genetic sex and testosterone on *Gsk3a* expression ($p=0.053$ Figure 13C); post hoc analysis revealed a difference between genetic females treated with testosterone and genetic males treated with testosterone (XX+T vs. XY+T; $p = 0.038$), as well as a difference between genetic males with and without testosterone treatment (XY+B vs. XY+T; $p = 0.052$). Finally, there was a significant interaction between gonadal sex and hormone on *Gsk3a* expression ($p=0.019$; Figure 13D); post hoc analysis revealed a difference between blank-treated gonadal females and blank-treated gonadal males (F+B vs. M+B; $p = 0.047$) as well as a difference between gonadal males with and without testosterone treatment (M+B vs. M+T; $p = 0.033$).

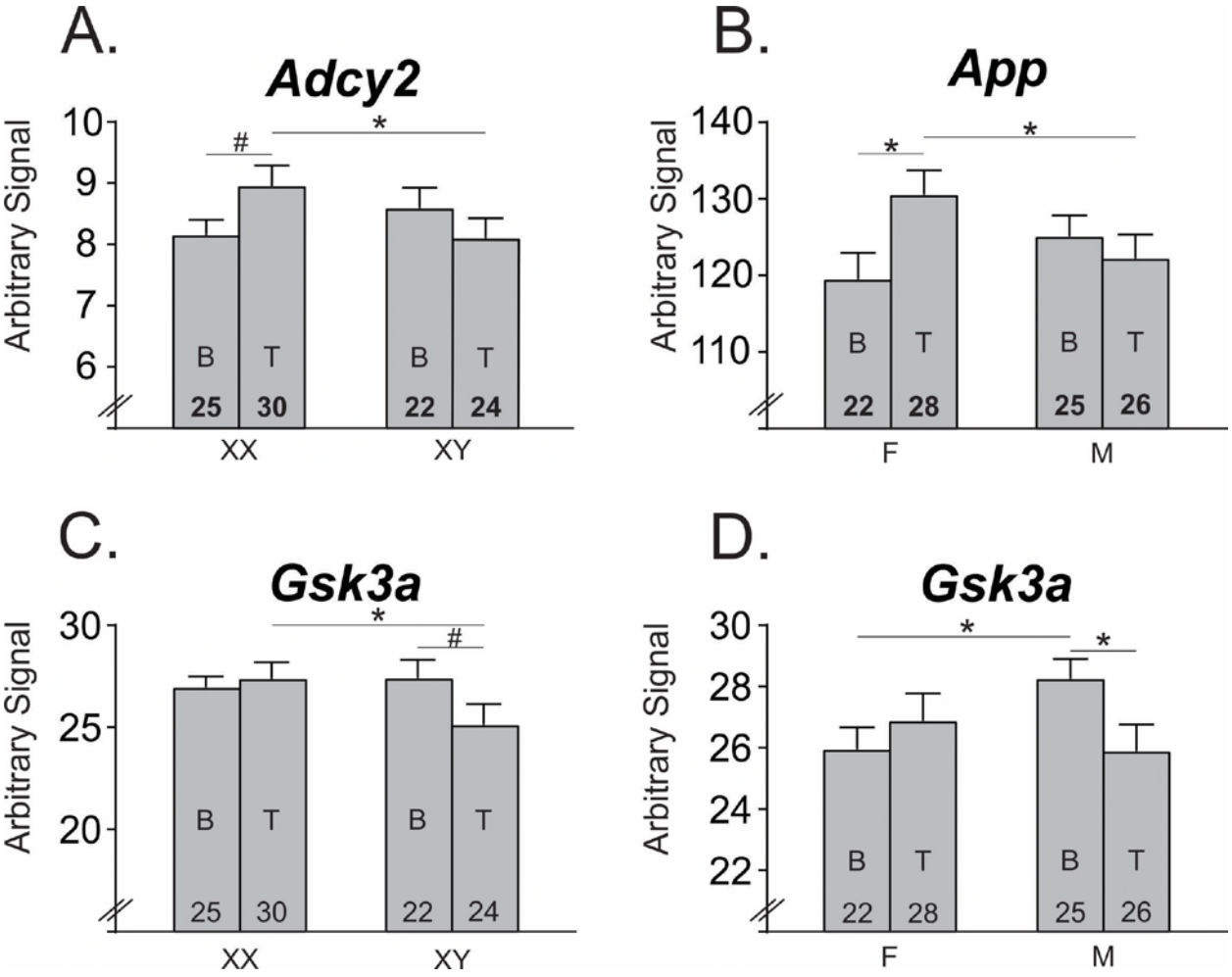

Figure 13: Interactions of sex-specific factors on serotonin- and dopamine-related gene expression in the BLA of adult stressed mice.

(A) There was a trend for testosterone to increase *Adcy2* expression in XX mice, but no effect of testosterone in XY mice (B) Testosterone increases *App* expression in gonadal females but not gonadal males. (C) There was a trend for testosterone to decrease *Gsk3a* expression in XY mice, but no effect of testosterone in XX mice. (D) Testosterone treatment decreases *Gsk3a* expression in gonadal males, but not gonadal females. Numbers at the base of the bars indicate *N*. *$p < 0.05$; #, $p < 0.1$. T, testosterone, B, blank, F, gonadal female, M, gonadal male.

A.4 DISCUSSION

We investigated the effects of genetic sex, developmental gonadal sex, and circulating testosterone on expression of several serotonin- and dopamine-related genes in the BLA of chronically stressed mice. We aimed to determine if sex-specific factors influenced serotonin- and dopamine-related gene expression in a brain-wide or region-specific fashion. We found a main effect of genetic sex on *App* expression in the BLA (Fig. 12A) with genetic females having higher expression than genetic males (XX > XY). We also found a trend for a main effect of gonadal sex on *Adrbk2* expression in the BLA (Fig. 12B) with gonadal males trending for higher expression than gonadal females. Testosterone was found to have a trend for increasing the expression of *Comt* and *Mapk1* in the BLA (Fig. 12C-D).

Depending on the gene, my results support both hypotheses. For instance, the effect of genetic sex on *App* expression in the BLA is consistent with the results from our frontal cortex study in [35]. Specifically, in both the frontal cortex and BLA, XY mice have lower expression of *App* than XX mice. Thus, my results for *App* expression in the BLA support the brain-wide hypothesis. However, my results for other genes support the region-specific hypothesis. In the frontal cortex, there was an effect of circulating testosterone (T > B) and a trend for an effect of genetic sex (XX > XY) on *Adcy2* expression [35]. In the BLA, however, there were no main effects in *Adcy2* expression. Similarly, in the frontal cortex, there was a main effect of gonadal sex (F > M), a main effect of circulating testosterone (B > T), and a trend for an effect of genetic sex (XX > XY) on *Akt3* expression [35]. There were no significant main effects found for *Akt3* expression in the BLA, thus supporting the region-specific hypothesis. My results further

illustrate the complexity of effects that sex-related factors have on expression of genes related to mood.

We also found several interesting two-way interactions between genetic sex and hormone as well as gonadal sex and hormone. There was a trend for an interaction between genetic sex and testosterone on *Adcy2* expression ($p = 0.053$; Fig. 13A). For instance, post hoc analysis revealed that testosterone treatment increased *Adcy2* expression only in XX mice. This is interesting to consider, as the same hormone exposure is having different effects depending on genetic sex. There was a significant interaction of gonadal sex and testosterone on *App* expression ($p = 0.031$; Fig. 13B). Post hoc analysis revealed that testosterone increases *App* expression in only gonadal females. Thus, the same hormone exposure is producing different effects depending on gonadal sex. There was a significant interaction between gonadal sex and hormone on *Gsk3a* expression ($p=0.019$; Fig. 13D). Post hoc analysis revealed that testosterone decreases *Gsk3a* expression in gonadal males only. There was also a trend for an interaction between genetic sex and testosterone on *Gsk3a* expression ($p=0.053$; Fig. 13C). Post hoc analysis revealed that testosterone decreases expression in XY mice only. These interactions are critical to understand, especially when considering future advancement in depression treatments. My studies demonstrate an important influence of genetic sex, developmental gonadal hormone, and adult circulating hormone on serotonin-, and dopamine-related gene expression in the BLA. Further, my results point out that the serotonin and dopamine systems in the BLA are not as affected by sex-specific factors as they are in the frontal cortex [35].

Interestingly, depressed women tend to respond better to SSRI treatment than depressed men[67]. Thus, it is tempting to speculate that the serotonin system is more dysregulated in women with depression compared to men. Due to inherent differences (genetic sex, gonadal sex,

circulating hormones), men and women might have different underlying biological mechanisms of MDD. We found that testosterone affects serotonin and dopamine genes differently based on genetic or gonadal sex. Since men have higher testosterone concentrations than women, testosterone may protect against serotonin and dopamine deficits in men. The interactions uncovered in my study, therefore, emphasize the potential difference in responses to drug treatments between the sexes, which provides further evidence for the need for sex-specific treatment for depression. Considering the evidence above, it is possible that the differential response to SSRI treatment between men and women is due to differential interactions of sex-specific factors. Treatment developers should take the interactions of these influences into consideration to ensure the effects novel drugs are consistent across all sex-specific factors.

There are a few limitations of this study that I want to address. First, the FCG mice model is an artificial system that is not equivalent to male and female mice or humans. This model is necessary for our studies, however, to differentiate the sex-related factors that underlie the sex differences that are observed in wild-type mice and in humans. We did not correlate BLA serotonin- and dopamine-related gene expression with the behavioral results from this cohort. Perhaps this data can give us a deeper understanding of the effects of stress exposure on the serotonin and dopamine systems in the BLA. Since we found interesting results involving a possible protective effect of testosterone in males, we could study a cohort of blank-treated and testosterone-treated male and female mice with UCMS. We predict that the females treated with testosterone would be protected from serotonin deficits. We also only studied BLA expression of serotonin- and dopamine-related genes in a stressed cohort of mice. In future studies, we will look at the serotonin- and dopamine-related genes in a non-stressed cohort of adult mice to add a control experiment. We may study serotonin- and dopamine-related gene expression in other

brain regions such as the hippocampus, dorsal striatum, and ventral striatum to gather further evidence for the two alternative hypotheses. It is possible that one brain region may drive another brain region to have a certain level of activity; however, it is impossible to determine this from the studies conducted so far. To determine if the frontal cortex drives the amygdala, for instance, a follow-up study can be conducted that chronically inhibits neurons in the frontal cortex to see the effect on the BLA neuronal activity.

To conclude, my study reveals the complex effects that sex-related factors have on expression of mood-related genes across brain regions. My results support both the brain-wide and region-specific hypotheses, depending on the gene of interest. Differential interactions of testosterone with both genetic and gonadal sex highlight the importance of considering inherent, physiological differences between men and women when developing treatments for depression to ensure therapeutic effects and proper treatment of underlying psychopathology.

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