THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

Insight Into Posttraumatic Stress Disorder: A Behavioral and Molecular Comparison Between Male and Female Mice Exposed to Modified Stress-Enhanced Fear Learning

ALEXANDRIA MCKENNA
SPRING 2024

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Biology

Reviewed and approved* by the following:

Dr. Janine Kwapis
Assistant Professor of Biology
Thesis Supervisor

Dr. Bernhard Lüscher
Professor of Biology, Biochemistry and Molecular Biology, and Psychiatry
Honors Adviser

* Electronic approvals are on file.

ABSTRACT

Posttraumatic stress disorder (PTSD), a trauma- and stress-related disorder, consists of emotional and behavioral changes in people following one or more traumatic experiences. According to epidemiological research, PTSD is *disproportionately* prevalent among the adult population. Specifically, more women are likely to develop PTSD than men – 8% of women compared to 4% of men at some point in their lives (U.S. Department of Veterans Affairs, 2023). Despite this sex difference persisting over several decades, minimal research provides insight into casual factors, with even less work focusing on molecular factors compared to situational factors. Consequently, this present study aimed to elucidate the roles of candidate genes behind the sex difference in fear learning through a reliable rodent model of PTSD known as the stress-enhanced fear learning (SEFL) procedure. Since the standard SEFL procedure elicits an effect strong enough to mask underlying sex differences, as indicted by some of our previous work, the SEFL procedure was first *modified* by decreasing the number and intensity of administered footshocks. Once we confirmed that the modified SEFL procedure engendered a sex difference in murine fear learning, another cohort of mice only underwent the first and second phases of the modified SEFL procedure to pinpoint any molecular effects of weak stress history on fear learning. From there, the mice were sacrificed for hippocampus and amygdala extractions due to the relevance of these brain regions in the SEFL research literature. With the brain tissues, RT-qPCR analyses were conducted to quantify the expressions of transcripts mapping to *BDNF* exons I, IV, and IX and of the *Gria1* gene; like the brain regions, these genes were selected due to their relevance in the SEFL research literature. We found that hippocampal *BDNF* transcripts are correlated with an increased SEFL efficacy regardless of sex while the roles of the amygdala *BDNF* transcripts and *Gria1* transcript,

in either brain region, remained unclear. Altogether, these results prompt future study with modified SEFL into establishing genes driving the observed sex difference, as well as validating the roles of *BDNF* and *Gria1* transcripts, to enhance understanding of PTSD for the sakes of millions struggling to benefit from current treatments and therapies.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

These next couple of pages are dedicated to everyone who shaped my undergraduate research experience. Without their support, I would not be the burgeoning researcher I am today.

First, I would like to thank my research supervisor, Dr. Janine Kwapis, for offering the opportunity to join her lab as a first-year undergraduate research assistant amidst uncertain circumstances because of the COVID-19 pandemic. I also would like to thank her for being an amazing role model in my life. She has taught me how to put forth dependable work and how to genuinely collaborate with others as I wade deeper into the professional realm of research. Next, I would like to thank one of my research mentors, graduate student Chad Brunswick, for teaching me how to perform tissue extractions, run RT-qPCR analyses, and analyze data. I tell everyone that if Chad had not been so present in my experience and so dedicated to my success, I would not have found my passion for research. I aspire to be that kind of person for someone one day. I would also like to thank another research mentor, Dr. Mark Urban, for teaching me how to run the modified SEFL procedure and for pushing me to further investigate it from a molecular perspective. I will never forget all the great advice he shared with me while we sat through hours of SEFL. Lastly, I would like to thank my other research mentor, Dr. Shoko Murakami, for teaching me how to design primers and probes for my RT-qPCR analyses. I will never forget her patience and guidance.

I would also like to thank my first honors adviser, Dr. Stephen Schaeffer, for guiding me through the process of joining a research lab and for connecting me with Dr. Kwapis. I would also like to thank my current honors adviser, Dr. Bernhard Lüscher, for not only providing feedback on my thesis, but also for letting me discuss future plans with him.

Thank you to the rest of the graduate and undergraduate students in the Kwapis Lab for making our lab a warm and welcoming space. Thank you to my friends and family for always pushing me to reach my goals and to do my best.

Most importantly, thank you to my Savior, Jesus Christ. My faith in you has brought me through every high and every low throughout my undergraduate experience. “That if thou shalt confess with thy mouth the Lord Jesus, and shalt believe in thine heart that God hath raised him from the dead, thou shalt be saved. For with the heart man believeth unto righteousness; and with the mouth confession is made unto salvation” – Romans 10:9-10.

This material is based upon work supported by the NIH/NIA under grant R01AG074041 and the PSU Department of Biology under start-up funds all to Janine L. Kwapis. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the NIH, NIA, and PSU Department of Biology.

Chapter 1

Introduction

In 1980, the American Psychological Association officially recognized posttraumatic stress disorder (PTSD) as a mental disorder in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM), provoking epidemiological research into PTSD (American Psychological Association, 1980). By the 1990s, the research consistently led to this finding: more women struggle with PTSD than men (Breslau & Davis, 1992; Breslau et al., 1998; Davidson et al., 1991; Kessler et al., 1995). Insight into causal factors behind this finding later came along through meta-analyses conducted by Tolin and Foa (2006). They found that women more frequently experience traumatic events of higher severity (e.g., sexual assault and child sexual abuse), *partially* engendering a greater susceptibility to PTSD. When they held the type of traumatic event constant, they still found a higher prevalence of PTSD among women compared to men, suggesting other unknown factors at play.

Fast forward to today, the fifth edition of the DSM considers PTSD a trauma- and stress-related disorder triggered by the experience of one or more traumatic events (e.g., military combat, physical attack, sexual abuse, natural disaster, severe accident, etc.). People with PTSD commonly experience the following symptoms: unprovoked, unsettling recollections of the trauma (also known as “flashbacks”), avoidance of trauma reminders, persistently negative perceptions, heightened emotional states, loss of interest in previously enjoyed activities, and reckless, self-destructive behaviors (American Psychological Association, 2013). Additionally, the higher prevalence of PTSD among women compared to men is widely recognized. According to the U.S. Department of Veterans Affairs (2023), about 8% of women will develop PTSD at some point in their life compared to 4% of men. Despite accepted clinical presentations and trends of PTSD,

minimal research addresses factors contributing to women's greater susceptibility to PTSD, with even less work focusing on molecular factors compared to situational factors (Olff, 2017).

On the level of animal research, we look to a reliable rodent model of PTSD known as the stress-enhanced fear learning (SEFL) procedure (Rau et al., 2005; Rau & Fanselow, 2009). For mice, the SEFL procedure consists of three phases: a strong stress event, subsequent mild fear conditioning, and a context test that assesses the extent of fear learning and memory (*Figure 1-1A*). Regarding PTSD, each phase respectively simulates the following: a "traumatic" event, a subsequent mild stress event in a new environment, and re-exposure to the environment of the mild stress event. This series of events represents a typical PTSD experience in which individuals display stronger emotional reactions to mildly arousing events following trauma. The ultimate goal of the SEFL procedure is to highlight that connection between stress exposure and responses to mild triggers through learning and memory; therefore, stressed mice should show a more intense memory for the subsequent mild fear conditioning during the context test. We have confirmed that mice indeed react more strongly to subsequent mild fear conditioning by freezing more (or spend more time holding extremely still) – a behavior commonly displayed by mice when feeling threatened – during the context test when they have a strong history of stress compared to unstressed mice (*Figure 1-1B*). However, upon further investigation of this experiment, the strong stress event masks underlying sex differences, complicating efforts to simulate the sex difference among people with PTSD (*Figure 1-1C*). When looking to the work of other researchers, few have attempted to modify the SEFL procedure to find any sex differences. Just recently, Gonzalez et al. (2021) modified the SEFL procedure for rats by decreasing the number of footshocks for the stress event and found important sex differences relating to fear learning, anxiety-related behavior, and voluntary alcohol intake, revealing the potential of this research direction.

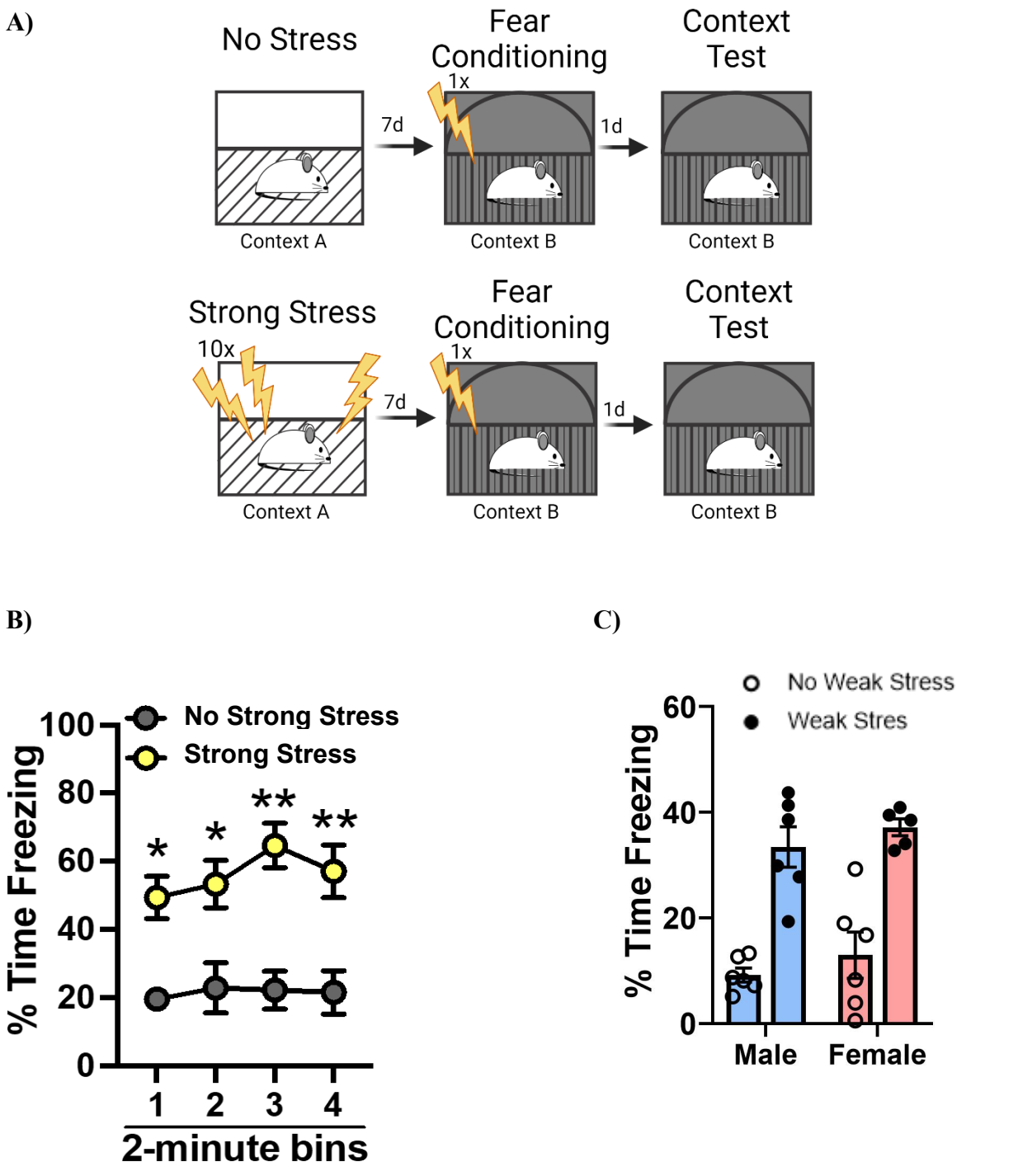


Figure 1-1. Results of an experiment using the standard SEFL procedure. (A) Schematic representation of the procedure. (B) Mice with a strong history of stress freeze more than unstressed mice upon re-exposure to the subsequent mild fear conditioning context. (C) At test, both male and female mice showed similar SEFL, revealing an inability to detect underlying sex differences with this procedure.

Although the SEFL procedure is a reliable model of PTSD with a potential to elucidate sex differences in murine fear learning and memory, little is known about the molecular mechanisms facilitating this process. In particular, the mechanisms of the brain responsible for encoding and integrating trauma into memories to drive behavior remain unknown. The mechanisms that make females more vulnerable to stress remain even more unclear. So far, some research points to a couple of brain regions associated with SEFL, one of them being the hippocampus. As examples, Jones et al. (2018) and Hersman et al. (2019) respectively found that pro-inflammatory cytokine interleukin-1β and muscarinic acetylcholine receptors in the hippocampus both attenuated sensitivity to SEFL when blocked. The other major brain region of interest is the amygdala. A study conducted by Perusini et al. (2016) found that blocking corticosterone in the amygdala worsened the efficacy of SEFL. Moving from the structural level to the genomic level, the research becomes sparser. A handful of studies have suggested a couple of candidate genes playing a role in SEFL, one of them being the brain-derived neurotrophic factor (*BDNF*). One study conducted by Takei and colleagues (2011) found that stressed rats expressed higher levels of hippocampal *BDNF* transcripts – particularly those containing exons I, IV, and IX – following fear conditioning compared to unstressed rats (this also supports further investigation into the hippocampus); importantly, the fear conditioning procedure used by Takei et al. aligns with SEFL, making this study a critical point of reference for the present study. The other gene of interest is *Gria1* coding for the GluA1 subunit of AMPA receptors due to the study conducted by Perusini et al. (2016), previously mentioned. In that study, Perusini and colleagues used western blot analyses of the amygdala to show that SEFL increased the expression of GluA1 containing AMPA receptors.

Here I investigated the potential roles of *BDNF* and *Gria1* in the hippocampus and the amygdala in driving sex differences in fear learning using a *modified* SEFL procedure. In the

modified SEFL procedure, the number and intensity of footshocks administered to the mice were decreased, similar to the protocol used by Gonzalez et al. (2021). To quantify the expressions of *BDNF* and *Gria1* gene-derived transcripts, I used reverse transcription-quantitative polymerase chain reactions (RT-qPCR). Because Takei et al. (2011) found that exons I, IV, and IX of *BDNF* increased in stressed rats following fear conditioning, we focused on the transcripts that include those exons: *BDNF I, BDNF IV*, and *BDNF IX*. It is important to note that each transcript contains exon IX, so *BDNF IX* represents the sum of all *BDNF* transcripts, while a probe that measures expression of exon I or IV measures the subset of *BDNF* transcripts that contain either exon together with exon IX (*Figure 1-2*). Here, we report that female mice with a weak history of stress react more negatively towards the subsequent mild fear conditioning compared to male mice in the modified SEFL procedure. This reveals the potential of using the modified SEFL procedure to further investigate the higher prevalence of PTSD among women compared to men. We also report that hippocampal *BDNF I, BDNF IV,* and *BDNF IX* show enhanced expression following SEFL regardless of sex whereas amygdala *BDNF I, BDNF IV,* and *BDNF IX* and *Gria1*, in either brain region, played unclear roles – significant changes in their expressions may require stronger stress events. Further research is needed to either validate or clarify the roles of *BDNF* and *Gria1* transcripts in SEFL, as well as to identify genes contributing to the sex difference brought about by the modified SEFL procedure.

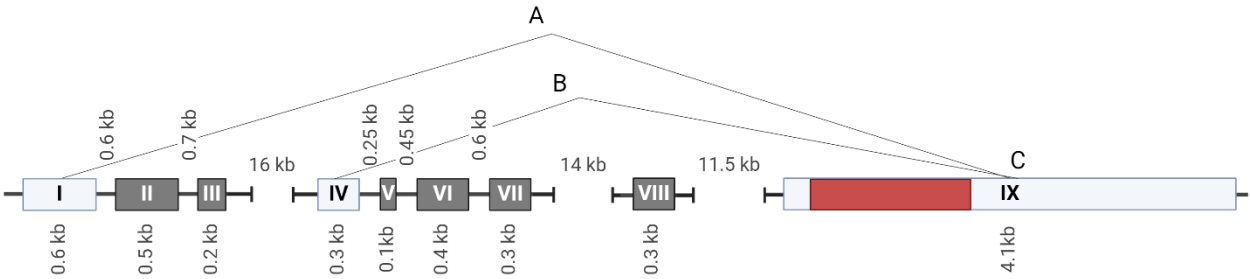


Figure 1-2. Schematic representation of *BDNF* transcripts originating from different promoters and corresponding untranslated 5' exons. The red colored region in exon IV represents the protein coding region. For the purposes of my experiment, *BDNF I* (A) contained a forward primer and a probe in exon I and a reverse primer in exon IX. *BDNF IV* (B) contained a forward primer and a probe in exon IV and a reverse primer in exon IX. *BDNF IX* (C) contained its own primers and probe. All the primers and probes are not shown. This figure was adapted from Aid et al. (2006).

Chapter 2

Materials and Methods

Subjects

Subjects were 8-week-old C57BL/6J mice obtained from Jackson Laboratories. Two cohorts of mice were separated into four groups: female no weak stress (n = 6), female weak stress (n = 6), male no weak stress (n = 6), and male weak stress (n = 6). One cohort underwent the entire modified SEFL procedure and the other only underwent the first two phases of the modified SEFL procedure (the weak stress event and the subsequent mild fear conditioning) before sacrifice for molecular analyses. All mice were individually housed during the experiment, were given free access to food and water, and were kept on a 12-hour light/dark cycle. All trials occurred during the light phase. The experiments were performed in accordance with the United States National Institutes of Health guidelines for animal care and were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

SEFL Apparatus

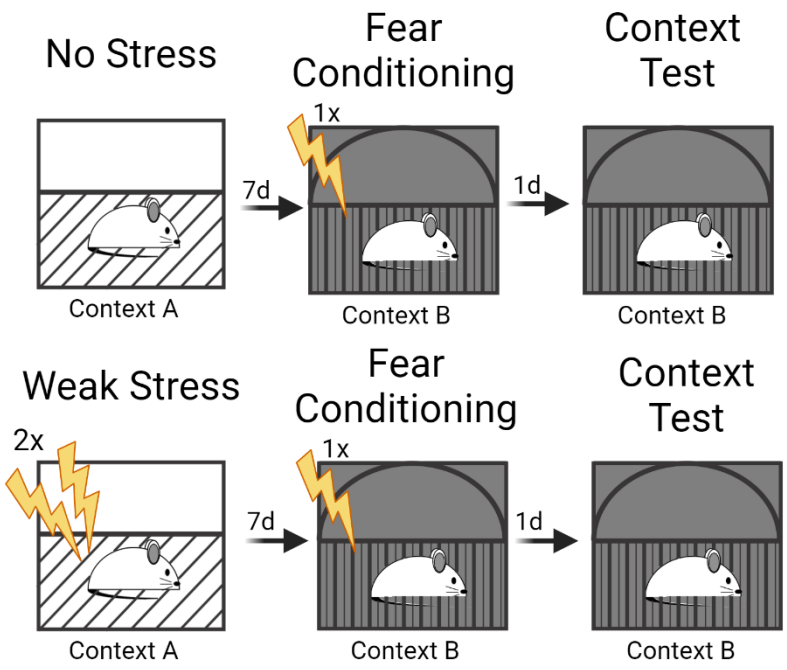
The SEFL procedure was performed in four identical plastic chambers housed within sound-dampening boxes. To distinguish the weak stress event from the subsequent mild fear conditioning and the context test, specific conditions were assigned to each context. For the weak stress event (Context A), the caged mice were transported to the SEFL room on a cart under normal lighting conditions and were placed into the plastic chambers with the following customizations: square walls with a plain appearance, evenly spaced floor bars for administering footshocks, dim white light, absence of background noise, and a 70% isopropyl alcohol scent. For the subsequent mild fear conditioning and the context test (Context B), the caged mice were carried under blankets

until they reached the SEFL room illuminated with red light and were placed into the plastic chambers with the following customizations: a U-shaped wall with a grid-like appearance, unevenly spaced floor bars for administering footshocks, absence of white light, constant dim white noise, and a scent of Windex.

Modified SEFL Procedure

After 5 days of handling for at least 1 minute each, the mice either underwent a mock or an actual weak stressor depending on their group assignment. The no weak stressor groups explored Context A for 14 minutes without footshocks while the weak stress groups explored Context A for 3 minutes before receiving 2 randomized 2-second 0.7mA footshocks over the course of 10 minutes followed by a 1-minute post-shock period without footshocks. 7 days following the mock or the actual weak stressor, all groups underwent mild fear conditioning in Context B which consisted of exploration for 3 minutes without footshocks before receiving a single, mild 2-second 0.35mA footshock followed by a 2-minute post-shock period without footshocks. 1 day after the fear conditioning, all groups underwent a context test in Context B which consisted of exploration for 5 minutes without footshocks. After every phase, the mice were immediately removed from the chambers, placed back into their cages, and returned to the vivarium the way they were brought to the SEFL room. See the figure below for a schematic representation of the modified SEFL procedure (*Figure 2-1*).

A)



B)

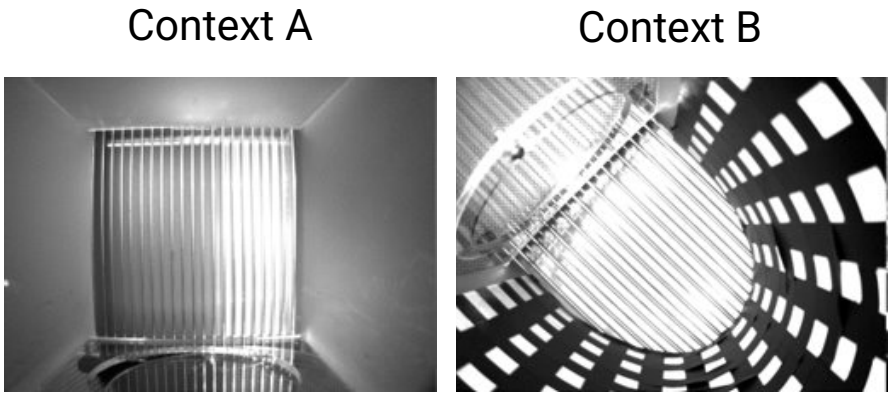


Figure 2-1. Schematic representation of the modified SEFL procedure. (A) Step-by-step layout of the modified SEFL procedure. (B) Images of each context to illustrate the different walls, floors, and lighting. Each context also had its own distinct scent to promote discrimination.

Tissue Extraction

Mice were euthanized via cervical dislocation and decapitated with surgical scissors (Fine Science Tools, Foster City, CA). Brains were removed from the skull with rongeurs and a surgical spatula (Fine Science Tools, Foster City, CA) and then flash-frozen in 2-methylbutane over dry ice at a temperature of -78ºC (Fisher Scientific, Waltham, MA). Brains were stored at -80ºC before being sectioned with a Leica CM150 Cryostat (Leica Biosystems, Wetzlar, Germany). 500µm coronal slices were taken from the dorsal hippocampus and the amygdala. Following this, the CA1 of the dorsal hippocampus and the basolateral amygdala were isolated via a punching tool and stored at -80ºC.

RT-qPCR

RNA was extracted from punches with RNeasy Mini Kits (Qiagen, Germantown, MD) and cDNA was generated with high-capacity cDNA Reverse Transcription Kits (ThermoFisher, Frederick, MD). PrimeTime primer/probe assays were generated with IDT PrimerQuest Design Tool (IDT, Coralville, IA) and used to quantify expression of *BDNF I*, *BDNF IV*, *BDNF IX*, *Gria1*, and *Gapdh*. Exact sequences: *BDNF I* left primer: 5'-GACACATTACCTTCCTGCATCT-3'; *BDNF I* right primer: 5'-GGATGGTCATCACTCTTCTCAC-3'; *BDNF I* probe: 5'/56-FAM/ACAGCAAAG/ZEN/CCACAATGTTCCACC/3IABkFQ/-3'; *BDNF IV* left primer: 5'-GCAGCTGCCTTGATGTTTAC-3'; *BDNF IV* right primer: 5'-TGCAACCGAAGTATGAAATAACC-3'; *BDNF IV* probe: 5'-/56-FAM/ACCAGGTGA/ZEN/GAAGAGTGATGACCA/3IABkFQ/-3'; *BDNF IX* left primer: 5'-TTCGGCCCAACGAAGAAA-3'; *BDNF IX* right primer: 5'-TCCTCCAGCAGAAAGAGTAGA-3'; *BDNF IX* probe: 5'-/56-FAM/ACTTGTACA/ZEN/CTTCCCGGGTGATGC/3IABkFQ/-3'; *Gria1* left primer: 5'-TCCGTATGGCTTCATTGATGG-3'; *Gria1* right

primer: 5'-ATCGAGTTCTGCTACAAATCCC-3'; *Gria1* probe: 5'-/56FAM/AACAGAAAC/ZEN/CCTTCATCCGCTTCGA/3IABkFQ/-3'; *Gapdh* left primer: 5'-GGAGAAACCTGCCAAGTATGA-3'; *Gapdh* right primer: 5'-TCCTCAGTGTAGCCCAAGA-3'; *Gapdh* probe: 5'-/5HEX/TCAAGAAGG/ZEN/TGGT GAAGCAGGCAT/3IABkFQ/-3'.

Statistics

For each phase of SEFL, behavioral responses were recorded as time spent freezing and were evaluated using EthoVision software. Freezing is presented as the mean of percent time freezing ± standard error of the mean (SEM). For each RT-qPCR analysis, LightCycler 96 (Roche, Basel, Switzerland) with Roche proprietary algorithms generated values representing the relative quantitative expression of the target gene (*Gapdh* serves as the reference gene) for each tissue sample. According to region of tissue extraction and group, these values were averaged, normalized to the group with the lowest expression of the target gene on average, and presented as the mean ± standard error of the mean (SEM). Any data point that differed from the mean by more than 2 standard deviations was considered an outlier and dropped. Two-way ANOVAs followed by Šídák's *post hoc* analyses were used to test for significance, with $p < 0.05$ being considered significant. Averages and standard deviations were calculated using Excel and statistical analyses were performed using GraphPad Prism 9 software.

Chapter 3

Results

Data are presented as mean ± standard error of the mean (SEM), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Modified SEFL Procedure Freezing Percentages

We assessed behavior during each session of our modified SEFL procedure to determine if there was a sex difference, as hypothesized. In the first session, the weak stress event, mice were exposed to Context A with or without presentation of 2 footshocks. Since the mice did not noticeably respond to the footshocks until the 12-minute mark, only the last three minutes of the weak stress event were analyzed. We found that the stressed male mice displayed more freezing than the unstressed male mice, although not to a significant extent according to two-way mixed ANOVA with Šídák's *post hoc* analyses. Likewise, the stressed female mice displayed more freezing than the unstressed female mice, yet significantly according to the same analyses (*Figure 3-1*; two-way repeated measures (RM) ANOVA, significant main effect of stress: $F_{(1,20)} = 1.55$, $p < 0.01$; *post hoc* tests: Males: $p > 0.05$, Females: $p < 0.01$, No Weak Stress: $p > 0.05$, Weak Stress: $p > 0.05$). This suggests that both the male and female mice responded normally to the weak stress footshocks; the shocked mice showed increased freezing, as expected. See *Figure 3-1* on the following page.

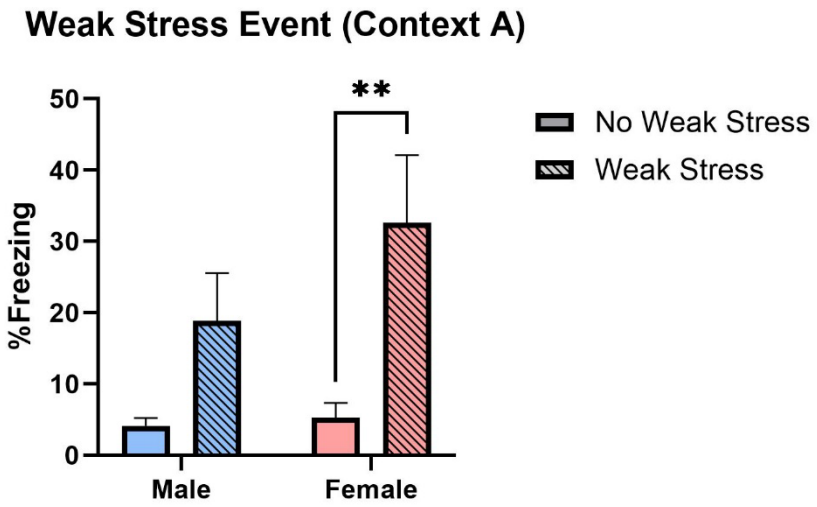


Figure 3-1. Average percentages of freezing during the last three minutes of the weak stress event. Two-way mixed ANOVA with Šídák's *post hoc* analyses.

Next, mice received mild fear conditioning in Context B, 7 days later. As expected, the stressed female mice displayed more freezing than the unstressed female mice during both the baseline and post-shock periods; two-way mixed ANOVA with Šídák's *post hoc* analyses revealed a significant difference only for the baseline period. Likewise, the stressed male mice displayed more freezing than the unstressed male mice during both the baseline and post-shock phases; no significant differences were detected by the same analyses (*Figure 3-2*; two-way RM ANOVA, significant main effect of period: $F_{(1,62)} = 8.288$, $p < 0.01$; significant main effect of stress: $F_{(1,62)} = 10.36$, $p < 0.01$; *post hoc* tests: Male Baseline: $p > 0.05$, Male Post-shock: $p > 0.05$, Female Baseline: $p < 0.05$, Female Post-shock: $p > 0.05$).

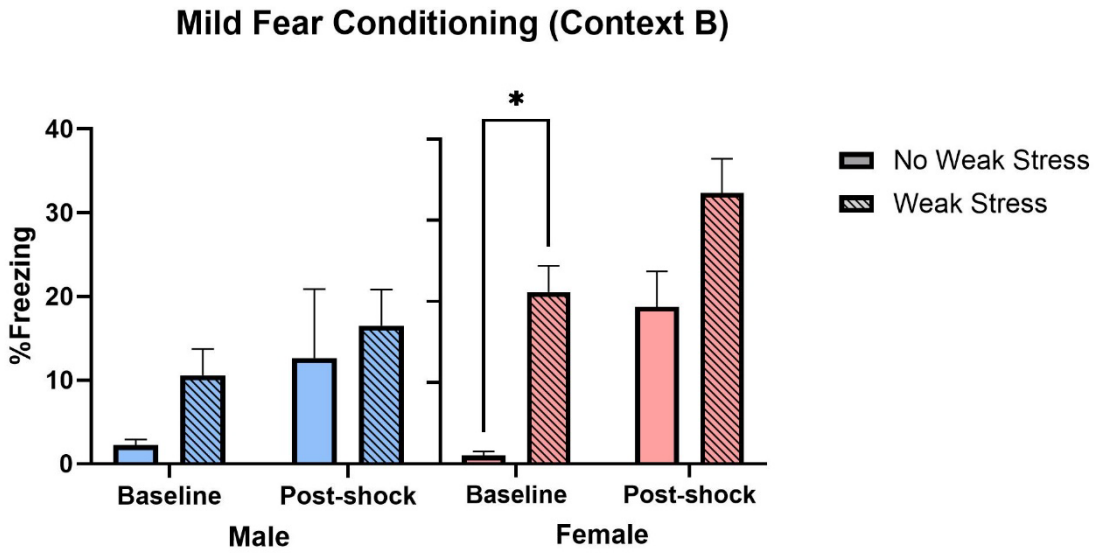


Figure 3-2. Average percentages of freezing during the baseline and post-shock periods of the mild fear conditioning. Two-way mixed ANOVA with Šídák's *post hoc* analyses.

Finally, fear to Context B was tested the following day to assess the strength of the memory for this mild fear conditioning. At test, the stressed male and female mice displayed more freezing than their unstressed counterparts, yet a two-way mixed ANOVA with Šídák's *post hoc* analyses only detected a significant difference for the female mice. For the groups without exposure to the weak stressor, the female mice displayed slightly more freezing than the males, although this was not significant according to the same analyses. Notably, among the weak stress groups, the female mice displayed significantly more freezing than the male mice according to the same analyses (*Figure 3-3*; two-way RM ANOVA, significant main effect of stress: $F_{(1,20)} = 10.82$, $p < 0.01$; significant main effect of sex: $F_{(1,20)} = 7.967$, $p < 0.05$; *post hoc* tests: No Weak Stress: $p > 0.05$, Weak Stress: $p < 0.05$. Males: $p > 0.05$, Females: $p < 0.01$). These results suggest that the female mice displayed a full SEFL response following exposure to weak stress whereas the male mice remained unaffected by the same level of stress. Therefore, our modified paradigm was sufficient to reveal sex differences in SEFL; male mice showed no lasting effects of stress on subsequent fear memory whereas female mice showed a robust, potentiated response to subsequent fear conditioning. See *Figure 3-3* on the following page.

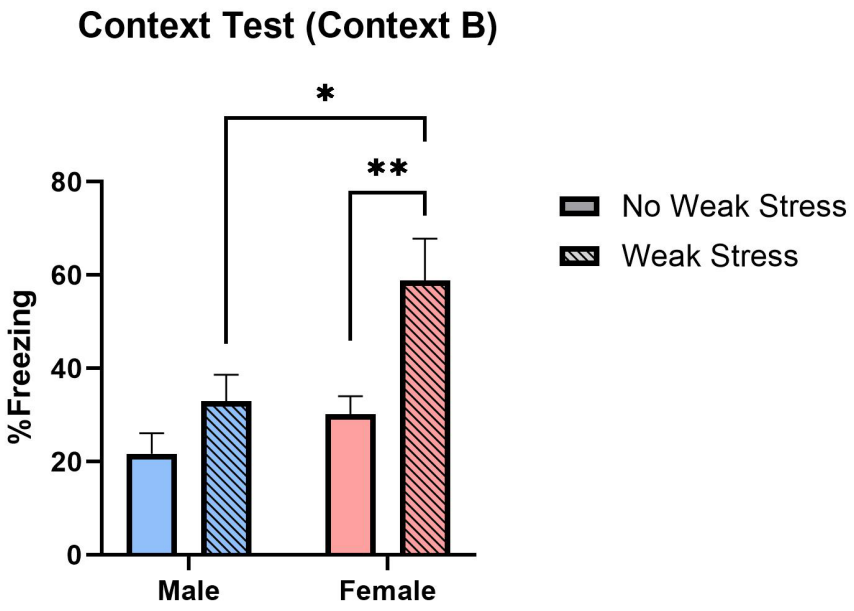


Figure 3-3. Average percentages of freezing during the context test. Two-way ANOVA with Šídák's *post hoc* analyses.

Expression of *BDNF I, BDNF IV, and BDNF IX*

Next, a separate cohort of mice underwent the modified SEFL procedure and were sacrificed following the subsequent mild fear conditioning in Context B to assess the expression of *BDNF* and *Gria1* transcripts and determine whether expression differed between male and female mice. One hour after mild fear conditioning, all mice were sacrificed for hippocampus and amygdala extractions. The tissues were used to assess the effect of the initial weak stressor on gene expression associated with fear learning. Specifically, we hypothesized that female mice (that show SEFL in response to this weak stress) would show exaggerated expression of *BDNF* and *Gria1* transcripts associated with fear learning. We further hypothesized that in male mice, the same behavioral procedure would not drive excessive gene expression, as male mice showed no lasting effects of weak stress. RT-qPCR analyses were performed to quantify the expression of *BDNF I*, *BDNF IV*, and *BDNF IX* in the hippocampus and the amygdala. All the *BDNF* transcripts were of interest because previous research has suggested that a history of stress induces their excessive expression following fear conditioning in rats (Takei et al., 2011).

I found that expression of each *BDNF* transcript noticeably increased in the hippocampus following weak stress exposure for both sexes, which means that transcripts containing either exon I or exon IV increased since exon IX represents the sum of all transcripts. Across sexes, the unstressed female mice expressed slightly higher levels of *BDNF I* and *BDNF IX* than their male counterparts, yet the stressed male and female mice expressed similar levels. For *BDNF IV*, the unstressed male and female mice expressed similar levels, yet the stressed male mice expressed slightly higher levels than their female counterparts. According to two-way mixed ANOVAs with Šídák's *post hoc* analyses, no main effects or interactions were significant (*Figure 3-4*). Therefore,

weak stress mildly increased fear-induced *BDNF* expression in the hippocampus and while this effect was consistent, it was not significant.

We also analyzed *BDNF* transcripts in the amygdala. In general, we saw no consistent changes in *BDNF* following weak stress in either males or females. Expression of each *BDNF* transcript was similar between the unstressed and stressed groups for each sex, except for *BDNF I* for the females. For that *BDNF* transcript, the stressed female mice expressed more than their unstressed counterparts, but not to a significant extent. Across sexes, both groups of female mice noticeably expressed lower levels than their respective male counterparts. According to two-way mixed ANOVAs with Šídák's *post hoc* analyses, no main effects or interactions were significant (*Figure 3-5*). Interestingly, the expression of the *BDNF* transcripts seemed to be more variable in the amygdala compared to the hippocampus. Overall, these results suggest that in the hippocampus, *BDNF I*, *BDNF IV*, and *BDNF IX* are enhanced, although not significantly, in response to fear learning in male and female mice with a weak history of stress. Amygdala *BDNF I*, *BDNF IV*, and *BDNF IX* play unclear roles. Thus, while *BDNF* may be a mechanism capable of supporting exaggerated fear learning in mice with a history of stress, it does not seem to be capable of supporting sex differences in this effect. See the following pages for the figures.

Expression of *Gria1*

We also quantified the expression of *Gria1* in the hippocampus and the amygdala. Similar to *BDNF*, *Gria1* is a gene of interest because research literature suggests that SEFL induces its expression in rats exposed to SEFL. In the hippocampus, *Gria1* expression showed a nonsignificant decrease following weak stress exposure in both sexes. Across the sexes, nothing noticeably changed (*Figure 3-6*). In the amygdala, *Gria1* expression modestly decreased following

weak stress exposure for only the male mice, although this was not significant. Across the sexes, the unstressed male and female mice expressed similar levels of *Gria1,* yet the stressed female mice expressed modestly higher levels of *Gria1* compared to their male counterparts (*Figure 3-7*). According to two-way mixed ANOVAs with Šídák's *post hoc* analyses, no main effects or interactions were significant. Interestingly, the expression of *Gria1* seemed to be more variable in the amygdala compared to the hippocampus. Overall, these results are not consistent with our hypothesis that *Gria1* in the hippocampus or amygdala may support exaggerated fear learning in male or female mice previously exposed to weak stress. See the following pages for the figures.

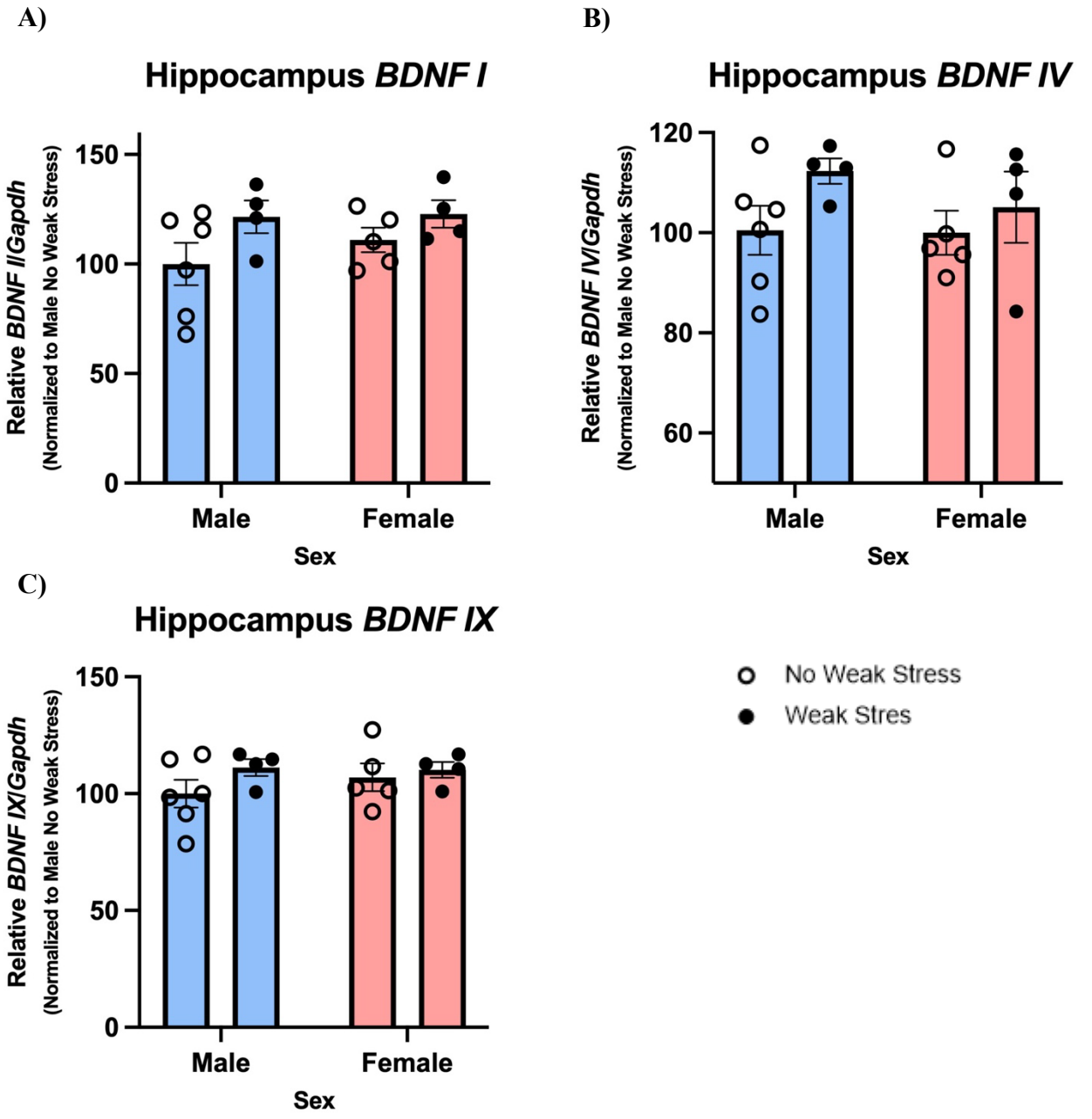


Figure 3-4. Expression of *BDNF* transcripts in the hippocampus. (A) *BDNF I*. (B) *BDNF IV*. (C) *BDNF IX*. Each group contained a variable number of mice due to removal of outliers (n = 4-6) and no main effects or interactions were found with two-way mixed ANOVAs with Šídák's *post hoc* analyses.

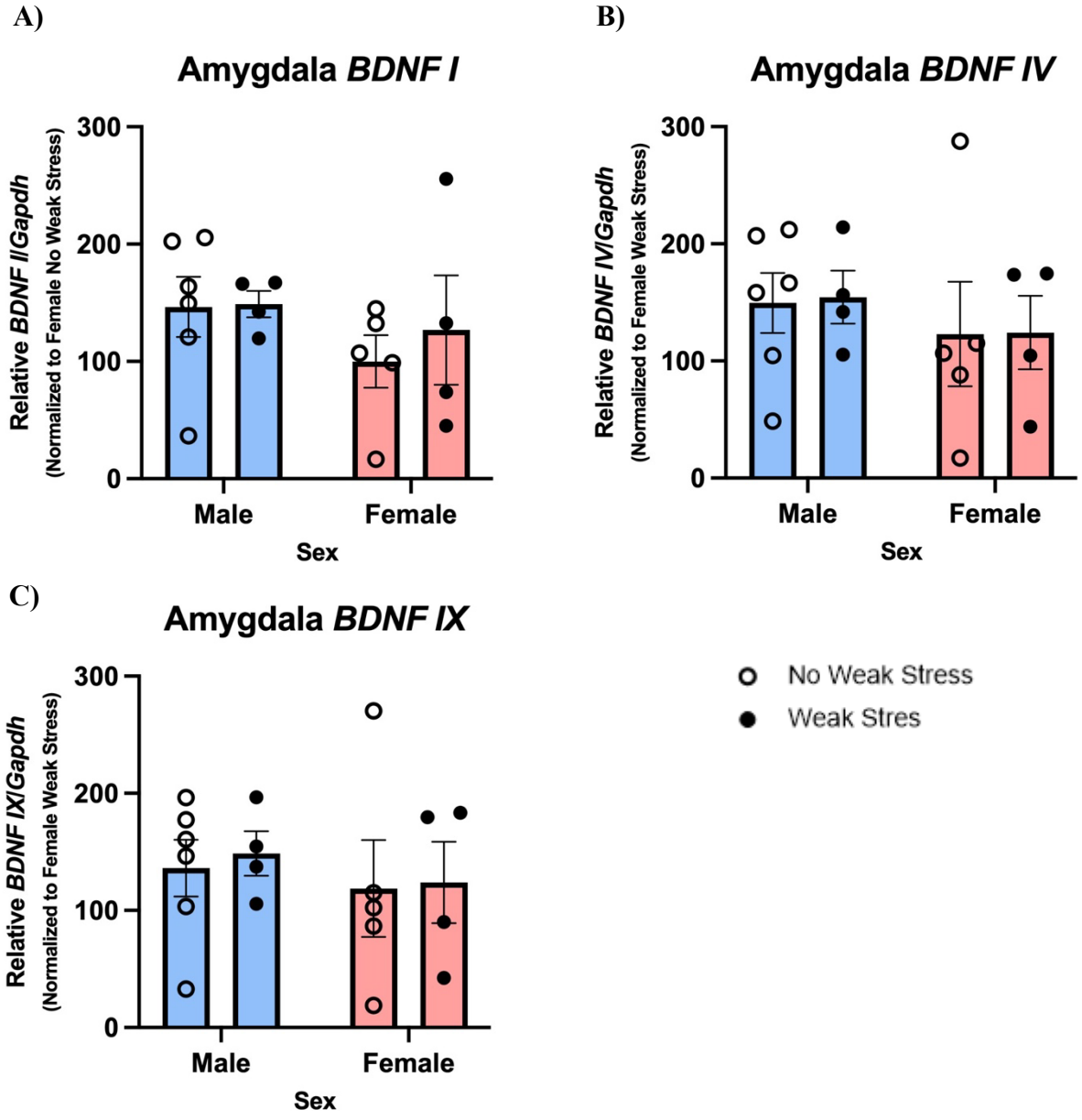


Figure 3-5. Expression of *BDNF* transcripts in the amygdala. (A) *BDNF I*. (B) *BDNF IV*. (C) *BDNF IX*. Each group contained a variable number of mice due to removal of outliers (n = 4-6) and no main effects or interactions were found with two-way mixed ANOVAs with Šídák's *post hoc* analyses.

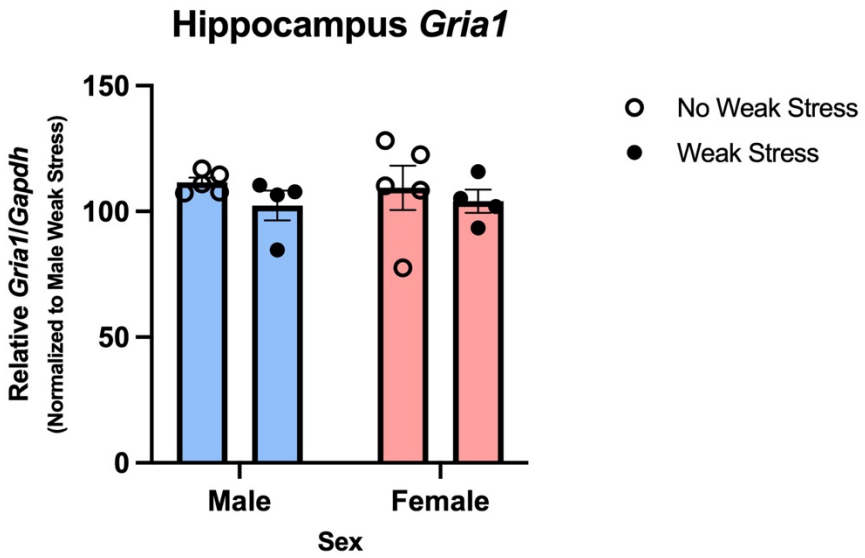


Figure 3-6. Expression of *Gria1* in the hippocampus. Each group contained a variable number of mice due to removal of outliers (n = 4-6) and no main effects or interactions were found with two-way mixed ANOVA with Šídák's *post hoc* analyses.

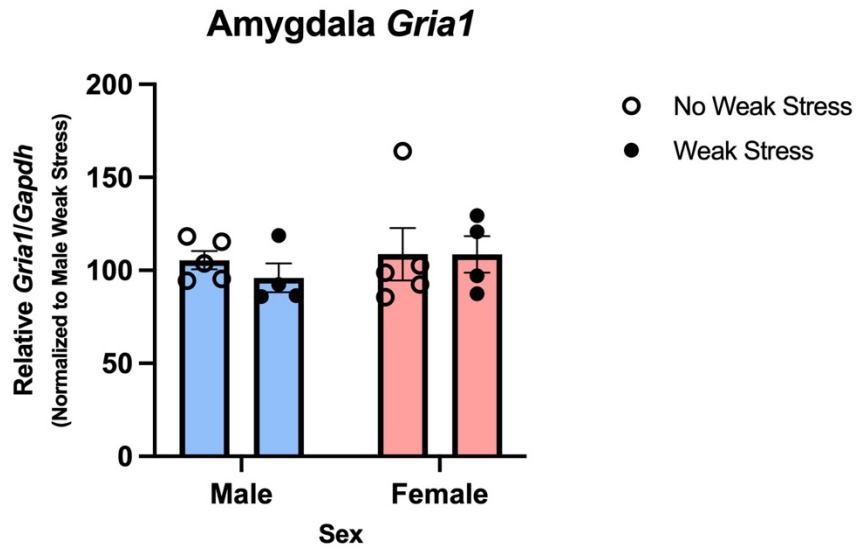


Figure 3-7. Expression of *Gria1* in the amygdala. Each group contained a variable number of mice due to removal of outliers (n = 4-6) and no main effects or interactions were found with two-way mixed ANOVA with Šídák's *post hoc* analyses.

Chapter 4

Discussion and Future Directions

Behaviorally, we found that female mice with a weak history of stress reacted strongly to a subsequent mild stressor compared to male mice with the same history, pinpointing a crucial sex difference in fear learning. The modified SEFL procedure modeled the higher prevalence of PTSD among women compared to men, revealing the potential of using this 2-shock paradigm to further investigate causal factors behind women's greater susceptibility to PTSD. Molecularly, we were unable to identify a mechanism that explains the sex difference in SEFL susceptibility, yet our work suggests that hippocampal *BDNF* transcripts may help facilitate SEFL. Specifically, we found that hippocampal *BDNF* transcripts were modestly increased by the modified SEFL procedure for both males and females. This upregulation following weak stress exposure seems to engender a more intense fear memory for the subsequent mild fear conditioning *regardless of sex* which, in turn, drives aversive behaviors. Coming across this finding with a weak stress event integrated into a SEFL procedure is promising, especially since Takei et al. (2011) reached the same finding with a strong stress event integrated into fear conditioning. Simply put, hippocampal *BDNF I*, *BDNF IV*, and *BDNF IX* appear to be sensitive to stress exposure. The amygdala *BDNF* transcripts and the *Gria1* transcript, however, played unclear roles, both showing no significant or consistent changes after SEFL. It is possible that a full 10-shock stress event would drive a significant upregulation of amygdala *BDNF* or *Gria1* in response to subsequent fear learning, something we will test in future studies.

Despite these intriguing findings, a couple limitations should be addressed when considering future study. First, the number of mice per group should be increased to help validate

or clarify the roles of *BDNF* and *Gria1* transcripts, as well as better compensate for any outliers. Second, home cage groups – mice not exposed to the modified SEFL procedure yet sacrificed for molecular analyses – should be incorporated to help simplify the RT-qPCR data normalizations.

The findings of this study inspire several other directions of future research, especially since the modified SEFL procedure brought about a sex difference in murine fear learning that coincides with the sex difference among people with PTSD. For example, the hippocampal *BDNF* gene could be manipulated to assess the effect of abnormally high or low transcript levels on responses to mild stressors subsequent to weak stress events. If the roles of the amygdala *BDNF* and *Gria1* transcripts are clarified, then the same could be done with these genes. Moreover, RNA-sequencing would be an excellent way to identify new genes upregulated by weak SEFL in female mice, but not male mice, to support our observed sex difference. Investigating molecular factors behind the sex difference in fear learning should go beyond the genetic level too. Consider the hormonal level as an example. According to a recent review, a handful of human and animal studies suggest that estradiol and progesterone differentially influence fear-related processes – depending on their concentrations – yet a majority of this research only used extinction learning and consolidation (Seligowski et al., 2020). This research aim could easily be incorporated into experiments with the modified SEFL procedure to help us better understand whether or not hormones contribute to the sex difference in fear learning.

In conclusion, adjusting the standard SEFL procedure by diminishing the number and intensity of administered footshocks created a modified SEFL procedure capable of unmasking a sex difference in fear learning and memory among mice. A weak history of stress heightened the vulnerability of female mice towards subsequent mild fear conditioning yet left the male mice unaffected. Furthermore, RT-qPCR analyses revealed that the levels of hippocampal *BDNF I,*

BDNF IV, and *BDNF IX* increased following subsequent mild fear conditioning for male and female mice with weak stress exposure, suggesting that these transcripts are sensitive to stress and help drive fear learning regardless of sex. The roles of amygdala *BDNF I, BDNF IV,* and *BDNF IX* and *Gria1*, in either brain region, remained unclear, prompting future study. Successfully modifying a reliable rodent model of PTSD into one that reflects the higher prevalence of PTSD among women compared to men enables researchers to further investigate this difference with a myriad of follow-ups (e.g. quantifying gene expressions, measuring hormonal levels, etc.), bringing about clarified understanding of PTSD. According to the U.S. Department of Veterans Affairs (2022), "53 of 100 patients who receive one of these three therapies [cognitive processing therapy, prolonged exposure therapy, and eye movement desensitization and reprocessing] will no longer have PTSD. With medication alone, 42 of 100 will achieve remission." Roughly *half* of the people suffering with PTSD come out of current treatments and therapies helpless, which is on a large scale; in 2020, about 13 million people developed PTSD in the United States alone (U.S. Department of Veterans Affairs, 2023). Understanding the factors contributing to PTSD is crucial for the better wellbeing of millions.

REFERENCES

Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., & Timmusk, T. (2007). Mouse and rat BDNF gene structure and expression revisited. *Journal of Neuroscience Research*, *85*(3), 525–535. https://doi.org/10.1002/jnr.21139

American Psychiatric Association. (1980). *Diagnostic and statistical manual of mental disorders*, (3rd ed.). Washington, DC: Author.

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders*, (5th ed.). Washington, DC: Author.

Breslau, N., & Davis, G. C. (1992). Posttraumatic stress disorder in an urban population of young adults: risk factors for chronicity. *The American Journal of Psychiatry*, *149*(5), 671-675. https://doi.org/10.1176/ajp.149.5.671

Breslau, N., Kessler, R. C., Chilcoat, H. D., Schultz, L. R., Davis, G. C., & Andreski, P. (1998). Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. *Archives of General Psychiatry*, *55*(7), 626-632. https://doi.org/10.1001/archpsyc.55.7.626

Davidson, J. R., Hughes, D., Blazer, D. G., & George, L. K. (1991). Post-traumatic stress disorder in the community: an epidemiological study. *Psychological Medicine*, *21*(3), 713-721. https://doi.org/10.1017/s0033291700022352

Gonzalez, S. T., Marty, V., Spigelman, I., Reise, S. P., & Fanselow, M. S. (2021). Impact of stress resilience and susceptibility on fear learning, anxiety, and alcohol intake. *Neurobiology of Stress*, *15*, Article 100335. https://doi.org/10.1016/j.ynstr.2021.100335

Hersman, S., Hoffman, A. N., Hodgins, L., Shieh, S., Lam, J., Parikh, A., Fanselow, M. S. (2019). Cholinergic signaling alters stress-induced sensitization of hippocampal contextual learning. *Frontiers in Neuroscience*, *13*, Article 251. https://doi.org/10.3389%2Ffnins.2019.00251

Jones, M. E., Paniccia, J. E., Lebonville, C. L., Reissner, K. J., & Lysle, D. T. (2018). Chemogenetic manipulation of dorsal hippocampal astrocytes protects against the development of stress-enhanced fear learning. *Neuroscience*, *388*, 45-56. https://doi.org/10.1016/j.neuroscience.2018.07.015

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of General Psychiatry*, *52*(12), 1048-1060. https://doi.org/10.1001/archpsyc.1995.03950240066012

Olff, M. (2017). Sex and gender differences in post-traumatic stress disorder: an update. *European Journal of Psychotraumatology*, *8*(sup4), Article 1351204. https://doi.org/10.1080/20008198.2017.1351204

Perusini, J. N., Meyer, E. M., Long, V. A., Rau, V., Nocera, N., Avershal, J., Maksymetz, J., Spigelman, I., & Fanselow, M. S. (2016). Induction and expression of fear sensitization caused by acute traumatic stress. *Neuropsychopharmacology*, *41*, 45-57. https://doi.org/10.1038/npp.2015.224

Rajbhandari, A. K., Gonzalez, S. T., & Fanselow, M. S. (2018). Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder. *Journal of Visualized Experiments*, *140*, Article 58306. https://doi.org/10.3791/58306

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: An animal model of posttraumatic stress disorder. *Neuroscience and Behavioral Reviews*, *29*(8), 1207-1223. https://doi.org/10.1016/j.neubiorev.2005.04.010

Rau, V. & Fanselow, M. S. (2009). Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress*, *12*(2), 125-133. https://doi.org/10.1080/10253890802137320

Seligowski, A. V., Hurly, J., Mellen, E., Ressler, K. J., & Ramikie, T. S. (2020). Translational studies of estradiol and progesterone in fear and PTSD. *European Journal of Psychotraumatology*, *11*(1), Article 1723857. https://doi.org/10.1080%2F20008198.2020.1723857

Takei, S., Morinobu, S., Yamamoto, S., Fuchikami, M., Matsumoto, T., & Yamawaki, S. (2011). Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. *Journal of Psychiatric Research*, *45*(4), 460-468. https://doi.org/10.1016/j.jpsychires.2010.08.009

Tolin, D. F. & Foa, E. B. (2006). Sex differences in trauma and posttraumatic stress disorder: A quantitative review of 25 years of research. *Psychological Bulletin*, *132*(6), 959-992. https://doi.org/10.1037/0033-2909.132.6.959

U.S. Department of Veterans Affairs. (2023, February 3). *How Common is PTSD in Adults?* https://www.ptsd.va.gov/understand/common/common_adults.asp

U.S. Department of Veterans Affairs. (2022, June 24). *Posttraumatic Stress Disorder (PTSD)*. https://www.research.va.gov/topics/ptsd.cfm